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Programa de Pós-graduação em Biotecnologia

Produção de biossurfactante comercial por *Candida lipolytica* UCP 0998 cultivada em resíduos agroindustriais para aplicação na indústria de petróleo e metais pesados

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Recife-PE

2017

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Tese apresentada ao Programa de Pós-graduação em Biotecnologia da Rede Nordeste de Biotecnologia (RENORBIO) do Ponto Focal de Pernambuco da Universidade Federal Rural de Pernambuco, como requisito parcial para obtenção do título de Doutor em Biotecnologia.

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DANYELLE KHADYDJA FELIX DOS SANTOS

PRODUÇÃO DE BIOSSURFACTANTE COMERCIAL POR *CANDIDA LIPOLYTICA* UCP 0998 CULTIVADA EM RESÍDUOS AGROINDUSTRIAIS PARA APLICAÇÃO NA INDÚSTRIA DE PETRÓLEO E METAIS PESADOS

Aprovada em 20/02/2017

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RESUMO

Os surfactantes são poderosos agentes anfipáticos com aplicação em vários segmentos industriais, especialmente nas indústrias petrolíferas. Muitos tipos de surfactantes quimicamente sintetizados são hoje utilizados; entretanto o desenvolvimento de produtos alternativos, biodegradáveis e menos tóxicos, como os chamados biossurfactantes, agentes obtidos por via microbiológica, torna-se uma estratégia importante na obtenção de produtos mais compatíveis com o meio ambiente e na ampliação das propriedades específicas e aplicações desses compostos. Muitos biossurfactantes têm sido produzidos, embora poucos sejam comercializados em virtude do alto custo de produção envolvido na obtenção desses compostos, principalmente no que se refere à utilização de substratos custosos e aos processos de purificação. Neste sentido, este trabalho propôs estudos para a produção de um biossurfactante de baixo custo para aplicação na despoluição de ambientes contaminados por derivados de petróleo e metais pesados. A maximização da produção do biossurfactante de *Candida lipolytica* UCP0988 cultivada em 5% de gordura animal e 2,5% de milhocina, foi inicialmente realizada em biorreator de 2L a partir de um planejamento fatorial 2^3 com ponto central. Os efeitos e interações da velocidade de agitação (200, 300 e 400rpm), aeração (0, 1 e 2vvm) e tempo de cultivo (48,96 e 144h) sobre a tensão superficial, o rendimento e a biomassa foram avaliados. Os resultados mostraram que o tempo de cultivo teve uma influência positiva na produção do biossurfactante, enquanto que o aumento da aeração e da agitação provocou um efeito negativo. A produção do biossurfactante em condições de cultivo maximizadas de 200 rpm e na ausência de aeração, alcançou valores em torno de 10,0 g/L em biossurfactante, com redução da tensão superficial para 28 mN/m após 96 horas. A curva de produção do biossurfactante demonstrou que a biomolécula foi produzida na fase estacionária de crescimento como metabólito secundário. Com o *scale-up* de produção do biossurfactante em reator de 50L, 40 g/L de biossurfactante foram produzidos, com tensão superficial de 25 mN/m. A biomassa celular foi quantificada e caracterizada para utilização como complemento nutricional em ração animal. A estrutura química do biossurfactante foi identificada utilizando Espectroscopia de infravermelho de Fourier (FTIR) e Espectroscopia de ressonância magnética nuclear (RMN). O biossurfactante bruto não apresentou toxicidade frente ao bivalve *Anomalocardia brasiliana* e ao microcrustáceo *Artemia salina* e nem frente a três espécies de sementes de hortaliças testadas. O biossurfactante formulado também não apresentou toxicidade frente ao peixe *Poecilia vivipara*. A adição de biossurfactante à água do mar estimulou a degradação do óleo de motor através da ação dos micro-organismos autóctones. Testes de dispersão demonstraram 80% de dispersão do óleo na água do mar, enquanto que os experimentos conduzidos em garrafas cilíndricas demonstraram valores em torno de 50% de dispersão para o biossurfactante isolado no dobro de sua concentração micelar crítica (1,6%). O biossurfactante mostrou-se eficiente em testes de detergência, com remoção de 70% de óleo de motor em tecido de algodão. A aplicação do biossurfactante bruto na remoção de metais pesados em amostras de areia padrão demonstrou 30 e 40% de remoção de Cu e Pb, respectivamente, enquanto que o biossurfactante isolado removeu cerca de 30% dos metais pesados. A solução de HCl removeu 60-50% de Cu, Pb e Zn e aumentou consideravelmente a remoção dos metais quando utilizada juntamente com o biossurfactante. A condutividade de soluções aquosas de efluente de mina preparado em laboratório contendo Cd e Pb foi drasticamente reduzida pelo biossurfactante. Com a finalidade de fornecer um produto comercial com vida de prateleira prolongada, o biossurfactante foi submetido ao método de conservação baseado na adição de sorbato de potássio a 0,2% e testado ao longo de 120 dias a

fim estimar a eficácia do produto a ser oferecido no mercado. O biossurfactante formulado foi então analisado quanto à emulsificação à tensão superficial, sob diferentes valores de pH, temperatura e a adição de NaCl. Os resultados mostraram que a formulação não causou alterações significativas na capacidade tensoativa da biomolécula, indicando a possibilidade da sua utilização em condições ambientais específicas. Os resultados obtidos nesse trabalho demonstraram a versatilidade do surfactante de *C. lipolytica* como agente de biorremediação de poluentes orgânicos e inorgânicos, bem como potencial para aplicação industrial como um agente comercial estável e seguro.

Palavras-chave: Biossurfactante, *Candida lipolytica*, poluente hidrofóbico, toxicidade, agente de biorremediação, petróleo.

ABSTRACT

Surfactants are amphipathic powerful agents with application in various industries, especially in the oil industry. Many types of chemically synthesized surfactants are used today, although the development of alternative products, biodegradable and less toxic as the so-called biosurfactants agents obtained by microbiological route, becomes an important strategy to achieve products adequate for use in the environment, and with specific properties and applications. Many biosurfactants have been produced, although few are marketed due to the high production cost involved in obtaining these compounds, especially as regards the use of expensive substrates and purification processes. In this sense, this project proposed studies directed towards maximizing the production of a low cost biosurfactant for application in environments contaminated by petroleum derivates and heavy metals. Experiments were conducted to maximize the production of the biosurfactant from *Candida lipolytica* UCP0988 cultivated on 5% animal fat and 2.5%corn steep liquor using a 2^3 full factorial design. The effects and interactions of the agitation speed (200, 300 and 400rpm), the variables aeration (0, 1 and 2vvm) and time of cultivation (48, 96 and 144h) on the surface tension, yield and biomass were evaluated. The results showed that the variable time of cultivation had positive influence on the production of biosurfactant, while the increase of the variables aeration and agitation showed a negative effect. This study investigated the large-scale production, characterization, evaluation of toxicity and economic analysis of the biosurfactant produced by *Candida lipolytica* UCP 0988 grown in amedium containing 5% animal fat and 2.5% corn steep liquor. The kinetics of biosurfactant production was described. The biosurfactant producedin the stationary growth phaseunder agitation of 200rpm andin the absence of aeration reducedthe surface tension of the medium to 28mN/m after 96 h, yielding 10.0 g/L ofisolated biosurfactant in a 2 L bioreactor. The production was maximized in a 50 L bioreactor, reaching 40 g/L biosurfactant and 25 mN/m. The cell biomasswas quantifiedand characterizedfor usein animal nutrition. Chemical structures of the biosurfactant were identified using Fourier transform infrared spectroscopy (FTIR) and Nuclear magnetic resonance spectroscopy (NMR). The crude biosurfactant was not toxic to the bivalve *Anomalocardia brasiliana*, to the microcrustacean *Artemia salina,* or three species of vegetables seeds. The formulated biosurfactant was also not toxic to the fish *Poecilia vivipara.* The addition of the biosurfactant to seawater stimulated the degradation of motor oil via the activity of the indigenous microorganisms. In tests carried out with seawater, the crude biosurfactant demonstrated 80% oil spreading efficiency in the screening dispersion test. Regarding the swirling bottle test, the dispersion rate was 50% for the isolated biosurfactant at a concentration twice the critical micelle concentration. The biosurfactant proved to be efficient in detergency tests, as it removed 70% of motor oil from contaminated cotton cloth. Application for the removal of heavy metals demonstrated that the crude biosurfactant removed 30 to 40% of Cu and Pb from standard sand, while the isolated biosurfactant removed approximately 30% of the heavy metals. The HCl solution tested removed 60 to 50% of Cu, Pb and Zn and greatly increased the removal of metals when used together with the biosurfactant. The conductivity of the solutions containing Cd and Pb was sharply reduced by the biosurfactant. To provide a commercial surfactant, the biosurfactant was subjected to a preservation method based on the addition of 0.2% potassium sorbate over 120 days to estimate the validity of the product to be offered to the market. The formulated biosurfactant was analysed for emulsification and surface tension under different pH values, temperatures and the addition of NaCl. The results showed that the formulation did not cause significant changes in the tensoactive capacity of the biomolecule, indicating the possibility of its use in specific

environmental conditions. The biosurfactant from *C. lipolytica* demonstrated versatility as a bioremediation agent of organic and inorganic pollutants as well as potential for industrial application as a stable, safe commercial agent.

Keywords: biosurfactant, *Candida lipolytica*, hydrophobic pollutants, toxicity, bioremediation agent, petroleum.

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LISTA DE SÍMBOLOS

$/kg	Valor por quilograma
$/mg	Valor por miligrama
°C	Grau Celsius
µg/mL	Microgramas por mililitro
COD/m^3	Demanda química de oxigênio por metro cúbico
Da	Dalton
g	Grama
g/L	Grama por Litro
kg	Quilo ou Quilograma
L	Litro
m	Metro
mg	Miligrama
mg/kg	Miligrama por quilograma
mg/L	Miligrama por litro
mL	Mililitro
mN/m	Mili Newton por metro
N	Newton
rpm	Rotação por minuto
U.S.	Dólar

LISTA DE ABREVIATURAS

ABNT	Associação Brasileira de Normas Técnicas
C	Carbono
$CaCO_3$	Carbonato de Cálcio
Cl	Cloro
CMC	Concentração Micelar Crítica
CMD	Diluição Micelar Crítica
CWO_4	Tungstato de carbono
DDT	Dicloro-Difenil-Tricloroetano
DNA	Ácido desoxirribonucléico
DPC	Surfactante catiônico
EOR	Enhanced oil recovery
FTIR	Espectroscopia de infravermelho de Fourier
Fe	Ferro
$FeCl_3.6H_2O$	Cloreto Férrico Hexa- hidratado
HPAS	Hidrocarbonetos aromáticos policíclicos
K	Potássio
KH_2PO_4	Di-Hidrogenofosfato de Potássio Monobásico
Mg	Magnésio
$MgSO_4.7H_2O$	Sulfato de Magnésio Hepta-Hidratado
N	Nitrogênio
NaCl	Cloreto de Sódio
NaOH	Hidróxido de Sódio
NH_2	Radical amino
NH_4Cl	Cloreto de Amônio
NI	Não informado
NO_2	Dióxido Nítrico
NPCIAMB	Núcleo de Pesquisas em Ciências Ambientais
O	Oxigênio
OH	Hidroxila
OOME	Efluente do Óleo de Oliva Verde
P	Fósforo
RMN	Espectroscopia de ressonância magnética nuclear

RNA	Ácido ribonucléico
SO_3	Anidrido sulfúrico ou óxido sulfúrico ou trióxido de enxofre
UNICAP	Universidade Católica de Pernambuco
YMA	Yeast Malt Agar
YMB	Yeast Mold Broth

SUMÁRIO

1. INTRODUÇÃO

As refinarias são grandes geradoras de poluição, produzem muitos despejos líquidos, liberam diversos gases nocivos para a atmosfera e produzem resíduos sólidos de difícil tratamento e disposição, além de, consomem enormes quantidades de água e de energia em decorrência de tais fatos, a indústria de refino de petróleo é uma grande degradadora do meio ambiente, pois tem potencial para afetar o mesmo em todos os níveis: ar, água e solo (GONZINI et al., 2010; ALMEIDA et al., 2016).

Os acidentes ocorridos com derramamentos de petróleo e seus derivados no Brasil, no período de 1975 a 2013, somaram milhões de litros que contaminaram solos, rios e mares. Diante dessa realidade, a possibilidade de contaminação ambiental é real e iminente, havendo necessidade urgente de desenvolvimento de novas tecnologias que possam conter possíveis contaminações (SILVA et al., 2014a).

A contaminação por petróleo e derivados, incluindo os metais pesados, normalmente é tratada através de metodologias físicas, químicas ou biológicas. Entretanto, as novas diretrizes de recuperação de águas e solos têm restringido o uso de produtos químicos (MUTHUSAMY et al., 2008; SARUBBO et al., 2015).

Dentre as técnicas de tratamento de petroderivados disponíveis, a tecnologia de biorremediação tem se destacado, embora a solubilidade reduzida dos hidrocarbonetos dificulte o acesso dos micro-organismos e a consequente biodegradação dos poluentes hidrofóbicos (CALVO et al., 2009). Os obstáculos associados à biodegradação dos hidrocarbonetos do petróleo surgem em função da ligação desses compostos hidrofóbicos às partículas do solo e à solubilidade reduzida em água, resultando em baixa biodisponibilidade para os micro-organismos e na consequente paralização do processo (CORTIS, GHEZZEHEI, 2007).

Nesse contexto, a utilização de compostos surfactantes surge como a tecnologia mais investigada para a resolução deste problema, permitindo a dessorção e consequente solubilização dos hidrocarbonetos, facilitando, assim, a assimilação desses compostos pelas células microbianas autóctones (VAN HAMME et al., 2006). Com relação à remediação de ambientes contaminados por metais pesados, os surfactantes podem ser adicionados em soluções, facilitando a solubilização, dispersão e dessorção desses contaminantes, permitindo ainda sua reutilização (SARUBBO et al., 2015; LUNA et al., 2016).

Por outro lado, a necessidade de substituição de compostos sintéticos por similares naturais tem levado a pesquisas para utilização de novos compostos tensoativos como alternativa aos produtos existentes (BANAT et al., 2010).

Os compostos anfipáticos de origem microbiana que exibem propriedades surfactantes, isto é, diminuem a tensão superficial e possuem capacidade emulsificante, são denominados biossurfactantes e consistem em subprodutos metabólicos de bactérias, fungos filamentosos e leveduras (SANTOS et al., 2016). Características como detergência, emulsificação, dispersão, ação umectante e solubilização atribuem grande versatilidade a essas biomoléculas, tornando-as candidatas em potencial na substituição dos surfactantes sintéticos (CAMPOS et al., 2013). A maioria dos biossurfactantes conhecidos é produzida em substratos insolúveis em água como hidrocarbonetos sólidos e líquidos, óleos e gorduras, embora muitos podem ser obtidos a partir de substratos solúveis (BANAT et al., 2010).

A possibilidade de produção dos biossurfactantes a partir de substratos renováveis e de diferentes espécies microbianas, além da possibilidade de variação de inúmeros parâmetros, como tempo de cultivo, velocidade de agitação, pH do meio e nutrientes adicionados, permite a obtenção de compostos com características estruturais e propriedades físicas distintas, o que os tornam comparáveis ou superiores aos surfactantes sintéticos em termos de eficiência, embora os custos de produção ainda não permitam uma maior competitividade com seus similares sintéticos (SILVA et al., 2014a).

Os primeiros estudos na área dos biossurfactantes ocorreram na década de 80 e, desde então, as pesquisas permitiram o desenvolvimento e a comercialização de dois produtos, a Surfactina, uma lipoproteína produzida pela bactéria *Bacillus subtilis*, e os Raminolipídeos, grupo de glicolipídeos produzidos pela bactéria *Pseudomonas aeruginosa* comercializados pela Jeneil Biosurfactants Company (USA). Esses dois biossurfactantes, embora extremamente eficientes, são comercializados a um alto custo em função das matérias-primas utilizadas para suas produções e do nível de pureza exigido para aplicações nas áreas farmacêutica e médica (SANTOS et al., 2016).

Nesse sentido, é de fundamental importância o desenvolvimento de estratégias que permitam a produção e consequente aplicação dos biossurfactantes em escala industrial. A seleção de micro-organismos superprodutores, os substratos de baixo custo utilizados na etapa de produção, e o aprimoramento dos processos

de purificação têm sido utilizados com essa finalidade (MARCHANT; BANAT, 2012b).

A disponibilidade e o tipo de matéria-prima podem contribuir consideravelmente para a redução do custo de produção dos biossurfactantes. Estima-se que a matéria-prima represente de 10 a 30% do custo total de um produto biotecnológico, enquanto que as etapas de purificação contribuem com 60% do processo (SARUBBO et al., 2015). Por outro lado, milhões de desperdícios em resíduos poluentes são jogados a cada ano por todo o mundo. O tratamento e a remoção destes resíduos também representam um alto custo para várias indústrias (MARCHANT; BANAT, 2012a).

Portanto, a presente Tese de Doutorado teve como objetivo maximizar a produção de um agente surfactante a partir de *Candida lipolytica* utilizando resíduos industriais como substratos de baixo custo, através do estudo da cinética de crescimento e de produção, bem como pelo estudo das propriedades do biossurfactante obtido, seu isolamento, quanto a caracterização, formulação comercial e sua aplicação na remoção de derivado de petróleo e metais pesados em ambientes terrestres e marinhos.

2. OBJETIVOS

2.1. Objetivo geral

- Produzir um biossurfactante comercial com potencial para aplicação na indústria visando à descontaminação ambiental de petróleo e derivados.

2.1.1. Objetivos específicos

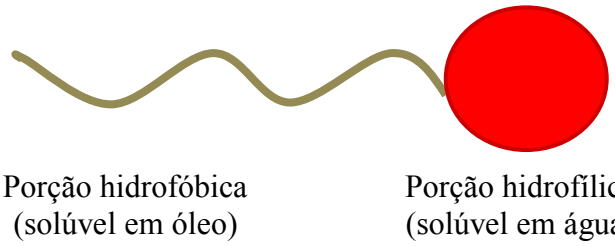
- Utilizar um planejamento fatorial como ferramenta para maximização das variáveis agitação, aeração e tempo de cultivo em biorreator para a produção do biossurfactante.
- Descrever a curva de crescimento do micro-orgainsmo e de produção do biossurfactante a partir da condição de cultivo previamente selecionada em biorreator.
- Realizar o *scale-up* de produção do biossurfactante.
- Realizar uma comparação da produção do biossurfactante com a literatura em birreator.
- Caracterizar bioquimicamente a biomassa celular para uso em ração animal.
- Caracterizar estruturalmente o biossurfactante.
- Avaliar a toxicidade do biossurfactante frente a sementes de vegetais; ao microcrustáceo *Artemia salina* e ao bivalve *Anomalocardia brasiliana*.
- Avaliar o potencial de aplicação do biossurfactante como agente de biorremediação em água do mar contaminada com derivado de petróleo
- Aplicar o biossurfactante como detergente de derivado de petróleo.
- Aplicar o biossurfactante como dispersante de derivado de petróleo em testes específicos.
- Aplicar o biossurfactante na remoção de metais pesados contidos em areia e em solução aquosa sintética.
- Formular o biossurfactante por adição de conservante para uso comercial no meio ambiente.
- Avaliar a estabilidade do biossurfactante formulado (efeitos do pH, da adição de NaCl e da temperatura).
- Avaliar a toxicidade do biossurfactante formulado frente ao peixe *Poecilia vivipara.*

3. REVISÃO DA LITERATURA

3.1. Surfactantes químicos e biológicos: definição e características principais

Os surfactantes são compostos anfipáticos contendo porções hidrofílicas e hidrofóbicas que se particionam, preferencialmente, na interface entre fases fluidas com diferentes graus de polaridade e pontes de hidrogênio, como interfaces óleo/água ou ar/água (SARUBBO et al., 2015). A porção apolar é frequentemente uma cadeia hidrocarbonada enquanto a porção polar pode ser iônica (catiônica ou aniônica), não-iônica ou anfotérica (MAO et al., 2015; SILVA et al., 2014a), como ilustrado na Figura 1.

Figura 1 - Molécula surfactante com porção apolar (hidrofóbica) e polar (hidrofílica)



A eficiência de um surfactante é determinada por sua habilidade em reduzir a tensão superficial, que é a força de atração existente entre as moléculas dos líquidos. Surfactantes aumentam a solubilidade aquosa de moléculas hidrofílicas, reduzindo a tensão superficial/interfacial de interfaces óleo-água. Bons surfactantes conseguem reduzir a tensão superficial da água de 72 mN/m para 35 mN/m e a tensão interfacial (tensão entre líquidos polares e apolares) da água e do n-hexadecano de 40 mN/m para 1 mN/m (BANAT et al., 2010).

A tensão superficial diminui quando a concentração de surfactante no meio aquoso aumenta, ocorrendo a formação de micelas, que são estruturas agregadas com as porções hidrofílicas posicionadas para a parte externa da molécula e as porções hidrofóbicas para a parte interna (Figura 2). A concentração de micelas é conhecida como a Concentração Micelar Crítica (CMC). Esta concentração corresponde ao ponto em que o agente tensoativo registra o menor valor de tensão superficial estável, ou seja, corresponde à mínima concentração de surfactante

necessária para que a tensão superficial seja reduzida ao máximo (Figura 3). Quando a CMC é atingida, várias micelas são formadas (CAMPOS et al., 2013).

Figura 2 - Representação esquemática de um tensoativo e formação da micela

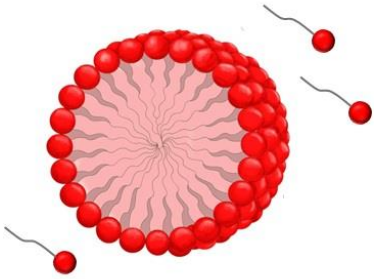
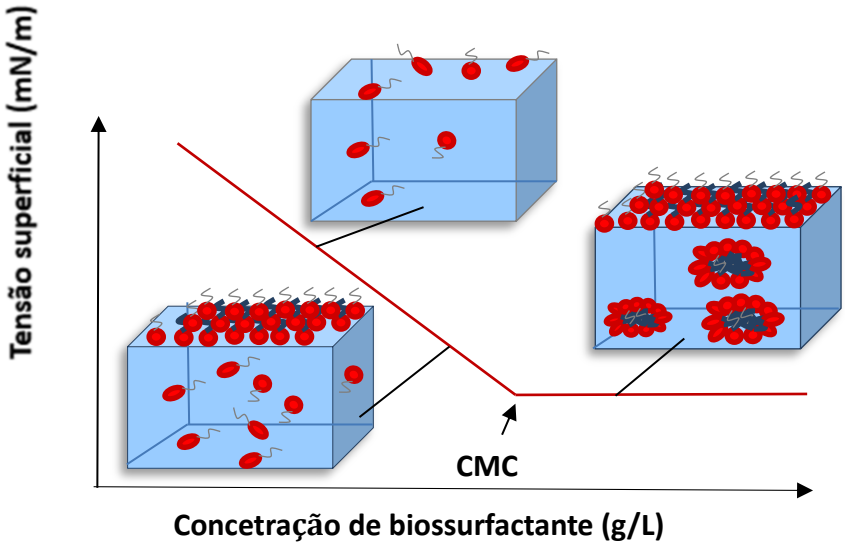


Figura 3 - Gráfico ilustrativo das regiões onde ocorre a formação de micelas



Fonte: PACWA-PLOCINICZAK et al., 2011

A seleção de surfactantes para uso em processos industriais é baseada nos custos de produção. Geralmente, surfactantes são utilizados para poupar energia e consequentemente para diminuir os custos da energia por exemplo, requerida pela bomba de ar e outras técnicas de tratamento. O tipo de carga, o comportamento físico-químico, a adsorção e a solubilidade, são os critérios mais importantes para selecionar surfactantes eficientes (CAMPOS et al., 2013).

A maioria dos surfactantes produzidos atualmente é quimicamente derivado de petróleo. No entanto, estes agentes tensoativos sintéticos são geralmente tóxicos e

dificilmente degradados por micro-organismos. Eles são, portanto, uma fonte potencial de contaminação e danos para o meio ambiente. Estes perigos associados têm, nos últimos anos, atraído a comunidade científica a buscar alternativas de obtenção de surfactantes mais compatíveis com o meio ambiente, como é o caso da produção microbiana de surfactantes (biossurfactantes) (VIJAYAKUMAR; SARAVANAN, 2015).

Aliado ao interesse científico, a preocupação ambiental entre os consumidores, combinada a novas legislações de controle ambiental têm levado ao desenvolvimento de surfactantes naturais como alternativa aos produtos existentes.

Os estudos relacionados aos biossurfactantes iniciaram-se em 1960 e a utilização desses compostos se estendeu nas últimas décadas como alternativa atrativa aos surfactantes sintéticos (CERQUEIRA et al., 2011; SILVA et al., 2014a). Os biossurfactantes são derivados de organismos vivos, principalmente de micro-organismos, e têm despertado interesse industrial em função de suas características vantajosas, como diversidade estrutural, toxicidade reduzida, maior biodegradabilidade, capacidade de ação em ambientes extremos de pH, temperatura e salinidade, maior seletividade, menor CMC e habilidade de serem produzidos a partir de fontes renováveis/resíduos industriais e de subprodutos (ROSA et al., 2015; PACWA- PLOCINICZAK et al., 2011). A redução da tensão superficial e a estabilidade da emulsão formada são características importantes em um biosurfactante, as quais tornam possível a utilização desses agentes em diferentes setores industriais, como será discutido nas próximas seções (BANAT et al., 2010; MAKKAR*;* CAMEOTRA, 2011; PACWA-PLOCINICZAK et al., 2011).

3.2. Micro-organismos produtores de biossurfactantes

Os micro-organismos utilizam uma série de fontes de carbono e energia para seu crescimento. A associação de fontes de carbono com substratos insolúveis facilita a difusão intracelular e a produção de várias substâncias, dentre elas os biossurfactantes (BANAT, 2010). Uma grande variedade de micro-organismos, incluindo bactérias, leveduras e uma minoria de fungos filamentosos são capazes de produzir biossurfactantes com diferentes estruturas moleculares e atividades superficiais (CAMPOS et al., 2013).

Nas últimas décadas houve um aumento no interesse científico em identificar e isolar novos micro-organismos produtores de moléculas tensoativas que apresentem

boas características surfactantes, como baixa CMC, baixa toxicidade e alta atividade de emulsificação, dentre outras (SILVA *et al*., 2014a).

As bactérias dos gêneros *Pseudomonas* e *Bacillus* são descritas na literatura como grandes produtoras de biossurfactantes (SILVA *et al*., 2014a). Entretanto, a grande maioria dos biossurfactantes de origem bacteriana é inadequada para utilização na indústria alimentícia devido a sua possível natureza patogênica (SHEPERD *et al*., 1995).

A *Candida bombicola* e *C. lipolytica* estão entre as leveduras mais comumente estudadas para a produção de biossurfactantes. Uma grande vantagem do uso de leveduras reside no status GRAS (Generally Regarded as Safe) que muitas delas apresentam, como *Yarrowia lipolytica*, *Saccharomyces cerevisiae* e *Kluyveromyces lactis*. Organismos com status GRAS não apresentam riscos de toxicidade e patogenicidade, o que permite sua utilização para aplicações nas indústrias de alimentos e farmacêutica (CAMPOS *et al*., 2013).

A Tabela 1 oferece uma lista de micro-organismos que produzem biossurfactantes.

Tabela 1 - Principais classes de biossurfactantes e respectivos micro-organismos produtores

Classes					
Glicolipídeos	**Surfactantes poliméricos**	**Lipopeptídeos**	**Ácidos graxos**	**Surfactantes Particulados**	**Fosfolipídeos**
Micro-organismos produtores					
Acinetobacter calcoaceticus (RAG1)	*Acinetobacter calcoaceticus*	*Acinetobacter* sp	*Arthrobacter paraffineus*	*Acinetobacter calcoaceticus*	*Acinetobacter* sp
Alcanivorax borkumensis	*Acinetobacter calcoaceticus*	*Bacillus licheniformis*	*Capnocytophaga sp*	*Cyanobacteria*	*Aspergillus*
Arthrobacter paraffineus	BD413	*Bacillus pumilus*	*Corynebacterium insidibasseosum*	*Pseudomonas marginalis*	*Corynebacterium lepus*
Arthrobacter sp	*Acinetobacter calcoaceticus* KA53	*Bacillus subtilis*	*Corynebacterium lepus*		
Candida antartica	*Acinetobacter calcoaceticus* RAG-1	***Candida lipolytica***	*Nocardia erythropolis*		
Candida apicola	*Bacillus stearothermophilus*	*Gluconobacter cerinus*	*Penicillium spiculisporum*		
Candida batistae	***Candida lipolytica***	*Pseudomonas fluorescens*	*Talaramyces trachyspermus*		
Candida bogoriensis	*Candida utilis*	*Serratia marcescens*			
Candida bombicola	*Halomonas eurihalina*	*Streptomyces sioyaensis*			
Candida ishiwadae	*Mycobacterium thermoautotrophium*	*Thiobacillus thiooxidans*			
Candida lipolytica	*Sphingomonas paucimobilis*				
Corynebacterium sp					
Lactobacillus fermentum					
Nocardia sp					
Pseudomonas aeruginosa					
Pseudomonas sp					
Rhodococcus erythropolis					
Rhodotorula glutinus					
Serratia marcescens					
Tsukamurella sp					

3.3. Classificação dos biossurfactantes

A maioria dos biossurfactantes é aniônica ou neutra. Apenas alguns são catiônicos, como os que contêm grupamentos amina. A parte hidrofóbica é caracterizada por ácidos graxos de cadeia longa, enquanto que a porção hidrofílica pode ser carboidrato, um aminoácido, um peptídeo cíclico, fosfato, um ácido carboxílico ou um álcool. A massa molar dos biossurfactantes geralmente varia de 500 a 1500 Da (BOGNOLO, 1999).

Os biossurfactantes são genericamente classificados de acordo com sua origem microbiana e composição química, como descrito abaixo (BANAT et al., 2010; RAHMAN; GAKPE, 2008; VIJAYAKUMAR; SARAVANAN, 2015):

Glicolipídeos: são carboidratos ligados a ácidos graxos alifáticos de cadeias longas ou ácidos graxos hidroalifáticos através de um éster. A maioria dos biossurfactantes são glicolipídicos. Dentre os glicolipídeos, os mais conhecidos são os raminolipídeos, os trealolipídeos e os soforolipídeos (Jarvis and Johnson, 1949). Os raminolipídeos foram inicialmente reportados como metabólitos extracelulares da patogênica oportunista *P. aeruginosa*. Eles são descritos como uma mistura de 04 homólogos: R1 [3-(α-L-raminopiranosil)-β-hidroxidecanoato] ou L-raminopiranosil-β-hidroxidecanoato (Rha-C_{10}), R2 [3-(2'-*O*-α-L-raminopiranosil-α-L-raminopiranosiloxi)-β-hidroxidecanoato] ou 2-*O*-L-raminopiranosil-β-L-raminopiranosil-β-hidroxidecanoil-β-hidroxidecanoato (Rha_2-C_{10}), R3 3-[3'-(α-L-raminopiranosil-hidroxidecanoil)-β-hidroxidecanoato] ou L-raminopiranosil-β-hidroxidecanoil-β-hidroxidecanoato (Rha-C_{10}-C_{10}) e R4 3-[3'-(2"-*O*-α-L-raminopiranosil-α-L-raminopiranosil-hidroxidecanoil)-β-hidroxidecanoato] ou (2-*O*-L-raminopiranosil-β-L-raminopiranosil-β- hidroxidecanoato (Rha_2-C_{10}-C_{10}). O desenvolvimento de técnicas analíticas mais sensíveis levaram à descoberta de uma maior diversidade de homólogos congêneros (cerca de 60), produzidos em diferentes concentrações por várias espécies de *Pseudomonas* e por outras bactérias pertencentes a outras famílias e classes. Várias espécies de *Burkholderia* têm demonstrado capacidade de produzir raminolipídeos de cadeias alquilas mais longas do que as produzidas pela *P. aeruginosa* (ABDEL-MAWGOUD et al., 2010; HITSATSUKA et al., 1971; NITSCHKE et al., 2011). Valores de tensão superficial de 29 mN/m são característicos destes componentes, que podem ser produzidos a partir de vários substratos, incluindo alcanos, piruvatos, citratos, frutoses, glicerol, óleo de oliva e glicose. Os glicolipídeos do tipo soforolipídeos são produzidos por espécies de

Candida (CORTÉS-SÁNCHEZ et al., 2013; DAVEREY; PAKSHIRAJAN, 2009). Esses glicolipídeos consistem de um carboidrato dimérico chamado soforose ligado a uma hidroxila de ácido graxo de cadeia longa por uma ligação glicosídica. Os soforolipídeos são geralmente uma mistura de pelo menos seis a nove tipos da porção hidrofóbica (GAUTAM; TYAGI, 2006) com a forma lactônica, a qual é preferível em muitas aplicações (HU; JU, 2001). Dentre os vários tipos de leveduras utilizadas na produção desses biossurfactantes, destaca-se a *C. bombicola.* A tensão superficial dessas biomoléculas apresenta valores em torno de 33 mN/m e reduções de 40 mN/m para 5 mN/m na tensão interfacial em n-hexadecano e água (DÍAZ DE RIENZO et al., 2015). Os lipídeos do tipo manosileritritol (MEL), glicolipídeos de leveduras, constituem um dos mais promissores grupos de biossurfactantes conhecidos e abundantemente produzidos a partir de óleos vegetais pela *Pseudozyma* (previamente *Candida*) *antarctica* (LANG, 2002). Os glicolipídeos do tipo trealolipídeos, por sua vez, estão associados com as espécies de *Mycobacterium, Nocardia* e *Corynebacterium*. Os trealolipídeos de *Rhodococcus erythropolis* e *Arthrobacter* spp. São capazes de reduzir a tensão superficial e interfacial do líquido metabólico para 25-40 e 1-5 mN/m, respectivamente (VIJAYAKUMAR; SARAVANAN, 2015).

Lipopeptídeos: a surfactina produzida pelo *B. subtilis* (BANAT et al., 2014b), é considerada um dos biossurfactantes mais poderosos já relatados na literatura, pois é capaz de reduzir a tensão superficial da água de 72 mN/m para 27 mN/m (LU et al., 2007). A solubilidade e a capacidade surfactante da surfactina, por outro lado, dependem do tipo de resíduo utilizado como substrato (HUE et al., 2001). O *B. licheniformis* produz vários biossurfactantes, similares à surfactina que exibem excelente estabilidade em condições extremas de temperature, pH e na presença de sal.

Ácidos graxos, fosfolipídios e lípideos neutros: várias bactérias e leveduras produzem grandes quantidades de ácidos graxos e agentes tensoativos fosfolipídicos durante o crescimento em n-alcanos. Em *Acinetobacter* spp. 1-N, vesículas ricas em fosfatidil-etanolamina são produzidas e formam micro-emulsões de água em alcanos. Esses biotensoativos são essenciais para aplicações médicas. Gautam e Tyagi (2006) relataram que a deficiência no complexo proteína fosfolipídica é a principal causa na falha respiratória nas crianças nascidas prematuramente. Eles também sugeriram que

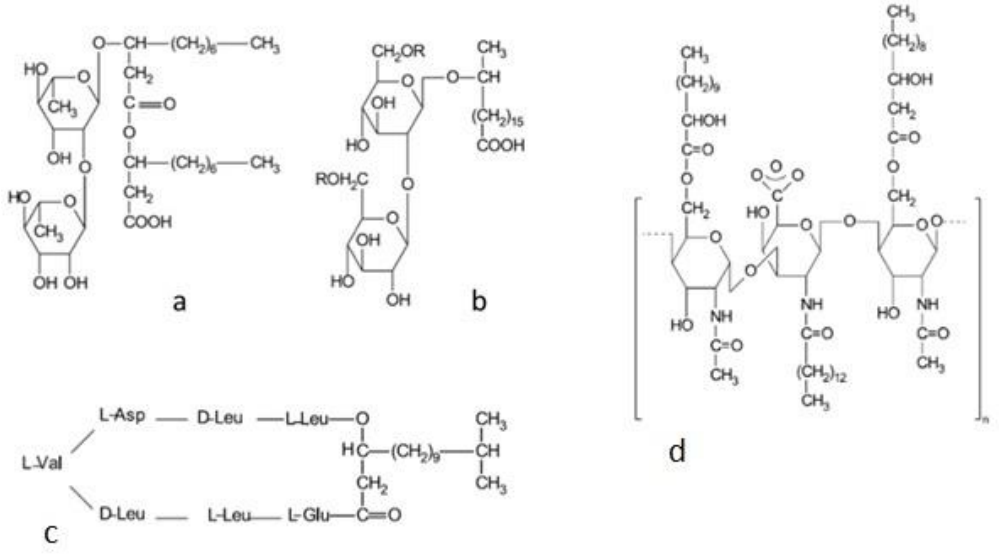
o isolamento e a clonagem dos genes responsáveis por esse tensoativo podem ser empregados na sua produção fermentativa.

Biosurfactantes poliméricos: os biossurfactantes poliméricos mais estudados incluem o emulsan, o liposan, o alasan, a lipomanana e outros complexos de polissacarídeo-proteína. O emulsan é um agente emulsificante eficaz de hidrocarbonetos, mesmo a uma concentração muito reduzida, como 0,001-0,01% (HATHA et al., 2007;. LANG, 2002). O liposan é um emulsificante extracelular solúvel em água sintetizado por *C. lipolytica* e é composto por 83% de carboidratos e 17% de proteínas. A aplicação de tais biossurfactantes poliméricos, como o liposan, como emulsificante em indústrias de alimentos e cosméticos foram discutidos por Chakrabarti (2012).

Biosurfactantes particulados: formam vesículas de membrana extracelulares que se particionam para formar uma microemulsão que desempenha um papel importante na absorção de alcano por células microbianas. Vesículas de *Acinetobacter* spp. HO1-N com diâmetro de 20-50 nm são compostas de proteínas, fosfolípidos e lipopolissacarídeo (CHAKRABARTI, 2012; VIJAYAKUMAR; SARAVANAN, 2015).

A estrutura química dos biossurfactantes microbiológicos mais estudados está ilustrada na Figura 4.

Figura 4 – Estrutura química dos compostos surfactantes microbiológicos mais estudados: (a) Raminolipídeo; (b) Soforolipídeo; (c) surfactina e (d) emulsan



3.4. Propriedades

Apesar da diversidade de composição química e de propriedades, algumas características são comuns à maioria dos biossurfactantes. Muitas dessas características representam vantagens sobre os surfactantes convencionais (VIJAYAKUMAR; SARAVANAN, 2015):

Atividade superficial e interfacial: a eficiência e a efetividade são características básicas essenciais que determinam um bom surfactante. A eficiência é medida através da CMC, enquanto que a efetividade está relacionada com as tensões superficiais e interfaciais (BARROS et al., 2007). Logo, os biossurfactantes são mais eficientes efetivos do que os surfactantes convencionais, pois produzem menor tensão superficial a menores concentrações. A CMC dos biossurfactantes (medida de sua eficiência) varia entre 1-2000 mg/L, enquanto que a tensão interfacial (óleo/água) e superficial fica em torno de 1 e 30 mN/m, respectivamente. Os bons surfactantes conseguem reduzir a tensão superficial da água de 72 para 35 mN/m e a tensão interfacial do n-hexadecano de 40 para 1 mN/m (VIJAYAKUMAR; SARAVANAN, 2015).

Tolerância à temperatura, pH e força iônica: muitos biossurfactantes podem ser utilizados sob condições extremas, que incluem elevadas temperaturas e valores de pH entre 2 e 12. Os biossurfactantes também suportam concentrações de até 10% de sal, enquanto que 2% de NaCl são suficientes para inativar os surfactantes sintéticos (VIJAYAKUMAR; SARAVANAN, 2015).

Biodegradabilidade: os biossurfactantes são facilmente degradados por micro-organismos na água e no solo, o que os torna adequados para aplicações na biorremediação e no tratamento de resíduos (VIJAYAKUMAR; SARAVANAN, 2015).

Toxicidade reduzida: os biossurfactantes têm recebido maior atenção devido à crescente preocupação da população com os efeitos alérgicos dos produtos artificiais; além disso, sua baixa toxicidade permite o uso em alimentos, em cosméticos e em produtos farmacêuticos. A ausência de toxicidade também é de fundamental importância para aplicações na área ambiental (VIJAYAKUMAR; SARAVANAN, 2015).

Disponibilidade: biossurfactantes podem ser produzidos a partir de matérias-primas largamente disponíveis, além da possibilidade de serem produzidos a partir de resíduos industriais (VIJAYAKUMAR; SARAVANAN, 2015).

Especificidade: biossurfactantes sendo moléculas orgânicas complexas com grupos funcionais específicos são muitas vezes específicos em sua ação. Isto seria de particular interesse na desintoxicação de poluentes específicos, na desmulsificação de emulsões industriais, em aplicações cosméticas, farmacêuticas e alimentares (VIJAYAKUMAR; SARAVANAN, 2015).

Biocompatibilidade e digestibilidade: o que garante a aplicação dessas biomoléculas nos mais diversos setores industriais, destacando as indústrias alimentícia, farmacêutica e cosmética (VIJAYAKUMAR; SARAVANAN, 2015).

Formação e quebra de emulsões: os biossurfactantes podem atuar como emulsificantes ou desemulsificantes. Uma emulsão pode ser descrita como um sistema heterogêneo, que consiste de um líquido imiscível disperso num outro, sob a forma de gotículas, cujo diâmetro, em geral, excede 0,1 mm. As emulsões são geralmente de dois tipos: óleo-em-água (O/W) ou água-em-óleo (W/O). Elas podem ser estabilizadas por meio de aditivos, tais como biossurfactantes, os quais permitem que elas possam ser mantidas como emulsões estáveis durante meses ou anos (VELIKONJA; KOSARIC, 1993). O liposan é um emulsificante solúvel em água sintetizado por *C. lipolytica*, que tem sido utilizado para emulsificar óleos comestíveis, formando emulsões estáveis. Estes liposans foram usados em cosméticos e indústrias de alimentos para fazer emulsões estáveis (CAMPOS et al., 2013;. CIRIGLIANO; CARMAN, 1985).

Apesar das inúmeras vantagens, há algumas deméritos associados aos biossurfactantes, como se segue (RAHMAN; GAKPE, 2008):

• A produção em larga escala pode ser cara. No entanto, este problema pode ser superado através da utilização de matérias-primas mais econômicas;

• A obtenção de biossurfactantes puros é difícil, uma vez que são necessários vários passos consecutivos do processo, como será discutido a seguir;

• Estirpes de bactérias superprodutoras são raras e as encontradas geralmente apresentam uma baixa produtividade. Além disso, meios complexos precisam ser aplicados a essas amostras;

• A regulação da síntese de biossurfactante é pouco compreendida, como será discutido a seguir;

• A melhoria do rendimento de produção é dificultada pela forte formação de espuma. Consequentemente, meios diluídos tem que ser aplicados ou agentes anti-espumantes devem ser incluídos no processo.

3.5. Fatores que afetam a produção de biossurfactantes

A produção de biossurfactantes pode ser espontânea ou induzida pela presença de compostos lipofílicos, por variações de pH, temperatura, aeração e velocidade de agitação, ou ainda, quando o crescimento celular é mantido sob condições de estresse, como baixas concentrações da fonte de nitrogênio (DESAI; BANAT, 1997). Os vários fatores físico-químicos são discutidos a seguir (BHARDWAJ et al., 2013):

Fonte de carbono: a fonte de carbono desempenha um importante papel não só no crescimento microbiano, como também na produção de biossurfactantes por vários micro-organismos. Quando apenas a glicose ou óleo vegetal foram utilizados individualmente para a produção de biossurfactante por *C. bombicola*, rendimentos muito reduzidos foram obtidos, mas quando ambas as fontes de carbono foram usadas em conjunto, o rendimento aumentou para 70 g/L (COOPER; PADDOCK, 1984). Quando concentrações de 80 e 40 g/L de glicose e óleo de soja respectivamente, foram usadas, atingiu-se o rendimento máximo de soforolipídeos pela *C. bombicola* (KIM et al., 1997); rendimentos ainda mais elevados, de 120 g/L de soforolípidos foram obtidos por *C. bombicola* quando o açúcar e o óleo foram usados em conjunto (CASAS et al., 1997). Quando o óleo de canola e a glicose foram usados como as fontes de carbono por *C. lipolytica* obteve-se 8 g/L de soforolípidos (SARUBBO et al., 2007). Embora a maior produção de bioemulsificante tenha sido observada com *C. lipolytica* em meio contendo 1,5% de glicose (SARUBBO et al., 2001), *C. antarctica* e *C. Apicola* produziram 13,4 e 7,3 g/L de soforolípidos, respectivamente, com a suplementação do meio com um resíduo da indústria de sabão a 5% de concentração (BEDNARSKI et al., 2004). As células de *Pseudozyma*

(*C. antarctica*) na fase estacionária foram capazes de converter n-alcanos de C12 a C18 em lipídeos. O rendimento de MEL foi de 140 g/L ao fim de 4 semanas (KITAMOTO et al., 2001).

Fonte de nitrogênio: é o segundo nutriente mais importante para a produção de biossurfactantes por micro-organismos. Em muitos processos de fermentação, a razão C/N é um parâmetro extremamente sensível, afetando, a quantidade de metabolitos produzidos. Uma alta relação de C/N, isto é, níveis reduzidos de nitrogênio, limitam o crescimento de bactérias e direcionam o metabolismo celular para a produção de metabólitos. Em contrapartida, um excesso da fonte de nitrogênio direciona o substrato para a síntese de material celular, limitando relativamente o acúmulo de produtos (ROBERT et al., 1989). Várias fontes orgânicas e inorgânicas de nitrogênio têm sido utilizadas na produção de biossurfactantes. Santa Anna et al. (2002) estudaram o efeito da fonte de nitrogênio na produção de um biossurfactante por *P. aeruginosa* PA1 cultivada em meio mineral contendo 3% de glicerol. Nesse trabalho, o $NaNO_3$ foi mais eficaz do que o $(NH_4)_2SO_4$, observando-se que as limitações nutricionais direcionaram o metabolismo para a formação de biossurfactante. De acordo com Mulligan e Gibbs (1989), *P. aeruginosa* utiliza amônia, nitratos e aminoácidos como fontes de nitrogênio. A amônia pode ser assimilada pela glutamato desidrogenase (EC 1.4.1.4) para formar glutamato ou, com o glutamato, pela ação da glutamina sintetase (EC 6.3.1.2.) para formar glutamina. A glutamina e o α–cetoglutarato são então convertidos em glutamato pela L-glutamina 2-oxiglutarato aminotransferase (EC 1.4.1.13). Os nitratos, por sua vez, devem ser reduzidos a nitrito e, em seguida, a amônia. Consequentemente, em comparação com a amônia, a assimilação de nitrato é mais lenta, simulando, assim, uma condição limitante de nitrogênio, que é favorável à produção do raminolipídeo. Por outro lado, durante a síntese de um raminolipídeo, a formação do lipídeo é a etapa determinante, e a limitação de nitrogênio pode promover o acúmulo de lipídeo. Os rendimentos mais elevados de soforolipídeos são observados utilizando-se extrato de levedura e ureia como fontes de nitrogênio (CASAS et al., 1997; DESHPANDE; DANIELS, 1995), Embora, os rendimentos mais elevados de MEL por *Candida* sp. SY16, *C. lipolytica* e *C. glabrata* UCP 1002 tenham sido observados com nitrato de amônio e extrato de levedura (KIM et al., 1999;. RUFINO et al., 2007; 2008; SARUBBO et al., 2006; 2007).

Condições de crescimento: as condições de crescimento, tais como pH, temperatura, agitação e disponibilidade de oxigênio também afetam a produção de biossurfactantes em função de seus efeitos sobre o crescimento ou atividade celular (DESAI; BANAT, 1997). Espécies de *Candida* produzem rendimentos máximos de biossurfactantes numa ampla faixa de pH, como pode ser visto com *C. glabrata* UCP 1002, que produziu quantidade máxima de biossurfactante em pH 5,7, com *Candida* sp. SY16 a pH 7,8, *C. lipolytica* a pH 5,0 e *C. batistae* a pH 6,0 (CIRIGLIANO; CARMAN, 1984;. KIM et al., 1999;. KONISHI, et al, 2008;. SARUBBO et al., 2006), enquanto que *Aspergillus ustus* e *Pichia anamola* produziram máximo rendimento em biossurfactante em pH 7,0 e 5,5, respectivamente (KIRAN et al., 2009; THANIYAVARN et al., 2008). Vários processos microbianos também são dependentes da temperatura. A temperatura mais favorável para a produção de biossurfactantes por vários fungos está em torno de 30 °C, como observado em várias espécies de *Candida, como Candida* sp. SY16, *C. bombicola* e *T. batistae* (COOPER; PADDOCK, 1984; DESHPANDE; DANIELS, 1995; KIM et al., 1999; KONISHI et al., 2008). O tempo de incubação também tem um efeito significativo sobre a produção de biossurfactantes. Diferentes micro-organismos são capazes de produzir biossurfactantes em diferentes intervalos de tempo. A máxima produção de biossurfactante por *Aspergillus ustus* MSF3 foi observada após 5 dias de incubação, enquanto que para a *C. bombicola*, os melhores períodos de incubação foram observados após 7, 8 e 11 dias (CASAS et al., 1997;. CAVALERO et al, 2003;. FELSE et al., 2007). Por outro lado, observou-se máxima produção de biossurfactante por *C. bombicola* usando gordura animal após 68 h de incubação (DESHPANDE; DANIELS, 1995). Um aumento na agitação favoreceu o acúmulo do biossurfactante de *P. aeruginosa* UCP0992 cultivada em glicerol (SILVA et al., 2010). A variação da velocidade de agitação entre 50 e 200 rpm foi estudado por OLIVEIRA et al. (2009) para a *P. alcaligenes* cultivada em óleo de palma. Os autores observaram que o aumento da velocidade de rotação favoreceu a redução da tensão superficial do líquido metabólico livre de células para 27,6 mN/m. Já Cunha et al. (2004) observaram que a agitação teve um efeito negativo sobre a redução da tensão superficial do biossurfactante produzido por *Serratia* sp. SVGG16 cultivada em hidrocarbonetos.

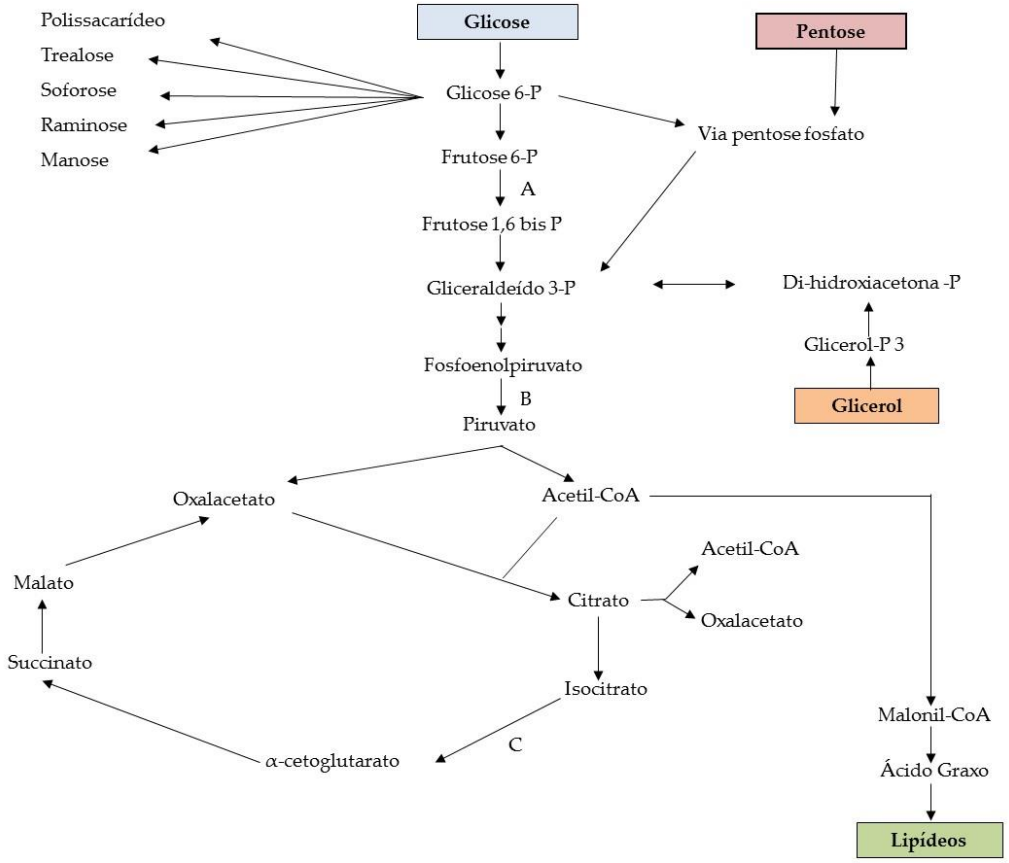
3.6. Vias metabólicas da produção de biossurfactantes

Os substratos hidrofílicos são utilizados primeiramente pelo micro-organismo para o metabolismo celular e para a síntese da porção polar da molécula de

biossurfactante, enquanto que os substratos hidrofóbicos são utilizados exclusivamente para a produção da porção hidrocarbônica do biossurfactante (DESAI; BANAT, 1997; WEBER et al., 1992).

As vias metabólicas envolvidas na síntese de precursores para produção de biossurfactante são diversas e dependem da natureza da principal fonte de carbono utilizada no meio de cultivo. Por exemplo, quando se utilizam carboidratos como única fonte de carbono no meio de cultivo para a produção de glicolipídeo, o fluxo de carbono é regulado de tal forma que ambas as vias lipogênicas (formação de lipídeos) e de formação da porção hidrofílica (através da via glicolítica) são especialmente supridas pelo metabolismo microbiano, como mostra a Figura 5 (HARITASH; KAUSHIK, 2009).

Figura 5 - Metabolismo intermediário relacionado à síntese de precursores de biossurfactantes a partir da utilização de carboidratos como substratos. Enzimas chaves para o controle do fluxo de carbono: a) fosfofrutoquinase; b) piruvato quinase; c) isocitrato desidrogenase

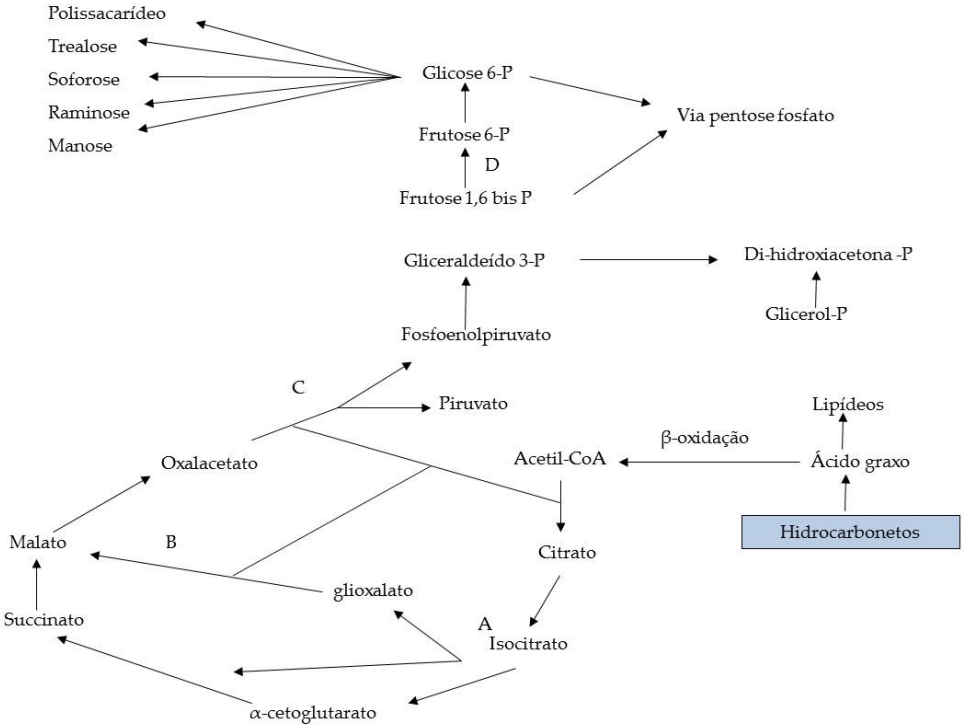


Fonte**:** SYLDATIK et al., 1987

O substrato hidrofílico utilizado, como por exemplo, glicose ou glicerol, é degradado até formar intermediários da via glicolítica, como a glicose 6-fosfato, que é um dos principais precursores dos carboidratos presentes na porção hidrofílica do biossurfactante. Para a produção de lipídeos, a glicose é oxidada a piruvato por meio da glicólise, sendo o piruvato então convertido a acetil-CoA, que unida ao oxalacetato produz malonil-CoA e, em seguida, ácido graxo, um dos precursores para a síntese de lipídeos (HOMMEL; HUSE, 1993).

Por outro lado, quando um hidrocarboneto é utilizado como fonte de carbono, o metabolismo microbiano se dirige principalmente à via lipolítica e à gliconeogênese (formação de glicose a partir de precursores diferentes das hexoses) podendo, desta forma, ser utilizado para produzir ácidos graxos ou sacarídeos. Para a produção dos sacarídeos, a via da gliconeogênese é ativada. Essa via consiste na oxidação dos ácidos graxos via β-oxidação a acetil-CoA (ou propionil-CoA, no caso de ácidos graxos de cadeia ímpar). A partir da formação do acetil-CoA, as reações envolvidas na síntese dos precursores do polissacarídeo, tal como glicose 6-fosfato, são essencialmente o inverso daquelas envolvidas na glicólise. Entretanto, as reações catalisadas pelo piruvato quinase e fosfofrutoquinase-1 são irreversíveis; desta forma, outras enzimas, as quais são exclusivas para gliconeogênese, são requeridas para contornar tais reações. As principais reações são apresentadas na Figura 6, até a formação da glicose 6-fosfato que é a principal precursora dos polissacarídeos, dissacarídeos a serem formados para produção da porção hidrofílica dos glicolipídeos (TOKUMOTO et al., 2009).

Figura 6 - Metabolismo intermediário relacionado à sintese de precursores de biossurfactantes a partir da utilização de hidrocarbonetos como substratos. As enzimas chaves são: a) isocitrato liase; b) malato sintase; c) fosfoenolpiruvato; d) frutose -1



Fonte**:** SYLDATIK et al., 1987

De acordo com Syldatik e Wagner (1987), a biossíntese de um biossurfactante pode acontecer de acordo com quatro caminhos diferentes: (a) síntese do carboidrato e do lipídeo; (b) síntese da metade de carboidrato, enquanto a síntese da metade lipídica dependerá do comprimento da cadeia do substrato carbônico presente no meio; (c) síntese da metade lipídica, enquanto a síntese da metade de carboidrato dependerá do substrato utilizado e (d) síntese das metades carboidrato e lipídica, dependendo do substrato. Portanto, um fator que altera a biossíntese do surfactante é o comprimento da cadeia do *n*-alcano utilizado como fonte de carbono. Kitamoto et al.(2001) estudaram a produção de manosileritritol lipídeo (MEL) pela levedura *C. antarctica* frente a diferentes *n*-alcanos. Observou-se que essa espécie não cresce nem produz biossurfactante em meio contendo *n*-alcanos de C_{10} a C_{18}. Entretanto, quando cultivada em meio contendo *n*-alcanos de C_{12} a C_{18} houve produção, sendo o octadecano o substrato que promoveu maior rendimento. Já em meio contendo *n*-alcanos com números de carbono maiores que 19, a produção foi mínima.

3.7. Fisiologia

Os biossurfactantes são produzidos por uma grande variedade de micro-organismos, excretados ou aderidos às células, especialmente quando cultivados em substratos insolúveis em água (DESAI; BANAT, 1997; TAN et al., 2000). Embora a função de células microbianas ainda não esteja totalmente compreendida, tem-se especulado o envolvimento dos biossurfactantes na emulsificação de substratos insolúveis. O contato direto entre as gotículas de células e de hidrocarbonetos tem sido descrita juntamente com as interações com as gotículas emulsionadas.

A principal função fisiológica do biossurfactantes reside no fato de que estes compostos permitem os micro-organismos crescerem em substratos insolúveis em água através da redução da tensão superficial entre as fases, tornando o substrato mais disponível para consumo e metabolismo. Os mecanismos moleculares da ingestão desses substratos (por exemplo, alcanos) ainda não foram completamente esclarecidos. A ingestão direta de hidrocarbonetos dissolvidos na fase aquosa tem sido descrita, bom como o contato direto entre as células e as gotículas de hidrocarbonetos e a interação com gotículas emulsionadas (emulsão). Além da emulsicação da fonte de carbono, os biossurfactantes também estão envolvidos na adesão das células microbianas aos hidrocarbonetos. A adsorção de células dos micro-organismos aos substratos insolúveis e a excreção de compostos tensoativos permitem o crescimento nestas fontes de carbono (DESAI; BANAT, 1997).

3.8. Cinética de produção de biossurfactantes

A cinética da produção de biossurfactantes apresenta muitas variações entre os diferentes sistemas. No entanto, por conveniência, os parâmetros cinéticos podem ser agrupados nos seguintes tipos: (a) produção associada ao crescimento, (b) produção sob condições limitantes de crescimento, (c) produção de células na fase estacionária ou imobilizadas, e (d) produção com a suplementação de um precursor (DESAI; BANAT, 1997):

Produção associada ao crescimento: para a produção de biossurfactante associada ao crescimento, existe uma relação paralela entre o crescimento, a utilização do substrato e a produção de biossurfactante.

Produção sob condições que limitam o crescimento: a produção sob condições limitantes de crescimento é caracterizada por um aumento acentuado no nível de biotensoativo como resultado de limitação de um ou mais componentes do meio.

Produção de células na fase estacionária ou imobilizadas: é um tipo de produção de biotensoativo em que não ocorre multiplicação celular. As células, no entanto, continuam a utilizar a fonte de carbono para a síntese de biosurfactantes.

Produção com suplementação de precursores: muitos investigadores relatam que a adição de precursores de biossurfactante adicionados ao meio de crescimento provocam mudanças qualitativas e quantitativas do produto.

3.9. Resíduos disponíveis para a produção de biossurfactantes

A sociedade atual caracteriza-se pelo aumento de despesas, a necessidade de reutilizar materiais e com a preocupação ambiental. Consequentemente vem dando uma ênfase maior a recuperação, reciclagem e reutilização de diversos resíduos.

A necessidade de preservação ambiental leva à reutilização de diversos resíduos industriais. Isso é particularmente válido para os alimentos e as indústrias de produção de alimentos, cujos resíduos, efluentes e co-produtos podem ser reutilizados (BANAT et al., 2014a).

Os resíduos industriais têm despertado o interesse de pesquisadores como substrato de baixo custo para a produção de biossurfactantes (MAKKAR; CAMEOTRA, 2002). A seleção de subprodutos, entretanto, deve garantir o equilíbrio adequado de nutrientes para permitir o crescimento microbiano e a consequente produção de biossurfactante. Os resíduos industriais com elevado teor de hidratos de carbono ou lipídeos é ideal para uso como substrato (BANAT et al., 2014a). De acordo com Barros et al. (2007), a utilização de resíduos agroindustriais é um dos primeiros passos para a viabilidade de implantação da produção de biossurfactantes em escala industrial, diante da qual os estudos sobre as diferentes variáveis envolvidas são necessários para otimizar o processo. A literatura descreve uma série de resíduos de produtos utilizados na produção de biossurfactantes, tais como óleos vegetais, efluentes oleosos (BATISTA et al., 2010; MERCADÉ et al., 1993; SARUBBO et al., 2007), efluentes ricos em amido (FOX; BALA, 2000;. THOMPSON et al., 2000), gordura animal (DESHPANDE; DANIELS, 1995; MANEERAT, 2005; SANTOS et al.,

2013; 2014), gordura vegetal (GUSMÃO et al., 2010), óleo vegetal residual de cozinha (ALCANTARA et. al., 2000;. CVENGROS; CVENGROSOVA, 2004;. Haba et al., 2000; Maneerat, 2005), borra (BENINCASA et al., 2002;. MANEERAT, 2005; SHABTAI, 1990), melaço (GHURYE et ai, 1994 ; KALOGIANNIS et al., 2003;. LAZARIDOU et al., 2002;. MAKKAR; CAMEOTRA, 1997), resíduos da indústria de laticínios (soro de leite) (SUDHAKAR-BABU et al., 1996), milhocina (LUNA et al., 2011a; RUFINO et al., 2007; 2008; 2011b; SOBRINHO et al., 2008), farinha de mandioca de águas residuais (NITSHKE et al., 2004), resíduos de destilaria de petróleo (LUNA et al., 2011a,b; 2013; RUFINO et al., 2007) e glicerol (SILVA et al., 2010).

Alguns dos resíduos industriais mais utilizados na produção de biossurfactantes são comentados em detalhes a seguir:

Efluente do Óleo de Oliva Verde (OOME): a extração do óleo de oliva envolve um consumo intenso de água produzindo grandes quantidades de efluente proveniente do moinho do óleo de oliva, causando efeitos nocivos ao ambiente. O Efluente do Óleo de Oliva Verde (OOME) é um licor preto com um elevado índice de matéria orgânica (20-60 kg COD/m^3), dependendo do procedimento da extração do óleo oliva verde. O OOME contém substâncias tóxicas, tais como polifenol, mas também possui valiosas substâncias orgânicas como açúcares, combinação de nitrogênio com ácidos orgânicos e óleos residuais (MERCADE et al., 1993;).

Gordura animal: A gordura animal ou sebo podem ser obtidos em grandes quantidades nas indústrias de processamento de carne e têm sido usados como meio para cozinhar alimentos. Recentemente, entretanto, estas gorduras têm perdido a maior parte do mercado para os óleos vegetais devido ao menor dano provocado por estes últimos à saúde (BANAT et al., 2014a). A gordura animal tem sido relatada como um estimulador para a produção de soforolipíeos por *C. bombicola* (Deshpande e Daniels, 1995). Recentemente, Santos et al. (2013; 2014) reportaram a produção máxima de um glicolipídeo usando gordura animal e milhocina em comparação com outras fontes de carbono usando a levedura *C. lipolytica* UCP0988.

Óleos de fritura: os resíduos de óleos e gorduras comestíveis são considerados ótimas fonte de carbono para a produção de biossurfactantes, tornando o seu descarte um desperdício de fonte energética contribuindo ainda para a poluição ambiental. Os óleos vegetais são uma fonte de carbono lipídica e são na sua maioria

compostos de ácidos graxos saturados ou insaturados com cadeias carbônicas de 16-18 átomos (MAKKAR; CAMEOTRA, 2011). Vários óleos, juntamente com fontes de carbono solúveis em água, são bons substratos para a produção de surfactantes microbianos. Isto é evidente a partir dos exemplos a seguir. O óleo de babaçu (5%) suplementado com glicose (1%) como fonte de carbono proporciona uma boa fonte para o crescimento e produção do biotensoativo. Este trabalho realizado por Sarubbo et al. (1999) sugeriram que duas estirpes de *C. lipolytica* (1055 e 1120) produziram biossurfactantes no final da fase exponencial de crescimento e no início da fase estacionária de crescimento. O azeite de girassol foi provado como um excelente fonte de carbono e de energia para a produção de tensoativos microbianos. Estirpes mutantes de *P. aeruginosa* EBN-8 produziram um biossurfactante utilizando óleo de canola, de soja, de milho e de refinaria de petróleo (RAZA et al., 2006; 2007). Resíduos de refinaria de óleo de canola suplementado com nitrato de sódio foi relatado para o crescimento microbiano e produção de raminolipídeo com rendimento de 8,50 g/L. A co-utilização de óleo de canola e glicose também tem sido relatada com sucesso para a produção de biossurfactantes por C. lipolytica (SARUBBO et al., 2007).

Borra de sabão ou "Soapstocks": borras oleosas ou "soapstocks" são produtos semi-sólidos ou com consistência de uma goma produzidos a partir de processamento de oleaginosas, onde os produtos químicos são utilizados na extração e refinação de sementes originadas de óleos comestíveis (MANEERAT, 2005). A borra, apesar de ser um substrato complexo, tem demonstrado aplicação na produção de maiores rendimentos de raminolipídeos, juntamente com diferentes substratos oleosos, ou seja, óleo de girassol e óleo de oliva. Rendimentos de até 15,9 g L foram relatados pela *P. aeruginosa* LBI cultivada em meio contendo borra (BENINCASA et al., 2002). A síntese de surfactantes com borra e resíduos de refinaria de petróleo foi demonstrada a partir de *C. antarctica* ou *C. Apicola,* com rendimentos muito mais elevados do que no meio sem adição de resíduos de refinaria de petróleo (BEDNARSKI et al., 2004). Shabtai (1990) também relatou a produção de dois heteropolissacarídeos extracelulares, emulsan e biodispersan, por *A. calcoaceticus* RAG-1 e *A. calcoaceticus* A2, respectivamente, usando borra de sabão como fonte de carbono.

Melaço: melaços são subprodutos do tipo xaropes concentrados de indústrias de cana de açúcar e processamento de beterraba. Este substrato de baixo custo contém 75% de matéria seca, 9-12% de matéria orgânica não glicosídica, 2,5% de proteínas, 1.5-5.0% de potássio e ≈1% de cálcio, magnésio e fósforo. Outros componentes, como biotina, ácido pantotênico, inositol, e tiamina em torno de 1-3% também estão presentes, dando-lhe uma aparência castanho espesso, de cor escura. O elevado teor de açúcar, que varia aproximadamente entre 48 e 56%, indica a viabilidade desse substrato para o crescimento, bem como a produção de compostos surfactantes microbianos por vários micro-organismos. Vários laboratórios de pesquisa estão envolvidos na utilização de melaço para a produção de vários metabolitos microbianos. Makkar e Cameotra (1997) relataram produção de biossurfactante por duas culturas de *Bacillus subtilis* em meio suplementado com melaço como fonte de carbono. Joshi et al. (2007) também usaram melaço e outras fontes de carbono para produzir biossurfactantes a partir de várias estirpes de *Bacillus* sob condições termofílicas.

Soro (laticínios e subprodutos de destilaria): indústrias de laticínios produzem grandes quantidades de soro de leite que incluem, soro de coalho, resíduos de soro de leite e soro de queijo, todos os quais estão facilmente disponíveis como substratos tratados para a produção microbiana de metabólitos (DUBEY et al., 2005; DUBEY; JUWARKAR, 2001; MAKKAR; CAMEOTRA, 2002; RODRIGUES; TEIXEIRA, 2006b). Altas quantidades (cerca de 75%) de lactose estão presentes no soro lático. Outros componentes, como proteínas e ácidos orgânicos, e vitaminas fornecem bons nutrientes para o crescimento microbiano e a produção de biossurfactantes (MANEERAT, 2005). O soro de leite representa um grave problema de poluição, especialmente para os países de forte economia leiteira, de modo a tornar o seu reaproveitmento ainda mais atrativo (HELMY et al., 2011).

Milhocina: a agroindustrialização de produtos à base de milho através de processamento úmido resulta em subprodutos sólidos e líquidos, que dispostos de forma inadequada tornam-se fontes de contaminação e agressão ao meio ambiente. A milhocina é um rejeito da água de lavagem e embebição dos grãos de milho quando do fracionamento em amido e gérmen (óleo), contendo 40% de sólidos. Possui entre 21% a 45% de proteínas, 20% a 26% de ácido lático, cerca de 8% de cinzas (contendo Ca^{2+}, Mg^{2+}, K^{+}, etc.), cerca de 3% de carboidratos e baixo teor de gordura

(0,9% - 1,2 %) (AKHTAR et al., 1997; CARDINAL; HEDRICK, 1948; HELMY et al., 2011). A milhocina é um resíduo de refinaria de petróleo relatado como nutrientes de baixo custo para a produção de um biossurfactante glicolipídico de *C. sphaerica* (UCD 0995). O biossurfactante recuperou cerca de 95% de óleo de motor adsorvido em amostra de areia e apresentou vastas aplicações em processos de biorremediação (LUNA et al, 2011a; 2013; 2015). Silva et al. (2014b) mostraram também a produção de um novo biossurfactante de *P. cepacia* cultivada em meio mineral suplementado com 2,0% de milhocina e 2,0% de óleo de fritura de soja.

Substratos ricos em amido: além dos substratos relativamente de baixo custo acima mencionados, uma série de substratos a base de amido estão abundantemente disponíveis e fornecem mais fontes de carbono renováveis alternativas. Um dos exemplos representativos é a indústria de processamento de batatas, que produz quantidades significativas de resíduos ricos em amido e adequados para a produção de biossurfactantes. Em adição ao conteúdo de água, de aproximadamente 80%, os resíduos de batata também têm hidratos de carbono (17%), proteínas (2%), gorduras (0,1%), vitaminas, minerais e oligoelementos (HELMY et al., 2011). Assim, os resíduos de batata são uma fonte rica de vários componentes que podem suportar o crescimento de micro-organismos para a produção de vários produtos comercialmente importantes. Como exemplo, um amido de batata comercialmente preparado em meio de sais minerais foi investigada por Fox e Bala (2000) para a produção de um biossurfactante por *B. subtilis* ATCC 21332. A mandioca, um outro resíduo rico em hidratos de carbono, que é gerado em grandes quantidades durante a preparação da farinha de mandioca, é também um substrato atraente e tem sido utilizada para a produção de surfactina de *B. subtilis* 35. Vários outros substratos de resíduos ricos em amido, como água de arroz (efluente da indústria de processamento de arroz de cozinha) e águas residuais da transformação de cereais, têm grande potencial para permitir o crescimento microbiano e a produção de biossurfactante (MUTHUSAMY et al., 2008).

3.10. Recuperação de biossurfactantes

A recuperação e concentração dos biossurfactantes a partir do líquido metabólico obtido no processo fermentativo é um dos fatores determinantes do alto custo de produção. As baixas concentrações obtidas e a natureza anfipática dos

surfactantes microbianos limitam sua recuperação. Vários métodos para isolamento de biossurfactantes são utilizados, incluindo centrifugação a altas rotações, ultrafiltração, precipitação com ácidos e sais, extração por solventes e cromatografia por adsorção (HELMY et al., 2011).

Uma grande variedade de solventes orgânicos como metanol, etanol, acetato de etila, pentano, acetona, clorofórmio e diclorometano têm sido utilizados, isoladamente ou combinados, na extração de biossurfactantes. As misturas de clorofórmio e metanol em diferentes proporções têm fornecido os melhores resultados, uma vez que facilitam o ajuste da polaridade do agente extrator ao material a ser extraído. Contudo, o uso de clorofórmio para extrações preparativas que demandam um grande volume de solvente não é economicamente garantido. Além disso, o clorofórmio é um composto clororgânico altamente tóxico considerado nocivo para o ambiente e para a saúde humana. Assim, existe uma necessidade de solventes de baixo custo e de baixa toxicidade para extração adequada para aplicações industriais. Por outro lado, outras técnicas de precipitação dos produtos como a precipitação por sulfato de amônio, centrifugação e adsorção têm sido reportadas, como mostra a Tabela 2.

A recuperação de biossurfactantes depende principalmente da carga iônica, solubilidade em água e localização (intracelular, extracelular ou ligado à célula). Mais pesquisas são necessárias a fim de maximizar e otimizar os processos de recuperação existentes para tornar os biossurfactantes mais competitivos e comercialmente viáveis.

Tabela 2 - Processos de recuperação de biossurfactantes e suas vantagens

Processo		Tipo de biossurfactante	Propriedade do biossurfactante responsável pela separação	Vantagens
Modo batelada	Precipitação ácida	Surfactina	Biossurfactantes se tornam insolúveis em valores baixos de pH	Custo reduzido, eficiente na recuperação de biossurfactantes na forma bruta
	Extração por solvente orgânico	Trealolipídeos Soforolipídeos Liposan	Biossurfactantes são solúveis em solventes orgânicos devido à presença da porção hidrofóbica	Eficiente na recuperação parcial ou total de biossurfactantes na forma bruta Natureza reutilizável
	Precipitação por sulfato de amônio	Emulsan Biodispersan Lipopeptídeos	Exclusão de biossurfactantes poliméricos ou à base de proteínas	Eficiente na recuperação total de alguns tipos de biossurfactantes poliméricos
	Adsorção em carbono ativado	Raminolipídeos Lipopeptídeos Glicolipídeos MEL	Biossurfactantes são adsorvidos em carbon ativado e podem ser dessorvidos usando solventes orgânicos	Biossurfactantes de elevada pureza, baixo custo, reutilizável, recuperação a partir de cultivo contínuo
	Adsorção em resinas de poliestireno	Raminolipídeos Lipopeptídeos Glicolipídeos MEL	Biossurfactantes são adsorvidos em resinas de poliestireno e subsequentemente dessorvidos usando solventes orgânicos	Biossurfactantes de elevada pureza, baixo custo, reutilizável, recuperação a partir de cultivo contínuo
Modo contínuo	Centrifugação	Glicolipídeos	Biossurfactantes insolúveis são precipitados devido à força centrífuga	Reutilizável, eficiente na recuperação de biossurfactante bruto
	Cromatografia de troca iônica	Glicolipídeos	Biossurfactantes são fixados a resinas de troca iônica e podem ser eluídos com tampões apropriados	Elevada pureza, reutilizável, rápida recuperação
	Fracionamento de espuma	Surfactina	Biossurfactantes se particionam na espuma	Recuperação a partir de cultivo contínuo, elevada pureza do produto, rápido, um único passo
	Ultrafiltração	Glicolipídeos	Biossurfactantes formam micelas acima de suas CMCs, as quais são retidas em membranas poliméricas	Alto grau de pureza, reutilizável

3.11. Aplicações industriais dos biossurfactantes

Os biossurfactantes têm uma ampla gama de aplicações biotecnológicas em petróleo, alimentos, bebidas, cosméticos, detergentes, têxteis, tintas, mineração, celulose, indústrias farmacêuticas e em novas aplicações, como a nanotecnologia (RODRIGUES; TEIXEIRA, 2006a).

Atualmente, o mercado principal para os biossurfactantes é a indústria do petróleo, onde esses compostos podem ser utilizados na limpeza de derramamento de óleos, na remoção de resíduos de óleo de armazenamento de tanques, na recuperação de petróleo e na biorremediação de solos e da água (SILVA et al., 2014a; SOBRINHO et al., 2013).

A Tabela 3 resume os campos de utilização dos biossurfactantes entre os diversos setores industriais, enquanto as principais aplicações biotecnológicas serão apresentadas em mais detalhes a seguir.

Tabela 3 – Aplicações dos biossurfactantes para usos industriais

Indústria	Aplicação	Função dos biossurfactantes	Referências
Meio Ambiente	Biorremediação Operações de limpezas de derramamentos de óleos Remediação de solos e lavagem	Emulsificação de óleos, redução da tensão interfacial, dispersão de óleos, solubilização de óleos, umidificação, detergência, espalhamento, formação de espumas, inibição de corrosão de óleos combustíveis em equipamentos, lavagem de solos.	SILVA et al. (2014a); PACWA-PLOCINICZAC et al. (2011)
Petróleo	Recuperação avançada de óleo Desemulsificação	Emulsificação de óleos, redução da tensão interfacial desemulsificação de emulsões oleosas, solubilização de óleos, redução de viscosidade, dispersão de óleos, umidificação de superfícies sólidas, dissolução de óleos, espalhamento, formação de espumas, inibição de corrosão de óleos combustíveis em equipamentos.	SOUZA et al. (2014); PACWA-PLOCINICZAC et al. (2011)
Mineração	Operação de remoção de metais pesados Remediação de solos Flotação	Umidificação e formação de espumas, coletores e espumantes, remoção de íons metálicos de soluções aquosas, solos e sedimentos, sequestradores de metais pesados, dispersão, inibição de corrosão de óleos	SARUBBO et al. (2015)
Alimentos	Emulsificação e desemulsificação Ingrediente funcional	Solubilização de odores de óleos, controle de consistência, emulsificação, agente umectante, dispersante, detergente, espumante, espessante.	CAMPOS et al. (2013)
Medicina	Microbiologia Farmácia and Terapêutica	Agentes antiadesivos, agentes antifúngicos, agentes antibacterianos, agentes antivirais, vacinas, moléculas imunomodulatórias.	MNIF; GHRIBI, (2015); BANAT et al. (2014b); CORTÉS-SÁNCHEZ et al. (2013)
Agricultura	Biocontrole Fertilizantes	Agentes umectantes, dispersão, suspensão de pesticidas e fertilizantes em pó, emulsificação de soluções de pesticidas, facilitação dos mecanismos de controle biológico de micróbios, eliminação de patógenos de plantas e para aumentar a	SACHDEV; CAMEOTRA (2013)

		biodisponibilidade de nutrientes para a planta associada a micróbios benéficos.	
Cosméticos	Saúde e produtos de beleza	Agentes de emulsificação, agentes espumantes, de solubilização, umectante, limpadores, agentes antimicrobianos, mediadores da ação de enzimas.	VIJAYAKUMAR; SARAVANAN (2015)
Limpeza industrial	Detergentes	Detergentes e sanitizantes para levanderias, agentes umectantes e de dispersão, inibidores de corrosão.	VIJAYAKUMAR; SARAVANAN (2015) BANAT *et al*. (2010)
Têxtil	Preparação de fibras Tingimento e impressão Acabamento de tecidos	Umidificação, penetração, solubilização, emulsificação, detergência e dispersão, molhabilidade e emulsificação no acabamento de formulações, amaciante.	HELMY et al. (2011); BANAT et al. (2010)
Nanotecnologia	Síntese de nanopartículas	Emulsificação, estabilização.	MULLIGAN (2009); VIJAYAKUMAR; SARAVANAN (2015)

3.11.1. Aplicações ambientais

Biorremediação: os derramamentos de petróleo ocorrem durante o transporte de carga ou na forma de derrames industriais de resíduos de óleo e derivados de petróleo. O petróleo é um hidrocarboneto hidrofóbico com efeitos negativos nas membranas celulares dos organismos vivos, oferecendo considerável risco de contaminação em ambos os ecossistemas marinhos e terrestres (SILVA et al. 2014a; SOUZA et al. 2014).

A Agência de Proteção Ambiental dos Estados Unidos (EPA, 2001) propõe diferentes tecnologias físicas, químicas e biológicas, para o tratamento de solos contaminados. Um dos métodos mais estudados é a biorremediação, que utiliza a capacidade de degradação natural de plantas e micro-organismos para converter parcialmente contaminantes em compostos menos tóxicos ou completamente converter tais substâncias em dióxido de carbono e água.

Quanto maior a população de micro-organismos degradadores, mais rápido e mais eficiente será o processo de biorremediação. Portanto, essa técnica pode ser efetuada por bioestimulação, que consiste na estimulação do crescimento de micro-organismos presentes no local contaminado. Este processo pode ser realizado através da introdução de oxigênio, nutrientes e receptores específicos de elétrons para a degradação do contaminante e substâncias, a fim de corrigir o pH. O estímulo também pode ser realizado através da bioaumentação, na qual os micro-organismos autóctones são adicionados ao ambiente contaminado para acelerar e completar a degradação do poluente (SOUZA et al., 2014).

A biorremediação teve importante papel na limpeza do derramamento de 41 milhões de litros de petróleo causado pelo navio Exxon Valdez, no Golfo do Alasca, em 1989, dando início ao desenvolvimento dessa tecnologia, demonstrando que há boas razões para se acreditar na aplicação efetiva deste método no tratamento de futuros derramamentos de óleo em circunstâncias apropriadas (SOUZA et al., 2014).

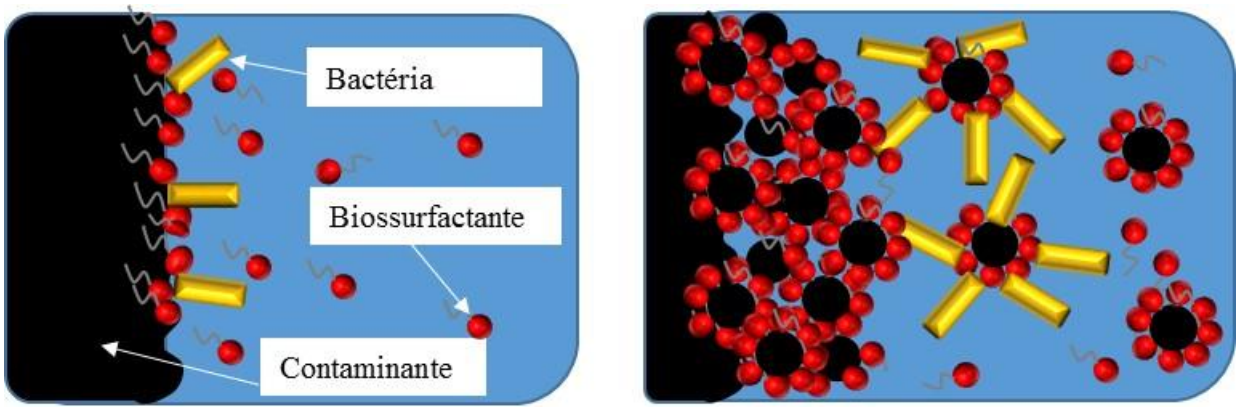
No citado acidente com o Exxon Valdez, a primeira medida tomada foi a lavagem física com jatos de água a alta pressão. Subsequentemente, surfactantes químicos foram aplicados nas áreas poluídas para acelerar a atividade dos micro-organismos degradadores de petróleo. Duas ou três semanas depois, as regiões tratadas com os surfactantes estavam significantemente mais limpas do que as áreas controle. Contudo, foi difícil avaliar os efeitos de tratamento devido à heterogeneidade da contaminação. De qualquer forma, outros estudos demonstraram a importância do

uso de surfactantes para aumentar a biodegradação do petróleo (SAPTURE et al., 2010; SOUZA et al., 2014).

Enquanto a biorremediação é um método eficaz e ambientalmente amigável, o tempo e os custos envolvidos tornam este processo inviável para o tratamento de grandes quantidades de resíduos (SHAVANDI et al., 2011). Assim, a utilização de biossurfactantes, surge como uma alternativa segura para melhorar a solubilidade dos compostos hidrofóbicos, permitindo a dessorção e solubilização de hidrocarbonetos e facilitando a assimilação destes compostos por células microbianas (KUYUKINA et al., 2005).

A biodegradação de hidrocarbonetos derivados do petróleo por biossurfactantes ocorre por dois mecanismos. O primeiro inclui o aumento da disponibilidade biológica do substrato hidrofóbico para os micro-organismos, com consequente redução da tensão superficial do meio em torno da bactéria e redução da tensão interfacial entre as moléculas da parede celular bacteriana e dos hidrocarbonetos. O outro mecanismo envolve a interação entre o biotensoativo e da superfície celular, promovendo modificações na membrana, o que facilita a adesão do hidrocarboneto (aumento de hidrofobicidade) e a redução do índice de lipopolissacárido da parede celular, sem danificar a membrana. Assim, os biossurfactantes bloqueiam a formação de pontes de hidrogênio e permitem as interações hidrofóbicas-hidrofílicas, as quais causam um rearranjo molecular e reduzem a tensão superficial do líquido, aumentando a sua área superficial e promovendo a biodisponibilidade e consequente biodegradabilidade (APARNA et al., 2011). A Figura 7 ilustra a ação de biossurfactantes no aumento da área superficial das gotas de óleo, facilitando o acesso de um maior número de bactérias, que consequentemente produzirão maior biomassa.

Figura 7 - Imagem ilustrativa da ação de biossurfactantes sobre o petróleo



Fonte: http://www.natureswaygreen.com/bioremediation.htm

Recuperação de petróleo: o petróleo continua a ser uma fonte essencial de energia e um motor de desenvolvimento econômico. De acordo com o Departamento de Energia dos Estados Unidos, 83% de todas as fontes de energia primária consumida em os EUA vem de combustíveis fósseis, com o petróleo que compreende 57% dos combustíveis fósseis consumidos. Em 2010, 19,2 milhões de m^3 de petróleo foram consumidos por dia (US ENERGY INFORMATION ADMINISTRATION, 2010).

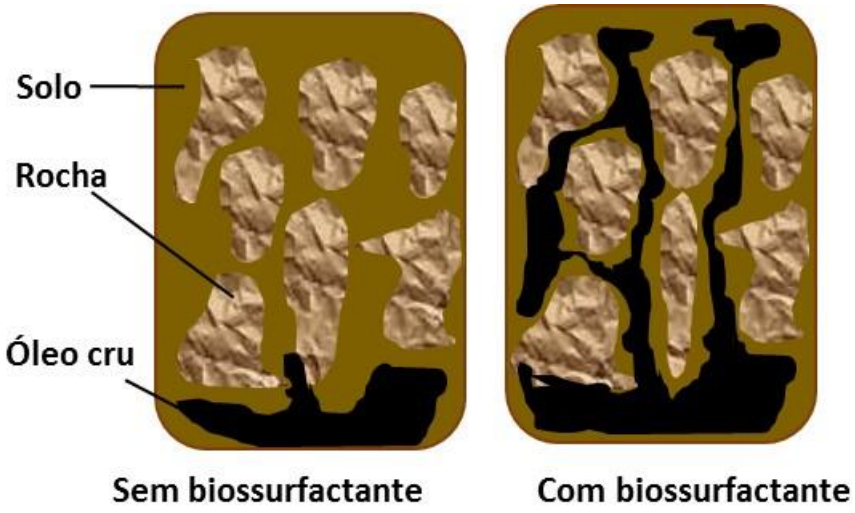
Os EUA produzem 0.870.000 m^3 de petróleo bruto por dia a partir de 530 mil poços de produção (US ENERGY INFORMATION ADMINISTRATION, 2010), 35% do que produzem 0,16 m^3/dia e 79% produzem <1,59 m^3/dia (SUMMERS, 2011). Estes poços de petróleo produzem apenas entre um terço e a metade do petróleo originalmente presente no local através de estágios de recuperação de petróleo. O óleo residual presente em pequenos poros dentro dos reservatórios compreende 50-65% de óleo (US DOE, 2012) e é aprisionado por altas forças capilares e tensão interfacial entre o hidrocarboneto e as fases aquosas. Várias ordens de magnitude na redução da tensão interfacial são necessárias para a mobilização desse hidrocarboneto (AUSTAD; TAUGBØL, 1995; WEST; HARWELL, 1992), o que é apenas conseguida com o uso de concentrações de surfactantes significativamente superior ao necessário para formar micelas (SABATINI et al., 1999, 2000). Na Enhanced Oil Recovery (EOR), por exemplo, o uso de calor, de agentes tensoativos, de processos microbianos e a injeção de gás pode recuperar uma porção significativa deste óleo retido. Os altos custos dos tensoativos químicos, entretanto, têm impedido o uso generalizado de surfactantes para a EOR.

Nesse sentido, os biossurfactantes têm sido aplicados na redução da tensão interfacial entre óleo/água e óleo/rocha. Isto reduz as forças capilares impedindo que o óleo se mova através dos poros de rocha (Figura 8). Os biossurfactantes também podem ligar-se fortemente na interface óleo-água formando a emulsão. Isso estabiliza o óleo dessorvido na água e permite a remoção do mesmo, juntamente com a água de injeção (MAO et al., 2015;. PACWA-PŁOCINICZAK et al., 2011).

Lavagem dos solos por biossurfactantes: a aplicação de biossurfactantes para a remoção de contaminantes de solos é menos conhecida do que a aplicação destes compostos em biorremediação, uma vez que a eficácia de remoção é conduzida principalmente pelas propriedades físico-químicas do biotensoativo e não através dos seus efeitos sobre a atividade metabólica ou alterações nas propriedades de superfície da célula. No entanto, os mecanismos que afetam a mobilização e a

solubilização de hidrocarbonetos no solo são semelhantes aos envolvidos no aumento de biodisponibilidade para a biorremediação (BANAT et al., 2010; FRANZETTI et al., 2009; MULLIGAN, 2009).

Figura 8 – Mecanismo de recuperação avançada de óleo por biossurfactantes



Fonte: PACWA-PLOCINICZAK et al., 2011

Os biossurfactantes melhoram a biodegradação e a remoção de hidrocarbonetos através da mobilização, solubilização ou emulsificação (PACWA-PŁOCINICZAK et al., 2011). A capacidade de solubilização depende da capacidade do tensoativo em aumentar a solubilidade dos componentes hidrofóbicos na fase aquosa. Um considerável aumento da capacidade ocorre acima da CMC, o que é atribuído à distribuição do hidrocarboneto no coração hidrofóbico das micelas. Neste processo, maiores concentrações de surfactantes são normalmente necessárias, uma vez que a solubilidade dos hidrocarbonetos na solução depende inteiramente da concentração de tensoativo (PACWA-PŁOCINICZAK et al., 2011).

A mobilização ocorre a concentrações abaixo da CMC e é dividida em deslocamento e dispersão. O deslocamento consiste na liberação das gotículas de hidrocarbonetos do meio poroso devido à redução na tensão interfacial. Usando uma explicação teórica, os hidrocarbonetos são removidos se a tensão interfacial entre as fases aquosa e oleosa for suficientemente reduzida para vencer as forças de capilaridade que causam a formação de saturação residual. A dispersão é o processo pelo qual um hidrocarboneto é disperso na fase aquosa, como emulsões minúsculas. As emulsões não são geralmente termodinamicamente estável, mas podem permanecer estáveis por longos períodos de tempo devido a restrições cinéticas. A

dispersão está relacionada com a tensão interfacial e a concentração de surfactante e difere do deslocamento, pois o processo de deslocamento está relacionado apenas com a tensão interfacial entre as fases aquosa e hidrofóbica, sem a formação de emulsões (BAI et al., 1997).

A eficácia de um surfactante na remoção de compostos hidrofóbicos também depende do pH e da força iônica da solução, que podem alterar a disposição dos agregados micelares e a sorção do agente tensoativo no solo, o que, por sua vez, limita o transporte do hidrocarboneto pelo surfactante.

Os biossurfactantes tem sido testados na remoção de petroderivados em solos e águas contaminados. Os Raminolipídeos têm sido aplicados com sucesso em processos biotecnológicos de descontaminação (ABDEL-MAWGOUD et al., 2010; SILVA et al., 2010; ROSA et al, 2015). Outros biossurfactantes produzidos por espécies de *Pseudomonas* (SILVA et al. 2013; 2014b), *Bacillus* (VIJAYAKUMAR; SARAVANANE 2015) e *Candida* (LUNA et al., 2015; SOBRINHO et al., 2013; RUFINO et al. 2011b; 2013; 2014) também têm sido aplicados com sucesso na remediação de solos.

Aplicação de biossurfactantes na limpeza de reservatórios de petróleo: a remoção do resíduos de óleo pesado requer a lavagem com solventes ou mesmo lavagem manual; esses processos são perigosos, lentos e caros, uma vez que os óleos pesados que se depositam no fundo dos tanques de armazenagem são muito viscosos e não podem ser removidos por bombeamento convencional. O uso de biossurfactantes neste processo de limpeza reduz a viscosidade do produto e promove a formação de emulsões de óleo/água para facilitar o bombeamento do resíduo e a recuperação do petróleo bruto na sequência da quebra da emulsão (MULLIGAN; WANG, 2004; SINGH et al., 2007). Mulligan (2009) relatou que o uso de biossurfactantes em substituição aos tensoativos convencionais na limpeza de tanques permitiu a limpeza e recuperação de 90% dos hidrocarbonetos.

Remoção de metais pesados: os contaminantes persistentes em solos incluem metais pesados e radionuclídeos. O aumento dos níveis de metais pesados no solo foram reportados em muitos países industrializados. Os metais e seus metalóides como cromo, cádmio, mercúrio e chumbo, podem afetar o ecossistema e a saúde humana através das cadeias alimentares por exposição direta aos solo/água contaminados (CHAKRABORTY; DAS, 2014; MAO et al., 2015).

Tendo em conta que algumas tecnologias podem ser utilizadas em conjunto no tratamento de poluentes orgânicos e metais pesados, os biossurfatantes, dependendo das suas propriedades, podem ser aplicados não só na remoção de compostos orgânicos hidrofóbicos, bem como na remoção de metais pesados (HAZRA et al., 2012).

Os metais pesados se adsorvem principalmente na superfície do solo sob a forma de íons ou precipitam na forma de compostos metálicos. Diferente dos contaminantes orgânicos, os metais pesados são removidos do solo principalmente através de sua complexação associada a um tensoativo (OCHOA-LOŽA et al., 2001) ou por troca iônica (SWARNKAR et al., 2012). Portanto, a lavagem melhorada por surfactantes ou a bioextração reforçada por surfactante podem ser aplicadas para a recuperação de solos contaminados por metais pesados.

Os surfactantes em soluções facilitam a solubilização, a dispersão e a dessorção dos contaminantes, além que permitem a reutilização do solo (HASHIM et al., 2011). Vários agentes tensoativos sintéticos já foram avaliados em testes de descontaminação (ASÇI et al., 2008;. NASH et al., 1987). No entanto, a necessidade de substituir compostos sintéticos por surfactantes naturais levou à investigação sobre o uso de biossurfactantes (Mulligan, 2009). Nesse contexto, vários estudos demonstraram o potencial da surfactina, de raminolipídeos e de soforolipídeos (HERMAN et al., 1995; MULLIGAN et al., 1999; OCHOA-LOŽA et al., 2007). A natureza iônica, a biodegradabilidade, o baixo grau de toxicidade e as excelentes propriedades superficiais fazem dos biossurfactantes excelentes candidatos à remoção de metais pesados em solos e sedimentos. De acordo com Mulligan (2009), a remoção é possível em diferentes concentrações de biossurfactantes. Das et al. (2009) relatou que a eliminação de cádmio utilizando uma solução aquosa também ocorreu em concentrações abaixo da CMC, enquanto uma concentração cinco vezes maior do que a CMC resultou na remoção quase completa de 100 ppm de íons metálicos. Wen et al. (2009) estudaram a degradação de um raminolipídeo em solos contaminados por cádmio e zinco e descobriu que este composto pode permanecer no solo por tempo suficiente para aumentar a fitoextracção do metal.

A remoção dos metais por biossurfactantes ocorre na seguinte sequência (Figura 9): 1) dessorção do biotensoativo à superfície do solo e a complexação com o metal; 2) separação do metal do solo para a solução; e 3) associação com as micelas do biotensoativo. Os metais pesados são aprisionados dentro das micelas através de

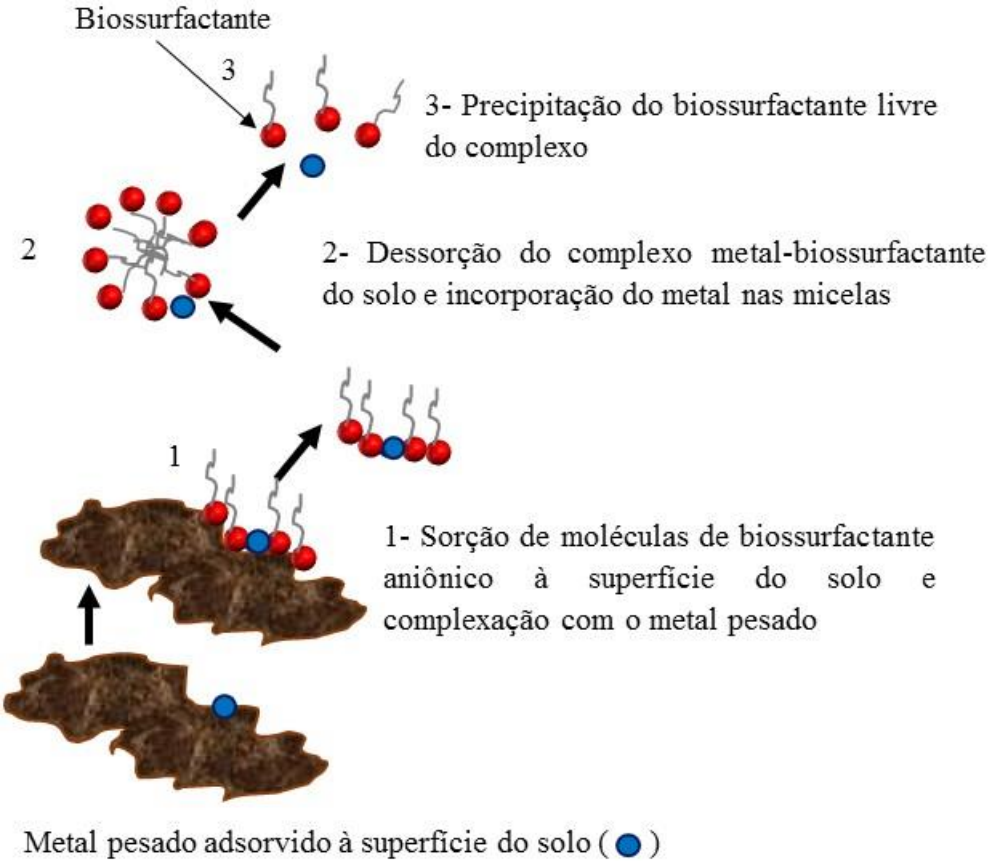
interações eletrostáticas e podem ser facilmente recuperados através de métodos de precipitação ou separação por membranas (KITAMOTO et al., 2002).

Os biossurfactantes aniônicos podem criar complexos não-iônicos com o metal através de ligações iônicas. Tais ligações são mais fortes do que aquelas entre o metal e o solo e o complexo de metal-biotensoativo é dessorvido da matriz do solo, devido à redução na tensão interfacial. Biossurfactantes catiônicos podem substituir íons metálicos através da concorrência pelas superfícies carregadas negativamente (troca de íons). Os íons metálicos podem também ser removidos da superfície do solo por micelas surfactantes.

Os biossurfactantes oferecem vantagens indiscutíveis, uma vez que os micro-organismos capazes de produzir compostos tensoativos não precisam sobreviver no solo contaminado por um metal pesado, embora a adição contínua de biossurfactante seja necessária no processo (PACKWA-PLOCINIKZAC et al., 2011). Biossurfactantes também são aplicados na indústria de mineração. Compostos tensoativos produzidos por *Pseudomonas* sp. e *Alcaligenes* sp. são utilizados para a separação de calcita e schelita, com as taxas de recuperação de 95% e 30% de $CaWO_4$ para $CaCO_3$, ao passo que os reagentes químicos convencionais não são capazes de separar estes dois minerais (NITSCHKE; PASTORE, 2002). Slizovskiy et al. (2011) estudaram a remediação melhorada de solos contaminados por metais pesados pelo surfactante catiônico DPC, pelo surfactante não iônico Ammonyx KP e pelo biossurfactante iônico JBR-425. Verificou-se que o JBR- 425 teve o melhor efeito de eluição de Zn (39%), Cu (56%), Pb (68%), e Cd (43%). Mao et al. (2015) descreveram a capacidade dos surfactantes em promover a dessorção de metais contidos em plantas e a posteiror absorção dos tensoativos pelas mesmas. Mais recentemente, biossurfactantes como uma saponina vegetal e raminolipídeos foram capazes de remover cromo e arsênio de solos ricos em resíduos de mineração (MAITY et al., 2013; OZTURK et al., 2012). Outros tipos de biossurfactantes produzidos por espécies do gênero *Candida* também foram empreguados com sucesso na flotação de metais pesados e demonstraram capacidade de remover mais de 90% de cátions em colunas de Flotação por Ar Dissolvido (ALBUQUERQUE et al., 2012; MENEZES et al., 2011; SARUBBO et al., 2015). O biossurfactante produzido pela levedura *C. lipolytica* também foi utilizado para a remoção de metais pesados e de derivados de petróleo usando um solo de formação barreira (RUFINO et al., 2011a). O biossurfactante reduziu significativamente a permeabilidade do solo, demonstrando sua aplicabilidade como

um aditivo em barreiras reativas, permitindo a remoção de cerca de 96% de Zn e Cu e a redução das concentrações de Pb e Cd nas águas subterrâneas.

Figura 9 – Mecanismo de remoção de metais pesados por biossurfactantes



Fonte: SARUBBO et al., 2015

3.12. Perspectivas de aplicação de biossurfactantes no mercado

Nos processos biotecnológicos a economia é sempre um fator importante, especialmente nos casos de produção de biossurfactantes. O sucesso da produção de biossurfactantes depende do desenvolvimento de processos de baixo custo e da utilização de substratos mais baratos, que representam de 10 a 30 % do custo total de produção. Os biossurfactantes têm que competir com os surfactantes petroquímicos considerando dois aspectos: custo e funcionalidade (BANAT et al., 2010; MUTHUSAMY et al., 2008).

Pattanath et al. (2008) sugerem quatro fatores para a redução dos custos dos biossurfactantes. Os micro-organismos (selecionados, adaptados ou criados para produção em larga escala), o processo (selecionado, adaptado ou criado para garantir baixo custo operacional), o meio de cultura (adaptado para baixo custo) e o

processamento de produtos reciclados (mínimos ou gerenciados para venda).

O custo de produção de biossurfactantes pode ser absorvido, caso os mesmos sejam utilizados em baixa concentração, para a produção de cosméticos, de medicamentos e de alimentos. Para aplicações mais abrangentes, por outro lado, como a recuperação de óleos, a qual requer grandes volumes de surfactantes, o custo de produção pode dificultar a utilização destes biocompostos (SILVA et al., 2014a).

Segundo Hazra et al. (2012), raminolipídeos estão comercialmente disponíveis na Jeneil biossurfactante Inc. (EUA), Ecover (França) e na Rhamnolipid holdings Inc., (EUA), enquanto que soforolipídeos são atualmente oferecidos pela Sophoron TM da Saraya (Japão) e Soliance (França). O preço atual dos soforolipídeos equivale a 2-5 €/kg, enquanto que os raminolipídeos custam US $ 5-20 /kg e quando produzidos em escala de 100 m^3 custam US $ 5/kg contra os etoxilados ou alquil poliglicosídeos, que custam US $ 1-3 / kg. Estudos de biorremediação apoiados pela empresa Exxon entre 1993 e 1997 geraram sete patentes e mostraram a eficência da biorremediação durante os primeiros anos da sua aplicação (HAZRA et al., 2012; SANTOS et al., 2011).

Embora se admita que o aperfeiçoamento da tecnologia de produção dos biossurfactantes já tenha possibilitado um aumento de 10-20 vezes da sua produtividade, é provável que novos e significativos progressos (ainda que de uma ordem de magnitude inferior) sejam necessários para tornar essa tecnologia comercialmente viável.

4. REFERÊNCIAS BIBLIOGRÁFICAS

ABDEL-MAWGOUD, A. M.; LÉPINE, F.; DEZIEL, E. Rhamnolipids: diversity of structures, microbial origins and roles. **Applied Microbiology and Biotechnology**, v. 86, n. 5, p. 1323-1336, 2010.

AKHTAR, M.; LENTZ, M.J.; BANCHETTE, R.A.; KIRK, T.K. Corn steep liquor lowers the amount of inoculum for biopulping. **TAPPI J.** v. 80, p.161–164, 1997.

ALBUQUERQUE, C. F.; LUNA-FINKLER, C. L.; RUFINO, R. D.; LUNA, J .M.; MENEZES, C. T. B.; SANTOS, V. A. ; SARUBBO, L. A. Evaluation of biosurfactants for removal of heavy metal ions from aqueous effluent using flotation techniques. **International Review of Chemical Engineering**, v. 4, p. 156-161, 2012.

ALCÂNTARA, R.; AMORES, J.; CANOIRA, L.; FIDALGO, E.; FRANCO, M. J.; NAVARRO, A. Catalytic production of biodiesel from soy-hean oil, used fruing oil and tallow. **Biomass and Bioenergy,** v. 18, p. 515-527, 2000.

ALMEIDA, D.G.; SOARES DA SILVA, R.C.F.; LUNA, J.M.; RUFINO, R.D.; SANTOS, V.A.; BANAT, I.M.; SARUBBO, L.A. **Biosurfactants: Promising Molecules for Petroleum Biotechnology Advances. Frontiers in Microbiology**, v. 7, p. 1718, 2016.

APARNA, A.; SRINIKETHAN, G.; HEDGE, S. Effect of addition of biosurfactant produced by *Pseudomonas* ssp. on biodegradation of crude oil. In International Proceedings of Chemical, Biological & Environmental Engineering,Proceedings of the 2nd International Proceedings of Chemical, Singapore, Singapore, 26–28 February; v. 6, p. 71–75, 2011.

ASÇI, Y.; NURBAS, M.; AÇIKEL, A. A comparative study for the sorption of Cd(II) by soils with different clay contents and mineralogy and the recovery of Cd(II) using rhamnolipid biosurfactant. **Journal of Hazardous Materials,** v. 154, p. 663-673, 2008.

AUSTAD, T.; TAUGBØL, K. Chemical flooding of oil reservoirs 1. Low tension polymer flood using a polymer gradient in the three-phase region. **Colloids Surface A: Physicochemical and Engineering Aspects,** v. 101, p. 87-97, 1995.

BAI, G.; BRUSSEAU, M.L.; MILLER, R. M. Biosurfactant-enhanced removal of residual hydrocarbon from soil. **Journal of Contaminant Hydrology**, v. 25, p. 157-170, 1997.

BANAT I.M.; DE RIENZO, M.A.D.; QUINN, GA. Microbial biofilms: biosurfactants as antibiofilm agentes. **Applied Microbiology and Biotchnology**, v. 98, p. 9915-9929, 2014b.

BANAT, I M. Microbial biosurfactants production, applications. **Applied Microbiology and Biotechnology**, v. 87, p.427-444, 2010.

BANAT, I.M.; SATPUTE, S.K.; CAMEOTRA, S.S.; PATIL, R.; NYAYANIT, N. Cost effective technologies and renewable substrates for biosurfactants' production. **Frontiers Microbiol,** v. 5, p. 697, 2014a.

BANAT, I.M.; FRANZETTI, A.; GANDOLFI, I.; BESTETTI, G.; MARTINOTTI, M.G.; FRACCHIA, L.; SMYTH, T.J.; MARCHANT, R. Microbial biosurfactants production, applications. **Applied Microbiology andBiotechnology**, v. 87, p. 427–444, 2010.

BARROS, F.F.C.; QUADROS, C.P.; MARÓSTICA, M.R.; PASTORE, M.G. Surfactina: propriedades químicas, tecnológicas e funcionais para aplicações em alimentos. **Química Nova,** v. 30, p. 01-14, 2007.

BATISTA, R.M.; RUFINO, R.D.; LUNA, J.M.; SOUZA, J.E.G.; SARUBBO, L.A. Effect of medium components on the production of a biosurfactant from *Candida tropicalis* applied to the removal of hydrophobic contaminants in soil. **Water Environmental Research**, v.82, p. 418-425, 2010.

BEDNARSKI, W.; ADAMCZAK, M.; TOMASIK, J.; PLASZVZYK, M. Application of oil refinery waste in the biosynthesis of glycolipids by yeast. **Bioresource Technology,**v. 95, p.15–18, 2004.

BENINCASA, M.; CONTIERO, J.; MANRESA, M. A.; MORAES, I. O. Rhamnolipid production by *Pseudomonas aeruginosa* LBI growing on soapstock as the sole carbon source.**Journal of Food Engineering**, v. 54, p. 283–288, 2002.

BHARDWAJ, G.; CAMEOTRA, S.S.; CHOPRA, H.K. Biosurfactants from Fungi: A Review. **Petroleum and Environmental Biotechnology**, v. 4, p. 1-6, 2013.

BOGNOLO, G. Biosurfactants as emulsifying agents for hydrocarbons. **Colloids and Surfaces A: Physicochemical Engineering Aspects,** v. 152, p. 41-52, 1999.

CALVO, C.; MANZANERA, M.; SILVA-CASTRO, G.A.; UAD, I.; GONZÁLEZ-LOPÉZ, J. Application of bioemulsifiers in soil oil bioremediation processes. Future prospects. **Science of the Total Environmental,** v. 407, p. 3634-3640, 2009.

CAMPOS, J. M.; STAMFORD, T.L.M.; RUFINO, R.D.; LUNA, J.M.; BANAT, I.; SARUBBO, L.A. Microbial biosurfactants as additives for food industries. **Biotechnology Progress**, v. 29, p.1097-1108, 2013.

CARDINAL, E.V.; HEDRICK, L.R. Microbiological assay of corn steep liquor for amino acid content. **Journalof Biological Chemistry,** v. 172, p. 609–612, 1948.

CASAS, J.A.; DE LARA, S.G.; GARCIA-OCHOA. Optimization of a synthetic medium for *Candida bombicola* growth using factorial design of experiments. **Enzyme Microbiol Technology**, v. 21, p. 221-229, 1997.

CAVALERO, D.A.; COOPER, D.G. The effect of medium composition on the structure and physical state of sophorolipids produced by *Candida bombicola* ATCC 22214. **Journal Biotechnology**, v. 103, p. 31-41, 2003.

CERQUEIRA, V.S.; HOLLENBACH, E.B.; MABONI, F.; VAINSTEIN, M.H.; CAMARGO, F.A.; DO CARMO, M.; PERALBA, R.; BENTO, F.M. Biodegradation potential of oily sludge by pure and mixed bacterial cultures. **Bioresource Technology,** v. 102, p.11003–11010, 2011.

CHAKRABARTI, S. Bacterial Biosurfactant: Characterization, Antimicrobial and Metal Remediation Properties. Ph.D. Thesis, National Institute of Technology, India, 2012.

CHAKRABORTY, J.; DAS, S. Biosurfactant-based bioremeditaion of toxic metals, 1st ed.; Das, S., Ed.; In Microbial Biodegradation and Bioremediation. **Elsevier:** India, p. 167-201, 2014.

CIRIGLIANO, M.C.; CARMAN, GM. Isolation of a bioemulsifier from *Candida lipolytica*. **Appliedand Environmental Microbiology**, v. 48, p. 747-750, 1984.

CIRILIANO, C.; CARMAN, G. Purification and characterization of liposan, a bioemulsifier from *Candida lipolytica*. **Applied Microbiology and Biotechnology**, v. 50, p.846-850, 1985.

COOPER, D.G.; PADDOCK, D.A. Production of a biosurfactant from *Torulopsis bombicola*. **Applied and Environmental Microbiology,** v**.** 47, p.173-176, 1984.

CORTÉS-SÁNCHEZ, A.J.; SÁNCHEZ, H.H.; JARAMILLO-FLORES, M.E. Biological activity of glycolipids produced by microorganisms: New trends and possible therapeutic alternatives. **Microbiological Research,** v.168, p. 22– 32, 2013.

CORTIS, A.; GHEZZEHEI, T.A. On the transport of emulsions in porous media. **Journal of Colloid and Interface Science**, v. 313, p. 1-4, 2007.

CUNHA, C.D.; ROSARIO, M.; ROSADO, A.S.; LEITE, G.F. *Serratia* sp. SVGG16: a promising biosurfactant producer isolated from tropical soil during growth with ethanol-blended gasoline. **Process Biochemistry**, v.39, p. 2277–2282, 2004.

CVENGROS, Z.; CVENGROSOVA, Z. Used fruing oils and fats and their utilization in the production of methyl ester of higher fatty acids. **Biomass Bioenergy,** v. 27, p.173-181, 2004.

DAS, P.; MUKHERJEE, S.; SEN, R. Antiadhesive action of a marine microbial surfactant. **Colloids and Surfaces B*:* Biointerfaces,** v. 71, p.183–186, 2009.

DAVEREY, A.; PAKSHIRAJAN, K. Production, characterization, and properties of sophorolipids from the yeast *Candida bombicola* using a low-cost fermentative medium. **Applied Biochemistry Biotechnology,** v. 158, p.663–674, 2009.

DESAI, J.D.; BANAT, I.M. Microbial production of surfactants and their commercial potential. **Microbiology and Molecular Biology Reviews,** v. 61, p. 47-64, 1997.

DESHPANDE, M.; DANIELS, L. Evaluation of sophorolipid biosurfactant production by *Candida bombicola* using animal fat. **Bioresource Technology,** v. 54, p. 143-150, 1995.

DÍAZ DE RIENZO, M.A.D.; BANAT, I.M.; DOLMAN, B.; WINTERBURN, J.; MARTIN , P.J. Sophorolipid biosurfactants: Possible uses as antibacterial and antibiofilm agent. **New Biotechnology**, v. 32, p. 1-7, 2015.

DUBEY, K.; JUWARKAR, A. Distillery and curd whey wastes as viable alternative sources for biosurfactant production. **World Journal of Microbiologyand Biotechnology,** v. 17, p. 61-69, 2001.

DUBEY, K.V.; JUWARKAR, A.A.; SINGH, S.K. Adsorption-desorption process using wood based activated carbon for recovery of biosurfactant from fermented distillery wastewater. **Biotechnology Progress**, v. 21, p. 860–867, 2005.

EPA, U.S. Treatment technologies for site cleanup: Annual status report, Office of Solid Waste and Emergency Response, 10th ed., USA, 2001.

FELSE, P.A.; SHAH, V.; CHAN, J.; RAO, K.J.; GROSS, R.A. **Enzyme Microbiology Technology**, v. 40, p. 316, 2007.

FOX, S. I.; BALA, G. A. Production of surfactant from *Bacillus subtilis* ATTCC 21332 using potato substrates. **Bioresource Technology,** v. 75, p. 235-240, 2000.

FRANZETTI, A.; CAREDDA, P.; RUGGERI, C.; LA COLLA, P.; TAMBURINI, E.; PAPACCHINI, M.; BESTETTI, G. Potential applications of surface active compounds by *Gordonia* sp. strain BS29 in soil remediation technologies. **Chemosphere,** v. 75,p. 810–807, 2009.

GAUTAM, K.K.; TYAGI, V.K. Microbial Surfactants: a review. **Journal of Oleo Science**, v. 55, p. 155-166, 2006.

GHURYE, G. L.; VIPULANANDA, C.; WILSON, R. C. A practical approach to biosurfactant production using nonaseptic fermantation of mixed cultures. **Biotechnology and Bioengineering,** v. 44, p. 661 - 666, 1994.

GONZINI, O.; PLAZA, A.; PALMA; D. I. L.; LOBO, M. C. Electrokinetic remediation of gasoil contaminated soil enhanced by rhamnolipids. **Journal of Applied Eletrochemistry,** v. 40, p. 1239-1248, 2010.

GUSMÃO, B.; RUFINO, R. D.; SARUBBO, L.A. Laboratory production and characterization of a new biosurfactant from *Candida glabrata* UCP1002 cultivated in vegetable fat waste applied to the removal of hydrophobic contaminant. **World Journal of Microbiology and Biotechnology,** v. 26, p. 1683-1692, 2010.

HABA, E; ESPUNY, M.J.; BUSQUETS, M.; MANRESA, A. Screening and production of rhamnolipids *Pseudomonas aeruginosa* 47T2 NCIB 40044 from waste flying oils. **Journal of Applied Microbiology,** v. 88, p. 379-387, 2000.

HARITASH, A.K.; KAUSHIK, C.P. Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. **Journal of Hazardous Materials** v. 169, p. 1–15, 2009.

HASHIM, M.A.; MUKHOPADHYAY, S.; SAHU, J. N.; SENGUPTA, B. Remediation technologies for heavy metal contaminated groundwater. **Journal of Environmental Management**, v. 92, p. 2355 -2388, 2011.

HATHA, A.A.M.; G. EDWARD, K.S.M.P.; RAHMAN. Microbial biosurfactants-review. **Journal Mar. Atmospheric Research,**v. 3, p. 1-17, 2007.

HAZRA, C.; KUNDU, D.; CHAUDHARI, A. Biosurfactant-assisted bioaugmentation in bioremediation. In Microorganisms in Environmental Management: Microbes and Environment, 1st ed.; Satyanarayana T.; Johri, B.N.; Prakash, A., Eds.; Springer: New York, EUA, p. 631-664, 2012.

HELMY, Q.; KARDENA, E.; FUNAMIZU, N.; WISJNUPRAPTO. Strategies toward commercial scale of biosurfactant production as potential substitute for it's chemically counterparts. **International Journal of Biotechnology,** v.12, p.66–86, 2011.

HERMAN, D.C.; ARTIOLA, J.F.; MILLER, R.M. Removal of cadmium, lead and zinc from soil by a rhamnolipid biosurfactant. **Environmental Science and Technology,** v. 29, p. 2280–2285, 1995.

HITSATSUKA, K.; NAKAHARA, T.; SANO, N.; YAMADA, K. Formation of a rhamnolipid by *Pseudomonas aeruginosa* and its function in hydrocarbon fermentation. **Agricultural and Biological Chemistry,** v. 35, p. 686–92, 1971.

HOMMEL, R.K.; HUSE, K. Regulation of sophorose lipid production by *Candida* (*Torulopsis*) *apicola*. **Biotechnology Letters**, v. 15, p.853-858, 1993.

HU, Y.; JU, L.K. Purification of lactonic sophorolipids by crystallization. **Journal Biotechnology,** v. 87, p.263-272, 2001.

HUE, N.; SEMNI, L.; LAPREVOTE, O. Structural investigation of cyclic peptidolipids from *Bacillus subtilis* by high energy tandem mass spectrometry. **Rapid Communications in Mass Spectrometry**, v. 15, p. 203-209, 2001.

JARVIS, F.G.; JOHNSON, M.J. A glycolipid produced by *Pseudomonas aeruginosa*. **Journal of the American Chemical Soc**iety, v. *71*, p.4124-4126, 1949.

JOSHI, S.; BHARUCHA, C.; J.H.A, S.; YADAV, S.; NERURKAR, A.; DESAI, A.J. Biosurfactant production using molasses and whey under thermophilic conditions. **Bioresource Technology**, v. 99, n.1, p.195–199, 2007.

KALOGIANNIS, S.; IAKOVIDOU, G.; LIAKOPOULOU-KYRIAKIDES, M.; KYRIAKIDIS, D. A.; SKARACIS, G. N. Optimization of xanthan gum production by xanthomonas campestris grown in molasses. **Process Biochemistry,** v. 39, p. 249-256, 2003.

KIM, H.S.; YOON, B.D.; CHOUNG, D.H.; OH, H.M.; KATSURAGI, T.; TANI, Y. Characterization of a biosurfactant mannosylerythritol lipid produced from *Candida* sp. SY 16. **Applied Microbiology and Biotechnology,** v.52, p. 713-721, 1999.

KIRAN, G.S.; HEMA, T.A.; GANDHIMATHI, R.; SELVIN, J.; THOMAS, T.A, RAJEETHA RAVIJI, T.; NATARAJASEENIVASAN, K.Optimization and production of a biosurfactant from the sponge-associated marine fungus *Aspergillus ustus* MSF3. **Colloids and Surfaces B*:* Biointerfaces,** v. 73, p. 250- 256, 2009.

KITAMOTO, D.; IKEGAMI, T.; SUZUKI, G.T.; SASAKI, A.; TAKEYAMA, Y.; IDEMOTO, Y.; KOURA, N.;YANAGISHITA, H. Microbial conversion of n-alkanes into glycolipid biosurfactants, mannosylerythritol lipids by *Pseudozyma* (*Candida antartica*), **Biotechnology Letter,** v. 23, n. 20, p.1709–1714, 2001.

KITAMOTO, D.; ISODA, H.; NAKAHARA T. Functions and potential applications of glycolipid biosurfactants: from energy-saving materials to gene delivery carriers. **Journal of Bioscience and Bioengineering**, v. 94, p. 187-201, 2002.

KONISHI, M.; FUKUOKA, T.; MORITA, T.; IMURA, T.; KITAMOTO, D. Production of new types of sophorolipids by *Candida batistae*. **Journal of Oleo Science**, v. 57, p.359-369, 2008.

KUYUKINA, M.S.; IVSHINA, I.B.; MAKAROV, S.O.; LITVINENKO, L.V.; CUNNINGHAM, C.J.; PHILIP, J.C. Effect of biosurfactants on crude oil desorption and mobilization in a soil system. **Environmental International,** v. 31, p.155–161, 2005.

LANG, S. Biological amphiphiles(microbial biosurfactants). **Current Opinion Colloid Interface Science**, v. 7: p.12-20, 2002.

LAZARIDOU, A.; ROUKAS, T.; BILIADERIS, C. G.; VAIKOUSI, H. Characterization of pullulan produced from beet molasses by *Aureobasidium pullulans* in a stirred tank reactor under varying agitation. **Enzyme Microbiology and Technology,** v. 31, p. 122-132, 2002.

LU, J. R.; ZHAO, X. B.; YASEEN, M. Biomimetic amphiphiles: biosurfactants. **Current Opinion in Colloid and Interface Science,** v. 12, p. 60-67, 2007.

LUNA, J.M.; RUFINO, R.D.; ALBUQUERQUE, C.D.C.; SARUBBO, L.A.; CAMPOS-TAKAKI, G.M. Economic optimized medium for tenso-active agentproduction by *Candida sphaerica* UCP0995 and application in the removal of hydrophobic contaminant from sand. **International Journal of Molecular Sacience,** v.12, p.2463 - 2476, 2011a.

LUNA, J.M.; RUFINO, R.D.; JARA, A.M.T.; BRASILEIRO, P.P.F.; SARUBBO, L.A. Environmental applications of the biosurfactant produced by *Candida sphaerica* cultivated in low-cost substrates. **Colloids and Surfaces A: Physicochememical and Engineering Aspects**, v.480, p.413–418, 2015.

LUNA, J.M.; RUFINO, R.D; SARUBBO, L.A. Biosurfactant from *Candida sphaerica* UCP0995 exhibiting heavy metal remediation properties. **Process Safety and Environmental Protection,** v. 1 0 2, p. 558–566, 2016.

LUNA, J.M.; RUFINO, R.D.; SARUBBO, L.A.; CAMPOS–TAKAKI, G.M. Characterization, surface properties and biological activity of a biosurfactant produced from industrial waste by *Candida sphaerica* UCP0995 for application in the petroleum industry**. Colloids and Surfaces B: Biointerfaces**, v.102, p. 202–209, 2013.

LUNA, J.M.; RUFINO, R.D.; SARUBBO, L.A.; RODRIGUES, L.R.M.; TEIXEIRA, J.A.C.; CAMPOS-TAKAKI, G.M. Evaluation antimicrobial and antiadhesive properties of the biosurfactant lunasan produced by *Candida sphaerica* UCP 0995. **Current Microbiology,** v. *62*, p.1527–1534, 2011b.

MAITY, J.P.; HUANG, Y.M.; FAN, C.-W.; CHEN, C.-C.; LI, C.-Y.; HSU, C.-M.; CHANG, Y.-F.; WU, C.-I.; CHEN, C.-Y.; JEAN, J.-S. Evaluation of remediation process with soapberry derived saponin for removal of heavy metals from contaminated soils in Hai-Pu Taiwan. **Journal Environmental Science,** v**.** 25, p.1180–1185, 2013.

MAKKAR, R. S.; CAMEOTRA, S. S. Utilization of molasses for biosurfactant production by two *Bacillus* strains at thermophilic conditions. **Journal American Oil Chemistry Society,** v. 74, p. 887-889, 1997.

MAKKAR, R.S.; CAMEOTRA, S.S.; BANAT, I.M. Advances in utilization of renewable substrates for biosurfactant production. AMB Exp. v.1, p.1-19, 2011.

MAKKAR, R.S.; CAMEOTRA, S.S. An update on the use of uncoventional substrates for biosurfactant production and their new applications. **Applied Micobiology and Biotechnology,** v. 58, p. 428-434, 2002.

MANEERAT, S. Production of biosurfactants using substrates from renewable-resources. Songklanakarin **Journal of Science and Technology,** v. 27, p. 675-683, 2005.

MAO, X.; JIANG, R.; XIAO, W.; YU, J. Use of surfactants for the remediation of contaminated soils: A review. **Journal of Hazardous Materials,** v.285, p.419–435, 2015.

MARCHANT, R.; BANAT, I.M. Microbial biosurfactants: challenges and opportunities for future exploitation. **Trends in Biotechnology**, v. 11, p. 558-565, 2012b.

MENEZES, C.T.B.; BARROS, E.C.; RUFINO, R.D.; LUNA, J.M.; SARUBBO, L.A. Replacing Synthetic with Microbial Surfactants as Collectors in the Treatment of Aqueous Effluent Produced by Acid Mine Drainage, Using the Dissolved Air Flotation Technique. **Applied Biochemistry and Biotechnology**, v. 163, p. 540-546, 2011.

MERCADE, M. E.; MANRESA, A.; ROBERT, M.; ESPUNY, M. J.; deANDRES, C.; GUINEA, J. Olive oil mill effluent (OOME). New substrat for biosurfactant production. **Bioresource and Tecnology**, v. 43, p. 1-6, 1993.

MNIF, I., GHRIBI, D. Microbial derived surface active compounds: properties and screening concept. **World Journal Microbiology Biotechnology,** v.31, p.1001-1020, 2015.

MULLIGAN, C.N. Recent advances in the environmental applications of biosurfactants. **Current Opinion In Colloids and Interface Science**, v 14, p. 372-378, 2009.

MULLIGAN, C.N.; GIBBS, B.F. Correlation of nitrogen metabolism with biosurfactant production by *Pseudomonas aeruginosa*. **Applied and Environmental Microbiology**, v. 55, p. 3016-3019, 1989.

MULLIGAN, C.N.; WANG, S.; Remediation of a heavy metal contaminated soil by a rhamnolipid foam. In: Geo environmental engineering. Integrated management of groundwater and contaminated land. London: Thomas Telford; p. 544-51, 2004.

MULLIGAN, C.N.; YONG, R.N.; GIBBS, B.F. Metal removal from contaminated soil and sediments by the biosurfactant surfactin. **Environmental Science and Technology,** v. 33, p. 3812–3820, 1999.

MUTHUSAMY, K.; GOPALAKRISHNAN, S.; RAVI, T. K.; SIVACHIDAMBARAM, P. Biosurfactants: properties, commercial production and application. **Current Science,** v. 94, p. 736-747, 2008.

NASH, J.; TRAVER, R.P.; DOWNEY, D.C. Surfactant enhanced in situ soil Washing. US.EPA, HWERL. Edilsin. New Jersey. 1987.

NITSCHKE, M.; COSTA, S.G.V.A.O.; CONTIERO, J. Rhamnolipids and PHAs: Recent reports on *Pseudomonas*-derived molecules of increasing industrial interest. **Process Biochemistry,** v. 46, p. 621–630, 2011.

NITSCHKE, M.; FERRAZ, C.; PASTORE, G. M. Selection of microorganisms for biosurfactant production using agroindustrial wastes. **Brazilian Journal of Microbiology**, v. 35, n. 1-2, 2004.

NITSCHKE, M.; PASTORE, G. M. Biossurfactantes: propriedades e aplicações. **Química Nova**, v. 25, p. 772-776, 2002.

OCHOA-LOZA, F.J.; ARTIOLA, J.F.; MAIER, R.M. Stability constants for the complexation of various metals with a rhamnolipid biosurfactant. **Journal of environmental quality,** v. 30, p. 479-485, 2001.

OCHOA-LOZA, F.J.; NOORDMAN, W.H.; JANNSEN, D.B.; BRUSSEAU, M.L.; MAIER, R.M. Effect of clays, metal oxides, and organic matter on rhaminolipid biosurfactant sorption by soil. **Chemosphere,** v.66,p.1634-1642, 2007.

OLIVEIRA, F.J.S.; VAZQUEZ L.; DE CAMPOS, N.P.; DE FRANCA, F.P. Production of rhamnolipids by a *Pseudomonas alcaligenes* strain. **Process Biochemistry,** v. 44, p. 383–389, 2009.

OZTURK, S.; KAYA, T.; ASLIM, B.; TAN, S. Removal and reduction of chromium by *Pseudomonas* spp. and their correlation to rhamnolipid production. **Journal Hazardous Materials,** p. 231–232, 2012.

PACWA-PLOCINICZAK, M.; PLAZA, G. A.; PIOTROWSKA-SEGET, Z.; CAMEOTRA, S. S. Environmental Applications of Biosurfactants: Recent Advances, International Journal of Molecular Sciences. 13, p. 633-654, 2011.

PATTANATH, K. M.; RAHMAN, K. S.; GAKPE, E. Production, characterization and applications of biosurfactants – reviem**. Biotechnology,** v.7, n. 2, p. 360-370, 2008.

RAHMAN, P. K. S. M.; GAKPE, E. Production, characterization and applications of biosurfactants – review. **Biotechnology,** v.7, p. 360-370, 2008.

RAZA, Z.A.; KHAN, M.S.; KHALID, Z.M.; REHMAN, A. Production kinetics and tensioactive characteristics of biosurfactant from a *Pseudomonas aeruginosa* mutant grown on waste frying oils. **Biotechnology Letter**, v.28, n. 20, p.1623–1631, 2006.

RAZA, Z.A.; REHMAN, A.; KHAN, M.S.; KHALID, Z.M. Improved production of biosurfactant by a *Pseudomonas aeruginosa* mutant using vegetable oil refinery wastes. **Biodegradation,** v. 18, n. 1, p.115–121, 2007.

ROBERT, M.; MERCADÉ, M. E.; BOSCH, M. P.; PARRA, J. L.; ESPINY, M. J.; MANRESA, M.A.; GUINEA, J. Effect of the carbon source on biosurfactant production by *Pseudomonas aeruginosa* 44T1. **Biotechnology Letters**, v. 11, p. 871-874, 1989.

RODRIGUES, L.R.; TEIXEIRA, J.A.; OLIVEIRA, R. Low-cost fermentative medium for biosurfactant production by probiotic bacteria. **Biochemical Engineering Journal,** v. 32, n. 3, p.135–142, 2006b.

ROSA, C.F.C.; FREIRE, D.M.G.; FERRAZ, E.C. Biosurfactant microfoam: Application in the removal of pollutants from soil. **Journal of EnvironmentalChemicalEngineering**, v.3, p. 89–94, 2015.

RUFINO, R.D.; LUNA, J.M.; CAMPOS-TAKAKI, G.M.; SARUBBO, L.A. Characterization and properties of the biosurfactant produced by *Candida lipolytica* UCP 0988. **Electronic Journal of Biotechnology**, v. 17, p. 34–38, 2014.

RUFINO, R. D.; LUNA, J. M.; MARINHO, P.H.C.; FARIAS, C. B. B.; FERREIRA, S. R. M.;

SARUBBO, L. A. Removal of petroleum derivative adsorbed to soil by biosurfactant Rufisan produced by *Candida lipolytica*. **Journal of Petroleum Science & Engineering**, v.109, p.117 - 122, 2013.

RUFINO, R. D.; LUNA, J. M.; RODRIGUES, G. I. B.; CAMPOS-TAKAKI, G. M.; SARUBBO, L. A.;FERREIRA, S. R. M.Application of a yeast biosurfactant in the removal of heavy metals and hydrophobic contaminant in a soil used as slurry barrier. **Applied and Environmental Soil Science**, p.1- 7, 2011a.

RUFINO, R.D.; LUNA, J.M.; SARUBBO, L.A.; RODRIGUES, L.R.M.; TEIXEIRA, J.A.C.; CAMPOS-TAKAKI, G.M. Antimicrobial and anti-adhesive potential of a biosurfactant Rufisan produced by *Candida lipolytica* UCP 0988. **Colloids and Surfaces B: Biointerfaces,** v. 84, p. 1-5, 2011b.

RUFINO, R.D.; SARUBBO, L.A.; BENICIO, B.N.; CAMPOS-TAKAKI, G.M. Experimental design for the production of tensio-active agent by *Candida lipolytica*. **Journal of Industrial Microbiology and Biotechnology,** v. 35, p. 907-914, 2008.

RUFINO, R.D.; SARUBBO, L.A.; CAMPOS-TAKAKI G.M. Enhancement of stability of biosurfactant produced by *Candida lipolytica* using industrial residue as substrate. **World Journal of Microbiology and Biotechnology**, v. 23, p. 729-734, 2007.

SABATINI, D.A.; HARWELL, J.H.; KNOX, R.C. Surfactant selection criteria for enhanced subsurface remediation. In *Innovative Subsurface Remediation*, Brusseau, M.L.; Sabatini, D.A.; Gierke, J.S.; Annable, M.D., Eds.; American Chemical Society, p. 8-23, 1999.

SABATINI, D.A.; KNOX, R.C.; HARWELL, J.H.; WU, B. Integrated design of surfactant enhanced DNAPL remediation: efficient super solubilization and gradient systems. **Journal of ContamaminatHydrology**, v. 45, p. 99-121, 2000.

SACHDEV, D.P.; CAMEOTRA, S.S. Biosurfactants in agriculture. **AppliedMicrobiology and Biotechnology, v.** 97, p.1005–1016, 2013.

SANTA ANNA I.M.; SEBASTIAN, G.V.; PEREIR, J.R.N.; ALVES, T.L.M.; MENEZES, E.P.; FREIRE, D.M.G. Production of biosurfactant from a new and producing strain of *Pseudomonas aeruginosa* P.A.1. **Applied Biochemistryand Biotechnology,** v. 91, p.459-467, 2002.

SANTOS, D.K.F.; BRANDÃO, Y.B.; RUFINO, R.D.; LUNA, J.M.; SALGUEIRO, A.A.; SANTOS, V.A.; SARUBBO, L.A. Optimization of cultural conditions for biosurfactant production from *Candida lipolytica*. **Biocatalysis and Agricultural Biotechnology,** v.3, p. 48–57, 2014.

SANTOS, D. K. F.; LUNA, J.M.; RUFINO, R. D.; SANTOS,V. A.; SALGUEIRO, A. A.; SARUBBO, L. A. Synthesis and evaluation of biosurfactant produced by *Candida lipolytica* using animal fat and corn steep liquor. **Journal of Petroleum Science & Engineering**, v.105, p.43 - 50, 2013.

SANTOS, H.F.; CARMO, F.L.; PAES, J.E.S.; ROSADO, A.S.; PEIXOTO, R.S. Bioremediation of mangroves impacted by petroleum.**Water, Air and Soil Pollution,** v. 216, p. 329–350, 2011.

SANTOS, D.K.F.; RUFINO, R.D.; LUNA, J.M.; SANTOS, V.A.; SARUBBO, L.A. Biosurfactants: Multifunctional Biomolecules of the 21st Century. **International Journal of Molecular Sciences**, v.17, p.401, 2016.

SAPTURE, S. K.; BANAT, I. M.; DHAKEPHALKAR, P. K.; BANPURKAR, A. G.; CHOPADE, B. A. Biosurfactants, bioemulsifiers and exopolysaccharides from marine microorganisms. **Biotechnology Advance** v.28, p. 436–450, 2010.

SARUBBO, L.A.; FARIAS, C.B.B.; CAMPOS-TAKAKI, G.M. Co-utilization of canola oil and glucose on the production of a surfactant by *Candida lipolytica*. **Current Microbiology,** v. 54, p. 68-73, 2007.

SARUBBO, L.A.; LUNA, J.M.; CAMPOS-TAKAKI, G.M. Production and stability studies of the bioemulsifier obtained from a new strain of *Candida glabrata* UCP 1002. **Eletronic Journal of Biotechnology**, v. 9, p. 400-406, 2006.

SARUBBO L.A.; MARÇAL, M.C.; NEVES, M.L.C.; PORTO, A.L.F.; CAMPOS-TAKAKI, G.M. The use of babassu oil as substrate to produce bioemulsifiers by Candida lipolytica. **Canadian Journal of Microbiology**, v. 45, p. 1-4, 1999.

SARUBBO L.A.; PORTO, A.L.F.; CAMPOS-TAKAKI, G.M. Bioemulsifier production in batch culture using glucose as carbon source by *Candida lipolytica*. **Applied Biochemistry and Biotechnology,** v. 95, p.59-67, 2001.

SARUBBO, L.A.; ROCHA JR., R.B.; LUNA, J.M.; RUFINO, R.D.; SANTOS, V.A.; BANAT, I. M. Some aspects of heavy metals contamination remediation and role of biosurfactants. **Chemistry and Ecology,** v. 31, p.707-723, 2015.

SHABTAI, Y. Production of exopolysaccharides by *Acinetobacter* strains in a controlled fed-batch fermentation process using soap stock oil (SSO) as carbon source. **International Journal of Biology Macromolecule,** v. 12, p. 145-152, 1990.

SHAVANDI, M.; MOHEBALI, G.; HADDADI, A.; SHAKARAMI, H.; NUHI, A. Emulsification potential of a newly isolated biosurfactant producing bacterium, *Rhodococcus* sp. strain TA6. **Colloids and Surfaces B: Biointerfaces**, v. 82, p.477–482, 2011.

SHEPHERD, R.; ROCKEY, J.; SHUTHERLAND, I.W.; ROLLER, S. Novel bioemulsifiers from microorganisms for use in foods. **Journal of Biotechnology,** v. 40, p. 207-217, 1995.

SILVA, N.M.P.R.; LUNA, J. M.; RUFINO, R. D.; SANTOS, V. A.; SARUBBO, L. A. Screening of *Pseudomonas* species for biosurfactant production using low-cost substrates. **Biocatalysis and Agricultural Biotechnology,** v. 3, p. 132-139, 2014b.

SILVA, R.C.F.S.; ALMEIDA D.G.; LUNA, J. M.; RUFINO, R. D.; SANTOS, V. A.;SARUBBO, L. A. Applications of biosurfactants in the petroleum industry and the remediation of oil spills. **Intenrational Journal of MolecularSciences,** v. 15, p. 12523-12542, 2014a.

SILVA, R. F. S.; FARIAS, C. B. B.; RUFINO, R. D.; LUNA, J. M.; FILHO, H. J. B.; SANTOS, V. A.; SARUBBO, L. A. Enhancement of biosurfactant production from *Pseudomonas cepacia* CCT6659 through optimisation of nutritional parameters using response surface methodology. **Tenside, Surfactants, Detergents,** v.50, p.137 - 142, 2013.

SILVA, S.N.R.L.; FARIAS, C.B.B.; RUFINO, R.D.; LUNA, J.M.; SARUBBO, L.A. Glycerol as substrate for the production of biosurfactant by *Pseudomonas aeruginosa* UCP0992. **Colloids and Surfaces B: Biointerfaces**, v.79, p.174 - 183, 2010.

SINGH, A.; VAN-HAMME, J.D.; WARD, O.P. Surfactants in microbiology and biotechnology: Part 2. Application aspects. **Biotechnology Advances**, v. 25, p. 99-121, 2007.

SLIZOVSKIY, I.B.; KELSEY, J.W.; HATZINGER, P.B. Surfactant-facilitated remediationof metal-contaminated soils: efficacy and toxicological consequences toearthworms. **Environmental Toxicology and Chemistry**. v. 30, p. 112–123, 2011.

SOBRINHO, H.B.S.; LUNA, J.M.; RUFINO, R.D.; PORTO, A.L.F.; SARUBBO, L.S. Assessment of toxicity of a biosurfactant from *Candida sphaerica* UCP 0995 cultivated with industrial residues in a bioreactor. **Electronic Journal of Biotechnology**, v. 16, n. 4, 2013.

SOBRINHO, H. B. S.; RUFINO, R. D.; LUNA, J. M.; SALGUEIRO, A. A.; CAMPOS-TAKAKI, G. M.; LEITE, L. F. C.; SARUBBO, L. A. Utilization of two agroindustrial by-products for the production of a surfactant by *Candida sphaerica* UCP0995. **Process Biochemistry***,* v. 43, p. 912-917, 2008.

SOUZA, E.C.; VESSONI-PENNA, T.C.; OLIVEIRA, R.P.S. Biosurfactant-enhanced hydrocarbon bioremediation: An overview. **International Biodeterioration & Biodegradation,** v. 89, p. 88-94, 2014.

SUDHAKAR-BABU, P.; VAIDYA, A.N.; BAL, A. S.; KAPUR, R., JUWARKA, A.; KHANNA, P. Kinetics of biosurfactants production by *Pseudomonas aeruginosa* strain from industrial wastes. **Biotechnology Letters**, v. 18*,* p.263-268,1996.

SUMMERS, D. Enhancing Oil Recovery: a Look at Stripper Wells [Online]. Available: www.oilprice.com (10 January 2011).

SWARNKAR, V.; AGRAWAL, N.; TOMAR, R. Sorption of chromate and arsenate bysurfactant modified erionite (E-SMZ). **Journal of Dispersion Science and Technology,** v. 33, p.919–927, 2012.

SYLDATIK, C.; WAGNER, F. IN: KOSARIC, N.; CAIRNS, W. L.; GRAY, N. C. C. (Eds.) **Biosurfactants and Biotechnology**, New York: Marcel Dekker, p. 89-120, 1987.

TAN, H.M. **Biosurfactants and their roles in bioremediation**. Cheong Jit Kong, p. 1-12, 2000.

THANIYAVARN, J.; CHIANGUTHAI, T.; SANGVANICH, P.; ROONGSAWANG, N.; WASHIO, K.; MORIKAWA, M. ; THANIYAVARN, S. Production of sophorolipid biosurfactant by *Pichia anomala*. **Bioscience, Biotechnology and Biochemistry,** v.72, p. 2061–2068, 2008.

THOMPSON, D. N.; FOX, S. L.; BALA, G. A. Biosurfactants from potato process effluents. **Applied Biochemistry and Biotechnology**, ed. 84, v. 84, p. 917-930, 2000.

TOKUMOTO, Y.; NOMURA, N.; UCHIYAMA, H.; IMURA, T.; MORITA, T. ;FUKUOKA T.; KITAMOTO, D. Structural characterization and surface-active properties of a succinoyl trehalose lipid produce by *Rhodococcus* sp. SD-74. **Journal of Oleo Science,** v.58, p. 97–102, 2009.

US Energy Information Administartion. Annual Energy Review, 2010. Available: 205.254.135.24/totalenergy/data/annual/pdf/aer.pdf., 2010.

VAN-HAMME, J.D.; SINGH, A.; WARD, O.P. Physiological aspects Part 1 in a series of papers devoted to surfactants in microbiology and biotechnology. **Biotechnology Advances,** v.24, p. 604-620, 2006.

VELIKONJA, J.; KOSARIC. Biosurfactants in Food Applications. In: Biosurfactants: Production: Properties: Applications, Kosaric, N. and F.V. Sukan (Eds.). Chapter 16, CRC Press, New York, ISBN-13: 9780824788117, p. 419-448, 1993.

VIJAYAKUMAR, S.; SARAVANAN, V. Biosurfactants-Types, Sources and Applications. **Research Journal of Microbiology,** v. 10, p. 181-192, 2015.

WEBER, L.; DOGE, C.; HAUFE, G.; HOMMEL, R.; KLEBER, H.P. Oxygenation of hexadecane in the biosynthesis of cyclic glycolipids in *Torulopsis apicola*. **Biocatalysis**, v. 5, p.267-292, 1992.

WEN, J.; STACEY, S.P.; MCLAUGHLIN, M.J.; KIRBY, J.K. Biodegradation of rhamnolipid, EDTA and citric acid in cadmium and zinc contaminated soils. **Soil Biology and Biochemistry**, v. 41, p.2214–2221, 2009.

WEST, C.C.; HARWELL, J.H. Surfactant and subsurface remediation. **Environmental Science &Technology, v.** 26, 2324-2330, 1992.

<http://www.natureswaygreen.com/bioremediation.htm> acessado no dia 15/03/2015 às 11hs.

CAPÍTULO 1

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Review

Biosurfactants: Multifunctional Biomolecules of the 21st Century

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Abstract: In the era of global industrialisation, the exploration of natural resources has served as a source of experimentation for science and advanced technologies, giving rise to the manufacturing of products with high aggregate value in the world market, such as biosurfactants. Biosurfactants are amphiphilic microbial molecules with hydrophilic and hydrophobic moieties that partition at liquid/liquid, liquid/gas or liquid/solid interfaces. Such characteristics allow these biomolecules to play a key role in emulsification, foam formation, detergency and dispersal, which are desirable qualities in different industries. Biosurfactant production is considered one of the key technologies for development in the 21st century. Besides exerting a strong positive impact on the main global problems, biosurfactant production has considerable importance to the implantation of sustainable industrial processes, such as the use of renewable resources and "green" products. Biodegradability and low toxicity have led to the intensification of scientific studies on a wide range of industrial applications for biosurfactants in the field of bioremediation as well as the petroleum, food processing, health, chemical, agricultural and cosmetic industries. In this paper, we offer an extensive review regarding knowledge accumulated over the years and advances achieved in the incorporation of biomolecules in different industries.

Keywords: biosurfactant; surface tension; critical micelle concentration; biodegradability; functional properties; physiology; kinetics; recovery; industrial applications

1. Introduction

Surfactants are amphipathic compounds with both hydrophilic and hydrophobic moieties that preferentially partition between liquid interfaces with different degrees of polarity and hydrogen bridges, such as oil/water or air/water interfaces. The apolar moiety is often a hydrocarbon chain, whereas the polar moiety may be ionic (cationic or anionic), non-ionic or amphoteric [1,2], as illustrated in Figure 1.

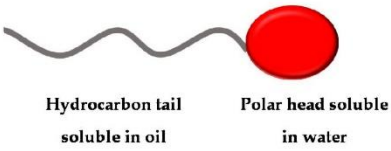

Figure 1. Surfactant molecule with apolar (hydrophobic) and polar (hydrophilic) moieties.

Surfactants increase the solubility of hydrophilic molecules, thereby reducing both surface and interfacial tensions at the oil/water interface [3]. The critical micelle concentration (CMC) is the concentration of surfactant at which organised molecular assemblies, known as micelles, are formed (Figure 2) and corresponds to the point at which the tensioactive agent achieves the lowest stable surface tension (Figure 3) [4].

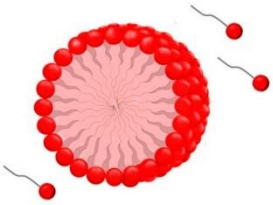

Figure 2. Schematic illustration of tensioactive agent and micelle formation.

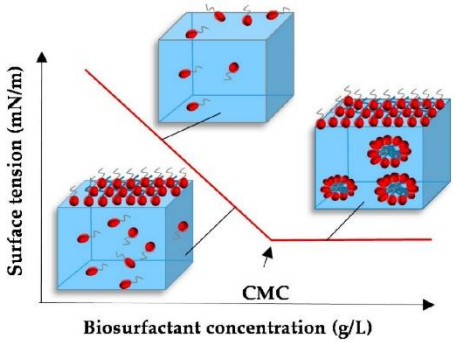

Figure 3. Illustration of regions in which micelle formation occurs (critical micelle concentration CMC).

Most currently produced surfactants are chemically derived from petroleum. However, such synthetic tensioactive agents are generally toxic and difficult to break down through the action of microorganisms. In recent years, such problems have motivated the scientific community to seek surfactants that are more environmentally friendly, such as those achieved through microbial production, known as biosurfactants [5]. Moreover, concerns regarding the environment on the part of consumers and new environmental control legislation have led to the development of natural surfactants as an alternative to existing products.

Studies involving biosurfactants began in the 1960s and the use of these compounds has expanded in recent decades [2,6]. Biosurfactants have drawn the interest of different industries due to advantages such as structural diversity, low toxicity, greater biodegradability, ability to function in wide ranges of pH, temperature and salinity as well as greater selectivity, lower CMC and production involving renewable sources/industrial waste and industrial by-products [7–9]. The present review demonstrates

the reasons for which biosurfactants are considered the multifunctional materials of the 21st century, with a description of concepts, properties, classification, modes of production, physiology and uses in the most diverse industries.

2. Producing Microorganisms

Microorganisms use a set of carbon sources and energy for growth. The combination of carbon sources with insoluble substrates facilitates the intracellular diffusion and production of different substances [10–12]. Microorganisms (yeasts, bacteria and some filamentous fungi) are capable of producing biosurfactants with different molecular structures and surface activities [4]. In recent decades, there has been an increase in scientific interest regarding the isolation of microorganisms that produce tensioactive molecules with good surfactant characteristics, such as a low CMC, low toxicity and high emulsifying activity [2].

The literature describes bacteria of the genera *Pseudomonas* and *Bacillus* as great biosurfactant producers [2]. However, most biosurfactants of a bacterial origin are inadequate for use in the food industry due to their possible pathogenic nature [13]. *Candida bombicola* and *Candida lipolytica* are among the most commonly studied yeasts for the production of biosurfactants. A key advantage of using yeasts, such as *Yarrowia lipolytica*, *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, resides in their "generally regarded as safe" (GRAS) status. Organisms with GRAS status do not offer the risks of toxicity or pathogenicity, which allows their use in the food and pharmaceutical industries [4]. Table 1 displays a list of microorganisms that produce biosurfactants.

3. Classification

Most biosurfactants are either anionic or neutral, whereas those that contain amine groups are cationic. The hydrophobic moiety has long-chain fatty acids and the hydrophilic moiety can be a carbohydrate, cyclic peptide, amino acid, phosphate carboxyl acid or alcohol. The molar mass of biosurfactants generally ranges from 500 to 1500 Da [14]. Biosurfactants are generally categorised by their microbial origin and chemical composition, as follows [3,5,15].

3.1. Glycolipids

Rhamnolipids, sophorolipids and trehalolipids are the best known glycolipids [16]. Rhamnolipids were found as exoproducts of the pathogen *P. aeruginosa* and are a combination of α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-Rha-C10-C10) and α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoate (Rha-Rha-C10) as well as their mono-rhamnolipid congeners (Rha-C10-C10 and Rha-C10) [17]. Sensitive analytical techniques have led to the discovery of rhamnolipid congeners and homologues (approximately 60) produced at different concentrations by species of *Pseudomonas* and bacteria belonging to other families, classes or even phyla [16]. For instance, various species of *Burkholderia* have been shown to produce rhamnolipids that have longer alkyl chains than those produced by *P. aeruginosa* [17–19]. Surface tensions values of 29 mN/m constitute a characteristic of such components, which can be produced using different substrates, such as alkanes, pyruvate, citrates, fructose, glycerol, olive oil and glucose [20]. Most studies involving rhamnolipids focus mainly on assessing the biodegradation efficiency of petroleum hydrocarbons [21,22]. Although researchers have found increased dissipation of target contaminant upon the addition of rhamnolipids, a decrease in biodegradation efficiency or no effect following rhamnolipid supplementation have also been reported [16,20]. The presence of surfactant molecules may induce changes in the microbial community, which, in turn, correspond to different degradation patterns. Interestingly, although rhamnolipids are considered biodegradable, few reports have demonstrated that these substances can be co-degraded or solely utilised as a carbon and energy source by various monocultures [18]. Rhamnolipids are described as potentially toxic to natural vegetation [23], but have also been found to reduce the toxicity of specific compounds by increasing hydrocarbon solubilisation, thereby facilitating biodegradation [24,25].

Table 1. Main classes of biosurfactants and respective producing microorganisms.

Biosurfactant Class					
Glycolipids	**Polymeric Surfactants**	**Lipopeptides**	**Fatty Acids**	**Particulate Surfactant**	**Phospholipids**
Producer microorganisms					
Acinetobacter calcoaceticus *Alcanivorax borkumensis* *Arthrobacter paraffineus* *Arthrobacter* sp. *Candida antartica* *Candida apicola* *Candida batistae* *Candida bogoriensis* *Candida bombicola* *Candida ishiwadae* *Candida lipolytica* *Lactobacillus fermentum* *Nocardia* sp. *Pseudomonas aeruginosa* *Pseudomonas* sp. *Rhodococcus erythropolis* *Rhodotorula glutinus* *Rhodotorula graminus* *Serratia marcescens* *Tsukamurella* sp. *Ustilago maydis*	*Acinetobacter calcoaceticus* *Acinetobacter calcoaceticus* *Acinetobacter calcoaceticus* *Acinetobacter calcoaceticus* *Bacillus stearothermophilus* *Candida lipolytica* *Candida utilis* *Halomonas eurihalina* *Mycobacterium thermoautotrophium* *Sphingomonas paucimobilis*	*Acinetobacter* sp. *Bacillus licheniformis* *Bacillus pumilus* *Bacillus subtilis* *Candida lipolytica* *Gluconobacter cerinus* *Pseudomonas fluorescens* *Serratia marcescens* *Streptomyces sioyaensis* *Thiobacillus thiooxidans*	*Arthrobacter paraffineus* *Capnocytophaga* sp. *Corynebacterium insidibasseosum* *Corynebacterium lepus* *Nocardia erythropolis* *Penicillium spiculisporum* *Talaramyces trachyspermus*	*Acinetobacter calcoaceticus* *Cyanobacteria* *Pseudomonas marginalis*	*Acinetobacter* sp. *Aspergillus* *Corynebacterium lepus*

Sophorolipids are produced by yeasts that belong to the genus *Candida* [26,27]. These glycolipids have a dimeric carbohydrate sophorose linked to a long-chain hydroxyl fatty acid through a glycosidic bond. Sophorolipids and lactone form a sophorolipid that is preferable in many applications [28,29]. *C. bombicola* stands out among the different types of yeasts used in the production of these biosurfactants. Surface tension values of approximately 33 mN/m and a reduction in the surface tension of *n*-hexadecane and water from 40 to 5 mN/m has been recorded for these agents [30]. Mannosylerythritol lipids (MEL), which are yeast glycolipids, are one of the most promising biosurfactants known and are abundantly produced from vegetable oils by *Pseudozyma* (previously *Candida*) *antarctica* [31]. Trehalolipids are produced by species of *Mycobacterium*, *Nocardia* and *Corynebacterium*. Trehalolipids from *Arthrobacter* spp. and *Rhodococcus erythropolis* are able to lower surface and interfacial tensions in culture broth to 25–40 and 1–5 mN/m, respectively [5].

3.2. Fatty Acids, Phospholipids and Neutral Lipids

Different bacteria and yeasts produce large amounts of fatty acids and phospholipid surfactants during growth on n-alkanes. Phosphatidyl ethanolamine-rich vesicles are produced from *Acinetobacter* spp. and form optically clear microemulsions of alkanes in water. These biosurfactants are essential to medical applications. According to Gautam and Tyagi [28], phospholipid protein complex deficiency is the major cause of respiratory failure in the children born prematurely. The authors also suggest that the isolation and cloning of genes involved in the production of surfactants can be used in fermentative processes [28].

3.3. Polymeric Biosurfactants

Emulsan, lipomanan, alasan, liposan and other polysaccharide protein complexes are the best-studied polymeric biosurfactants. Emulsan is an emulsifier for hydrocarbons in water at concentrations as low as 0.001% to 0.01% [31,32]. Liposan is an extracellular water soluble emulsifier synthesised by *C. lipolytica* and is made up of 83% carbohydrates and 17% proteins. Chakrabarti [33] discuss the application of liposan as an emulsifier in the food and cosmetic industries.

3.4. Particulate Biosurfactants

Particulate biosurfactants partition extracellular membrane vesicles to form a microemulsion that exerts an influence on alkane uptake in microbial cells. The *Acinetobacter* spp. has vesicles with a diameter of 20 to 50 nm and a buoyant density of 1.158 cubic gcm composed of proteins, phospholipids and lipo-polysaccharides [5,33].

Figure 4 illustrates the chemical structure of the most studied microbial surfactants. Table 1 also displays biosurfactant classes and their producers.

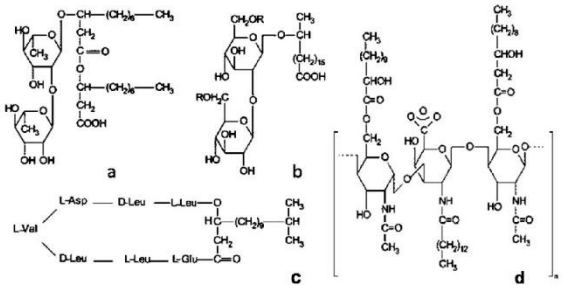

Figure 4. Chemical structure of most studied microbial surface-active compounds. (**a**) Rhamnolipid; (**b**) Sophorolipid; (**c**) Surfactin and (**d**) Emulsan.

4. Properties

It is necessary to submit a biosurfactant to conservation methods to evaluate its properties (surface tension and dispersion) over a period of 120 days to estimate the commercial validity of the product. Thus, heating methods are used separately or in combination with potassium sorbate, which is a conservative that inhibits the growth of mould that is widely used in the production and conservation of foods. Some characteristics are common to the majority of biosurfactants and have advantages over conventional surfactants, as described below [5].

4.1. Surface and Interfacial Activity

Efficiency and effectiveness are essential characteristics of a good surfactant. Efficiency is measured by the CMC, whereas effectiveness is related to surface and interfacial tensions [34]. The CMC of biosurfactants ranges from 1 to 2000 mg/L, whereas interfacial (oil/water) and surface tensions are respectively approximately 1 and 30 mN/m. Good surfactants are able to reduce water surface tension from 72 to 35 mN/m and the interfacial tension of *n*-hexadecane from 40 to 1 mN/m.

4.2. Tolerance to Temperature, pH and Ionic Strength

Many biosurfactants can be used at high temperatures and pH values ranging from 2 to 12. Biosurfactants also tolerate a salt concentration up to 10%, whereas 2% NaCl is enough to inactivate synthetic surfactants.

4.3. Biodegradability

Biosurfactants are easily degraded by microorganisms in water and soil, making these compounds adequate for bioremediation and waste treatment.

4.4. Low Toxicity

Low degree of toxicity allows the use of biosurfactants in foods, cosmetics and pharmaceuticals. Low toxicity is also of fundamental importance to environmental applications.

Biosurfactants can be produced from largely available raw materials as well as industrial waste.

4.5. Specificity

Biosurfactants are complex molecules with specific functional groups and therefore often have specific action. This is of particular interest in the detoxification of different pollutants and the de-emulsification of industrial emulsions as well as specific food, pharmaceutical and cosmetic applications.

4.6. Biocompatibility and Digestibility

These properties allow the use of biomolecules in different industries, especially the food, pharmaceutical and cosmetic industries.

4.7. Emulsion Forming/Breaking

Biosurfactants can be either emulsifiers or de-emulsifiers. An emulsion is a heterogeneous system consisting of an immiscible liquid dispersed in another liquid in the form of droplets, the diameter of which generally exceeds 0.1 mm. There are two basic types of emulsion: oil-in-water (o/w) and water-in-oil (w/o). Emulsions have minimal stability, but the addition of biosurfactants can lead to an emulsion that remains stable for months or even years [35]. Liposan, which is a water-soluble emulsifier synthesised by *C. Lipolytica*, has been used with edible oils to form stable emulsions. Liposan is commonly used in the cosmetic and food industries for producing stable oil/water emulsions [4,36].

5. Factors Affecting Biosurfactant Production

The production of biosurfactants can be either spontaneous or induced by the presence of lipophilic compounds, variations in pH, temperature, aeration and agitation speed or when cell growth is maintained under conditions of stress, such as a low concentration of nitrogen [37]. The various physicochemical factors are discussed below [38].

5.1. Carbon Source

The carbon source plays an important role in the growth and production of biosurfactants by microorganisms and varies from species to species. A very low yield was found when only either glucose or vegetable oil was used for the production of a biosurfactant by *T. bombicola*, but the yield increased to 70 g/L when both carbon sources were provided together [39]. At a concentration of 80 and 40 g/L of glucose and soybean oil, respectively, the maximum yield of sophorose lipids was obtained by *T. bombicola* [40]. Even higher yields of sophorolipids (120 g/L) were produced with *C. bombicola* in eight days when sugar and oil were used as carbon sources [41]. When canola oil and glucose were used as carbon sources at concentrations of 10% each, maximum yield of sophorolipids (8 g/L) was obtained from *C. lipolytica* [42]. Moreover, when industrial waste was used for the production of a biosurfactant by *C. lipolytica*, the yield of the protein-lipid-carbohydrate complex was 4.5 g/L, with a reduction in the surface tension of distilled water from 71 to 32 mN/m [43]. A high production of bioemulsifier was obtained with *C. lipolytica* when supplemented with 1.5% glucose (*w*/*v*) [44]. *C. antarctica* and *C. apicola* yielded 13.4 and 7.3 g/L of sophorolipids, respectively, when soapstock was used at a concentration of 5% (*v*/*v*) [45]. The resting cells of *Pseudozyma (C. antarctica)* were found to covert C_{12} to C_{18} n-alkanes into mannosylerythritol lipids (MEL); the yield was 140 g/L after four weeks and the biosurfactant was able to emulsify soybean oil [46]. A change in the fatty acid constitution of the final biosurfactant occurred when the fatty acid composition was changed in the fermentation medium containing *C. ingens* [47].

5.2. Nitrogen Sources

This is the second most important supplement for the production of biosurfactants by microorganisms. In fermentative processes, the C/N ratio affects the buildup of metabolites. High C/N ratios (*i.e.*, low nitrogen levels) limit bacterial growth, favouring cell metabolism towards the production of metabolites. In constrast, excessive nitrogen leads to the synthesis of cellular material and limits the buildup of products [48]. Different organic and inorganic nitrogen sources have been used in the production of biosurfactants. Santa Anna *et al.* [49] describe the importance of nitrogen for the production of a biosurfactant by *P. aeruginosa* cultivated in a mineral medium containing 3% glycerol. As $NaNO_3$ proved more effective than $(NH_4)_2SO_4$, nutritional limitations clearly guide the cell metabolism to the formation of the product. Mulligan and Gibbs [50] report that *P. aeruginosa* uses nitrates, ammonium and amino acids as nitrogen sources. Nitrates are first reduced to nitrite and then ammonium. Ammonium is assimilated either by glutamate dehydrogenase (EC 1.4.1.4) to form glutamate or glutamine synthetase (EC 6.3.1.2) to form glutamine. Glutamine and α-ketoglutarate are then converted to glutamine by L-glutamine 2-oxoglutarate aminotransferase (EC 1.4.1.13). However, lipid formation rather than sugar is the rate-determining factor in the biosynthesis of rhamnolipids and nitrogen limitation can lead to the accumulation of lipids. In comparison to ammonium, the assimilation of nitrate is slower and simulates nitrogen limitation, which is favourable to the production of rhamnolipids. High yields of sophorose lipids, which are biosurfactants produced by the fungi *T. bombicola* and *C. Bombicola*, have been achieved using yeast extract and urea as the nitrogen source [51]. Moreover, high yields of mannosylerythritol lipid by *Candida* sp. SY16, *C. lipolytica* and *C. glabrata* have been achieved with ammonium nitrate and yeast extract [42,43,46,52–54].

5.3. Growth Conditions

Growth conditions (temperature, pH, agitation speed and oxygen) also influence biosurfactant production [37]. Species of the genus *Candida* produce maximum biosurfactant yields in a wide pH range, such as pH 5.7 for *C. glabrata* UCP 1002, pH 7.8 for *Candida* sp. SY16, pH 5.0 for *C. Lipolytica* and pH 6.0 for *C. batistae* [52,54–56]. Moreover, *Pichia anamola* and *Aspergillus ustus* produce maximum biosurfactant yield at pH 5.5 and 7.0, respectively [57,58]. Different microbial processes are affected by even a small change in temperature. The most favourable temperature for the production of biosurfactants by different fungi is 30 °C, as observed for different species of *Candida*, viz. *Candida* sp. SY16, *C. bombicola*, *C. batistae* and *T. bombicola* [39,51,52,56]. In case of *C. lipolytica*, 27 °C has been found to be the best temperature. Incubation time also exerts a significant effect on biosurfactant production. Microorganisms produce biosurfactants in different time intervals. Maximum biosurfactant production by *Aspergillus ustus* was found after five days of incubation, whereas the incubation periods for *C. bombicola* were seven, eight and 11 days [59,60]. Maximum biosurfactant production by *C. bombicola* grown in animal fat was found after 68 h of incubation [49]. Moreover, an increase in agitation speed favoured the accumulation of a biosurfactant by *P. aeruginosa* UCP 0992 grown in glycerol [61]. Oliveira *et al.* [62] studied the effect of a change in agitation speed of cultures from 50 to 200 rpm on *P. alcaligenes* cultivated in palm oil. The authors found that the increase in rotation velocity favoured a reduction in the surface tension of the cell-free broth to 27.6 mN/m. In contrast, Cunha *et al.* [63] found that agitation had a negative effect regarding a reduction in surface tension using a biosurfactant from *Serratia* sp. SVGG16 grown in a hydrocarbon culture.

6. Metabolic Pathways of Biosurfactant Production

Hydrophilic substrates are primarily used by microorganisms for cell metabolism and the synthesis of the polar moiety of a biosurfactant, whereas hydrophobic substrates are used exclusively for the production of the hydrocarbon portion of the biosurfactant [37,64]. Diverse metabolic pathways are involved in the synthesis of precursors for biosurfactant production and depend on the nature of the main carbon sources employed in the culture medium. For instance, when carbohydrates are the only carbon source for the production of a glycolipid, the carbon flow is regulated in such as way that both lipogenic pathways (lipid formation) and the formation of the hydrophilic moiety through the glycolytic pathway are suppressed by the microbial metabolism, as illustrated in Figure 5 [65].

A hydrophilic substrate, such as glucose or glycerol, is degraded until forming intermediates of the glycolytic pathway, such as glucose 6-phosphate, which is one of the main precursors of carbohydrates found in the hydrophilic moiety of a biosurfactant. For the production of lipids, glucose is oxidised to pyruvate through glycolysis and pyruvate is then converted into acetyl-CoA, which produces malonyl-CoA when united with oxaloacetate, followed by conversion into a fatty acid, which is one of the precursors for the synthesis of lipids [66]. When a hydrocarbon is used as the carbon source, however, the microbial mechanism is mainly directed to the lipolytic pathway and gluconeogenesis (the formation of glucose through different hexose precursors), thereby allowing its use for the production of fatty acids or sugars. The gluconeogenesis pathway is activated for the production of sugars. This pathway consists of the oxidation of fatty acids through β-oxidation to acetyl-CoA (or propionyl-CoA in the case of odd chain fatty acids). Beginning with the formation of acetyl-CoA, the reactions involved in the synthesis of polysaccharide precursors, such as glucose 6-phosphate, are essentially the inverse of those involved in glycolysis. However, reactions catalysed by pyruvate kinase and phosphofructokinase-1 are irreversible. Thus, other enzymes exclusive to the process of gluconeogensis are required to avoid such reactions. Figure 6 illustrates the main reactions through to the formation of glucose 6-phosphate, which is the main precursor of polysaccharides and disaccharides formed for the production of the hydrophilic moiety of glycolipids [67].

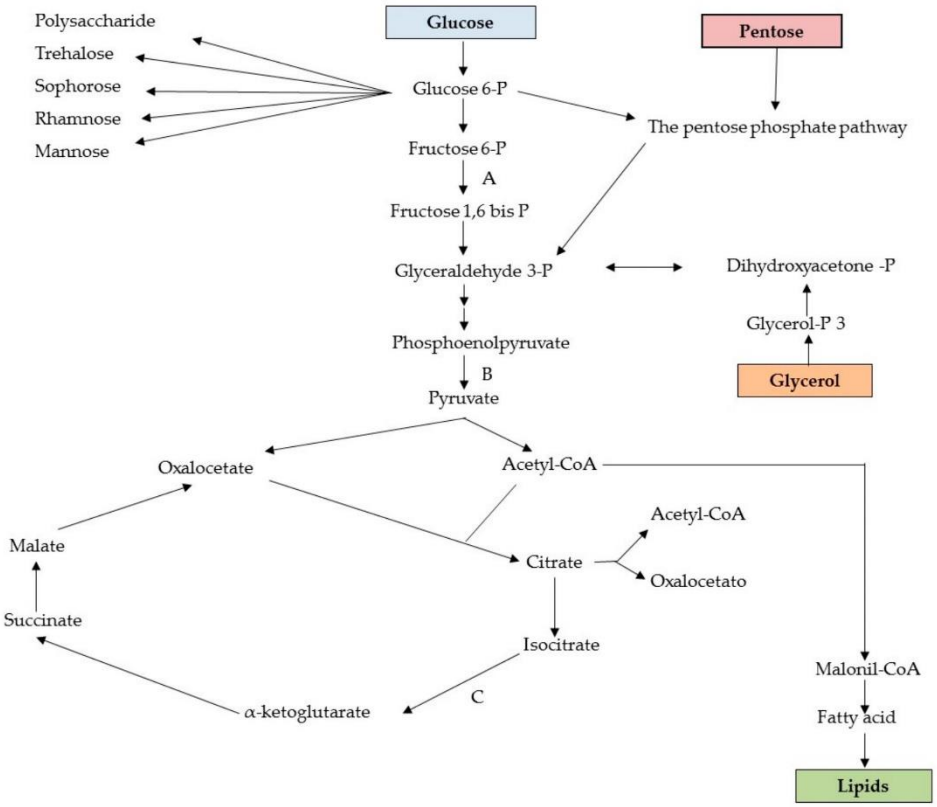

Figure 5. Intermediate metabolism related to synthesis of biosurfactant precursors with use of carbohydrates as substrate. Enzyme keys for control of carbon flow: (**A**) phosphofructokinase; (**B**) pyruvate kinase; (**C**) isocitrate dehydrogenase.

According to Sydatk and Wagner [68], the biosynthesis of a surfactant occurs through four different routes: (a) carbohydrate and lipid synthesis; (b) synthesis of the carbohydrate half while the synthesis of the lipid half depends on the length of the chain of the carbon substrate in the medium; (c) synthesis of the lipid half while the synthesis of the carbon half depends on the substrate employed; and (d) synthesis of the carbon and lipid halves, which are both dependent on the substrate. Therefore, the length of the n-alkane chain used as the carbon source alters the biosynthesis of a surfactant. Kitamoto *et al*. [46] studied the production of manosilerythritol lipid (MEL) by the yeast *C. antarctica* in the presence of different n-alkanes and found that this species does not grow or produce a biosurfactant in media containing C_{10} to C_{18}. However, production occurred when the species was grown in a medium containing C_{12} to C_{18} and octadekane as substrate led to the greatest yield. In contrast, production was minimal in media containing n-alkanes with more than 19 carbons.

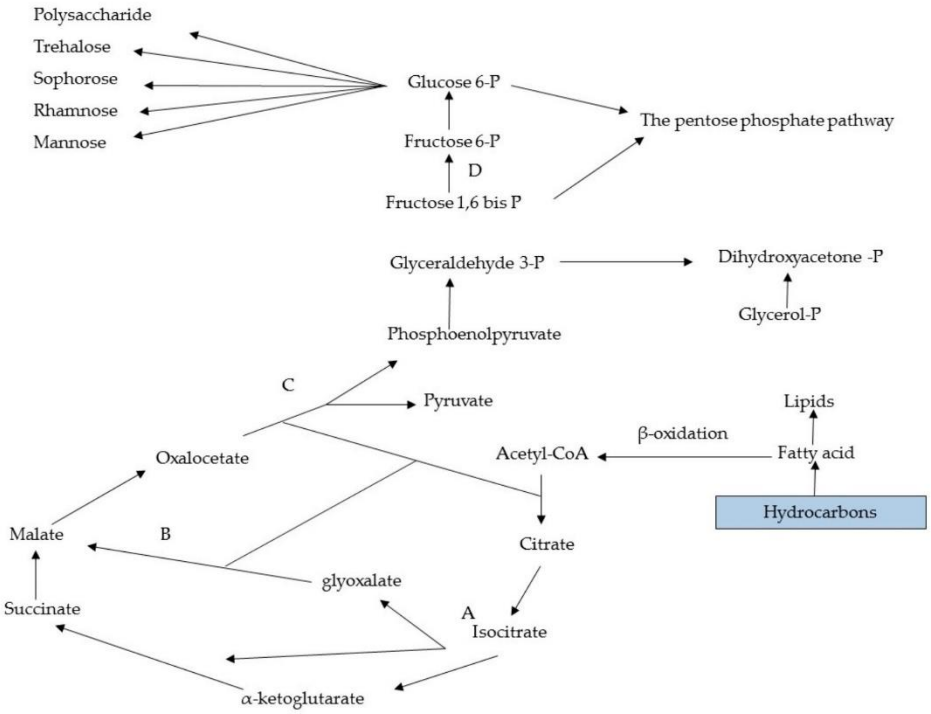

Figure 6. Intermediate metabolism related to synthesis of precursors of biosurfactant using hydrocarbons as substrate. Key enzymes: (**A**) isocitrate lyase; (**B**) malate synthase; (**C**) phosphoenolpyruvate; (**D**) fructose-1.

7. Physiology

Biosurfactants are produced by microorganisms either through excretion or adhesion to cells, especially when cultivated on substrates that are insoluble in water. While the function in microbial cells is not yet fully understood, it has been speculated that biosurfactants are involved in the emulsification of insoluble substrates [37,69].

The main physiological role of biosurfactants is to allow microorganisms to grow on substrates that are insoluble in water through a reduction in surface tension between phases, making the substrate more available for uptake and metabolism. The uptake mechanisms of these substrates (such as, alkanes) are not yet fully clarified. The direct uptake of dissolved hydrocarbons in the aqueous phase, direct contact between cells and large hydrocarbon droplets, and the interaction with emulsified droplets (emulsion) have been described. Besides the emulsification of the carbon source, biosurfactants are also involved in the adhesion of microbial cells to hydrocarbons, as discussed in the following sections. Microorganism cell adsorption to insoluble substrates and the excretion of surfactant compounds allow growth on carbon sources [37].

8. Fermentation Kinetics

Biosurfactant production kinetics has considerable variation among different systems. For convenience, kinetic parameters are grouped as follows: (a) growth-associated production; (b) production under growth-limiting conditions; (c) production by resting or immobilised cells; and (d) production with precursor supplementation [37]. In growth-associated production, parallel relationships are found between growth, the use of the substrate and biosurfactant production. Production under growth-limiting conditions is characterised by an accentuated increase in biosurfactant concentration as a result of the limitation of one or more medium components. Production by resting or immobilised cells is a type of biosurfactant production in which there is no cell multiplication; the cells nonetheless continue to use the carbon source for biosurfactant synthesis. Investigators report that the addition of biosurfactant precursors to the medium leads to qualitative and quantitative changes in the final product.

9. Raw Materials for Biosurfactant Production

Current society is characterised by an increase in expenditures, the need to reuse materials and environmental concerns. Consequently, greater emphasis has been given to recovery, recycling and reuse. Indeed, the need for environmental preservation has led to the reuse of different industrial wastes. This is particularly valid for the food production industry, the waste products, effluents and by-products of which can be reused [70]. Industrial waste had piqued the interest of researchers as a low-cost substrate for biosurfactant production [71]. The selection of waste products should ensure the proper balance of nutrients to allow microbial growth and consequent biosurfactant production. Industrial waste with a high content of carbohydrates or lipids is ideal for use as substrate [71]. According to Barros *et al.* [34], the use of agro-industrial waste is one of the steps towards the implantation of feasible biosurfactant production on an industrial scale, for which the optimisation of the different variables involved is required.

The literature describes a number of waste products employed in biosurfactant production, such as vegetable oils, oily effluents [42,72,73], starchy effluents [74,75], animal fat [51,76–78], vegetable fat [79], vegetable cooking oil waste [72,80–82], soapstock [76,83,84] molasses [85–88], dairy industry waste (whey) [89], corn steep liquor [43,71,90–92], cassava flour wastewater [93], oil distillery waste [43,90,94,95] and glycerol [61]. Some of the most commonly employed industrial waste products for biosurfactant production are detailed below.

9.1. Olive Oil Mill Effluent (OOME)

Olive Oil Mill Effluent (OOME) is a concentrated black liquor with a water-soluble portion of ripe olives and water that is used for the extraction of olive oil. OOME has polyphenols that represent a challenge in terms of the environment disposal. However, it also contains nitrogen compounds (12 to 24 g/L), sugars (20 to 80 g/L), residual oil (0.3 to 5 g/L) and organic acids (5 to 15 g/L). Mercade *et al.* [73] successfully employed OOME for the strain *Pseudomonas* sp. to produce rhamnolipids.

9.2. Animal Fat

Animal fat and lard can be obtained in large quantities from the meat processing industry and have been used as a medium for cooking foods. Recently, however, such fats have lost a large part of the market to vegetable oils due to the lower degree of harm to health caused by the latter [70]. Animal fat stimulates the production of sophorolipids by the yeast *C. bombicola* [51]. Using animal fat and corn steep liquor, Santos *et al.* [77,78] achieved maximum glycolipid production by the yeast *C. lipolytica* UCP 0988. The authors also report that the product has uses in bioremediation as well as oil mobilisation and recovery.

9.3. Frying Oils

Fry oil and edible fats are considered great carbon sources for biosurfactant production. Vegetable oils constitute a lipid carbon source and are mainly comprised of saturated or unsaturated fatty acids with chains of 16 to 18 carbon atoms [9]. Different oils are adequate substrates for biosurfactants. Babassu oil (5% *v*/*v*) with a carbon source (1% glucose *w*/*v*) is a good medium for biosurfactant production. Sarubbo *et al.* [96] found that two strains of *C. lipolytica* (1055 and 1120) produce biosurfactants toward the final of the exponential growth phase and onset of the stationary phase. Sunflower and olive oils have proven to be adequate energy and carbon sources for the production of biosurfactants. *P. aeruginosa* strains produce a biosurfactant on residue from corn, soybean and canola oil plants [97,98]. Canola oil residue and sodium nitrate has been reported adequate for microbial growth and the production of up to 8.50 g/L of rhamnoipids. The combination of glucose and canola oil has been used for the successful production of a biosurfactant by *C. lipolytica* [42].

9.4. Soapstocks

Oil cakes or soapstocks are produced from oilseed processing involving the refining of seed-based edible oils with the use of chemicals [76]. Soapstock has been used together with sunflower oil, olive oil or soybean oil as substates to produce rhamnolipids. Yields as high as 15.9 g/L have been reported using *P. aeruginosa* in a soapstock medium [83]. Soapstock and oil refinery wastes have been used with *C. antarctica* or *C. apicola* for biosurfactant production, achieving a greater yield than that in the medium without oil refinery residue [45]. Shabtai [84] also report the production of two extracellular heteropolysaccharides (emulsan and biodispersan) by *A. calcoaceticus* and *A. calcoaceticus*, respectively, using soapstock as a carbon source.

9.5. Molasses

Molasses is a by-product of sugarcane and beet processing. This inexpensive substrate has dry matter (75%), non-sugar organic matter (9%–12%), protein (2.5%), and potassium (1.5%–5.0%), as well as magnesium, phosphorus and calcium (≈1%). The inositol, biotin, thiamine and pantothenic acid contents (1%–3%) give molasses its thick consistency and brown colour. The high sugar content (48%–56%) makes molasses adequate for biosurfactant production by different microorganisms. Laboratories have used molasses for the production of different microbial metabolites. According to Makkar and Cameotra [88], *Bacillus subtilis* in a minimal medium supplemented with molasses as the carbon source produces a biosurfactant. Joshi *et al.* [99] used molasses as well as other carbon sources to produce biosurfactants from strains of *Bacillus*.

9.6. Whey

The dairy industry produces large quantities of whey, such as whey waste, cheese whey, curd whey and lactic whey, all of which can be used as substrates for the microbial production of metabolites [70,100–102]. A high amount of lactose (approximately 75%) is found in lactic whey. Other components, such as proteins, vitamins and organic acids, are good sources for microbial growth and biosurfactant production [75]. Moreover, whey disposal represents a major pollution problem, especially in countries that depend on a dairy economy [103]. Thus, the disposal of this by-product represents a waste of a widely available substrate and an environmental hazard.

9.7. Corn Steep Liquor

The agro-industry of corn-based products through wet processing results in both solid and liquid by-products, which, when disposed improperly, become a source of contamination and harm to the environment. Corn steep liquor is a by-product of the washing water and soaking of kernels as well as fractioning into starch and germen (oil) that contains 40% solid matter. This by-product consists of 21% to 45% proteins, 20% to 26% lactic acid, approximately 8% ash (containing Ca^{2+}, Mg^{2+},

K^+, *etc.*), approximately 3% carbohydrates and a low fat content (0.9% to 1.2%) [103–105]. Nut oil refinery residue and corn steep liquor are low-cost nutrients for the production of glycolipids by *C. sphaerica* (UCP 0995). The biosurfactant of this strain mobilises and removes up to 95% of motor oil on sand, making it useful for bioremediation [89,94,106]. Silva *et al.* [107] also report the production of a biosurfactant from *P. cepacia* grown in mineral medium supplemented with 2.0% corn steep liquor and 2.0% soybean waste frying oil.

9.8. Starchy Substrates

Abundant starch-based substrates also constitute renewable carbon sources. The potato processing industry produces significant amounts of starch-rich waste that are adequate for biosurfactant production. Besides the approximately 80% water content, potato waste has carbohydrates (17%), proteins (2%) and fats (0.1%) as well as inorganic minerals, trace elements and vitamins [103]. As an example, Fox and Bala investigated a commercially prepared potato starch in a mineral salt medium for the production of a biosurfactant by *B. subtilis* [74]. Cassava wastewater, which is another carbohydrate-rich waste product generated in large amounts, has been used for the production of surfactin by *B. Subtilis* 35. Other starchy wastes, such as rice water and cereal processing wastewater, have the potential to permit microbial growth and biosurfactant production [108].

10. Recovery of Biosurfactants

The production of low-coast biosurfactants is unlikely due to the complicated recovery process. Process development is conduted in order to obtain biosurfactants that can be recovered easily and inexpensively. In many biotechnological processes, downstream processing accounts for 70%–80% of production costs. For economic reasons, most biosurfactant production processes need to involve spent whole-cell culture broths or other crude preparations [11,103,109]. Extraction with chloroform-methanol, dichloromethane-methanol, butanol, ethyl acetate, pentane, hexane, acetic acid, ether, *etc.* constitutes the most commonly used method in biosurfactant downstream processing. The most widely employed products are different ratios of chloroform and methanol, which facilitate the adjustment of the polarity of the extraction agent to the extractable target material. The disadvantages of using organic solvents for biosurfactant recovery include the large amount of solvent required and the increase in production costs due to the price of expensive solvents. Chloroform is a toxic chloro-organic compound that is harmful to human health and the environment. Thus, there is a need for inexpensive solvents with low toxicity for biosurfactant extraction processes that are suitable for industrial applications. Other product precipitation techniques have also been reported, such as precipitation with ammonium sulphate, centrifugation and adsorption. Biosurfactant recovery depends mainly on the ionic charge, water solubility and location (intracellular, extracellular or cell bond) [103,109]. Foam fractionation is a solvent-free method that separates biosurfactant molecules adsorbed to air bubbles in the culture medium. Biosurfactant production involves continuous foam formation due to the high surface activity. Foam in the broth interferes with mass and heat transfer processes, thereby affecting productivity. However, foam is beneficial to biosurfactant production, as it assists in the continuous removal of product, and therefore production and recovery processes can be accomplished in a single stage [110]. Continuous foam fractionation in the fermentation process helps prevent the accumulation of product that could otherwise inhibit biomass growth and product formation and also facilitates extended biosurfactant production in fed-batch or continuous mode operations. Moreover, biosurfactants do not readily undergo denaturation due to their small size and simple structure [111].

More research and development are required to optimise existing recovery processes to make such processes both commercially viable and more competitive [39,103]. Table 2 lists the most common biosurfactant recovery techniques and their advantages.

Table 2. Downstream processes for recovery of important biosurfactants and respective advantages.

Process		Biosurfactant Type	Biosurfactant Property Responsible for Separation	Advantages
Batch mode	Acid precipitation	Surfactin	Biosurfactants become insoluble at low pH values	Low cost, efficient in crude biosurfactant recovery
	Organic solvent extraction	Trehalolipids; Sophorolipids; Liposan	Biosurfactants are soluble in organic solvents due to the hydrophobic end	Efficient in crude biosurfactant recovery and partial purification, reusable nature
	Ammonium sulphate precipitation	Emulsan; Biodispersan; Lipopeptides	Salting-out of polymeric or protein-rich biosurfactants	Effective in isolation of certain type of polymeric biosurfactants
Continuous mode	Adsorption to wood-activated carbon	Rhamnolipids; Lipopeptides; Glycolipids; Mannosylerythritol Lipids (MEL)	Biosurfactants are adsorbed to activated carbon and can be desorbed using organic solvents	Highly pure biosurfactants, cheaper, reusability, recovery from continuous culture
	Adsorption to polystyrene resines	Rhamnolipids; Lipopeptides; Glycolipids; MEL	Biosurfactants are adsorbed to polystyrene resins and subsequently desorbed using organic solvents	Highly pure biosurfactants, cheaper, reusability, recovery from continuous culture
	Centrifugation	Glycolipids	Insoluble biosurfactants are precipitated due to centrifugal force	Reusable, effective in crude biosurfactant recovery
	Ion-exchange chromatography	Glycolipids	Charged biosurfactants are attached to ion-exchange resins and can be eluted with buffer	High purity, reusability, fast recovery
	Foam fractionation	Surfactin	Biosurfactant form and partition into foam	Useful in continuous recovery processes, high purity of product
	Ultrafiltration	Glycolipids	Biosurfactants form micelles above their critical micelle concentration (CMC), which are trapped by polymeric membranes	Fast, one-step recovery, high level of purity, reusability

11. Industrial Applications of Biosurfactants

Biosurfactants have a wide range of biotechnological applications in petroleum, foods, beverages, cosmetics, detergents, textiles, paints, mining, cellulose, pharmaceutics and nanotechnology [112]. Currently, the main market is the petroleum industry. Biosurfactants can be used for oil residue recovery from storage tanks, other oil recovery processes, the cleanup of oil spills and the bioremediation of both soil and water [2,113]. Table 3 offers a summary of the uses of biosurfactants in different industries. The main biotechnological applications are detailed in the following sections.

11.1. Petroleum Recovery

Petroleum is an essential energy source and driving force of economic development. The US Department of Energy reports that fossil fuels constitute 83% of all primary energy sources in the country and petroleum accounts for 57% of such products. Indeed, 19.2 million cubic metres of petroleum were consumed per day in 2010 [114]. The USA produces 870,000 m^3 of crude oil from 530 thousand production wells, 35% of which produce 0.16 m^3/day and 79% produce < 1.59 m^3/day [115]. Through oil recovery processes, these oil wells produce only one third to half of the petroleum originally present at the sites. Oil residue in small pores within petroleum reservoirs accounts for 50% to 65% of oil and is trapped by high forces of capillarity as well as interfacial tension between the hydrocarbon and aqueous phases. Different reductions in interfacial tension are needed for the mobilisation of this hydrocarbon [116,117], which is only achieved with the use of surfactant concentrations significantly higher than that required for the formation of micelles [118,119]. In enhanced oil recovery, the use of heat, tensioactive agents, microbial processes and gas injection leads to the recovery of a significant portion of the retained oil. However, the high cost of chemical tensioactive agents hinders widespread use of surfactants in oil recovery processes. Thus, biosurfactants have been employed to reduce the interfacial tension between oil/water and oil/rock, which leads to a reduction in the capillary forces that impede oil from moving through rock pores (Figure 7). Biosurfactants also form an emulsion at the oil-water interface, which stabilises the desorbed oil in water and allows oil removal along with the injection water [1,8].

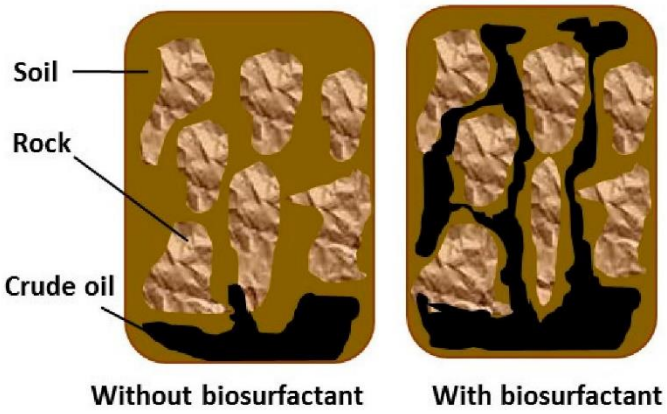

Figure 7. Enhanced oil recovery mechanism by biosurfactants.

Table 3. Applications of biosurfactants for industrial uses.

Industry	Application	Role of Biosurfactants	References
Environment	Bioremediation; Oil spill cleanup operations; Soil remediation and flushing	Emulsification of oils, lowering of interfacial tension, dispersion of oils, solubilisation of oils, wetting, spreading, detergency, foaming, corrosion inhibition in fuel oils and equipment, soil flushing.	[2,8]
Petroleum	Enhanced oil recovery; De-emulsification	Emulsification of oils, lowering of interfacial tension, de-emulsification of oil emulsions, solubilisation of oils, viscosity reduction, dispersion of oils, wetting of solid surfaces, spreading, detergency, foaming, corrosion inhibition in fuel oils and equipment.	[8,120]
Mining	Heavy metal cleanup operations; Soil remediation; Flotation	Wetting and foaming, collectors and frothers, removal of metal ions from aqueous solutions, soil and sediments, heavy metals sequestrants, spreading, corrosion inhibition in oils.	[121]
Food	Emulsification and de-emulsification; Functional ingredient	Solubilisation of flavoured oils, control of consistency, emulsification, wetting agent, spreading, detergency, foaming, thickener.	[4]
Medicine	Microbiological; Pharmaceuticals and therapeutics	Anti-adhesive agents, antifungal agents, antibacterial agents, antiviral agents, vaccines, gene therapy, immunomodulatory molecules.	[20,122,123]
Agriculture	Biocontrol; Fertilisers	Wetting, dispersion, suspension of powdered pesticides and fertilisers, emulsification of pesticide solutions, facilitation of biocontrol mechanisms of microbes, plant pathogen elimination and increased bioavailability of nutrients for beneficial plant-associated microbes.	[124]
Cosmetics	Health and beauty products	Emulsification, foaming agents, solubilisation, wetting agents, cleansers, antimicrobial agents, mediators of enzyme action.	[5]
Cleaning	Washing detergents	Detergents and sanitisers for laundry, wetting, spreading, corrosion inhibition.	[3,5]
Textiles	Preparation of fibres; Dyeing and printing; Finishing of textiles	Wetting, penetration, solubilisation, emulsification, detergency and dispersion, wetting and emulsification in finishing formulations, softening.	[3,103]
Nanotechnology	Synthesis of nanoparticles	Emulsification, stabilisation.	[5,125]

11.2. Bioremediation

Oil spills occur during cargo transportation or in the form of industrial oil and by-product spills. Petroleum exerts a negative effect on cell membranes in living organisms, offering considerable risk of contamination to both marine and terrestrial ecosystems [2,114].

The US Environmental Protection Agency proposes different physical, chemical and biological technologies for the treatment of contaminated soil [126], one of the most studied of which is bioremediation. This process involves the natural degradation capacity of plants and microorganisms for either the partial conversion of contaminants into less toxic compounds or the complete conversion of such substances into carbon dioxide and water.

Larger degrading microorganism populations lead to a quicker, more efficient bioremediation processes. Therefore, this technique can be conducted through biostimulation, which consists of stimulating the growth of microorganisms present at the contaminated site. The process involves the introduction of specific electron receptors, oxygen and nutrients for the degradation of the contaminant as well as substances to correct the pH. Bioremediation can also be performed through bioaugmentation, in which indigenous (allochthonous) microorganisms are added to the contaminated environment to accelerate and complete the degradation of the pollutant [114].

Bioremediation played an important role in the cleanup of the 41 million litre oil spill caused by the oil tanker Exxon Valdez in the Gulf of Alaska in 1989, giving rise to the development of this technology and demonstrating that there are good reasons to believe in the effective application of this treatment method in future oil spills under the appropriate circumstances [114]. In the accident with the Exxon Valdez, the first measure taken was physical washing with high-pressure water. Chemical surfactants were then applied in polluted areas to accelerate the growth and activity of petroleum-degrading microorganisms. Two or three weeks later, the regions treated with surfactants were significantly cleaner than control areas. However, it was difficult to evaluate the exact effects of the treatment due to the heterogeneity of the contamination. Nonetheless, subsequent studies have demonstrated the importance of the use of surfactants to enhance the biodegradation of oil [114,127].

While bioremediation is an effective, environmentally friendly method, the time and costs involved make this process unviable for the treatment of large amounts of waste [128]. Thus, the use of biosurfactants emerges as a safe alternative for improving the solubility of hydrophobic compounds by allowing the desorption and solubilisation of hydrocarbons and facilitating the assimilation of these compounds by microbial cells [129].

The biodegradation of oil-derived hydrocarbons by biosurfactants occurs through two mechanisms. The first involves an increase in the bioavailability of the hydrophobic substrate to microorganisms, with a consequent reduction in surface tension of the medium around the bacterium as well as a reduction in interfacial tension between the cell wall and hydrocarbon molecules. The other mechanism involves the interaction between the biosurfactant and cell surface, leading to changes in the membrane, facilitating hydrocarbon adherence (increase in hydrophobicity) and reducing the lipopolysaccharide index of the cell wall without damaging the membrane. Thus, biosurfactants block the formation of hydrogen bridges and allow hydrophobic-hydrophilic interactions, which cause molecular rearrangements and reduce the surface tension of the liquid by increasing its surface area as well as promoting bioavailability and consequent biodegradability [130,131]. Figure 8 illustrates the action of biosurfactants in increasing the surface area of oil droplets as well as facilitating access to a greater number of bacteria and consequently producing a greater biomass.

11.3. Removal of Hydrophobic Organic Pollutants

The application of biosurfactants for the removal of contaminants from soil is less well known than the advanced application of these compounds in bioremediation processes, since removal efficiency is driven mainly by the physicochemical properties of the biosurfactant rather than the effects on metabolic activity or changes in the properties of the cell surface. However, the mechanisms that

affect the mobilisation and solubilisation of hydrocarbons in soils are similar to those involved in the enhancement of bioavailability for bioremediation [131,132].

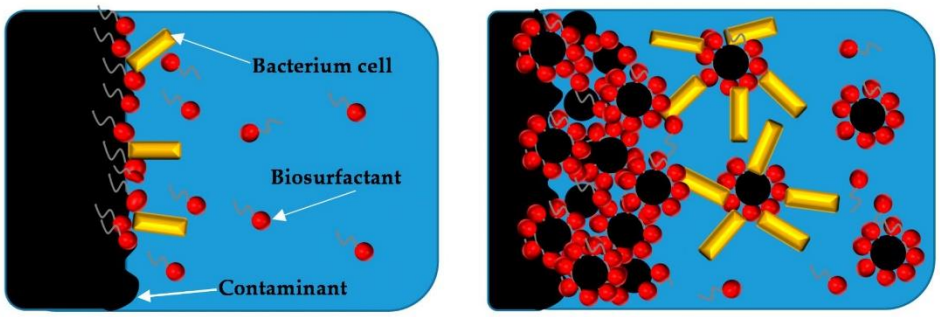

Figure 8. Illustration of biosurfactant action on petroleum.

Biosurfactants enhance the removal of hydrocarbons through biodegradation, solubilisation, mobilisation or emulsification [8]. Solubilisation capacity depends on the ability of the surfactant to increase the solubility of hydrophobic components in the aqueous phase. A considerable increase in this capacity occurs above the CMC due to the partitioning of the hydrocarbon in the hydrophobic portion of the micelles. In this process, greater concentrations of surfactants are normally required, since the solubility of the hydrocarbon components in the solution depends wholly on the concentration of the surfactant [8]. Mobilisation occurs at concentrations below the CMC and is divided into displacement and dispersion. Displacement consists of the release of hydrocarbon droplets from the porous medium due to the reduction in interfacial tension. Using a theoretical explanation, hydrocarbon removal is possible when the interfacial tension between the aqueous and oil phases is sufficiently reduced to overcome the forces of capillarity that cause the formation of residual saturation. Dispersion is a process by which a hydrocarbon is dispersed in the aqueous phase as tiny emulsions. Emulsions are not generally thermodynamically stable, but may remain stable for significant periods of time due to kinetic restrictions. Dispersion is related to interfacial tension and surfactant concentration and differs from displacement, which is related only to interfacial tension between the aqueous and hydrophobic phases, with no formation of emulsion [132].

The efficiency of a surfactant in the removal of hydrophobic compounds also depends on the pH and ionic strength of the solution, which can alter the arrangement of the aggregated micelles and sorption of the surfactant to the soil, which, in turn, limits the transport of the hydrocarbon by the surfactant. Different biosurfactants have been tested for the removal of petroleum-derived products from contaminated soil and water. Rhaminolipids have been successfully used in biotechnological decontamination processes [2,7,17]. Other surfactants produced by species of *Pseudomonas* [110,133], *Bacillus* [5,134], and *Candida* [109,113,135–137], have also been successfully used in the remediation of soil.

11.4. Removal of Heavy Metals

Heavy metals and radionuclides are persistent soil contaminants. Increases in levels of heavy metals in soil have been reported in many industrialised countries. Metals and metalloids, such as chromium, cadmium, mercury and lead, can threaten ecosystems and human health through either the food chain or direct exposure to contaminated soil and water [1,12]. As different technologies can be used in combination for the treatment of organic pollutants and heavy metals, biosurfactants can be used in the removal of hydrophobic organic compounds and heavy metals [138–140]. Heavy metals mainly adsorb to the surface of soil in the form of ions or the precipitation of metal compounds. Unlike organic contaminants, heavy metals are removed

from soil through surfactant-associated complexation [141] and ion exchange [142]. Therefore, surfactant-enhanced washing and surfactant-enhanced bio-extraction can be applied to the remediation of soils contaminated with heavy metals.

Surfactants in solutions facilitate the solubilisation, dispersion and desorption of contaminants and allow the reuse of the soil [143]. Decontamination tests have been performed with different synthetic surfactants [144,145], but the desire to replace such compounds with natural surfactants has led to research into the use of biosurfactants [119]. Studies have demonstrated the potential of surfactin, rhamnolipids and sophorolipids [146–148]. The ionic nature, biodegradability, low toxicity and excellent surface properties make biosurfactants adequate for the removal of heavy metals from sediment and soil. According to Mulligan [119], removal is possible with different concentrations of biosurfactants. Das *et al.* [149] found that the removal of cadmium using an aqueous solution also occurred at concentrations below the CMC, while a concentration fivefold greater than the CMC resulted in the virtually complete removal of 100 ppm of metal ions. Wen *et al.* [150] studied the degradation of a rhamnolipid in soils contaminated by cadmium and zinc and found that this compound could remain in the soil long enough to enhance the phytoextraction of the metals.

The removal of metals by ionic biosurfactants is thought to occur in the following order (Figure 9): (1) sorption of the biosurfactant to the soil surface and complexation with the metal; (2) detachment of the metal from the soil to the solution; and (3) association with micelles. Heavy metals are trapped within the micelles through electrostatic interactions and can be easily recovered through precipitation or membrane separation methods [151].

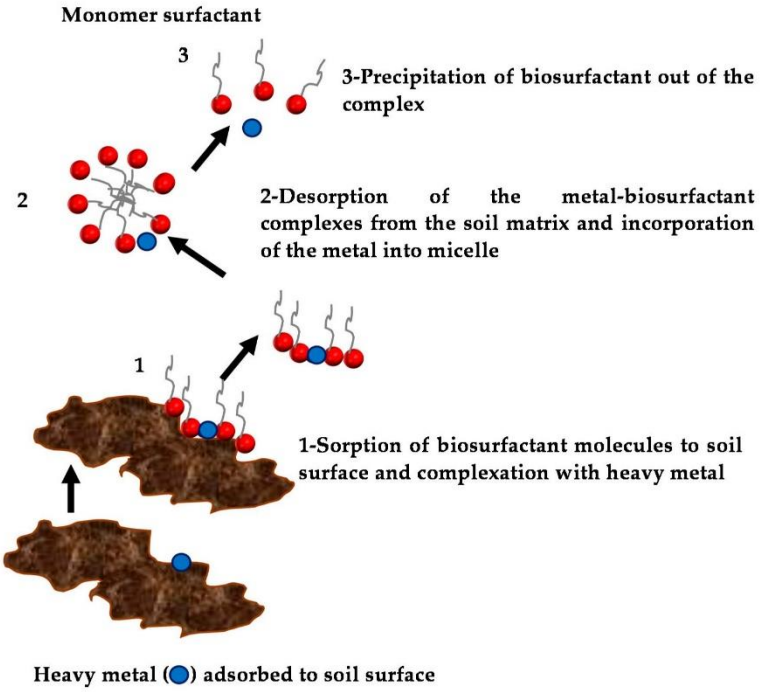

Figure 9. Mechanism of heavy metal removal by biosurfactants.

Anionic biosurfactants create non-ionic complexes with metals through ionic bonds. As such bonds are stronger than those between the metal and soil, the metal-biosurfactant complex is detached from the soil due to the reduction in interfacial tension. Cationic biosurfactants can replace similarly

charged metal ions through ion exchange (competition for negatively charged surfaces). Surfactant micelles can also be used to remove metal ions from the soil surface [115].

Biosurfactants offer indisputable advantages, since surfactant-producing microorganisms do not need to survive in contaminated soil, although the continual addition of biosurfactant is required in the process [8]. Biosurfactants have also been applied in mining processes. Tensioactive compounds produced by *Pseudomonas* sp. and *Alcaligenes* sp. have been used for the floatation and separation of calcite and scheelite, with recovery rates of 95% for $CaWO_4$ and 30% for $CaCO_3$, whereas conventional chemical reagents are not capable of separating these two minerals [152]. Slizovskiy *et al.* [153] studied the enhanced remediation of soils contaminated with heavy metals using the cationic surfactant 1-dodecylpyridinium chloride (DPC), the non-ionic surfactant oleyl dimethyl benzyl ammonium chloride (trade name Ammonyx KP) and the ionic rhamnolipid biosurfactant (trade name JBR-425); the latter substance exhibited the best elution with regard to Zn (39%), Cu (56%), Pb (68%) and Cd (43%). Almeida *et al.* [154] studied the impact of surfactants on the removal of Cu by the salt marsh plant *Halimione portulacoides*. TX-100 and SDS (sodium dodecyl sulfate) were favourable to Cu harvesting and transportation in plant roots, but did not affect the transportation of Cu in the stem and leaves. The results of this study suggest that surfactants promote phytoremediation through a change in the membrane permeability of root cells. Therefore, surfactants can promote the desorption of metals and uptake by plants [1].

Rhamnolipids and soapberry-derived saponin have recently been found to assist in the removal of chromium and arsenic oxyanions from soils or the ore waste of mines [155,156]. Biosurfactants produced by yeast of the genus *Candida* have been successfully used in heavy metal flotation, demonstrating the ability to remove more than 90% of cations in columns and air-dissolved flotation processes [157,158]. *C. lipolytica* produces a biosurfactant that has also been used for the removal of heavy metals and petroleum byproducts using a soil barrier [90]. The biosurfactant significantly reduced soil permeability, thereby demonstrating its usefulness in reactive barriers, with the removal of approximately 96% of Zn and Cu as well as a reduction in Pb and Cd concentrations in groundwater.

11.5. Food Industry

Emulsification is important for the formation of consistency and texture in foods as well as phase dispersion and the solubilisation of aromas [4,159]. The general function of emulsifiers in food products is to stabilise the emulsion by controlling the agglomeration of fat globules and stabilising aerated systems [4,152]. An emulsion has at least one immiscible liquid (discontinuous internal phase) dispersed in another (continuous outer phase) in the form of droplets. The stability of this system is minimal, but can be enhanced by the addition of a surfactant, which reduces surface energy between the two phases by a reduction in interfacial tension, thereby preventing particle coalescence through the formation of steric and electrostatic barriers. Examples of processed foods that are emulsions include heavy cream, butter, mayonnaise, salad dressings, fillings,*etc.* [35]. Other uses for emulsifiers have been described, such as improving the texture and shelf life of products containing starch, the formation of complexes, altering the rheological properties of wheat flour and interactions with gluten as well as improving the consistency and texture of fat-based products through the control of polymorphism and the crystalline structure of fats [4].

Biosurfactants can also be used as emulsifiers in the processing of raw materials, the control of fat globule agglomeration, the stabilisation of aerated systems and an improvement in the consistency of fat-based products. The use of rhaminolipids to improve the emulsifying properties of frozen desserts, butter and croissants has also been reported [4,111]. For instance, *Candida utilis* produces a bioemulsifier used in processed salad dressings [160]. A manoprotein produced by *Saccharomyces cerevisiae* stabilises water/oil emulsions in cookies, mayonnaise and ice cream, *etc.* [13,161]. However, the food industry has not yet made widescale use of biosurfactants. Many of the properties of biosurfactants and their regulation as new ingredients for foods are pending approval.

11.6. Medicine

Biosurfactants have also been used in different biological (therapeutic) applications due to their fungicidal, bactericidal, insecticidal and anti-viral properties as well as use as anti-adhesive agents and enzyme inhibitors [111,116,117]. A number of rhaminolipids exhibit antibacterial activity. For instance, Abalos *et al.* [162] identified six rhaminolipids in cultures of *P. aeruginosa* AT10 grown on soybean oil refinery residue and evaluated the antimicrobial properties of the solution. These rhaminolipids exhibited excellent antifungal properties against different fungi at concentrations ranging from 16 to 32 μg/mL. *C. bombicola*-derived sophorolipids inhibited the growth of both Gram-negative and Gram-positive bacteria with a minimum inhibitory concentration of approximately 30 and 1 mg/mL in a contact time of 2 and 4 h, respectively, for *E. coli* (ATCC 8739) and *P. aeruginosa* (ATCC 9027) as well as 6 and 1 mg/mL in a contact time of 4 h for *S. aureus* (ATCC 6358) and *B. subtilis* (ATCC6633), respectively [163].

Despite the number of publications describing the antimicrobial activity of biosurfactants and patents related to their usage, real applications in the pharmaceutical, biomedical and health industries remains quite limited [164]. Some lipopeptides, such as daptomycin, have reached a commercial antibiotic status [165]. Daptomycin, is a branched cyclic lipopeptide isolated from *Streptomyces roseosporus* cultures and is produced by Cubist Pharmaceuticals under the name Cubicin® [166]. This drug was approved in 2003 for the treatment of skin infections caused by methicillin-resistant *Staphylococcus aureus* and other Gram-positive pathogens and approved in 2006 for the treatment of endocarditis and bacteraemia caused by *S. aureus*. Daptomycin had also been reported to display strong antibacterial activity against other important pathogens, such as penicillin-resistant *Streptococcus pneumoniae*, coagulase-negative *Staphylococci*, glycopeptide-intermediate-susceptible *S. aureus* and vancomycin-resistant *Enterococci* [166].

Biofilms are groups of bacteria and other organic matter that has colonised/accumulated on a given surface [117]. Bacterial adherence to the surface is the first step in the establishment of biofilm and is affected by various factors, such as the type of microorganism, hydrophobicity, electrical charges of the surface, environmental conditions and the ability of microorganisms to produce extracellular polymers that assist the cells in anchoring to the surface [167]. Anti-adherent activity, which is the ability to inhibit the adherence of pathogenic microorganisms to solid surfaces or infectious sites, has also been reported for biosurfactants, leading to a reduction in hospital infections with no need for drugs or synthetic chemical agents [112]. Meylheuc *et al.* [168] studied a biosurfactant obtained from *P. fluorescens* with inhibitory properties regarding the adherence of *Listeria monocytogenes* to stainless steel and polytetrafluoroethylene surfaces. *C. sphaerica*-derived lunasan inhibited the adherence of *P.aeruginosa, Streptococcus agalactiae* and *S. sanguis* between 80% and 92% at a concentration of 10 mg/mL [95], while the biosurfactant rufisan produced by *C. lipolytica* UCP 0988 demonstrated antimicrobial activities against *S. agalactiae*, *S. mutans*, *S. mutans* NS, *S. mutans* HG, *S. sanguis* 12, *S. oralis* J22 at a concentration above the CMC (0.3%). Moreover, the biosurfactant showed anti-adherent activity against most of the microorganisms tested [137].

Deficiency of lung surfactant, which is a protein-phopholipid complex, is responsible for respiratory failure in premature infants. Gene isolation for protein molecules in this surfactant and cloning in bacteria allow fermentative production for medical applications [108]. Sophorolipids from *C. bombicola* have been studied due to their spermicidal and cytotoxic activities as well as anti-HIV action that can reduce the proliferation of acquired immunodeficiency syndrome (AIDS). Sophorolipids have also been studied as anti-inflammatory agents for patients with immune diseases [108,169]. Iturin is a lipopeptide produced by *B. subtilis* that has demonstrated antifungal activity by affecting the morphology and structure of the cell membrane of yeasts. *In vitro* experiments have demonstrated that surfactin can effectively inactivate the virus that causes herpes as well as the retrovirus and other compact RNA and DNA viruses. The antiviral activity of surfactin has been determined for a broad spectrum of viruses. Moreover, surfactin has been found to exert an effect on insulin absorption in the lungs of laboratory rats [108].

11.7. Nanaotechnology

Biosurfactants have been used in nanotechnology and nanoparticle synthesis is emerging as part of green chemistry [118,170]. Nickel oxide (NiO) nano-rods can be produced by water-in-oil microemulsions [171]. In one experiment, two microemulsions were formed with the addition of a nickel chloride solution to a biosurfactant and heptane solution, with the addition of ammonium hydroxide to the same hydrocarbon mixture. The centrifuged microemulsions and ethanol was then used to wash the precipitates and remove the biosurfactant and heptane. The use of biosurfactants is a more ecofriendly approach [119]. Reddy *et al.* [172] found that silver nanoparticle synthesis could be stabilised for two months using surfactin, which is a biodegradable, renewable stabilising agent with low toxicity [3,119]. A biosurfactant produced by *P. Aeruginosa* grown in a low-cost medium has been employed to stabilise silver nanoparticles in the liquid phase [173]. The effect of a rhamnolipid on the electrokinetic and rheological behaviour of nano-zirconia particles has also been analysed, although this is not strictly an environmental application [174]. The biosurfactant increasingly adsorbs to zirconia with the increase in concentration and can serve as an ecofriendly product for the flocculation and dispersion of high solid contents of microparticles. New applications for biosurfactants are being developed in the field of nanotechnology. Future research should focus on the stabilisation of the nanoparticles by biosurfactants before addition during remediation processes [119].

12. Future Directions and Concluding Remarks

The biosurfactant industry has demonstrated remarkable growth in recent decades, although the large-scale production of these biomolecules remains a challenge from the economic standpoint. This is mainly due to the enormous difference between the financial investment required and viable industrial production. Thus, the following are the main criteria to be considered for biosurfactant production to become truly viable: (a) type of raw materials; (b) continuous provision of the same composition of ingredients; (c) types of microorganisms; (d) the adequate design of industrial fermentors; (e) financial investments; (f) the target market; (g) purification processes; (h) biosurfactant properties; (i) production conditions, especially the time required for fermentation; (j) adequate production yields; and (k) the processing of recycled products (minimal or able to sell for more than the drop in value).

The target market is of fundamental importance to the implantation of an industrial biosurfactant production project. For cosmetic, medicinal and food products, production is only viable on a small-scale, as the column chromatography methods required to separate molecules are not economical on a large scale. Thus, the use of crude fermentation broths could be a viable solution, especially if the application is in an environmental context, as biosurfactants in such cases do not need to be pure and can be synthesized using a blend of inexpensive carbon sources, which would allow the creation of an economically and environmentally viable technology for bioremediation processes.

According to Hazra *et al.* [140], sophorolipids are offered as sophoron TM from Saraya (Osaka, Japan) and Soliance (Pomacle, France), whereas rhamnolipids are available from Ecover (Boulogne-sur-Mer, France), Jeneil Biosurfactant Inc. (Saukville, Wisconsin, USA) and Rhamnolipid Holdings Inc. (New york, USA). Sophorolipid production costs run from 2 to 5 €/kg. Rhamnolipid production costs US$ 20/kg at a volume of 20 m^3, but only US$ 5/kg when produced on a scale of 100 m^3, placing it closer to ethoxylate or alkyl polyglycoside (US$ 1 to 3/kg). The Exxon Company spent more than US$ 10 million in bioremediation studies between 1993 and 1997 after the spillage of petroleum (41 million litres) by the oil tanker Exxon Valdez in Alaska in 1989, leading to the generation of seven patents and making bioremediation second only to enhanced oil recovery within the initial years of use. Distribution in specific biosurfactant areas of the oil industry includes 17 patents for soil and water bioremediation as well as 20 for enhanced oil recovery [140,175].

Although improvements in biosurfactant technology have enabled a 10-to-20-fold increase in the production of these biomolecules, it is likely that further, significant advances (even if of a smaller magnitude) are needed to make this technology commercially viable.

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References

1. Mao, X.; Jiang, R.; Xiao, W.; Yu, J. Use of surfactants for the remediation of contaminated soils: A review. *J. Hazard. Mater.* **2015**, *285*, 419–435. [CrossRef] [PubMed]
2. Silva, R.C.F.S.; Almeida, D.G.; Luna, J.M.; Rufino, R.D.; Santos, V.A.; Sarubbo, L.A. Applications of biosurfactants in the petroleum industry and the remediation of oil spills. *Int. J. Mol. Sci.* **2014**, *15*, 12523–12542. [CrossRef] [PubMed]
3. Banat, I.M.; Franzetti, A.; Gandolfi, I.; Bestetti, G.; Martinotti, M.G.; Fracchia, L.; Smyth, T.J.; Marchant, R. Microbial biosurfactants production, applications. *Appl. Microbiol. Biotechnol.* **2010**, *87*, 427–444. [CrossRef] [PubMed]
4. Campos, J.M.; Stamford, T.L.M.; Sarubbo, L.A.; Luna, J.M.; Rufino, R.D.; Banat, I.M. Microbial biosurfactants as additives for food industries. *Biotechnol. Prog.* **2013**, *29*, 1097–1108. [CrossRef] [PubMed]
5. Vijayakumar, S.; Saravanan, V. Biosurfactants-types, sources and applications. *Res. J. Microbiol.* **2015**, *10*, 181–192.
6. Cerqueira, V.S.; Hollenbach, E.B.; Maboni, F.; Vainstein, M.H.; Camargo, F.A.; do Carmo, M.; Peralba, R.; Bento, F.M. Biodegradation potential of oily sludge by pure and mixed bacterial cultures. *Bioresour. Technol.* **2011**, *102*, 11003–11010. [CrossRef] [PubMed]
7. Rosa, C.F.C.; Freire, D.M.G.; Ferraz, E.C. Biosurfactant microfoam: Application in the removal of pollutants from soil. *J. Environ. Chem. Eng.* **2015**, *3*, 89–94. [CrossRef]
8. Pacwa-Plociniczak, M.; Plaza, G.A.; Piotrowska-Seget, Z.; Cameotra, S.S. Environmental applications of biosurfactants: Recent advances. *Int. J. Mol. Sci.* **2011**, *13*, 633–654. [CrossRef] [PubMed]
9. Makkar, R.S.; Cameotra, S.S.; Banat, I.M. Advances in utilization of renewable substrates for biosurfactant production. *Appl. Microbiol. Biotechnol.* **2011**, *1*, 1–19. [CrossRef] [PubMed]
10. Deleu, M.; Paquot, M. From renewable vegetables resources to microorganisms: new trends in surfactants. *C. R. Chimie* **2004**, *7*, 641–646.
11. Marchant, R.; Funston, S.; Uzoigwe, C.; Rahman, P.K.S.M.; Banat, I.M. Production of biosurfactants from nonpathogenic bacteria. In *Biosurfactants*; Taylor & Francis: New York, USA, 2014; pp. 73–81.
12. Chakraborty, J.; Das, S. Biosurfactant-based bioremeditaion of toxic metals. In *Microbial Biodegradation and Bioremediation*, 1st ed.; Das, S., Ed.; Elsevier: Rourkela Odisha, India, 2014; pp. 167–201.
13. Shepherd, R.; Rockey, J.; Shutherland, I.W.; Roller, S. Novel bioemulsifiers from microorganisms for use in foods. *J. Biotechnol.* **1995**, *40*, 207–217. [CrossRef]
14. Bognolo, G. Biossurfactants as emulsifying agents for hydrocarbons. *Coll. Surf. A Physicochem. Eng. Asp.* **1999**, *152*, 41–52. [CrossRef]
15. Pattanath, K.M.; Rahman, K.S.; Gakpe, E. Production, characterization and applications of biosurfactants—Review. *Biotechnology* **2008**, *7*, 360–370.
16. Chrzanowski, L.; Ławniczak, L.; Czaczyk, K. Why do microorganisms produce rhamnolipids? *World J. Microbiol. Biotechnol.* **2012**, *28*, 401–419. [CrossRef] [PubMed]
17. Abdel-Mawgoud, A.M.; Lépine, F.; Deziel, E. Rhamnolipids: Diversity of structures, microbial origins and roles. *Appl. Microbiol. Biotechnol.* **2010**, *86*, 1323–1336. [CrossRef] [PubMed]
18. Hitsatsuka, K.; Nakahara, T.; Sano, N.; Yamada, K. Formation of a rhamnolipid by *Pseudomonas aeruginosa* and its function in hydrocarbon fermentation. *Agric. Biol. Chem.* **1971**, *35*, 686–692. [CrossRef]
19. Nitschke, M.; Costa, S.G.V.A.O.; Contiero, J. Rhamnolipids and PHAs: Recent reports on *Pseudomonas*-derived molecules of increasing industrial interest. *Process Biochem.* **2011**, *46*, 621–630. [CrossRef]
20. Ławniczak, L.; Marecik, R.; Chrzanowski, L. Contributions of biosurfactants to natural or induced bioremediation. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 2327–2339. [CrossRef] [PubMed]

21. Chrzanowski, L.; Dziadas, M.; Ławniczak, L.; Cyplik, P.; Białas, W.; Szulc, A.; Lisiecki, P.; Jelen, H. Biodegradation of rhamnolipids in liquid cultures: Effect of biosurfactant dissipation on diesel fuel/B20 blend biodegradation efficiency and bacterial community composition. *Bioresour. Technol.* **2012**, *111*, 328–335. [CrossRef] [PubMed]
22. Szulc, A.; Ambrozewicz, D.; Sydow, M.; Ławniczak, L.; Piotrowska-Cyplik, A.; Marecik, R.; Chrzanowski, L. The influence of bioaugmentation and biosurfactant addition on bioremediation efficiency of diesel-oil contaminated soil: Feasibility during field studies. *J. Environ. Manag.* **2014**, *132*, 121–128. [CrossRef] [PubMed]
23. Marecik, M.; Wojtera-Kwiczor, J.; Ławniczak, L.; Cyplik, P.; Szulc, A.; Piotrowska-Cyplik, A.; Chrzanowski, L. Rhamnolipids increase the phytotoxicity of diesel oil towards four common plant species in a terrestrial environment. *Water Air Soil Pollut.* **2012**, *223*, 4275–4282. [CrossRef] [PubMed]
24. Chrzanowski, L.; Wick, L.Y.; Meulenkamp, R.; Kaestner, M.; Heipieper, H.J. Rhamnolipid biosurfactants decrease the toxicity of chlorinated phenols to *Pseudomonas putida* DOT-T1E. *Lett. Appl. Microbiol.* **2009**, *48*, 756–762. [PubMed]
25. Chrzanowski, L.; Owsianiak, M.; Szulc, A.; Marecik, R.; Piotrowska-Cyplik, A.; Olejnik-Schmidt, A.K.; Staniewski, J.; Lisiecki, P.; Ciesielczyk, F.; Jesionowski, T.; *et al.* Interactions between rhamnolipid biosurfactants and toxic chlorinated phenols enhance biodegradation of a model hydrocarbon-rich effluent. *Int. Biodeterior. Biodegrad.* **2011**, *65*, 605–611. [CrossRef]
26. Cortés-Sánchez, A.J.; Sánchez, H.H.; Jaramillo-Flores, M.E. Biological activity of glycolipids produced by microorganisms: New trends and possible therapeutic alternatives. *Microbiol. Res.* **2013**, *168*, 22–32. [CrossRef] [PubMed]
27. Daverey, A.; Pakshirajan, K. Production, characterization, and properties of sophorolipids from the yeast *Candida bombicola* using a low-cost fermentative medium. *Appl. Biochem. Biotechnol.* **2009**, *158*, 663–674. [CrossRef] [PubMed]
28. Gautam, K.K.; Tyagi, V.K. Microbial Surfactants: A review. *J. Oleo Sci.* **2006**, *55*, 155–166. [CrossRef]
29. Hu, Y.; Ju, L.K. Purification of lactonic sophorolipids by crystallization. *J. Biotechnol.* **2001**, *87*, 263–272. [CrossRef]
30. Díaz De Rienzo, M.A.D.; Banat, I.M.; Dolman, B.; Winterburn, J.; Martin, P.J. Sophorolipid biosurfactants: Possible uses as antibacterial and antibiofilm agent. *New Biotechnol.* **2015**. [CrossRef] [PubMed]
31. Lang, S. Biological amphiphiles (microbial biosurfactants). *Curr. Opin. Coll. Interface Sci.* **2002**, *7*, 12–20. [CrossRef]
32. Hatha, A.A.M.; Edward, G.; Rahman, K.S.M.P. Microbial biosurfactants-review. *J. Mar. Atmos. Res.* **2007**, *3*, 1–17.
33. Chakrabarti, S. Bacterial Biosurfactant: Characterization, Antimicrobial and Metal Remediation Properties. Ph.D. Thesis, National Institute of Technology, Surat, India, March 2012.
34. Barros, F.F.C.; Quadros, C.P.; Maróstica, M.R.; Pastore, G.M. Surfactina: Propriedades químicas, tecnológicas e funcionais para aplicações em alimentos. *Quím. Nova* **2007**, *30*, 1–14. [CrossRef]
35. Velikonja, J.; Kosaric, N. Biosurfactants in food applications. In *Biosurfactants: Production, Properties, Applications*; Kosaric, N., Sukan, F.V., Eds.; CRC Press: New York, NY, USA, 1993; pp. 419–448.
36. Cirigliano, M.C.; Carman, G.M. Purification and characterization of liposan, a bioemulsifier from *Candida lipolytica*. *Appl. Microbiol. Biotechnol.* **1985**, *50*, 846–850.
37. Desai, J.D.; Banat, I.M. Microbial production of surfactants and their commercial potential. *Microbiol. Mol. Biol. Rev.* **1997**, *61*, 47–64. [PubMed]
38. Bhardwaj, G.; Cameotra, S.S.; Chopra, H.K. Biosurfactants from Fungi: A Review. *Pet. Environ. Biotechnol.* **2013**, *4*, 1–6. [CrossRef]
39. Cooper, D.G.; Paddock, D.A. Production of a biosurfactant from *Torulopsis bombicola*. *Appl. Environ. Microbiol.* **1984**, *47*, 173–176. [PubMed]
40. Kim, S.Y.; Oh, D.K.; Lee, K.H.; Kim, J.H. Effect of soybean oil and glucose on sophorose lipid fermentation by *Torulopsis bombicola* in continuous culture. *Appl. Microbiol. Biotechnol.* **1997**, *48*, 23–26. [CrossRef] [PubMed]
41. Casas, J.A.; de Lara, S.G.; Garcia-Ochoa, F. Optimization of a synthetic medium for *Candida bombicola* growth using factorial design of experiments. *Enzyme Microb. Technol.* **1997**, *21*, 221–229. [CrossRef]
42. Sarubbo, L.A.; Farias, C.B.B.; Campos-Takaki, G.M. Co-utilization of canola oil and glucose on the production of a surfactant by *Candida lipolytica*. *Curr. Microbiol.* **2007**, *54*, 68–73. [CrossRef] [PubMed]

43. Rufino, R.D.; Sarubbo, L.A.; Campos-Takaki, G.M. Enhancement of stability of biosurfactant produced by *Candida lipolytica* using industrial residue as substrate. *World J. Microbiol. Biotechnol.* **2007**, *23*, 729–734. [CrossRef]
44. Sarubbo, L.A.; Porto, A.L.F.; Campos-Takaki, G.M. Bioemulsifier production in batch culture using glucose as carbon source by *Candida lipolytica*. *Appl. Biochem. Biotechnol.* **2001**, *95*, 59–67. [CrossRef]
45. Bednarski, W.; Adamczak, M.; Tomasik, J.; Plaszvzyk, M. Application of oil refinery waste in the biosynthesis of glycolipids by yeast. *Bioresour. Technol.* **2004**, *95*, 15–18. [CrossRef] [PubMed]
46. Kitamoto, D.; Ikegami, T.; Suzuki, G.T.; Sasaki, A.; Takeyama, Y.; Idemoto, Y.; Koura, N.; Yanagishita, H. Microbial conversion of n-alkanes into glycolipid biosurfactants, mannosylerythritol lipids by *Pseudozyma* (*Candida antartica*). *Biotechnol. Lett.* **2001**, *23*, 1709–1714. [CrossRef]
47. Amezcua-Veja, C.; Poggi-Varaldo, H.M.; Esparza-Garcia, F.; Rodriguez-Vazquez, R. Effect of culture conditions on fatty acids composition of a biosurfactant produced by *Candida ingens* and of surface tension of culture media. *Bioresour. Technol.* **2006**, *98*, 237–240. [CrossRef] [PubMed]
48. Robert, M.; Mercadé, M.E.; Bosch, M.P.; Parra, J.L.; Espiny, M.J.; Manresa, M.A.; Guinea, J. Effect of the carbon source on biosurfactant production by *Pseudomonas aeruginosa* 44T1. *Biotechnol. Lett.* **1989**, *11*, 871–874. [CrossRef]
49. Santa Anna, I.M.; Sebastian, G.V.; Pereira, N., Jr.; Alves, T.L.M.; Menezes, E.P.; Freire, D.M.G. Production of biosurfactant from a new and promissing strain of *Pseudomonas aeruginosa* PA1. *Appl. Biochem. Biotechnol.* **2002**, *91*, 459–467.
50. Mulligan, C.N.; Gibbs, B.F. Correlation of nitrogen metabolism with biosurfactant production by *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* **1989**, *55*, 3016–3019. [PubMed]
51. Deshpande, M.; Daniels, L. Evaluation of sophorolipid biosurfactant production by*Candida bombicola* using animal fat. *Bioresour. Technol.* **1995**, *54*, 143–150. [CrossRef]
52. Kim, H.S.; Yoon, B.D.; Choung, D.H.; Oh, H.M.; Katsuragi, T. Characterization of a biosurfactant mannosylerythritol lipid produced from *Candida* sp. SY 16. *Appl. Microbiol. Biotechnol.* **1999**, *52*, 713–721. [CrossRef] [PubMed]
53. Rufino, R.D.; Sarubbo, L.A.; Benicio, B.N.; Campos-Takaki, G.M. Experimental design for the production of tensio-active agent by *Candida lipolytica*. *J. Ind. Microbiol. Biotechnol.* **2008**, *35*, 907–914. [CrossRef] [PubMed]
54. Sarubbo, L.A.; Luna, J.M.; Campos-Takaki, G.M. Production and stability studies of the bioemulsifier obtained from a new strain of *Candida glabrata* UCP 1002. *Electr. J. Biotechnol.* **2006**, *9*, 400–406.
55. Cirigliano, M.C.; Carman, G.M. Isolation of a bioemulsifier from *Candida lipolytica*. *Appl. Environ. Microbiol.* **1984**, *48*, 747–750. [PubMed]
56. Konishi, M.; Fukuoka, T.; Morita, T.; Imura, T.; Kitamoto, D. Production of new types of sophorolipids by *Candida batistae*. *J. Oleo Sci.* **2008**, *57*, 359–369. [CrossRef] [PubMed]
57. Kiran, G.S.; Hema, T.A.; Gandhimathi, R.; Selvin, J.; Thomas, T.A. Optimization and production of a biosurfactant from the sponge-associated marine fungus *Aspergillus ustus* MSF3. *Coll. Surf. B Biointerfaces* **2009**, *73*, 250–256. [CrossRef] [PubMed]
58. Thaniyavarn, J.; Chianguthai, T.; Sangvanich, P.; Roongsawang, N.; Washio, K.; Morikawa, M.; Thaniyavarn, S. Production of sophorolipid biosurfactant by *Pichia anomala*. *Biosci. Biotechnol. Biochem.* **2008**, *72*, 2061–2068. [CrossRef] [PubMed]
59. Cavalero, D.A.; Cooper, D.G. The effect of medium composition on the structure and physical state of sophorolipids produced by *Candida bombicola* ATCC 22214. *J. Biotechnol.* **2003**, *103*, 31–41. [CrossRef]
60. Felse, P.A.; Shah, V.; Chan, J.; Rao, K.J.; Gross, R.A. Sophorolipid biosynthesis by *Candida bombicola* from industrial fatty acid residues. *Enzyme Microb. Technol.* **2007**, *40*, 316–323. [CrossRef]
61. Silva, S.N.R.L.; Farias, C.B.B.; Rufino, R.D.; Luna, J.M.; Sarubbo, L.A. Glycerol as substrate for the production of biosurfactant by *Pseudomonas aeruginosa* UCP0992. *Coll. Surf. B Biointerfaces* **2010**, *79*, 174–183. [CrossRef] [PubMed]
62. Oliveira, F.J.S.; Vazquez, L.; de Campos, N.P.; de Franca, F.P. Production of rhamnolipids by a *Pseudomonas alcaligenes* strain. *Process Biochem.* **2009**, *44*, 383–389. [CrossRef]
63. Cunha, C.D.; Rosario, M.; Rosado, A.S.; Leite, G.F. *Serratia* sp. SVGG16: A promising biosurfactant producer isolated from tropical soil during growth with ethanol-blended gasoline. *Process Biochem.* **2004**, *39*, 2277–2282. [CrossRef]

64. Weber, L.; Doge, C.; Haufe, G.; Hommel, R.; Kleber, H.-P. Oxygenation of hexadecane in the biosynthesis of cyclic glycolipids in *Torulopsis apicola*. *Biocatal* **1992**, *5*, 267–292. [CrossRef]
65. Haritash, A.K.; Kaushik, C.P. Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. *J. Hazard. Mater.* **2009**, *169*, 1–15. [CrossRef] [PubMed]
66. Hommel, R.K.; Huse, K. Regulation of sophorose lipid production by *Candida* (*Torulopsis*) *apicola*. *Biotechnol. Lett.* **1993**, *15*, 853–858. [CrossRef]
67. Tokumoto, Y.; Nomura, N.; Uchiyama, H.; Imura, T.; Morita, T.; Fukuoka, T.; Kitamoto, D. Structural characterization and surface-active properties of a succinoyl trehalose lipid produce by *Rhodococcus* sp. SD-74. *J. Oleo Sci.* **2009**, *58*, 97–102. [CrossRef] [PubMed]
68. Syldatk, C.; Wagner, F. Production of biosurfactants. In *Biosurfactants and Biotechnology*; Kosaric, N., Cairns, W.L., Gray, N.C.C., Eds.; Marcel Dekker: New York, NY, USA, 1987; pp. 89–120.
69. Tan, H.M. Biosurfactants and their roles in bioremediation. *Cheong Jit Kong* **2000**, 1–12.
70. Banat, I.M.; Satpute, S.K.; Cameotra, S.S.; Patil, R.; Nyayanit, N. Cost effective technologies and renewable substrates for biosurfactants' production. *Front. Microbiol.* **2014**, *5*. [CrossRef] [PubMed]
71. Makkar, R.S.; Cameotra, S.S. An update on the use of unconventional substrates for biosurfactant production and their new applications. *Appl. Micobiol. Biotechnol.* **2002**, *58*, 428–434.
72. Batista, R.M.; Rufino, R.D.; Luna, J.M.; Souza, J.E.G.; Sarubbo, L.A. Effect of Medium components on the production of a biosurfactant from *Candida tropicalis* applied to the removal of hydrophobic contaminants in soil. *Water Environ. Res.* **2010**, *82*, 418–425. [CrossRef] [PubMed]
73. Mercade, M.E.; Manresa, A.; Robert, M.; Espuny, M.J.; Deandres, C.; Guinea, J. Olive oil mill effluent (OOME). New substrat for biosurfactant production. *Bioresour. Technol.* **1993**, *43*, 1–6. [CrossRef]
74. Fox, S.I.; Bala, G.A. Production of surfactant from *Bacillus subtilis* ATTCC 21332 using potato substrates. *Bioresour. Technol.* **2000**, *75*, 235–240. [CrossRef]
75. Thompson, D.N.; Fox, S.L.; Bala, G.A. Biosurfactants from potato process effluents. *Appl. Biochem. Biotechnol.* **2000**, *84*, 917–930. [CrossRef]
76. Maneerat, S. Production of biosurfactants using substrates from renewable-resources. *Songklanakarin J. Sci. Technol.* **2005**, *27*, 675–683.
77. Santos, D.K.F.; Brandão, Y.B.; Rufino, R.D.; Luna, J.M.; Salgueiro, A.A.; Santos, V.A.; Sarubbo, L.A. Optimization of cultural conditions for biosurfactant production from *Candida lipolytica*. *Biocatal. Agric. Biotechnol.* **2014**, *3*, 48–57. [CrossRef]
78. Santos, D.K.F.; Rufino, R.D.; Luna, J.M.; Santos, V.A.; Salgueiro, A.A.; Sarubbo, L.A. Synthesis and evaluation of biosurfactant produced by *Candida lipolytica* using animal fat and corn steep liquor. *J. Pet. Sci. Eng.* **2013**, *105*, 43–50. [CrossRef]
79. Gusmão, C.A.B.; Rufino, R.D.; Sarubbo, L.A. Laboratory production and characterization of a new biosurfactant from *Candida glabrata* UCP 1002 cultivated in vegetable fat waste applied to the removal of hydrophobic contaminant. *World J. Microbiol. Biotechnol.* **2010**, *26*, 1683–1692. [CrossRef]
80. Alcântara, R.; Amores, J.; Canoira, L.; Fidalgo, E.; Franco, M.J.; Navarro, A. Catalytic production of biodiesel from soy-hean oil, used frying oil and tallow. *Biomass Bioenergy* **2000**, *18*, 515–527. [CrossRef]
81. Cvengros, Z.; Cvengrosova, Z. Used fruing oils and fats and their utilization in the production of methyl ester of higher fatty acids. *Biomass Bioenergy* **2004**, *27*, 173–181. [CrossRef]
82. Haba, E.; Espuny, M.J.; Busquets, M.; Manresa, A. Screening and production of rhamnolipids *Pseudomonas aeruginosa* 47T2 NCIB 40044 from waste flying oils. *J. Appl. Microbiol.* **2000**, *88*, 379–387. [CrossRef] [PubMed]
83. Benincasa, M.; Contiero, J.; Manresa, M.A.; Moraes, I.O. Rhamnolipid production by *Pseudomonas aeruginosa* LBI growing on soapstock as the sole carbon source. *J. Food Eng.* **2002**, *54*, 283–288. [CrossRef]
84. Shabtai, Y. Production of exopolysaccharides by *Acinetobacter* strains in a controlled fed-batch fermentation process using soap stock oil (SSO) as carbon source. *Int. J. Biol. Macromol.* **1990**, *12*, 145–152. [CrossRef]
85. Ghurye, G.L.; Vipulananda, C.; Wilson, R.C. A practical approach to biosurfactant production using nonaseptic fermantation of mixed cultures. *Biotechnol. Bioeng.* **1994**, *44*, 661–666. [CrossRef] [PubMed]
86. Kalogiannis, S.; Iakovidou, G.; Liakopoulou-Kyriakides, M.; Kyriakidis, D.A.; Skaracis, G.N. Optimization of xanthan gum production by *Xanthomonas campestris* grown in molasses. *Process Biochem.* **2003**, *39*, 249–256. [CrossRef]

87. Lazaridou, A.; Roukas, T.; Biliaderis, C.G.; Vaikousi, H. Characterization of pullulan produced from beet molasses by *Aureobasidium pullulans* in a stirred tank reactor under varying agitation. *Enzyme Microbiol. Technol.* **2002**, *31*, 122–132. [CrossRef]
88. Makkar, R.S.; Cameotra, S.S. Utilization of molasses for biosurfactant production by two *Bacillus* strains at thermophilic conditions. *J. Am. Oil Chem. Soc.* **1997**, *74*, 887–889. [CrossRef]
89. Sudhakar-Babu, P.; Vaidya, A.N.; Bal, A.S.; Kapur, R.; Juwarka, A.; Khanna, P. Kinetics of biosurfactants production by *Pseudomonas aeruginosa* strain from industrial wastes. *Biotechnol. Lett.* **1996**, *18*, 263–268. [CrossRef]
90. Luna, J.M.; Rufino, R.D.; Albuquerque, C.D.C.; Sarubbo, L.A.; Campos-Takaki, G.M. Economic optimized medium for tenso-active agent production by *Candida sphaerica* UCP 0995 and application in the removal of hydrophobic contaminant from sand. *Int. J. Mol. Sci.* **2011**, *12*, 2463–2476. [CrossRef] [PubMed]
91. Rufino, R.D.; Luna, J.M.; Rodrigues, G.I.B.; Campos-Takaki, G.M.; Sarubbo, L.A.; Ferreira, S.R.M. Application of a yeast biosurfactant in the removal of heavy metals and hydrophobic contaminant in a soil used as slurry barrier. *Appl. Environ. Soil Sci.* **2011**, *2011*. [CrossRef]
92. Sobrinho, H.B.S.; Rufino, R.D.; Luna, J.M.; Salgueiro, A.A.; Campos-Takaki, G.M.; Leite, L.F.C.; Sarubbo, L.A. Utilization of two agroindustrial by-products for the production of a surfactant by *Candida sphaerica* UCP 0995. *Process Biochem.* **2008**, *43*, 912–917. [CrossRef]
93. Nitschke, M.; Ferraz, C.; Pastore, G.M. Selection of microorganisms for biosurfactant production using agroindustrial wastes. *Braz. J. Microbiol.* **2004**, *435*, 81–85. [CrossRef]
94. Luna, J.M.; Rufino, R.D.; Sarubbo, L.A.; Campos-Takaki, G.M. Characterisation, surface properties and biological activity of a biosurfactant produced from industrial waste by *Candida sphaerica* UCP 0995 for application in the petroleum industry. *Coll. Surf. B Biointerfaces* **2013**, *102*, 202–209. [CrossRef] [PubMed]
95. Luna, J.M.; Rufino, R.D.; Sarubbo, L.A.; Rodrigues, L.R.M.; Teixeira, J.A.C.; Campos-Takaki, G.M. Evaluation antimicrobial and antiadhesive properties of the biosurfactant Lunasan produced by *Candida sphaerica* UCP 0995. *Curr. Microbiol.* **2011**, *62*, 1527–1534. [CrossRef] [PubMed]
96. Sarubbo, L.A.; Marçal, M.C.; Neves, M.L.C.; Porto, A.L.F.; Campos-Takaki, G.M. The use of babassu oil as substrate to produce bioemulsifiers by *Candida lipolytica*. *Can. J. Microbiol.* **1999**, *45*, 1–4. [CrossRef]
97. Raza, Z.A.; Khan, M.S.; Khalid, Z.M.; Rehman, A. Production kinetics and tensioactive characteristics of biosurfactant from a *Pseudomonas aeruginosa* mutant grown on waste frying oils. *Biotechnol. Lett.* **2006**, *28*, 1623–1631. [CrossRef] [PubMed]
98. Raza, Z.A.; Rehman, A.; Khan, M.S.; Khalid, Z.M. Improved production of biosurfactant by a *Pseudomonas aeruginosa* mutant using vegetable oil refinery wastes. *Biodegradation* **2007**, *18*, 115–121. [CrossRef] [PubMed]
99. Joshi, S.; Bharucha, C.; Jha, S.; Yadav, S.; Nerurkar, A.; Desai, A.J. Biosurfactant production using molasses and whey under thermophilic conditions. *Bioresour. Technol.* **2007**, *99*, 195–199. [CrossRef] [PubMed]
100. Dubey, K.V.; Juwarkar, A.A. Distillery and curd whey wastes as viable alternative sources for biosurfactant production. *World J. Microbiol. Biotechnol.* **2001**, *17*, 61–69. [CrossRef]
101. Dubey, K.V.; Juwarkar, A.A.; Singh, S.K. Adsorption-desorption process using woodbased activated carbon for recovery of biosurfactant from fermented distillery wastewater. *Biotechnol. Prog.* **2005**, *21*, 860–867. [CrossRef] [PubMed]
102. Rodrigues, L.R.; Teixeira, J.A.; Oliveira, R. Low-cost fermentative medium for biosurfactant production by probiotic bacteria. *Biochem. Eng. J.* **2006**, *32*, 135–142. [CrossRef]
103. Helmy, Q.; Kardena, E.; Funamizu, N.; Wisjnuprapto, W. Strategies toward commercial scale of biosurfactant production as potential substitute for it's chemically counterparts. *Int. J. Biotechnol.* **2011**, *12*, 66–86.
104. Akhtar, M.; Lentz, M.J.; Banchette, R.A.; Kirk, T.K. Corn steep liquor lowers the amount of inoculum for biopulping. *TAPPI J.* **1997**, *80*, 161–164.
105. Cardinal, E.V.; Hedrick, L.R. Microbiological assay of corn steep liquor for amino acid content. *J. Biol. Chem.* **1948**, *172*, 609–612. [PubMed]
106. Luna, J.M.; Rufino, R.D.; Jara, A.M.T.; Brasileiro, P.P.F.; Sarubbo, L.A. Environmental applications of the biosurfactant produced by *Candida sphaerica* cultivated in low-cost substrates. *Coll. Surf. A Physicochem. Eng. Asp.* **2015**, *480*, 413–418. [CrossRef]
107. Silva, N.M.P.R.; Rufino, R.D.; Luna, J.M.; Santos, V.A.; Sarubbo, L.A. Screening of *Pseudomonas* species for biosurfactant production using low-cost substrates. *Biocatal. Agric. Biotechnol.* **2014**, *3*, 132–139.

108. Muthusamy, K.; Gopalakrishnan, S.; Ravi, T.K.; Sivachidambaram, P. Biosurfactants: Properties, commercial production and application. *Curr. Sci.* **2008**, *94*, 736–747.
109. Marchant, R.; Banat, I.M. Biosurfactants: A sustainable replacement for chemical surfactants? *Biotechnol. Lett.* **2012**, *34*, 1597–1605. [CrossRef] [PubMed]
110. Winterburn, J.B.; Russell, A.B.; Martin, P.J. Integrated recirculating foam fractionation for the continuous recovery of biosurfactant from fermenters. *Biochem. Eng. J.* **2011**, *54*, 132–139. [CrossRef]
111. Rangarajan, V.; Sem, R. An inexpensive strategy for facilitated recovery of metals and fermentation products by foam fractionation process. *Coll. Surf. B Biointerfaces* **2013**, *104*, 99–106. [CrossRef] [PubMed]
112. Rodrigues, L.R.; Banat, I.M.; Teixeira, J.A.; Oliveira, R. Biosurfactants: Potential applications in medicine. *J. Antimicrob. Chemother.* **2006**, *57*, 609–618. [CrossRef] [PubMed]
113. Sobrinho, H.B.S.; Luna, J.M.; Rufino, R.D.; Porto, A.L.F.; Sarubbo, L.A. Assessment of toxicity of a biosurfactant from *Candida sphaerica* UCP 0995 cultivated with industrial residues in a bioreactor. *Electron. J. Biotechnol.* **2013**, *16*. [CrossRef]
114. US Energy Information Administration. Annual Energy Review, 2010. Available online: http://large.stanford.edu/courses/2012/ph241/druzgalski2/docs/aer.pdf (accessed on 05 September 2015).
115. Summers, D. Enhancing Oil Recovery: A Look at Stripper Wells. Available online: http//www.oilprice.com (accessed on 10 January 2011).
116. Austad, T.; Taugbøl, K. Chemical flooding of oil reservoirs 1. Low tension polymer flood using a polymer gradient in the three-phase region. *Coll. Surf. A Phys. Eng. Asp.* **1995**, *101*, 87–97. [CrossRef]
117. West, C.C.; Harwell, J.H. Surfactant and subsurface remediation. *Environ. Sci. Technol.* **1992**, *26*, 2324–2330. [CrossRef]
118. Sabatini, D.A.; Harwell, J.H.; Knox, R.C. Surfactant selection criteria for enhanced subsurface remediation. In *Innovative Subsurface Remediation*; Brusseau, M.L., Sabatini, D.A., Gierke, J.S., Annable, M.D., Eds.; American Chemical Society: Washington, WA, USA, 1999; pp. 8–23.
119. Sabatini, D.A.; Knox, R.C.; Harwell, J.H.; Wu, B. Integrated design of surfactant enhanced DNAPL remediation: Efficient super solubilization and gradient systems. *J. Contam. Hydrol.* **2000**, *45*, 99–121. [CrossRef]
120. Souza, E.C.; Vessoni-Penna, T.C.; Souza Oliveira, R.P. Biosurfactant-enhanced hydrocarbon bioremediation: An overview. *Int. Biodeterior. Biodegrad.* **2014**, *89*, 88–94. [CrossRef]
121. Sarubbo, L.A.; Rocha, R.B., Jr.; Luna, J.M.; Rufino, R.D.; Santos, V.A.; Banat, I.M. Some aspects of heavy metals contamination remediation and role of biosurfactants. *Chem. Ecol.* **2015**. [CrossRef]
122. Mnif, I.; Ghribi, D. Microbial derived surface active compounds: Properties and screening concept. *World J. Microbiol. Biotechnol.* **2015**, *31*, 1001–1020. [CrossRef] [PubMed]
123. Banat, I.M.; De Rienzo, M.A.D.; Quinn, G.A. Microbial biofilms: Biosurfactants as antibiofilm agents. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 9915–9929. [CrossRef] [PubMed]
124. Sachdev, D.P.; Cameotra, S.S. Biosurfactants in agriculture. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 1005–1016. [CrossRef] [PubMed]
125. Mulligan, C.N. Recent advances in the environmental applications of biosurfactants. *Curr. Opin. Coll. Interface Sci.* **2009**, *14*, 372–378. [CrossRef]
126. Environmental Protection Agency (EPA), U.S. *Treatment Technologies for Site Cleanup: Annual Status Report*, 10th ed.; Office of Solid Waste and Emergency Response: Cincinnati, Ohio, USA, 2001.
127. Satpute, S.K.; Banat, I.M.; Dhakephalkar, P.K.; Banpurkar, A.G.; Chopade, B.A. Biosurfactants, bioemulsifiers and exopolysaccharides from marine microorganisms. *Biotechnol. Adv.* **2010**, *28*, 436–450. [CrossRef] [PubMed]
128. Shavandi, M.; Mohebali, G.; Haddadi, A.; Shakarami, H.; Nuhi, A. Emulsification potential of a newly isolated biosurfactantproducing bacterium, *Rhodococcus* sp. strain TA6. *Coll. Surf. B Biointerfaces* **2011**, *82*, 477–482. [CrossRef] [PubMed]
129. Kuyukina, M.S.; Ivshina, I.B.; Makarov, S.O.; Litvinenko, L.V.; Cunningham, C.J.; Philip, J.C. Effect of biosurfactants on crude oil desorption and mobilization in a soil system. *Environ. Int.* **2005**, *31*, 155–161. [CrossRef] [PubMed]

130. Aparna, A.; Srinikethan, G.; Hedge, S. Effect of addition of biosurfactant produced by *Pseudomonas* ssp. on biodegradation of crude oil. In International Proceedings of Chemical, Biological & Environmental Engineering, Proceedings of the 2nd International Proceedings of Chemical, Singapore, Singapore, 26–28 February 2011; Volume 6, pp. 71–75.
131. Franzetti, A.; Caredda, P.; Ruggeri, C.; La Colla, P.; Tamburini, E.; Papacchini, M.; Bestetti, G. Potential applications of surface active compounds by *Gordonia* sp. strain BS29 in soil remediation technologies. *Chemosphere* **2009**, *75*, 810–807. [CrossRef] [PubMed]
132. Bai, G.; Brusseau, M.L.; Miller, R.M. Biosurfactant-enhanced removal of residual hydrocarbon from soil. *J. Contam. Hydrol.* **1997**, *25*, 157–170. [CrossRef]
133. Silva, R.C.F.S.; Rufino, R.D.; Luna, J.M.; Farias, C.B.B.; Filho, H.J.B.; Santos, V.A.; Sarubbo, L.A. Enhancement of biosurfactant production from *Pseudomonas cepacia* CCT6659 through optimisation of nutritional parameters using Response Surface Methodology. *Tenside Surf. Det.* **2013**, *50*, 137–142. [CrossRef]
134. Chaprão, M.J.; Ferreira, I.N.S.; Correa, P.F.; Rufino, R.D.; Luna, J.M.; Silva, E.J.; Sarubbo, L.A. Application of bacterial and yeast biosurfactants for enhanced removal and biodegradation of motor oil from contaminated sand. *Electron. J. Biotechnol.* **2015**, *18*, 471–479. [CrossRef]
135. Rufino, R.D.; Luna, J.M.; Campos Takaki, G.M.; Sarubbo, L.A. Characterization and properties of the biosurfactant produced by *Candida lipolytica* UCP 0988. *Electron. J. Biotechnol.* **2014**, *17*, 34–38. [CrossRef]
136. Rufino, R.D.; Luna, J.M.; Marinho, P.H.C.; Farias, C.B.B.; Ferreira, S.R.M.; Sarubbo, L.A. Removal of petroleum derivative adsorbed to soil by biosurfactant Rufisan produced by *Candida lipolytica*. *J. Pet. Sci. Eng.* **2013**, *109*, 117–122. [CrossRef]
137. Rufino, R.D.; Luna, J.M.; Sarubbo, L.A.; Rodrigues, L.R.M.; Teixeira, J.A.C.; Campos-Takaki, G.M. Antimicrobial and anti-adhesive potential of a biosurfactant Rufisan produced by *Candida lipolytica* UCP 0988. *Coll. Surf. B Biointerfaces* **2011**, *84*, 1–5. [CrossRef] [PubMed]
138. Mulligan, C.N.; Wang, S. Remediation of a heavy metal contaminated soil by a rhamnolipid foam. In *Geoenvironmental Engineering: Integrated Management of Groundwater and Contaminated Land*; Thomas Telford: London, UK, 2004; pp. 544–551.
139. Singh, A.; Van-Hamme, J.D.; Ward, O.P. Surfactants in microbiology and biotechnology: Part 2. Application aspects. *Biotechnol. Adv.* **2007**, *25*, 99–121. [CrossRef] [PubMed]
140. Hazra, C.; Kundu, D.; Chaudhari, A. Biosurfactant-assisted bioaugmentation in bioremediation. In *Microorganisms in Environmental Management: Microbes and Environment*, 1st ed.; Satyanarayana, T., Johri, B.N., Prakash, A., Eds.; Springer: New York, NY, USA, 2012; pp. 631–664.
141. Ochoa-Loza, F.J.; Artiola, J.F.; Maier, R.M. Stability constants for the complexation of various metals with a rhamnolipid biosurfactant. *J. Environ. Qual.* **2001**, *30*, 479–485. [CrossRef] [PubMed]
142. Swarnkar, V.; Agrawal, N.; Tomar, R. Sorption of chromate and arsenate bysurfactant modified erionite (E-SMZ). *J. Dispers. Sci. Technol.* **2012**, *33*, 919–927. [CrossRef]
143. Hashim, M.A.; Mukhopadhyay, S.; Sahu, J.N.; Sengupta, B. Remediation technologies for heavy metal contaminated groundwater. *J. Environ. Manag.* **2011**, *92*, 2355–2388. [CrossRef] [PubMed]
144. Asçi, Y.; Nurbas, M.; Açikel, Y. A comparative study for the sorption of Cd(II) by soils with different clay contents and mineralogy and the recovery of Cd(II) using rhamnolipid biosurfactant. *J. Hazard. Mater.* **2008**, *154*, 663–673. [CrossRef] [PubMed]
145. Nash, J.; Traver, R.P.; Downey, D.C. Surfactant enhanced *in situ* soil washing. US.EPA HWERL: Edilsin, NJ, USA, 1987.
146. Herman, D.C.; Artiola, J.F.; Miller, R.M. Removal of cadmium, lead and zinc from soil by a rhamnolipid biosurfactant. *Environ. Sci. Technol.* **1995**, *29*, 2280–2285. [CrossRef] [PubMed]
147. Mulligan, C.N.; Yong, R.N.; Gibbs, B.F. Metal removal from contaminated soil and sediments by the biosurfactant surfactin. *Environ. Sci. Technol.* **1999**, *33*, 3812–3820. [CrossRef]
148. Ochoa-Loza, F.J.; Noordman, W.H.; Jannsen, D.B.; Brusseau, M.L.; Maier, R.M. Effect of clays, metal oxides, and organic matter on rhaminolipid biosurfactant sorpition by soil. *Chemosphere* **2007**, *66*, 1634–1642. [CrossRef] [PubMed]
149. Das, P.; Mukherjee, S.; Sen, R. Antiadhesive action of a marine microbial surfactant. *Coll. Surf. B Biointerfaces* **2009**, *71*, 183–186. [CrossRef] [PubMed]
150. Wen, J.; Stacey, S.P.; Mclaughlin, M.J.; Kirby, J.K. Biodegradation of rhamnolipid, EDTA and citric acid in cadmium and zinc contaminated soils. *Soil Biol. Biochem.* **2009**, *41*, 2214–2221. [CrossRef]

151. Kitamoto, D.; Isoda, H.; Nakahara, T. Functions and potential applications of glycolipid biosurfactants: From energy-saving materials to gene delivery carriers. *J. Biosci. Bioeng.* **2002**, *94*, 187–201. [CrossRef]
152. Nitschke, M.; Pastore, G.M. Biossurfactantes: Propriedades e aplicações. *Química Nova* **2002**, *25*, 772–776. [CrossRef]
153. Slizovskiy, I.B.; Kelsey, J.W.; Hatzinger, P.B. Surfactant-facilitated remediationof metal-contaminated soils: Efficacy and toxicological consequences to earth worms. *Environ. Toxicol. Chem.* **2011**, *30*, 112–123. [CrossRef] [PubMed]
154. Almeida, C.M.R.; Dias, A.C.; Mucha, A.P.; Bordalo, A.A.; Vasconcelos, M.T.S.D. Influence of surfactants on the Cu phytoremediation potential of a saltmarsh plant. *Chemosphere* **2009**, *75*, 135–140. [CrossRef] [PubMed]
155. Maity, J.P.; Huang, Y.M.; Fan, C.-W.; Chen, C.-C.; Li, C.-Y.; Hsu, C.-M.; Chang, Y.-F.; Wu, C.-I.; Chen, C.-Y.; Jean, J.-S. Evaluation of remediation process with soapberry derived saponin for removal of heavy metals from contaminated soils in Hai-Pu Taiwan. *J. Environ. Sci.* **2013**, *25*, 1180–1185. [CrossRef]
156. Ozturk, S.; Kaya, T.; Aslim, B.; Tan, S. Removal and reduction of chromium by *Pseudomonas* spp. and their correlation to rhamnolipid production. *J. Hazard. Mater.* **2012**, *231*, 64–69. [PubMed]
157. Albuquerque, C.F.; Luna-Finkler, C.L.; Rufino, R.D.; Luna, J.M.; Menezes, C.T.B.; Santos, V.A.; Sarubbo, L.A. Evaluation of biosurfactants for removal of heavy metal ions from aqueous effluent using flotation techniques. *Int. Rev. Chem. Eng.* **2012**, *4*, 156–161.
158. Menezes, C.T.B.; Barros, E.C.; Rufino, R.D.; Luna, J.M.; Sarubbo, L.A. Replacing synthetic with microbial surfactants as collectors in the treatment of aqueous effluent produced by acid mine drainage using the dissolved air flotation technique. *Appl. Biochem. Biotechnol.* **2011**, *163*, 540–546. [CrossRef] [PubMed]
159. Campos, J.M.; Stamford, T.L.M.; Sarubbo, L.A. Production of a Bioemulsifier with Potential Application in the Food Industry. *Appl. Biochem. Biotechnol.* **2014**, *172*, 3234–3252. [CrossRef] [PubMed]
160. Campos, J.M.; Stamford, T.L.M.; Rufino, R.D.; Luna, J.M.; Stamford, T.C.M.; Sarubbo, L.A. Formulation of mayonnaise with the addition of a bioemulsifier isolated from *Candida utilis*. *Toxicol. Rep.* **2015**, *2*, 1164–1170. [CrossRef]
161. Torabizadeh, H.; Shojaosadati, S.A.; Tehrani, H.A. Preparation and characterization of bioemulsifier from *Saccharomyces cerevisiae*. *Lebensm. Wiss. Technol.* **1996**, *29*, 734–737. [CrossRef]
162. Abalos, A.; Pinazo, A.; Infante, M.R.; Casals, M.; García, F.; Manresa, A. Physicochemical and antimicrobial properties of new rhamnolipids produced by *Pseudomonas aeruginosa* AT10 from soybean oil refinery wastes. *Langmuir* **2001**, *17*, 1367–1371. [CrossRef]
163. Joshi-Navare, K.; Prabhune, A. A biosurfactant sophorolipid acts in synergy with antibiotics to enhance their efficiency. *BioMed. Res. Int.* **2013**, *2013*, 1–8. [CrossRef] [PubMed]
164. Fracchia, L.; Banat, J.J.; Cavallo, M.; Ceresa, C.; Banat, I.M. Potential therapeutic applications of microbial surface-active compounds. *Bioengineering* **2015**, *2*, 144–162.
165. Robbel, L.; Marahiel, M.A. Daptomycin, a bacterial lipopeptide synthesized by a nonribosomal machinery. *J. Biol. Chem.* **2010**, *285*, 27501–27508. [CrossRef] [PubMed]
166. Tally, F.P.; Zeckel, M.; Wasilewski, M.M. Daptomycin, A novel agent for Gram-positive infections. *Expert Opin. Investig. Drug* **1999**, *8*, 1223–1238. [CrossRef] [PubMed]
167. Zottola, E.A. Microbial attachment and biofilm formation: A new problem for the food industry? *Food Technol.* **1994**, *48*, 107–114.
168. Meylheuc, T.; Van Oss, C.J.; Bellon-Fontaine, M.N. Adsorption of biosurfactants on solid surfaces and consequences regarding the bioahesion of *Listeria monocytogenes* LO28. *J. Appl. Microbiol.* **2001**, *91*, 822–832. [CrossRef] [PubMed]
169. Kitamoto, D.; Morita, T.; Fukuoka, T.; Konishi, M.; Imura, T. Self-assembling properties of glycolipid biosurfactants and their potential applications. *Curr. Opin. Coll. Interface Sci.* **2009**, *14*, 315–328. [CrossRef]
170. Kiran, G.S.; Sabu, A.; Selvin, J. Synthesis of silver nanoparticles by glycolipid biosurfactant produced from marine *Brevibacterium casei* MSA19. *J. Biotechnol.* **2010**, *148*, 221–225. [CrossRef] [PubMed]
171. Palanisamy, P. Biosurfactant mediated synthesis of NiO nanorods.*Mater. Lett.* **2008**, *62*, 743–746. [CrossRef]
172. Reddy, A.S.; Chen, C.-Y.; Baker, S.C.; Chen, C.-C.; Jean, J.-S.; Fan, C.-W.; Chen, H.-R.; Wang, J.-C. Synthesis of silver nanoparticles using surfactin: A biosurfactant stabilizing agent. *Mater Lett.* **2009**, *63*, 1227–1230. [CrossRef]

173. Farias, C.B.B.; Silva, A.F.; Rufino, R.D.; Luna, J.M.; Souza, J.E.; Sarubbo, L.A. Synthesis of silver nanoparticles using a biosurfactant produced in low-cost medium as stabilizing agent. *Electron. J. Biotechnol.* **2014**, *17*, 122–125. [CrossRef]
174. Biswas, M.; Raichur, A.M. Electrokinetic and rheological properties of nano zirconia in the presence of rhamnolipid biosurfactant. *J. Am. Ceram. Soc.* **2008**, *91*, 3197–3201. [CrossRef]
175. Santos, H.F.; Carmo, F.L.; Paes, J.E.S.; Rosado, A.S.; Peixoto, R.S. Bioremediation of mangroves impacted by petroleum. *Water Air Soil Pollut.* **2011**, *216*, 329–350. [CrossRef]

CAPÍTULO 2

Artigo publicado no Periódico Internacional Biocatalysis and Agricultural Biotechnology

Optimization of Cultural Conditions for Biosurfactant Production from *Candida lipolytica*

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Abstract

The maximizationof the production ofmetabolitesof industrial interest from fermentative processes, such as biosurfactants, requires standardizationof the medium andthe cultivation conditions. In this sense, experiments were conductedto maximize theproductionof the biosurfactantfrom*Candidalipolytica*UCP0988cultivatedon 5%animal fatand 2.5% corn steep liquor using a 2^3 full factorial design. The effects and interactions of the agitation speed (200, 300 and 400 rpm), the variables aeration (0, 1 and 2 vvm) and time of cultivation (48, 96 and 144 hours) on the surface tension, yield and biomass were evaluated.The results showed thatthe variabletime of cultivationhad positive influenceon the production ofbiosurfactant, while the increase of the variablesaerationand agitation showed a negative effect. These results indicate that the biosurfactant has a strong potential to be applied as a clean-up of oil spills at sea and on shorelines.

Keywords*:*Biosurfactant; *Candida lipolytica*; animal fat; corn steep liquor; response surface methodology; surface tension

1. Introduction

Interest in microbially produced biosurfactants has increased recently, due mainly to their potential as agents in enhanced oil recovery. A variety of microbes and their products have been assessed for their surface-active properties, and it has been suggested that biosurfactants may prove useful in a broad spectrum of potential applications which presently utilize synthetic surfactants (Banat et al., 2010). Biosurfactants can be as effective as some widely-used synthetics and have conceivable advantages, often being more biodegradable than many synthetics, reducing pollutant loads, and sometimes being less sensitive to extremes of temperature, Ph, or salinity (Pacwa-Plociniczak et al., 2011). In economic terms there are prospects of their being cheaper than the synthetics, if production by fermentation technology from cheap renewable substrates is compared with production by petrochemical routes from a diminishing hydrocarbon resource (Marchant and Banat, 2012). One of the strategies to improve production is to optimize the growth media and cultivation conditions in order to get maximum production (Mukherjee et al., 2006). Statistical experimental designs such as Response Surface Methodology (RSM) and

Contour Curve (CC), factorial and Taguchi designs have been used to increase the product yields (Sen, 1997; Rodrigues et al., 2006; Rivera et al., 2007; Whei et al., 2007). These statistical optimization procedures minimize the number of experiments saving time and labor.

Statistical techniques commonly referred to as Response Surface Methodology and Contour Curve are powerful experimental design tools that have been used to optimise and evaluate the performance of complex systems (Aleboyeh et al.,2008; Barros Neto et al., 2010; Chaves, 2008).

In the present study, the production of the biosurfactant from the yeast *Candida lipolytica* UCP0988 using low-cost fermentative medium based on animal fat and corn steep liquor was improved by using a bioreactor and a full factorial design. One method of modelling, i.e., RSM/CC, applying a 2^3full-factorial, was used to determine the relationship and influence between input and output variables on the process efficiency. The input variables were agitation, aeration and time of the process, and the output variables were the surface tension, yield and biomass. The importance of each input variable on the variation of the output response was determined and compared with the results obtained by RSM and CC. The effect of the three factors (agitation, aeration and time) was investigated. The factorial *k* (2^3) includes eight points and three repetitions at the centre point ($2^3 + 3$), totalling 11 runs.

2. Materials and Methods

2.1 Materials

All chemicals reagent were of analytical grade. Growth media were purchased from Difco Laboratories, USA. The animal fat used was white choice grease from a bovine processing plant located in the city of Recife (Brazil) and was used without any further processing.

Corn steep liquor was obtained from Corn Products from Brazil in the city of Cabo de Santo Agostinho (Brazil). According to Akhtar et al. (1997) and Cardinal and Hedrick (1948), corn steep liquor is 21 to 45% protein, 20 to 26% lactic acid, 8% ash (containing Ca^{2+}, Mg^{2+}, K^{+}), 3% sugar and has low fat content (0.9 to 1.2%).

2.2 Microorganism

Candida lipolytica UCP0988 was obtained from the culture collection of the *Universidade Católica de Pernambuco* (Brazil). The microorganism was maintained at

5 °C on yeast mould agar slants containing (w/v) yeast extract (0.3%), malt extract (0.3%), tryptone (0.5%), D-glucose (1.0%) and agar (5.0%). Transfers were made to fresh agar slants each month to maintain viability.

2.3 Growth conditions

The inoculum of *Candida lipolytica* was prepared by transferring cells grown on a slant to 50 ml of yeast mould broth. The seed culture was incubated for 24 h at 28 °C and agitated at 150 rpm. The yeast was cultivated in a submerged culture with agitation in a New Bruswick C-24 shaker. The production medium contained 5% animal fat and 2.5% corn steep liquor.The medium was sterilised by autoclaving at 121 °C for 20 min (all components were sterilised together). The final pH of the medium was 5.3. The inoculum (1% v/v) was introduced in the amount of 10^4 cells/ml to a cool medium yeast.

2.4 Biosurfactant production

Biosurfactant production was performed in a 2 L bioreactor (BioStat B-plus, Sartorius) with a working volume of 1.2 L operated in a batch mode, with controlled pH (5.3) and temperature (28 °C). The reactor was aerated by a sparger. The culture medium was inoculated with a 24 h inoculum. Values of aeration, agitation speed and time of cultivation were established according to a factorial design. All assays were carried out in triplicate and did not vary more than 5%.

2.5 Factorial design

Response Surface Methodology and Contour Curve were used for the experimental design and optimisation. The most popular class of first-order designs was used for RSM and CC in the experimental design. The full-factorial is well suited for fitting a linear surface, which usually works well for process optimisation (Rodrigues and Lemma, 2009; Calado and Montgomery, 2003; Myers and Montgomery, 2002).

Therefore, a 2^3full-factorial with three replicates at the centre point was applied as the experimental design. The parameters mentioned (time, aeration and agitation) were chosen as independent variables and the surface tension, yield and biomass were chosen as the output variables or responses. Independent variables and their experimental ranges that were determined by experiments are given in Table 1.

Experimental data were analysed using the response surface regression and contour curve regression procedure of a statistical analysis software package (Statistic 8.0) and fitted to a first-order polynomial model.

Table 1
Experimental range and levels of the process independent variables

Factor	Range and level		
Independent Variable x_i	**-1**	**0**	**+1**
Time (h) X_1	48	96	144
Aeration (vvm) X_2	0	1	2
Agitation(rpm) X_3	200	300	400

2.6 Biomass estimation

Biomass was monitored by estimating the cell dry weight after removal of the hydrophobic substrates at 10000 *g* for 15 min (Felse et al., 2007). Briefly, a 10-mL aliquot of the fermentation broth was centrifuged to obtain a cell pellet. The supernatant was discarded and the cell pellet was washed twice with distilled water to remove the medium components. The pellet was then washed with 10 mL of ethyl acetate to remove hydrophobic substances. The washed cells were centrifuged to obtain a pellet, which was dried in vacuum to a constant weight (indicated by a variation of less than ± 0.2 g). Suitable calculations were made to express cell mass in terms of g/L.

2.7 Surface tension

The surface tension of the culture supernatants obtained by centrifuging the cultures at 5000 g for 20 minutes was measured using a Sigma 700 digital surface tensiometer (KSV Instruments LTD - Finland) working on the principle of the Du Nuoy ring method. Ten millilitres of each sample were transferred to a clean 20-mL beaker and placed on the tensiometer platform. A platinum wire ring was submerged in the solution and then slowly pulled through the liquid-air interface to measure surface tension (mN/m). Between each measurement, the platinum wire ring was rinsed with chromic acid, deionised water and acetone and was then flamed and allowed to dry. Calibration was performed using Mill-Q-4 ultrapure distilled water (surface tension = 71.5 mN/m ±0.5) before taking measurements of the samples.

2.8 Biosurfactant isolation

The biosurfactant was recovered from the cell-free broth by cold acetone precipitation, as described by Ilori et al. (2005). Three volumes of chilled acetone were added and allowed to stand for 10 h at 4 ºC. The precipitate was collected by centrifugation and evaporated to dryness to remove residual acetone. The precipitate was then re-dissolved in sterile water. The yield in isolated biosurfactant was expressed in g/L.

3. Results and Discussion

Maximizing the production of metabolites of interest from industrial fermentation processes, such as biosurfactants, however, requires standardization of the medium and cultivation conditions (Sarubbo, 2006). Therefore, the use of statistical modeling is an important tool that can be used to explain the influence not only more relevant, but also the interaction parameters involved in fermentation performance of a given process (Rufino, 2007; Luna et al., 2011). According to Davila et al. (1992), is an efficient way to generate information from a few experimental tests, thus reducing the costs and time required for development of experimental procedures.

In this sense, tests performed to optimize the production of the surfactant were evaluated by means of a 2^3 full-factorial design, in order to analyze the main effects and interactions of variables: aeration, agitation speed and time of cultivation on the response variables surface tension, biosurfactant yield and biomass concentration.

3.1 Study of the surface tension

The following response equation was used to correlate the dependent and independent variables given by eq 1:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \text{ ,} \qquad (01)$$

where*Y* is the response variable or surface tension efficiency; b_0 is a constant; b_1, b_2 and b_3 are regression coefficients for the linear effects and b_{12}, b_{13} and b_{23} are interaction coefficients.

To analyse the mathematical model, adjustments to the points were made by nonlinear regression methods. Table 2 shows the adjustments made between the observed (experimental) and predicted values (simulated) of the model to obtain the best response in relation the lower surface tension (<20 mN/m). At this rate, the

experimental points varied from 11 to 55% and predicted points varied from 9.5 to 53.28% (Figure 1).

Table 2

2^3full-factorial with three replicates of the centre point for the surface tension efficiency

Run	Agitation (rpm)	Aeration (vvm)	Time (h)	Surface Tension(mN/m)	
				Experimental	Predicted
1	-1	-1	-1	36.09	34.58
2	+1	-1	-1	41.20	39.48
3	-1	+1	-1	55.00	53.28
4	+1	+1	-1	48.77	47.26
5	-1	-1	+1	21.20	19.48
6	+1	-1	+1	46.87	45.36
7	-1	+1	+1	11.01	9.50
8	+1	+1	+1	26.19	24.47
9	0	0	0	30.64	34.18
10	0	0	0	30.00	34.17
11	0	0	0	29.00	34.18

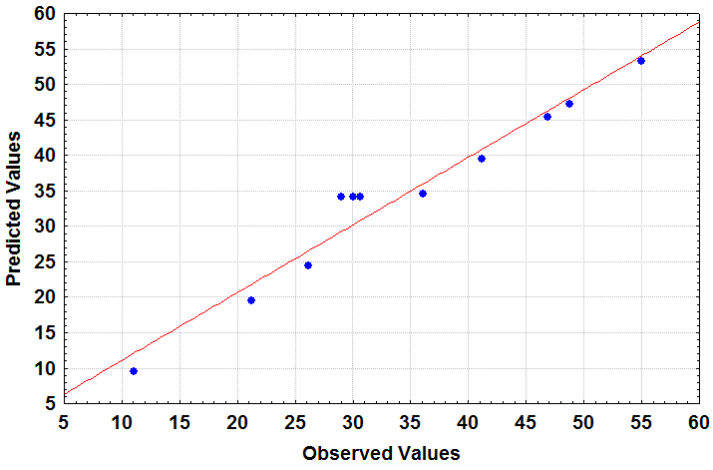

Fig. 1. Observed values *versus* predicted values by model for the answer surface tension

The regression coefficient values, standard deviation, t_{exp} and significance level are presented in Table 3. It can be seen that b_2 and b_3, as well as the interaction coefficients b_{12}, b_{13} and b_{23}, are significant. Therefore, the linear effect of the variables (the coefficients b_2 and b_3) and the interaction of the agitation with aeration (coefficients b_{12}), agitation with time (coefficients b_{13}) and aeration with time (coefficients b_{23}) are the most influential parameters. The significance of these interaction effects between variables would have been lost if the experiments were conducted using conventional methods.

The application of RSM offers, on the basis of parameter estimation (Table 3), the following empirical relationship (eq 02) between the surface tension (Y) and independent variables studied:

$$Y = (47.730) + (-0.028)x_1 + (21.975)x_2 + (-0.376)x_3 + (-0.027)x_1x_2 + (0.001)x_1x_3 + (-0.149)x_2x_3 , \quad (02)$$

Table 3

Estimated regression coefficients and corresponding t_{exp} as well as significance levels for the surface tension

Coefficient	Value	Standard deviation	$t_{exp.}$	Significance level (%)
b_0	47.730	2.333	20.460	<0.01
b_1	-0.028	0.007	-3.908	0.06
b_2	21.975	1.093	20.097	<0.01
b_3	-0.376	0.020	-18.617	<0.01
b_{12}	-0.027	0.003	-9.338	0.011
b_{13}	0.001	0.000	17.952	<0.01
b_{23}	-0.149	0.006	-24.531	<0.01

The results predicted by eq 03 indicate good agreement between the experimental and predicted values for the process efficiency. An analysis of variance (ANOVA) showed that the predictive model represented the experimental data at a level of approximately 95.39% because the significance level calculated from the ratio

of mean squares obtained from the regression was 0.68. Additionally, the regression model had a high coefficient of determination ($R(2) = 0.890$).

This implies that 89% of the variation in the process efficiency is explained by the independent variables and also that only approximately 11% of the variation was not explained by the model. The model in eq 03 was then optimised. The optimal values of the process parameters were obtained in coded units, converted to uncoded units by using eq 3 and then experimentally validated, as shown in Table 4.

Table 4

Optimum values of the process parameters for the maximum process efficiency

Parameter	Optimum value	
	Predicted	Experimental
Time (h)	144	150
Aeration (vvm)	0	0
Agitation(rpm)	200	200
Surface tension (mN/m)	19.48	18.53

Silva et al. (2013) optimised the production of a biosurfactant by *Pseudomonas cepacia*CCT6659 with aid of a combination of a Rotational Central Composite Design (RCCD) and Response Surface Methodology (RSM). The empirical forecast model developed through RSM regarding effective nutritional factors was adequate for explaining 89% of the variation observed in biosurfactant production. Maximal reduction in surface tension of 26 mN m^{-1} was obtained under the optimal conditions

Figures 2, 3 and 4 show the response surfaces and contour curves determined. In these experiments, the surface tensionwas assessed varying the time between 48 and 144 h, the aeration between 0 and 2 vvm, and the agitation between 200 and 400 rpm.

Figure 2 shows that an agitation below than 200 rpm and an aeration equal to 0 vvm, a lower surface tension is obtained (<20 mN/m). However, when the agitation is greater than 320 rpm and the aeration is below 1 vvm, the highest surface tension values are obtained (>40 mN/m).

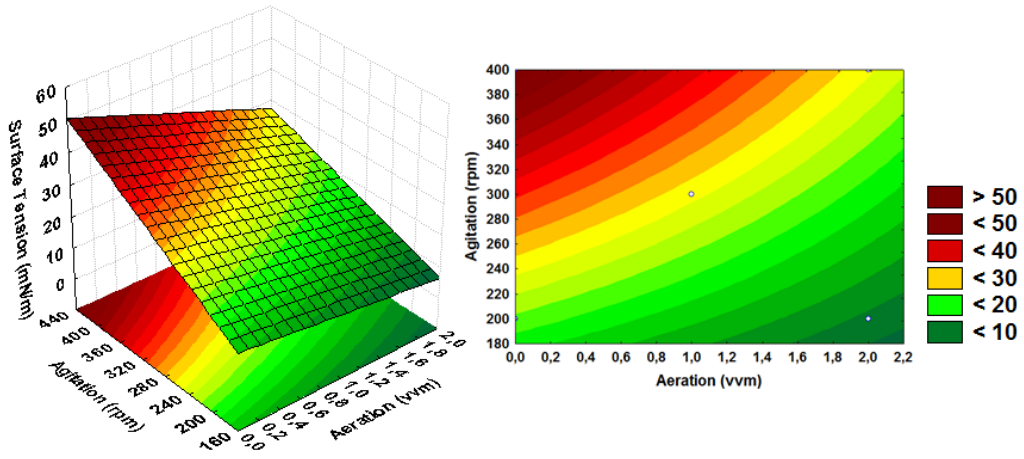

Fig. 2. Response surface and contour curves for thesurface tension. (time) = 144 h

Figure 3 shows that an agitation below than 200 rpm and a time of the process higher than 120 h, a lower surface tension is obtained (<20 mN/m). However, when the agitation is greater than 380 rpm to a time of the process higher than 120 h, the highest surface tension values are obtained (>50 mN/m).

Oliveira et al. (2006) observed, for *P. aeruginosa*, the decrease of the surface tension to 35 mN/m at 150 rpm, while the velocities of 50,100 and 200 rpm increased the surface tension to 55.39 and 51mN/m, respectively. Cunha et al. (2004) observed that agitation had a negative effect on surface tension reduction by the biosurfactant produced by *Serratia* sp. SVGG16, and that best results were obtained with the lowest value, of 100 rpm, when compared to 200 and 300rpm.

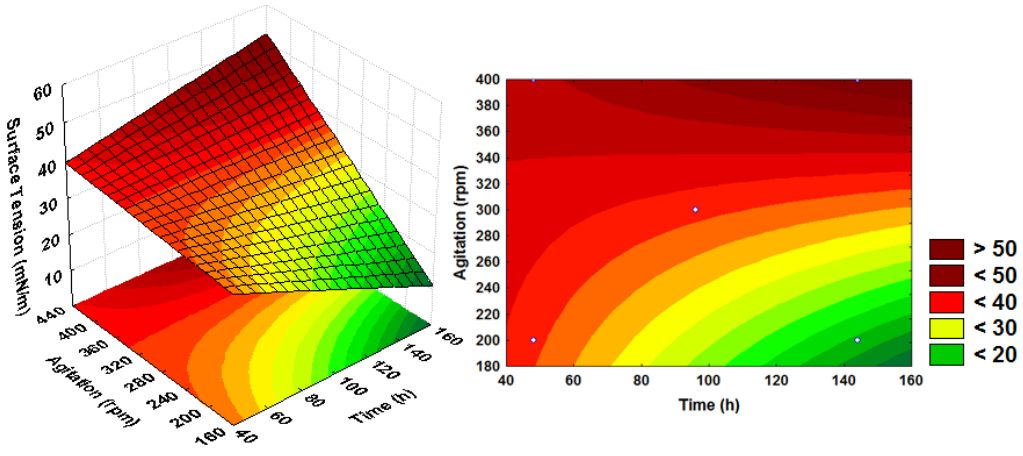

Fig. 3. Response surface and contour curves for thesurface tension. (Aeration) = 0 vvm

Figure 4 shows that an aeration greater than 1 vvm and a time of the process higher than 120 h, a lower surface tension values is obtained (<10 mN/m). When the aeration is equal to 0 vvm and a time higher than 120 h, the surface tension is below

(<20 mN/m).However, an aeration greater than 1.4 vvm and a time than 40 h, the highest surface tension values are obtained (>50 mN/m).

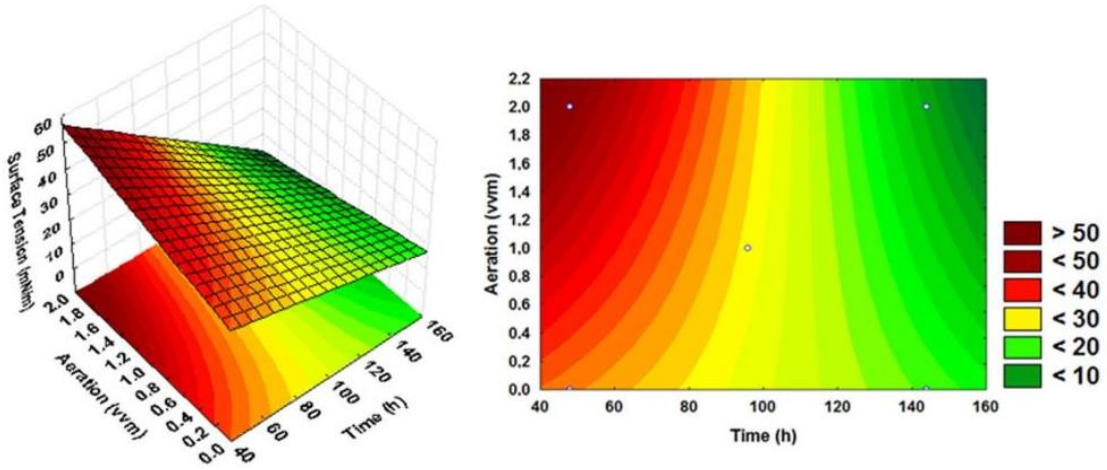

Fig. 4. Response surface and contour curves for thesurface tension. (Agitation) = 200 rpm

The reduction in surface tension is used as a primary criterion for selecting microorganisms producing biosurfactants, although emulsifiers and dispersants do not necessarily have ability to reduce surface tension (Yossef et al., 2004).

According to literature, the bacterial biosurfactants are more effective inreducing surface tension. In particular, the bacterium *Pseudomonasaeruginosa* have been the most studied microorganism to produce potent biosurfactants. Most biosurfactant produced by this bacterium has demonstrated the ability to reduce surface tension to values around 28-27mN/m (Santa Anna et al., 2001; Silva et al., 2010).Although biosurfactants produced by yeasts described in the literature in the past decades have demonstrated ability to reduce surface tension to values around 35 mN/m (Kitamoto, 2002), more recent research has revealed values consistent with bacterial biosurfactants, as the result obtained in this work for the biosurfactant from *C.lipolytica* UCP0988. Rufino e tal.(2008) noted that the biosurfactant produced by the yeast *C.lipolytica* grown on industrial waste as substrate reduced the surface tension of the medium to 26 mN/m within 72 hours of culture, while Luna e tal.(2009), using cotton seed oil, glucose and yeast extractin the production of a surfactant from *C.glabrata*, found a reduction of surface tension to 31 mN/m after 144 hours of cultivation.

3.2 Study of the biomass

The following response equation was used to correlate the dependent and independent variables given by eq 3:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{23}x_2x_3 \text{ ,} \quad (03)$$

where *Y* is the response variable or biomass efficiency; b_0 is a constant; b_1, b_2 and b_3 are regression coefficients for the linear effects and b_{12} and b_{23} are interaction coefficients.

To analyse the mathematical model, adjustments to the points were made by nonlinear regression methods. Table 5 shows the adjustments made between the observed (experimental) and predicted values (simulated) of the model to obtain the best response in relation the highest biomass values (>6 g/L). At this rate, the experimental points varied from 1 to 9.7% and predicted points varied from 1.10 to 9.19% (Figure 5).

Table 5

2^3full-factorial with three replicates of the centre point for the biomass efficiency

Run	Agitation (rpm)	Aeration (vvm)	Time (h)	Biomass(g/L)	
				Experimental	Predicted
1	-1	-1	-1	1.58	1.10
2	+1	-1	-1	1.03	1.21
3	-1	+1	-1	5.55	5.73
4	+1	+1	-1	3.39	2.91
5	-1	-1	+1	7.32	7.50
6	+1	-1	+1	6.53	6.05
7	-1	+1	+1	9.68	9.19
8	+1	+1	+1	4.61	4.79
9	0	0	0	4.37	4.80
10	0	0	0	4.31	4.81
11	0	0	0	4.30	4.81

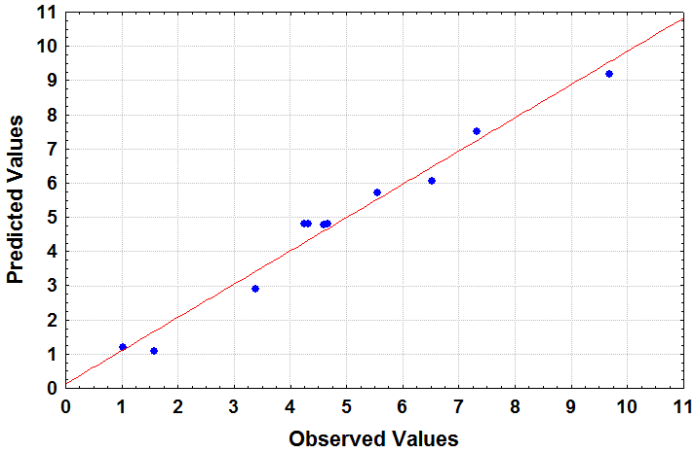

Fig. 5. Observed values *versus* predicted values by model for the answer biomass

The regression coefficient values, standard deviation, t_{exp} and significance level are presented in Table 6. It can be seen that b_2 and b_3, as well as the interaction coefficients b_{12} and b_{23}, are significant. Therefore, the linear effect of the variables (the coefficients b_2 and b_3) and the interaction of the agitation with aeration (coefficients b_{12}) and aeration with time (coefficients b_{23}) are the most influential parameters.

Table 6

Estimated regression coefficients and corresponding t_{exp} as well as significance levels for the biomass

Coefficient	Value	Standard deviation	$t_{exp.}$	Significance level (%)
b_0	-3.023	0.642	-4.710	0.04
b_1	0.005	0.002	2.306	0.15
b_2	4.530	0.301	15.060	<0.01
b_3	0.083	0.006	14.977	<0.01
b_{12}	-0.007	0.001	-9.159	0.01
b_{23}	-0.015	0.002	-9.169	0.01

The application of RSM offers, on the basis of parameter estimation (Table 6), the following empirical relationship (eq 04) between the biomass (Y) and independent variables studied:

$$Y = (-3.023) + (0.005)x_1 + (4.530)x_2 + (0.083)x_3 + (-0.007)x_1x_2 + (-0.015)x_2x_3 , \quad (04)$$

The results predicted by eq 05 indicate good agreement between the experimental and predicted values for the process efficiency. An analysis of variance (ANOVA) showed that the predictive model represented the experimental data at a level of approximately 97.27% because the significance level calculated from the ratio of mean squares obtained from the regression was 0.05. Additionally, the regression model had a high coefficient of determination ($R(2) = 0.932$).

This implies that 93% of the variation in the process efficiency is explained by the independent variables and also that only approximately 7% of the variation was not explained by the model. The model in eq 05 was then optimised. The optimal values of the process parameters were obtained in coded units, converted to uncoded units by using eq 5 and then experimentally validated, as shown in Table 7.

Figures 6, 7 and 8 show the response surfaces and contour curves determined. In these experiments, the biomasswas assessed varying the time between 48 and 144 h, the aeration between 0 and 2 vvm, and the agitation between 200 and 400 rpm.

Table 7

Optimum values of the process parameters for the maximum process efficiency

Parameter	Optimum value	
	Predicted	**Experimental**
Time (h)	144	150
Aeration (vvm)	0	0
Agitation(rpm)	200	200
Biomass (g/L)	7.50	7.90

Figure 6 shows that an agitation until 270 rpm and an aeration equal to 0 vvm, a lower biomass values is obtained (<8 g/L). However, when the aeration is greater than 1 vvm and the agitation is below than 200 rpm, the highest biomass values are obtained (>9 g/L).

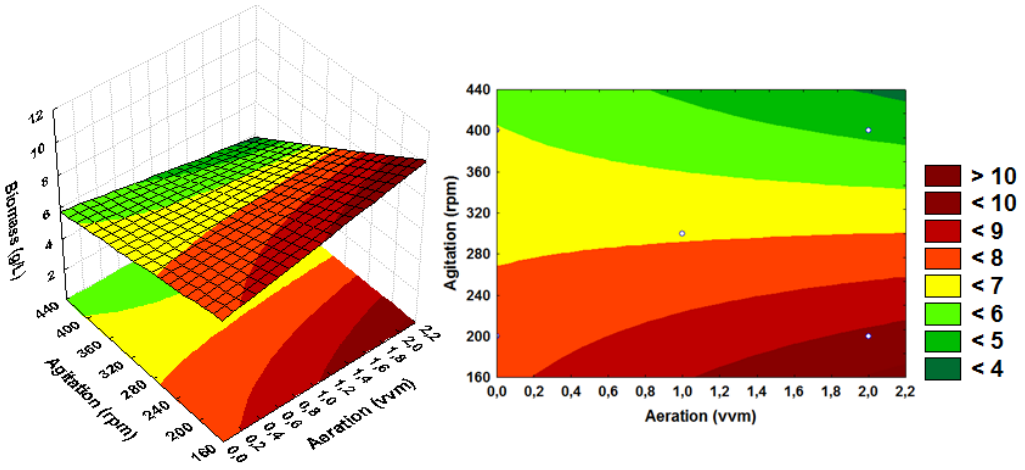

Fig. 6. Response surface and contour curves for the biomass. (Time) = 144 h.

Figure 7 shows that independent of the agitation (varying between 200 and 400 rpm) to a time of the process lower than 70 h, a lower biomass is obtained (<2 g/L). However, when the agitation is lower than 200 rpm to a time of the process higher than 120 h, the highest biomass values are obtained (<8 g/L).

Figure 8 shows that an aeration equal to 0 vvm and a time of the process higher than 120 h, the biomass values is observed (<8 g/L), being efficientfor the process. However, an aeration below than 1 vvm and a time lower than 70 h, the lower biomass values are obtained (<2 g/L).

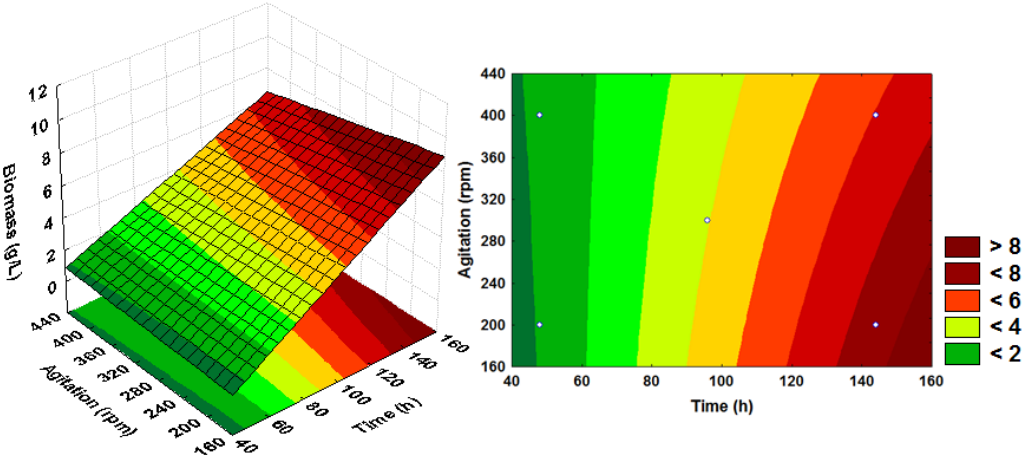

Fig. 7. Response surface and contour curves for the biomass. (Aeration) = 0 vvm

The results obtained by Konishi et al. [31] can explain the ones obtained in our work. Agitation speeds seemed to negligibly affect the cell growth of *Pseudozyma hubeinensis* SY62 cultivated in glucose. On the other hand, agitation speed markedly affected mannosylerythritol lipid (MEL) biosurfactant production. Agitation speed of

less than 150 rpm resulted in MEL production one-half of those at higher speeds. Therefore, aeration with high agitation speeds seemed to stimulate the MEL production. However, excess of agitation, i.e., 250 rpm, seemed to stimulate the growth on the wall of flasks, and resulted in a slight decrease in MEL production. The aggregation on wall would limit the material transfer between the medium and cells, resulting in the production limitations. Therefore, the agitation speed was set at 200 rpm during the following experiments. This was also the best velocity value found in our experiments.

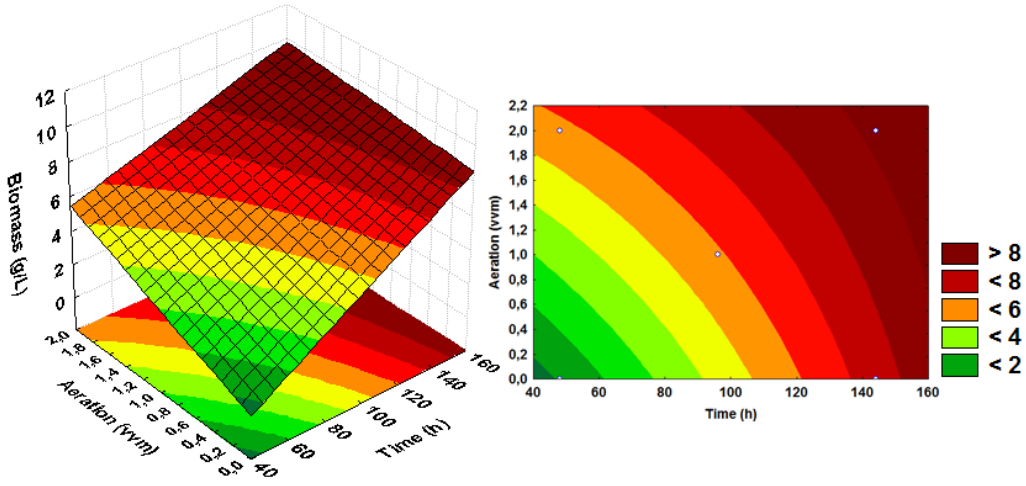

Fig. 8. Response surface and contour curves for the biomass. (Agitation) = 200 rpm

3.3 Study of the yield

The following response equation was used to correlate the dependent and independent variables given by eq 5:

$$Y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3, \qquad (05)$$

where Y is the response variable or biomass efficiency; b_0 is a constant; b_1, b_2 and b_3 are regression coefficients for the linear effects.

To analyse the mathematical model, adjustments to the points were made by nonlinear regression methods. Table 8 shows the adjustments made between the observed (experimental) and predicted values (simulated) of the model to obtain the best response in relation the highest yield (>6 g/L).At this rate, the experimental points varied from 0.7 to 7.2% and predicted varied from 0.58 to 7.09% (Figure 9).

Table 8

2^3full-factorial with three replicates of the centre point for the yield efficiency

Run	Agitation (rpm)	Aeration (vvm)	Time (h)	Yield(g/L)	
				Experimental	Predicted
1	-1	-1	-1	4.03	4.03
2	+1	-1	-1	0.60	0.63
3	-1	+1	-1	4.05	4.08
4	+1	+1	-1	0.59	0.58
5	-1	-1	+1	7.05	7.09
6	+1	-1	+1	2.60	2.59
7	-1	+1	+1	7.10	7.09
8	+1	+1	+1	2.47	2.50
9	0	0	0	3.54	3.57
10	0	0	0	3.57	3.57
11	0	0	0	3.63	3.57

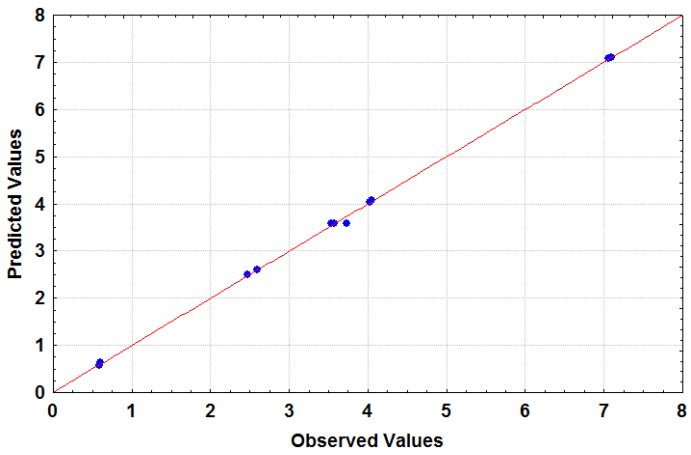

Fig. 9. Observed values *versus* predicted values by model for the answer yield

The regression coefficient values, standard deviation, t_{exp} and significance level are presented in Table 9. It can be seen that b_1 and b_3 are significant. Therefore, the linear effect of the variables are the most influential parameters.

Table 9

Estimated regression coefficients and corresponding t_{exp} as well as significance levels for the yield

Coefficient	Value	Standard deviation	$t_{exp.}$	Significance level (%)
b_0	5.338	0.286	18.672	<0.01
b_1	-0.014	0.001	-16.213	<0.01
b_2	0.088	0.134	0.657	0.579
b_3	0.043	0.003	17.516	<0.01

The application of RSM offers, on the basis of parameter estimation (Table 9), the following empirical relationship (eq 06) between the yield (*Y*) and independent variables studied:

$$Y = (5.338) + (-0.014)x_1 + (0.088)x_2 + (0.043)x_3 \ , \qquad (06)$$

The results predicted by eq 07 indicate good agreement between the experimental and predicted values for the process efficiency. An analysis of variance (ANOVA) showed that the predictive model represented the experimental data at a level of approximately 99.94% because the significance level calculated from the ratio of mean squares obtained from the regression was 0.01. Additionally, the regression model had a high coefficient of determination ($R(2) = 0.998$).

This implies that 99.8% of the variation in the process efficiency is explained by the independent variables and also that only approximately 0.2% of the variation was not explained by the model. The model in eq 07 was then optimised. The optimal values of the process parameters were obtained in coded units, converted to uncoded units by using eq 7 and then experimentally validated, as shown in Table 10.

Figures 10, 11 and 12 show the response surfaces and contour curves determined. In these experiments, the yieldwas assessed varying the time between 48 and 144 h, the aeration between 0 and 2 vvm, and the agitation between 200 and 400 rpm.

Figure 10 shows that an agitation below than 200 rpm and an aeration equal to 0 vvm, a highest yield is obtained (>6.5 g/L). However, when the agitation is greater than 360 rpm, independent of the aeration (varying between 0 and 2 vvm), the lower yield values are obtained (<2.5 g/L).

Table 10

Optimum values of the process parameters for the maximum process efficiency

Parameter	Optimum value	
	Predicted	Experimental
Time (h)	144	150
Aeration (vvm)	0	0
Agitation(rpm)	200	200
Yield (g/L)	7.08	7.27

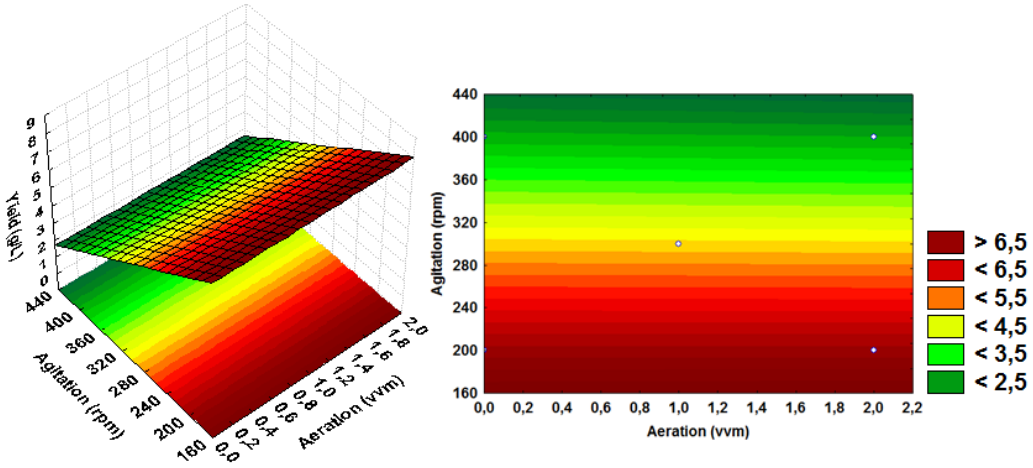

Fig. 10. Response surface and contour curves for the yield. (Time) = 144 h

Figure 11 shows that a time of the process higher than 120 h and an agitation below than 200 rpm, the highest yield is obtained (>8 g/L). However, when the agitation is greater than 320 rpm and the time is below than 60 h, the lower yield are observed (<2 g/L).

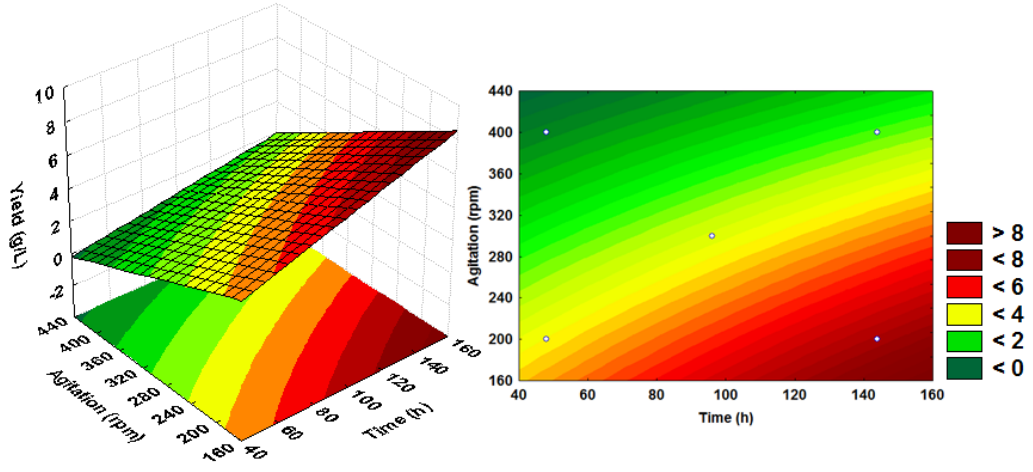

Fig. 11. Response surface and contour curves for the yield. (Aeration) = 0 vvm.

Figure 12 shows that an aeration equal to 0 vvm and a time of the process higher than 120 h, the highest yield is observed (>7 g/L). However, when the time is below than 80 h, the lower yield are obtained (<4 g/L), independent of the aeration (varying between 0 and 2 vvm).

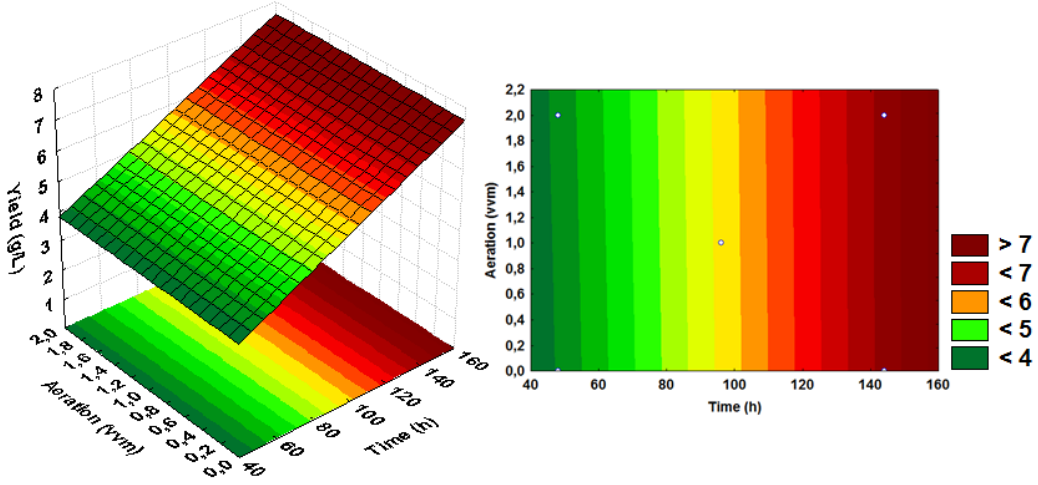

Fig. 12. Response surface and contour curves for the yield. (Agitation = 200 rpm).

An analysis of variance ANOVA showed that the model predicted represented the experimental data at levels ranging from approximately 95.39%, 97.27% and 99.94% for surface tension, yield and biomass, respectively.

The importance of oxygenation for biosurfactant production has been well documented (Silva et al., 2010). In previous studies, the agitation speed of the medium

is also a determining factor in the oxygen mass transfer into the cultures using agitated flasks (Oliveira, 2006; Cunha, 2004; Silva et al., 2010).

Syldatik and Wagner (1987) described a decrease in surfactant production by *Nocardia erythropolis* with the increase of agitation speed from 250 to 500 rpm due to a shear rate effect on the growth kinetics of the microorganism. Results obtained from agitation speed tests indicated that this factor had no significant effect on the maximum biosurfactant produced by a microbial consorptium (Darvishi, 2011).

Chen et al. (2012) found that the yield of biosurfactant production from *B. licheniformis* TKU004 is significantly correlated with oxygen content. Under anaerobic conditions, *B. licheniformis* TKU004 cannot produce biosurfactant.

Oliveira et al. (2012), on the other hand, describes that the increase in rotary velocity had a positive effect on the biosurfactant production from *Pseudomonas alcaligenes*. According to these authors, *P. alcaligenes* is an aerobic bacterium, and as such, higher rotary velocities may increase the oxygen mass transfer to the aqueous medium yielding the best conditions for microbial growth and biosurfactant production, once the biosurfactant is produced during the exponential growth phase. As previously presented in our results, the biosurfactant from *C. lipolytica*is mainly produced in the stationary growth phase. Thus, less oxygen must be required for surfactant biosynthesis, explaining the negative effect of this cultural parameter in the biosurfactant production.

4. Conclusions

Sensitivity analysis was performed on the matematical parameters to determine those that are most influential in the reduction of the surface tension and biomass as well as the increase in the yield of the production biosurfactant. As expected, the most influential parameters were optimized involving these studied conditions. Parameter estimation was performed to best fit the experimental data and the correlation coefficient obtained was 0.95 for surface tension, 0.97 for biomass and yield 0.99.

The present developments include the integrated optimization of the process, as well as the extension of the model to address the cultural conditions for biosurfactant production from *Candida lipolytica*.

After analysis of the results obtained in complete factorial planning, the selected condition and optimized was the smallest value of agitation (200 rpm), the greater time of the process (144 h) and an aeration equal to 0 vvm, since under these conditions

the production of biosurfactant showed that the variables established favoured the reduction of the surface tension and biomass, in turn enabled the increase in the yield of the production biosurfactant by obtaining a biopolymer promising for applications in bioremediation processes.

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References

Aleboyeh, A., Kasiri, M.B., Olya, M.E., 2008. Aleboyeh, H. Prediction of azo dye decolorization by UV/H_2O_2 using artificial neural networks. Dyes and Pigments 288, 277–294.

Barros Neto, B., Scarminio, I.S., Bruns, R.E., 2010. Como fazer experimentos, 4 ed., Bookman, Porto Alegre.

Banat, I.M., Franzetti, A., Gandolfi, I., Bestetti, G., Martinotti, M.G., Fracchia, L., Smyth, T.J., Marchant, R., 2010. Microbial biosurfactants production, applications. Applied Microbiology and Biotechnology 87, 427–444.

Calado, V., Montgomery, D., 2003. Planejamento de experimentos usando o statistica, 1 ed., Rio de Janeiro: E-Papers Serviços Editoriais.

Chaves, A.T.C.A., 2008. Otimização do processo de biodiesel etílico do óleo de girassol (*Hellianthusannus*) aplicando um delineamento composto central rotacional (DCCR), Master Dissertation. João Pessoa-PB.

Chen, Y-C., Chiang, T-J., Liang, T-W., Wang, I-L., Wang, S-L., 2012. Reclamation of squid pen by *Bacillus licheniformis* TKU004 for the production of thermally stable and antimicrobial biosurfactant. Biocatalysis and Agricultural Biotechnology 1, 62–69.

Cunha, C.D. et al., 2004. Serratia sp. SVGG16: a promising biosurfactant producer isolated from tropical soil during growth with ethanol-blended gasoline. Process Biochemistry 39, 2277–2282.

Davila, A.M., Marchal, R., Vandecasteele, J.P., 1992. Kinetics and balance of a fermentation free from product inhibition: sophorose lipid production by *Candida bombicola*. Applied Microbiology and Biotechnology 38, 6-11.

Darvishi, P., Ayatollahi, S., Mowlaa, D., Niazi, A., 2011. Biosurfactant production under extreme environmental conditions by an efficient microbial consortium, ERCPPI-2, Colloids and Surfaces B: Biointerfaces 84, 292–300.

Felse, P.A., Shah, V., Chan, J., Rao, K. J., Gross, R.A., 2007. Sophorolipid biosynthesis by Candida bombicola from industrial fatty acid residues. Enzyme and Microbial Technology 40 (2), 316-323.

Gautam, K.K., Tyagi, V.K., 2006. Microbial Surfactants: a review. Journal of Oleo Science 55, 155-166.

Ilori M.O., Amobi C.J., Odocha A.C., 2005. Factors affecting biosurfactant production by oil degrading Aeromonas spp. Isolated from a tropical environment. Chemosphere 61, 985-992.

Kitamoto, D., Isoda, H., Nakahara, T., 2002. Functions and potential applications of glycolipid biosurfactants: from energy saving materials to gene delivery carriers. Journal Bioscience and Bioengineering 94, 187-201.

Konishi, M., Nagahama, T., Fukuoka, T., Morita, T. Imura, T., Kitamoto, D., Hatada, Y., 2011. Yeast extract stimulates production of glycolipid biosurfactants, mannosylerythritol lipids, by *Pseudozyma hubeiensis* SY62. Journal of Bioscience and Bioengineering 111 (6), 702–705.

Luna, J.M., Rufino, R.D., Albuquerque, C.D.C., Sarubbo, L.A., Campos-Takaki, G.M., 2011. Economic optimized medium for tenso-active agentproduction by *Candida sphaerica* UCP0995 and application in the removal of hydrophobic contaminant from sand. International Journal of Molecular Science 12, 2463-2476.

Luna, J.M., Sarubbo, L., de Campos-Takaki, G.M., 2009. A New Biosurfactant Produced by *Candida glabrata* UCP 1002: Characteristics of Stability and Application in Oil Recovery. Brazilian Archives of Biology and Technology 52, 785- 793.

Marchant, R. and Banat, I., 2012. Microbial biosurfactants: challenges and opportunities for future exploitation. Trends in Biotechnology 30, 558-565.

Myers, R.H., Montgomery, D.C., 2002. Response Surface Methodology: Process and Product Optimization Using Designed Experiments, 2 ed., John Wiley and Sons, New York.

Mukherjee, S., Das, P., Sen, R., 2006. Towards commercial production of microbial surfactants, Trends in Biotechnology 24, 509–515.

Oliveira, F.J.S., Vazquez, L., Campos, N.P.,X França, F.P., 2009. Production of rhamnolipids by a *Pseudomonas alcaligenes* strain. Process Biochemistry 44, 383-389.

Oliveira, F.J.S., Vazquez, L., Campos, N.P., França, F.P., 2006. Biosurfactants production by *Pseudomonas aeruginosa* FR using palm oil. Applied Biochemistry and Biotechnology 131, 727–737.

Pacwa-Plociniczak, M., Plaza, G.A., Piotrowska-Seget, Z. and Cameotra, S.S., 2011. Environmental Applications of Biosurfactants: Recent Advances, International Journal of Molecular Sciences 13, 633-654.

Rivera, O.M.P., Moldes, A.B., Torrado, A.M., Domínguez, J.M., 2007. Lactic acid and biosurfactants production from hydrolyzed distilled grape marc, Process Biochemistry 42, 1010–1020.

Rodrigues, M. I., Lemma, A. F., 2009. Planejamento de experimentos & otimização de processos, 2 ed., Campinas, SP: Casa do Espírito Amigo Fraternidade Fé e Amor.

Rodrigues, L., Teixeira, J., Oliveira, R., Mei, H.C.V., 2006. Response surface optimization of the medium components for the production of biosurfactants by probiotic bacteria, Process Biochemistry 41, 1–10.

Rufino, R.D., Sarubbo, L.A., Campos-Takaki, G.M., 2007. Enhancement of stability of biosurfactant produced by *Candida lipolytica* using Industrial residue as substrate. World Journal of Microbiology and Biotechnology 23, 729–734.

Rufino, R.D., Sarubbo, L.A., Benicio, B.N., Campos-Takaki, G.M., 2008. Experimental design for the production of tensio-active agent by *Candida lipolytica*. Journal of Industrial Microbiology and Biotechnology 35, 907-914.

Sarubbo L.A., Luna J.M., Campos-Takaki G.M., 2006. Production and stability studies of the bioemulsier obtained from a new strain of *Candida glabrata* UCP1002. Electronic Journal Biotechnology 9, 400– 406.

Santa Anna, I.M., Sebastian, G.V., Pereira Jr, N., Alves, T.L.M., Menezes, E.P., Freire, D.M.G., 2001. Production of biosurfactant from a new and promissing strain of

Pseudomonas aeruginosa P. A. 1. Applied Biochemistry and Biotechnology 91-93, 459-467.

Sen, R., 1997. Response surface optimization of the critical media components for the production of surfactin, Journal of Chemical Technology and Biotechnology 68, 263–270.

Silva, R.C.F.S., Rufino, R.D., Farias, C.B.B., Filho, H.J.B., Santos, V.A., Sarubbo, L.A., 2013. Enchancement of biosurfactant production from *Pseudomonas cepacia* CCT6659 through optimisation of nutricional parameters using response surface methodology. Tenside, Surfactans and Detergents 50, 137-142.

Silva, S.N.R.L., Farias, C.B.B., Rufino, R.D., Luna, J.M., Sarubbo, L.A., 2010. Glycerol as subtrate for the production of biosurfactant by *Pseudomonas aeruginosas* UCP 0992. Colloids and Surfaces B: Biointerfaces.79, 174-183.

Syldatk, C., Wagner, F., 1987. Production of biosurfactants. In N. Kosaric, W. L. Cairns, & N. C. C. Gray (Eds.), Biosurfactants andBiotechnology. New York: Marcel Decker. 89-120.

Whei, Y.H., Lai, C.C., Chang, J.S., 2007. Using taguchi experimental designmethods to optimize trace element composition for enhanced surfactin production by *Bacillussubtilis* ATCC 21332, Process Biochemistry 42, 40–45.

Youssef, N.H., Duncan K.E., Nagle D.P., Savage K.N., Hnapp R.M., Mcinerney M.J., 2004. Comparison of methods to detect biosurfactants production by diverse microorganisms. Journal of Microbiological Methods 56, 339-347.

CAPITULO 3

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Biosurfactant production from *Candida lipolytica* in bioreactor and evaluation of its toxicity for application as a bioremediation agent

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Abstract

This large-scale production, toxicity, characterization and economic analysis of the biosurfactant from *Candida lipolytica* UCP 0988 produced in the low-medium formulated with animal fat and corn steep liquor was investigated. The biosurfactant was produced in the stationary phase under 200 rpm in the absence of aeration and reduced the surface tension of the medium from 50 to 28 mN/m after 96 h, yielding 10.0 g/L of isolated biosurfactant in a 2 L bioreactor. The production was maximized in a 50 L bioreactor, reaching 40 g/L biosurfactant and 25 mN/m. The cell biomass was quantified and characterized for use in animal nutrition. Chemical structures of the biosurfactant were identified using FTIR and NMR. The crude biosurfactant was not toxic to the bivalve *Anomalocardia brasiliana*, to the microcrustacean *Artemia salina,* or three species of vegetables seeds. The biosurfactant stimulated the degradation of motor oil by the seawater indigenous microorganisms. The results obtained indicate that the biosurfactant produced has great potential to be applied as a bioremediation agent for cleaning oil spills.

Keywords: Biosurfactant; *Candida lipolytica*; animal fat; corn steep liquor; surface tension; bioreactor

1. Introduction

Biosurfactants have been successfully applied in recent years as remediation agents in soil and aquatic environments [1,2]. Regarding their ability to solubilize hydrocarbons by partitioning them into the surfactant micelles above the CMC, biosurfactants are used in different remediation processes originated form the oil industry [3]. One of the main obstacles to the expansion of the surfactant market for *in situ* remediation is the lack of knowledge about its effects on the environment and the toxicity of these substances [4]. The presence of synthetic surfactants in the aquatic environment in the last 30 years has resulted in great toxicity. As a consequence, an extensive database of laboratory toxicity tests of various commercial surfactants has been constructed over the years. On the other hand, the toxicity of biosurfactants in the environment has been little studied. Edwards et al. [5] in a comparison of the toxicity of three synthetic surfactants and three biosurfactants, concluded that biosurfactants were less toxic than synthetic surfactants against some invertebrate species [6].

Many *Candida* biosurfactants with potential to be used industrially have been produced in our laboratories [7-11]. The biosurfactant from the yeast *Candida lipolytica* UCP0988 described here was previously produced in shake flasks and properties of the produced biosurfactant based on its emulsification activity and its stability with different oils and effect of environmental factors on the emulsification activity and stability were reported [12]. The optimization of cultural conditions for the biosurfactant production using surface response methodology (SRM) was also described [13].

In this work, the production of the biosurfactant from *Candida lipolytica* UCP0988 cultivated in a low-cost fermentative medium based on animal fat and corn steep liquor was evaluated in flasks scale and bioreactors. The characterization and the toxicity of the biomolecule against vegetables and marine indicators and its application as a bioremediation agent are reported. Finally, an economic analysis of the biosurfactant produced in bioreactor was described.

2. Materials and methods

2.1. Materials

All chemicals were of reagent grade. Growth media were purchased from Difco Laboratories, USA. The animal fat used was white choice grease from a bovine processing plant located in the city of Recife (Brazil) and was used without any further processing. The animal fat is composed by the following fatty acids: stearic (43.41%), palmitic (26.40%), oleic (24.16%) and miristic (6.03%) [12].

Corn steep liquor was obtained from Corn Products do Brasil in the city of Cabo de Santo Agostinho (Brazil). According to Akhtar et al. [14] and Cardinal and Hedrick [15] corn steep liquor is 21 to 45% protein, 20 to 26% lactic acid, 8% ash (containing Ca^{2+}, Mg^{2+}, K^{+}), 3% sugar and has low fat content (0.9 to 1.2%).

2.2. Microorganism

Candida lipolytica UCP0988 was obtained from the culture collection of the *Universidade Católica de Pernambuco* (Brazil). The microorganism was maintained in the asexual phase at 5 °C on yeast mould agar slants containing (w/v) yeast extract (0.3%), malt extract (0.3%), tryptone (0.5%), D-glucose (1.0%) and agar (5.0%). Transfers were made to fresh agar slants each month to maintain viability.

2.3. Growth conditions

The inoculum of *Candida lipolytica* was prepared by transferring cells grown on a slant to 50 mL of yeast mould broth. The seed culture was incubated for 24 h at 28 °C and agitated at 150 rpm. The inoculum (1% v/v) was introduced in the amount of 10^4 cells/mL to the cool production medium. The production medium contained 5% animal fat and 2.5% corn steep liquor. The medium was sterilised by autoclaving at 121 °C for 20 min (all components were sterilised together). The final pH of the medium was 5.3 and its surface tension was 50 mN/m.

2.4. Biosurfactant production

Biosurfactant production was first performed in a 2 L bioreactor (Tec-Bio-Plus, Tecnal Ltda., Brazil) with a working volume of 1.2 L operated in a batch mode, with controlled pH (5.3) and temperature (28 °C). The reactor was aerated by a sparger. The culture medium was inoculated with a 24 h inoculum at 200 rpm in the absence of aeration during 144 h. For larger-scale production, i.e., production maximization, a 50 L bioreactor (MA502, Marconi Ltda., Brazil) was used. All assays were carried out in triplicate and did not vary more than 5%. The Growth and biosurfactant production were monitored along fermentation. At regular intervals, different process parameters such as growth, pH, surface tension and biosurfactant concentration were evaluated.

2.5. Biomass estimation

Biomass was monitored by estimating the cell dry weight after removal of the hydrophobic substrates at 10000 *g* for 15 min [16]. Briefly, a 10 mL aliquot of the fermentation broth was centrifuged to obtain a cell pellet. The supernatant was discarded and the cell pellet was washed twice with distilled water to remove the medium components. The pellet was then washed with 10 mL of ethyl acetate to remove hydrophobic substances. The washed cells were centrifuged to obtain a pellet, which was dried in vacuum to a constant weight (indicated by a variation of less than ± 0.2 g). Suitable calculations were made to express cell mass in terms of g/L.

2.6. Biomass composition

To determine protein, carbohydrate and lipid, cells obtained by centrifuging one volume of whole broth were resuspended in 1 volume each of saline and ethyl acetate, centrifuged again, resuspended in saline, then analysed. Protein concentration was estimated using a total protein test kit (Labtest Diagnostica S.A., Brazil). Total

carbohydrate content was estimated using the phenol-sulphuric acid method [17]. Lipid content was determined based on the method described by Manocha et al. [18].

2.7. Surface tension

The surface tension of the culture supernatants obtained by centrifuging the cultures at 5000 g for 20 min was measured using a Sigma 700 digital surface tensiometer (KSV Instruments LTD - Finland) working on the principle of the Du Nuoy ring method. Ten millilitres of each sample were transferred to a clean 20 ml beaker and placed on the tensiometer platform. A platinum wire ring was submerged in the solution and then slowly pulled through the liquid-air interface to measure surface tension (mN/m). Between each measurement, the platinum wire ring was rinsed with chromic acid, deionised water and acetone and was then flamed and allowed to dry. Calibration was performed using Mill-Q-4 ultrapure distilled water (surface tension = 71.5 mN/m ±0.5) before taking measurements of the samples.

2.8. Biosurfactant isolation

The biosurfactant was recovered from the cell-free broth by cold acetone precipitation, as described by Ilori et al. [19]. Three volumes of chilled acetone were added and allowed to stand for 10 h at 4 ºC. The precipitate was collected by centrifugation and evaporated to dryness to remove residual acetone. The precipitate was then re-dissolved in sterile water. The yield in isolated biosurfactant was expressed in g/L.

2.9. Fourier transform infrared spectroscopy

The solid biosurfactant extract recovered from the supernatants of *C. lipolytica* was subjected to Fourier Transform Infra Red spectrometry (FT-IR) to elucidate the chemical nature by identifying the types of functional groups and chemical bonds. One-milligram biosurfactant was ground with 100 mg KBr pellet and pressed with 7500 kg for 30 s to obtain translucent pellet. The FT-IR spectrum was recorded in 4000–400 cm^{-1} region on a GX-FTIR system (Perkin Elmer, USA) with a spectral resolution and wave number accuracy of 4 and 0.01 cm^{-1}, respectively. All the measurements consisted of 500 scans and a KBr pellet was used as background reference.

2.10. Nuclear magnetic resonance spectroscopy

The extracted biosurfactant was re-dissolved in deuterated chloroform ($CDCl_3$) and the respective ^1H and 13C NMR spectra were recorded at 25 °C using an Agilent 300Mz spectrometer operating at 300.13 MHz. Chemical shifts (δ) are given on the ppm scale relative to tetramethylsilane (TMS).

2.11. Phytotoxicity assay

The phytotoxicity of the biosurfactant was evaluated in a static test based on seed germination and root elongation of the vegetables *Brassica oleracea var. botrytis* L., *Lactuca sativa* L. and *Brassica oleracea* var. capitata L. following the methods described by Tiquia et al. [20]. Solutions of the isolated biosurfactant were prepared with distilled water at concentrations of ½ the CMC (0.04%), the CMC (0.08%) and 2 x the CMC (1.6%). Toxicity was determined in sterilised Petri dishes (1 × 10 cm) containing Whatman N^o 1 filter paper. The seeds were pre-treated with sodium hypochlorite. Ten seeds were inoculated in each Petri dish with 5 mL of the test solution at 27 °C. After five days of incubation in the dark, seed germination, root elongation (≥ 5 mm) and the germination index (a factor of relative seed germination and relative root elongation) were determined as follows:

Relative seed germination (%) = (number of seeds germinated in the extract/number of seeds germinated in the control) x 100

Relative root length (%) = (mean root length in the extract/mean root length in the control) x 100

Germination index = [(% of seed germination) x (% of root growth)]/100%

2.12. Artemia assay

The toxicity assay was performed using brine shrimp (*Artemia salina*) as the toxicity indicator. Brine shrimp eggs were obtained from a local store. Larvae were used within one day of hatching. Following dilutions of a biosurfactant solution at the CMC (0.08%) with saline water (33 g/L) to give concentrations of 0.02, 0.04, 0.06 and 0.08%, the assays were conducted in 10 mL penicillin tubes containing 10 brine shrimp larvae in 5 mL of saline water per tube. The brine shrimp larvae in each tube were tested using 5 mL of each concentration of the biosurfactant solution. The samples were observed for 24 h for the calculation of the mortality rate [21]. The toxicity threshold concentration, expressed as biosurfactant concentration per 100 ml of saline

water, was defined as the lowest concentration that killed all brine shrimp within 24 h. Each test was run in triplicate and saline water was used as the control.

2.13. Toxicity against Anomalocardia brasiliana

Bioassays were performed using the native bivalve mollusc *Anomalocardia brasiliana* according to the method mentioned in the Standard Methods for the Examination of Water and Wasterwater [22]. The species is widely distributed along the Brazilian coast and inhabits areas protected from the action of waves and currents [23,24].

Assays were performed at room Quarantine Laboratory of Sustainable Mariculture of the Fisheries and Aquaculture Department from the Federal Rural University of Pernambuco (UFRPE), Pernambuco, Brazil. The animals used in the tests were collected at Mangue Seco beach, in the city of Igarassu, Pernambuco, transported to the laboratory and kept in Boxes glass fiber with a volume of 400 L in seawater with salinity of 26 and an average temperature of 26 ± 0.5 °C under constant aeration and fed with a mixture of algal *Chaetoceros gracilis* and *Navicula*, at a concentration of 10^4 cells/ml for 10 days prior to the experiment. Before performing each test, the specimens were transferred to a 100 L container, acclimated and kept without food for 24 h. For the tests of acute toxicity during 96 h, 2 L glass beakers were filled with 1.0 to 2.0 L of test solution, with variable dilutions employing water with salinity of 26, maintained with constant aeration, in a room with an average temperature of 26 ± 0.5 °C, and natural photoperiod of approximately 12/12 h. During the duration of the experiments, the animals were not fed. Five dilutions of the crude biosurfactant (cell-free broth) were tested: surfactant:brine (1:10, 1:20, 1:40, 1:160 and 1:80, v/v) with three replicates each. Controls contained just seawater. The result of the experiment was based on determination of lethal concentration for 50% of subjects employed (LC50), expressed in terms of mortality mean of three replicates for each dilution tested with the biosurfactant

2.14. Bioremediation test

Bioremediation tests were performed according to the method mentioned in the Standard Methods for the Examination of Water and Wasterwater [22]. In brief, 250-ml Erlenmeyer flasks were filled with 100 ml fresh seawater obtained from the Suape Petrochemical Complex, Pernambuco State, Brazil, 1% of motor oil, and biosurfactant solutions at concentrations of ½ the CMC (0.04%), the CMC (0.08%) and 2 x the CMC

(1.6%). Control flasks contained seawater and motor oil. The flasks were incubated at 28°C on an orbital shaker rotating at 150 rpm. Triplicate shake flasks were sacrificed on days 1, 5, 7, 21 and 30 of incubation and then analysed for the number of microorganisms by using the most probable number (MPN).

2.15. Statistical analysis

The determination of all surface tensions, biosurfactant and biomass concentrations was performed at least three times. Mean and standard error values were calculated using the Microsoft Office Excel 2003 (Version 7).

3. Results and discussion

3.1. Biosurfactant production

Industrial wastes have attracted great interest from researchers as alternative for the provision of low cost substrates for the production of biosurfactants [25]. Distillery waste [26], whey [27], molasses [28] and wastewater of olive oil [29], among others, have been described as substrates for the production of biosurfactants. Thus, based on the promising results previously obtained for the cultivation of the yeast *C. lipolytica* in media containing animal fat [12,13], we have studied the production of the biosurfactant obtained in a low-cost medium composed of 5% animal fat and 2.5% corn steep liquor in bioreactors in order to maximize its production.

Growth and biosurfactant production by *C. lipolytica* on medium containing 5% animal fat and 2.5% corn steep liquor substrate were followed for 6 days in a 2 L bioreactor. The time course of this process is shown in Fig. 1. The maximum biosurfactant production (10.0 g/L) was reached in the stationary growth phase at 96 h. This observation suggests that biosurfactant production by *C. lipolytica* is a secondary microbial metabolic process. The initial pH after inoculation was adjusted to 5.5. Thereafter it did not change along cultivation, showing a stable profile along 144 h. Growth started without a lag phase and cells reached a maximum concentration (7.8 g/L) at 144 h. Other biosurfactants from yeasts are produced in the stationary growth phase, as described by Amézcua-Vega et al. [30], Desphande and Daniels [31] and Luna et al. [10] for cultivations of *C. igens, C. bombicola* and *C. sphaerica*, respectively.

Biosurfactant production was markedly higher than previously reported [12], i.e., 2.0 g/L in shake flasks after 144 h against 10.0 g/L in a 2 L bioreactor after 96 h.

Benincasa et al. [32] improved the production of rhamnolipids from soapstock as the sole carbon source to 15.9 g/L using a 2 L bioreactor. The production of another rhamnolipid from waste frying oil reached over 20 g/L in a 50 L bioreactor against 12.47 g/L in shake flasks [33]. Marti et al. [34] produced two biosurfactants from genetically-modified strains of *Bacillus subtilis* and observed that biosurfactant yields were higher in shake flasks than in bioreactors.

The production profiles for the biosurfactant from *C. lipolytica* were very similar in both 2 and 50 L bioreactors, although the biosurfactant yield and surface tension had reached 40 g/L and 25 mN/m in the 50 L bioreactor. Marti et al. [34] compared the production of biosurfactants in 5 and 15 L bioreactors. They observed that the final concentration of biosurfactants were lower in residual broth of larger fermentation vessels, as most of the surfactant was removed with the foam and concentrated in this fraction. Since there was no foam resulting from biosurfactant production in the conditions of this work, the production was higher in the larger vessel.

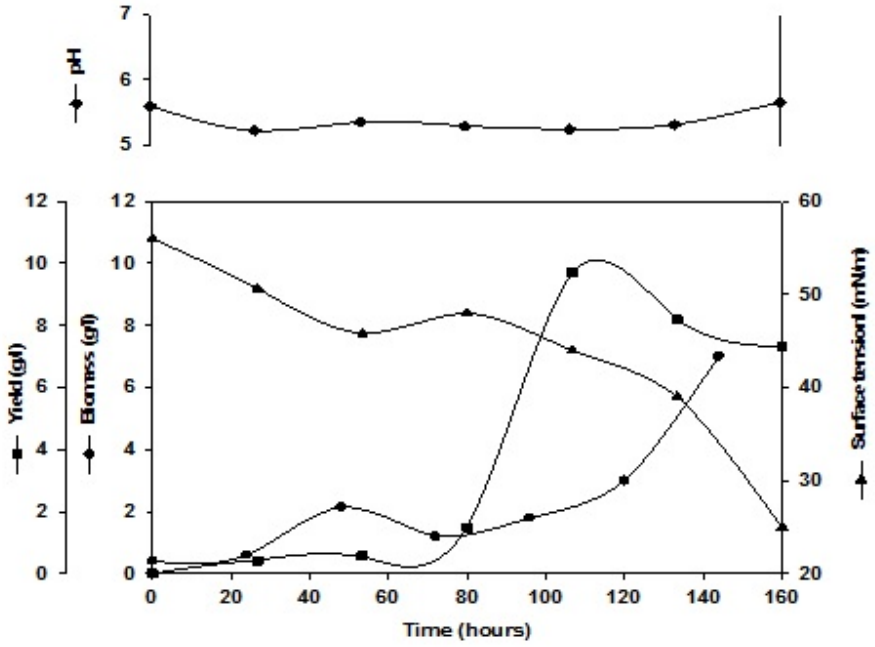

Figure 1. Growth, pH surface tension and biosurfactant yield profiles from *C. lipolytica*cultivated in distilled water supplemented with 5.0% animal fat and 2.5% corn steep liquor during 144 h at 200 rpm and 28ºC.

3.2. Chemical analysis of cells

In fermentation processes, the biomass obtained is generally sold as a component of animal nutrition. In this sense, the nutritional characteristics of the cells from *C. lipolytica* were analysed after production of the biosurfactant from animal fat

and corn steep liquor. The content of proteins, carbohydrates and lipids represented 25, 15 and 20% dry weight, respectively. This content is higher in lipids when compared to the yeasts that grow in the presence of carbohydrates (10.5%) but not as high as to oleaginous yeasts, for which are reported values around 32-72% [31]. Considering FAO (Food and Agriculture Organization of the United Nations) guidelines for high-content protein, the results obtained suggest the possibility of applying the biomass of *C. lipolytica* for animal nutrition.

3.3. Biosurfactant characterization

The biosurfactant isolated from *C. lipolytica* was characterized by FT-IR and NMR analyses. The IR spectrum (Fig. 2) showed a stretch region between the ranges of 3000-35000 cm^{-1}, indicating the presence of hydroxyl groups in a carboxylic acid junction. The carbonyl group (C=O) was detected in 1711.29 cm^{-1} and aliphatic carbon bonds in 2855-3010 cm^{-1} regions.

The H^1NMR (Fig. 3) suggested the presence of hydrogen closed to the carboxylic acid groups in 10-11 ppm, close to double bonds in 5-6 ppm and aliphatic carbons in 1-3 ppm range. The C^{13} NMR (Fig. 4) confirms previous results showing a characteristic pick of carboxylic acid in 180 ppm, double bonds picks between 120-140 ppm and aliphatic carbons in 10-40 ppm region. This result suggests that the biomolecule of surfactant is a kind of metabolized carboxylic acid probably linked to carbohydrates, as described for other biosurfactants produced by yeasts, which are glycolipids.

Our results are similar to the ones obtained for the characterization of biosurfactants isolated from yeast species cultivated in diesel oil [35]. The broad band observed for the biosurfactants from *C. rugosa* and *Rhodotorula muciliginosa* was at 3410-3434 cm^{-1} corresponding to the O-H stretch. The asymmetrical stretching (V_{as} CH_2) of methylene occurred at 2920-2926 cm^{-1} and the band 1620-1627 cm^{-1} was from stretching of unsaturated C=C bonds.

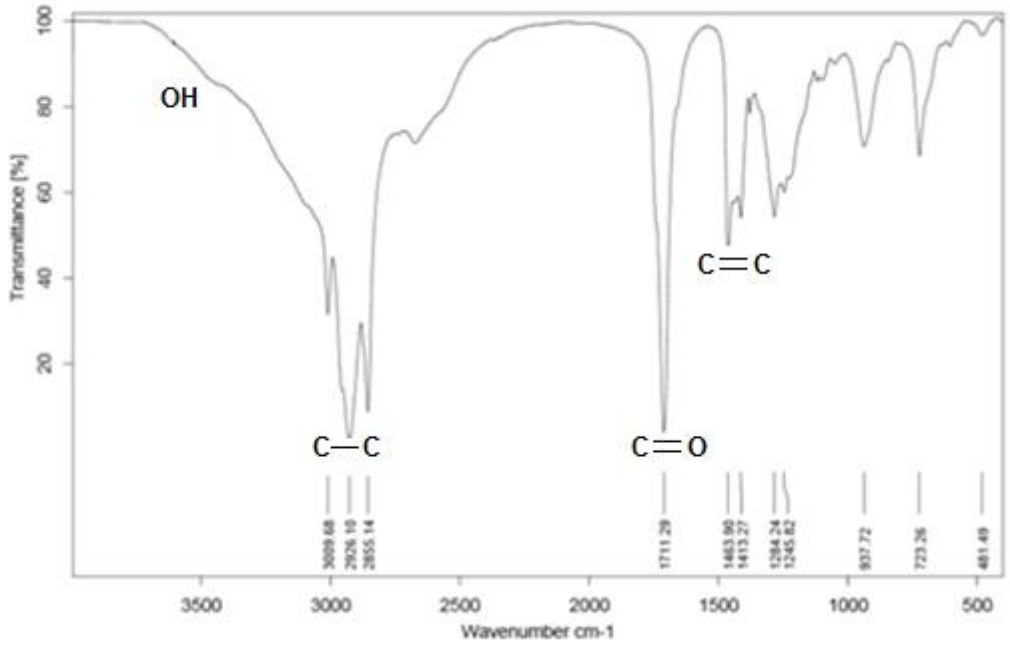

Fig. 2. Transmission FT-IR spectrum of solid surfactant obtained after purification and sample preparation in KBr tablets from 400 to 4000cm−1.

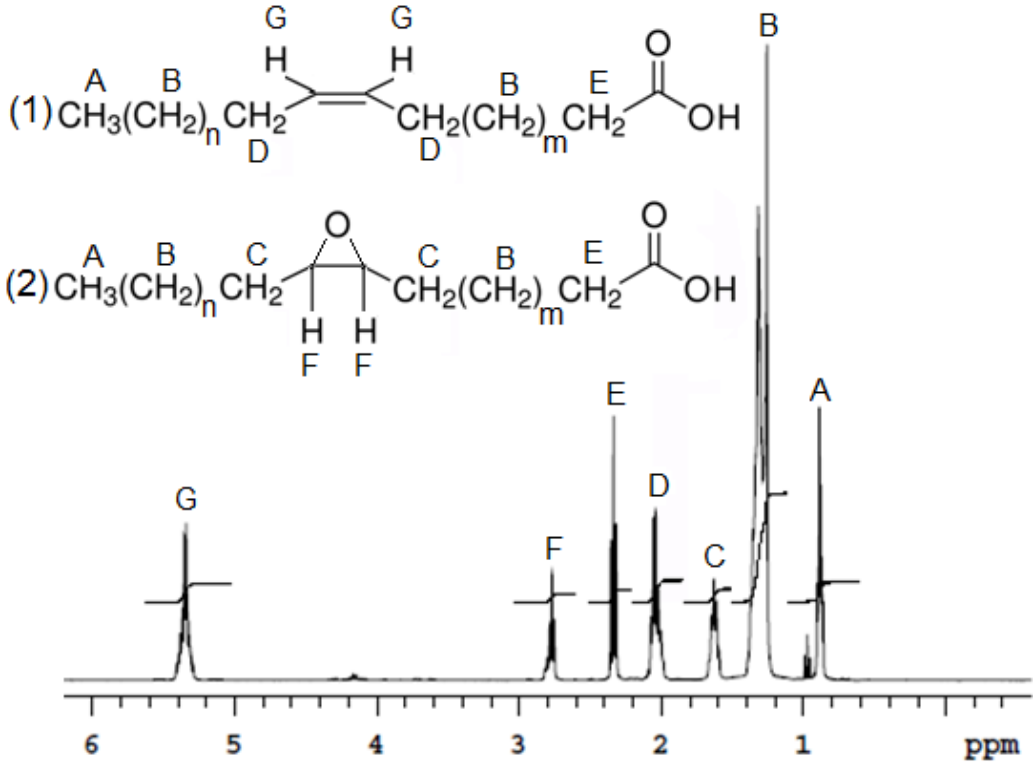

Fig. 3. 1H NMR spectrum for biosurfactant extract recorded in CDCl3.

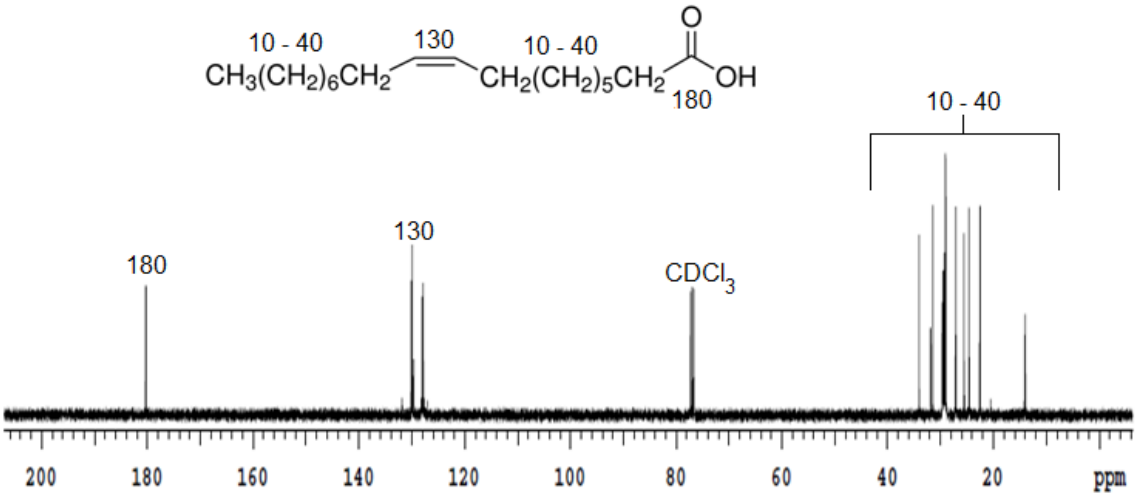

Fig. 4. 13C NMR spectrum for biosurfactant extract recorded in CDCl3.

3.4. Comparison of biosurfactant production by Candida species in bioreactors

The limiting factor for the commercialization of fermented products, including biosurfactants, is the economics of large-scale production, although the economy of biosurfactant production has not been detailed in the literature [36]. For the commercial success of biosurfactants, they must compete with synthetic surfactants, which are typically sold at lower prices to $ 2/kg [2]. Several approaches can be established to achieve this goal. The economic strategies extensively described include the selection of low cost substrates, increased product yields and reducing total processing time, which includes the preparation of materials, fermentation, extraction and purification. All this translates into material costs, capital and labour reduced and hence reduced production costs.

Many researchers have studied the preparation of culture media for production of biosurfactants [31,37,38]. Several researches describe media containing glucose, yeast extract, urea and a hydrophobic compound such as a vegetable oil or a fatty acid or a long chain alkane [7,39]. The components used in this work are industrially attractive: corn steep liquor, which has a greatly reduced cost compared to yeast extract, and animal fat compared to pure fatty acids or vegetagle oils.

In this sense, the influence of culture conditions including agitation, aeration and time of cultivation were evaluated in the production of biosurfactant from a culture medium with composition previously established [12]. The temperature was maintained at 28 °C, which is favourable industrially since the costs associated with cooling are

larger, and hinder the mixing of fat in half. The maintenance of pH during the cultivation also helps reduce the cost of maintenance thereof, as well as a lower cell lysis. The absence of aeration contributes decisively to a further reduction in process costs.

Table 1 describes the data obtained over the years to biosurfactants produced by *Candida* species in bioreactors. Comparing our results with those detailed in this Table, it is observed that the maximum yield and rate of production obtained in this study are inferior, while the cultivation time is within the ranges stated. Moreover, the surface tension is favourable when compared to the literature, indicating more efficient production of a surfactant when compared to the others. Importantly, also, is that the ability of bioreactors and agitation speeds differ greatly from those used in this work, and that the media described include glucose, yeast extract, vegetable oils and fatty acids, which inevitably will favour the results, although an extremely cost up compared to the medium formulated with only two residues and distilled water described in our work. The inclusion of molasses as a low-cost substrate would be interesting to increase yields.

Biosurfactant production is still not considered competitive against detergents like sodium lauryl sulphates and alkyl benzene sulfonates, but it is likely that microbial surfactants will compete, in a near future, with the synthetic surfactants used in many industries [2]. Although the investments necessary to research, development, and capital expenditures are high, there is the possibility of creating an industry based on biosurfactants produced from animal fat and corn steep liquor.

Table 1

Comparison of biosurfactant production from *Candida* species in bioreactors described in the literature

Media components (%)	Time (h) to reach max. level	Production rate (g/L/h)	Max. yield (g/L)	Surface tension (mN/m)	Reference
5% animal fat, 2.5% corn steep liquor (2 L bioreactor)	96	0.1	10	28	This work
5% animal fat, 2.5% corn steep	120	0.3	40	25	This work

liquor (50 L bioreactor)					
10% glucose, 3.6% oleic acid, 1% yeast extract (30 L bioreactor)	120	0.5	34	ND	[40]
13.6% oleic acid, 0.1% yeast extract (30 L bioreactor)	160	1.0	74	ND	[40]
10.0% glucose, 9.5% sunflower oil, 0.5% yeast extract (7 L bioreactor)	130	1.0	28	37	[37]
10% glucose, 10.5% canola oil, 0.4% yeast extract (1 L bioreactor)	192	1.1	160	34	[41]
10.0% glucose, 10.0% animal fat, 0.4% corn steep liquor (15 L bioreactor)	60	1.7	97	ND	[31]
10.0% glucose, 4% tallow fatty acid residue, 0.1% yeast extract, 0.01% urea (fed batch and lasks)	240	0.5	120	ND	[42]
3-4% glucose, rapeseed oil (fed batch and 2.5 L bioreactor)	192	1.9	365	ND	[33]

ND: not described

3.5. Toxicity of the biosurfactant

Toxicity can be defined as the capacity of a substance to cause a harmful effect to a living organism. It depends on the concentration and properties of the chemical to which the organism is exposed and on the exposure time [43].

The toxicity of the biosurfactant from *C. lipolytica* on three vegetable species is shown in Table 2. The results indicated that the seeds germination occurred even in the presence of high concentrations of the isolated biosurfactant. The crude biosurfactant also did not show inhibitory effects to the vegetable species (data not shown). This result shows the possibility of using the biosurfactant in soil remediation. The results obtained also indicated that increasing the concentration of the surfactant reduced the percentage of seed germination.

According to the protocols described by the APHA [22] for testing oil spill dispersants, acute toxicity bioassay studies were conducted using brine shrimp and a bivalve.

The results obtained for the brine shrimp larvae *Artemia salina* and the bivalve *Anomalocardia brasiliana* subjected to different dilutions of the crude biosurfactant for 24 and 96 h, respectively, showed 100% survival, demonstrating the potential of the crude biosurfactant for use in aquatic environments. No mortality of the *Artemia salina* was observed when the brine shrimp was treated with the isolated biosurfactant solutions from 0.02 to 0.06%, while the biosurfactant solution at 0.08% killed 100% of the larvae.

Acute toxicity bioassay studies were conducted by Saeki et al. [44] with the surfactant JE1058BS produced by the bacterium *Gordonia* sp. Two marine larval species (*Mysidopsis bahia* and *Menidia beryllina*) were tested. The shrimp larvae were exposed to the samples for 48 h, while the fish larvae were exposed for 96 h. The results showed that JE1058BS had a considerably low toxicity. Franzetti et al. [45] also conducted ecotoxicity tests by the bioemulsifiers from *V. paradoxus* 7bCT5. Acute toxicity test on crustaceans showed a 100% survival of the organisms. The Germination Indexes calculated from seed germination and root elongation tests did not significantly differ from their controls, showing that the bioemulsifiers were not toxic to any tested organisms (crustaceans, bacteria or plants). The same solution was used for contact tests on earthworms, showing 100% of survival.

Table 2

Phytotoxicity of the isolated biosurfactant from *C. lipolytica* UCP0988 cultivated in medium formulated with 5% animal fat and 2.5% corn steep liquor on three vegetable species seeds

Vegetable Seeds	Germination index (GI) (%)		
	Isolated biosurfactant at 0.04%	**Isolated biosurfactant at 0.08%**	**Isolated biosurfactant at 1.6%**
Brassica oleracea var. botrytis L.	60±0.25	55±0.13	40±0.11
Brassica oleracea var.capitata	75±0.19	56±0.29	41±0.09
Lactuca sativa L.	75±0.30	60±0.15	40±0.21

3.6. Bioremediation test

The effect of the biosurfactant on the biodegradation of motor oil via the activity of indigenous marine bacteria and fungi was evaluated during 30 days. The presence of the biosurfactant from *C. lipolytica* favoured the growth of microorganisms in seawater at the concentrations evaluated (½ the CMC, CMC and 2 x CMC) (Fig. 5a,b). It was observed increased growth of bacteria in the presence of the highest concentration of the biosurfactant, while the solution of biosurfactant in the lower concentration stimulated fungi growth. Moreover, in the presence of petroleum-based motor oil, higher concentrations of the biosurfactant favoured the growth of all microorganisms in sea water in the conditions studied in this work (Fig. 5c,d). It could be also observed that the growth of the indigenous fungi in the control formulated with sea water and petroleum was poor in the absence of the biosurfactant. The stimulation of oil degradation by the biosurfactant could be attributed to the dispersion of oil and the increase in the number of cells that utilized the biosurfactant as well as the nutrients derived from the culture broth.

Saeki et al. [44], in a similar experiment, found that the degradation of the total saturated hydrocarbons and total target PAHs (polycyclic aromatic hydrocarbons) was stimulated by the biosurfactant from *Gordonia* sp. in sea water samples.

Lima et al. [46] evaluated the effects of different concentrations (2 x CMC, 4 x CMC and 8 x CMC) of bacterial surfactants on the growth of pure cultures of bacteria in mineral medium supplemented with glucose. They observed that the influence of the biosurfactants at twice the CMC in the growth of the bacterial isolates was not significant. However, an inhibition of the bacterial isolates growth occurred when the biosurfactants were tested at 8 x CMC, showing the toxicity of these biosurfactants at high concentrations. The literature describes the higher effectiveness of surfactants at concentrations slightly above their CMC than at concentrations well above the CMC values [47,48].

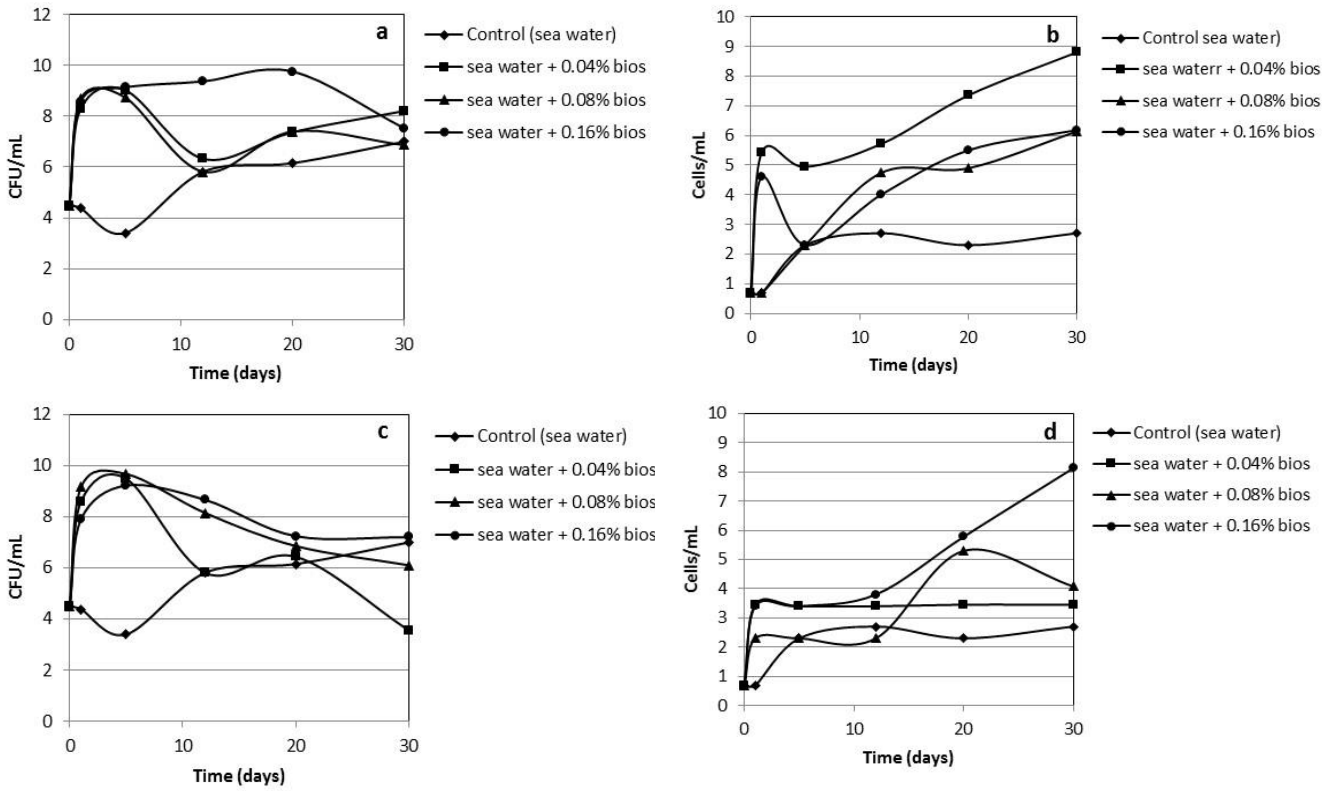

Fig. 5. Influence of the isolated biosurfactant (0.04, 0.08 and 0.16%) from *C. lipolytica* UCP0988 on the growth of indigenous microorganisms. (a) bacteria in seawater; (b) fungi in seawater; (c) bacteria in seawater supplemented with motor oil; (d) fungi in seawater supplemented with motor oil.

4. Conclusions

In the present work the production of the biosurfactant by *C. lipolytica* cultivated in a low-cost medium have been described in bioreactors. Good surface tension

reduction and biosurfactant yield were obtained. The biosurfactant not only has low toxicity but also a significant potential to be applied as an oil spill remediation agent in marine environments. The remediation experiments showed that the biosurfactant can be used as an oil spill dispersant. The biosurfactant could also stimulate the degradation of the spilled oil by the native microorganisms. The economic analysis shows that the biosurfactant and the biomass produced from inexpensive raw materials have potential to be used not only in the petroleum industry but also in other industries.

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References

[1] Banat IM, Satpute SK, Cameotra SS, Patil R, Nyayanit N. Cost effective technologies and renewable substrates for biosurfactants' production. Front Microbiol 2014;5:1-18.

[2] Santos DKF, Rufino RD, Luna JM, Santos, VA, Sarubbo LA. Biosurfactants: multifunctional biomolecules of the 21st Century. Int J Mol Sci 2016;17: 401.

[3] Sarubbo LA, Rocha Júnior RB, Luna JM, Rufino RD, Santos VA, Banat IM. Some aspects of heavy metals contamination remediation and role of biosurfactants. Chem Ecol 2015;31:707–723.

[4] Franzetti A, Di Gennaro P, Bevilacqua A, Papacchini M, Bestetti G. Environmental features of two commercial surfactants widely used in soil remediation. Chemosphere 2006;62:1474–1480.

[5] Edwards KR, Lepo JE, Lewis MA. Toxicity comparison of biosurfactants and synthetic surfactants used in oil spill remediation to two estuarine species. Marine Poll Bull 2003;46:1309–1316.

[6] Silva RCFS, Almeida DG, Luna JM, Rufino RD, Santos VA, Sarubbo LA. Applications of biosurfactants in the petroleum industry and the remediation of oil spills. Int J Mol Sci 2014;15:12523–12542.

[7] Coimbra CD, Rufino RD, Luna JM, Sarubbo LA. Studies of the cell surface properties of *Candida* Species and relation with the production of biosurfactants for environmental applications. Curr Microbiol 2009;58:245–251.

[8] Gusmão CAB, Rufino RD, Sarubbo LA. Laboratory production and characterization of a new biosurfactant from *Candida glabrata* UCP1002 cultivated in vegetable fat waste applied to the removal of hydrophobic contaminant. World J Microbiol Biotechnol 2010;26:1683–1692.

[9] Luna JM, Sarubbo LA, Campos-Takaki GM. A new biosurfactant produced by *Candida glabrata* UCP 1002: characteristics of stability and application in oil recovery. Braz Arch Biol Technol *2*009;52:785-793.

[10] Luna JM, Rufino RD, Sarubbo LA, Campos–Takaki GM. Characterization, surface properties and biological activity of a biosurfactant produced from industrial waste by *Candida sphaerica* UCP0995 for application in the petroleum industry. Colloids Surf. B. 2012;102:202–209.

[11] Rufino RD, Rodrigues GIB, Campos-Takaki GM, Sarubbo LA, Ferreira SRM. Application of a yeast biosurfactant in the removal of heavy metals and hydrophobic contaminant in a soil used as slurry barrier. Appl Environ Soil Sci 2011;1-7.

[12] Santos DKF, Rufino RD, Luna JM, Santos VA, Salgueiro AA, Sarubbo LA. Synthesis and evaluation of biosurfactant produced by *Candida lipolytica* using animal fat and corn steep liquor. J Petroleum Sci Eng 2013;105:43–50.

[13] Santos DKF, Brandão YB, Rufino RD, Luna JM, Salgueiro AA, Santos VA, Sarubbo LA. Optimization of cultural conditions for biosurfactant production from *Candida lipolytica.* Biocatal Agric Biotechnol 2014;3:48–57.

[14] Akhtar M, Lentz MJ, Blanchette RA, Kir TK. Corn steep liquor lowers the amount of inoculum for biopulping. TAPPI J 1997;80:161–4.

[15] Cardinal EV, Hedrick LR. Microbiological assay of corn steep liquor for amino acid content. J Biol Chem 1984;172:609–12.

[16] Felse PA, Shah V, Chan J, Rao KJ, Gross RA. Sophorolipid biosynthesis by *Candida bombicola* from industrial fatty acid residues. Enzyme Microb Tech 2007;40:316-323.

[17] Dubois M, Gilles KA, Hamilton JK. Colorimetric method for determination of sugar and related substances. Anal Chem 1956;28:350–6.

[18] Manocha MS, San-Blas G, Centeno S. Lipid composition of *Paracoccidioides brasilienses*: possible correlation with virulence of different strains. J Gen Microbiol 1980;117:147–54.

[19] Ilori MO, Amobi CJ, Odocha AC. Factor's affecting biosurfactant production by oil degrading *Aeromonas* spp. isolated from a tropical environment. Chemosphere 2005;61:985-992.

[20] Tiquia SM, Tam NFY, Hodgkiss IJ. Effects of composting on phytotocicity of spent pig-manure sawdust litter. Environ Pollut 1996;93:249-256.

[21] Kajs TM, Vanderzant CV. Batch-scale utilization studies of tallow by food yeasts. Dev Ind Microbiol 1980;21:481-8.

[22] APHA. Standard methods for the examination of water and wastewater. 21st Ed. Washington: American Public Health Association; 2005.

[23] Boehs G, Magalhães ARM. Simbiontes associados com *Anomalocardia brasiliana* (Gmelin) (Mollusca, Bivalvia, Veneridae) na Ilha de Santa Catarina e região continental adjacente, Santa Catarina, Brasil. Revista Brasileira de Zoologia 2004;21:865-869.

[24] Quayle DB, Newkirk GF. Farming bivalve molluscs: methods for study and development. The World Aquaculture Society. Louisiana; 1989. p. 285.

[25] Van Bogaert INA, Zhang J, Soetaert W. Microbial synthesis of sophorolipids. Process Biochem 2011;46:821-833.

[26] Dubey K, Juwarkar A. Determination of genetic basis for biosurfactant production in distillery and curd whey wastes utilizing *Pseudomonas aeruginosa* strain BS2. Indian J Biotechnol 2004;3:74–81.

[27] Koch AK, Reiser J, Kappeli O, Fiechter A. Genetic construction of lactose-utilizing strains of *Pseudomonas aeruginosa* and their application in biosurfactant production. Biotechnol 1998;51:1335-1339.

[28] Patel P, Desai A. Biosurfactant production by *Pseudomonas aeruginosa* GS3 from molasses. Lett Appl Microbiol 1997;25:91-96.

[29] Mercade ME, Manresa MA. The use of agro industrial by products for biosurfactant production. J Am Oil Chem Soc 1994;1: 61-64.

[30] Amézcua-Veja C, Poggi-Varaldo HM, Esparza-Garcıa F, Rıos-Leal E, Rodrıguez-Vazquez R. Effect of culture conditions on fatty acids composition of a

biosurfactant produced by *Candida ingens* and changes of surface tension of cultura media. Bioresour Technol *2*007; 98:237–240.

[31] Deshpand M, Daniels L. Evaluation of sophorolipid biosurfactant production by *Candida bombicola* using animal fat. Bioresour Technol 1995;54:143-150.

[32] Benincasa M, Contiero J, Manresa MA, Moraes IO. Rhamnolipid production by *Pseudomonas aeruginosa* LBI growing on soapstock as the sole carbon source. J Food Eng 2002;54:283–288.

[33] Zhu Y, Gan J-J, Zhang G-L, Yau B, Zhu W-J, Meng Q. Reuse of waste frying oil for production of rhamnolipids using *Pseudomonas aeruginosa* zju.u1M. J Zhejiang Univ Sci A 2007;8:1514-1520.

[34] Marti ME, Colonna WJ, Patra P, Zhang H, Greene C, Reznik G, Pynn M, Jarrell K, Nyman JA, Somasundaran P, Glatz CE, Lamsal BP. Production and characterization of microbial biosurfactants forpotential use in oil-spill remediation. Enzyme Microb Tech 2014;55:31– 39.

[35] Chandran P, Das N. Characterization of sophorolipid biosurfactant produced by yeast species grown on diesel oil. Int J Sci Nat 2011;2:63-71.

[36] Muthusamy K, Gopalakrishnan S, Ravi TK, Sivachidambaram P. Biosurfactants: properties, commercial production and application. Curr Sci 2008;94:736-747.

[37] Cooper DG, Paddock DA. Production of a biosurfactant from *Torulopsis bombicola.* Appl Environ Microbiol 1984;47:173-176.

[38] Sarubbo LA, Farias CBB, Campos-Takaki GM. Co-utilization of canola oil and glucose on the production of a surfactant by *Candida lipolytica*. Curr Microbiol 2007;54:68–73.

[39] Batista RM, Rufino RD, Luna JM, Souza JEG, Sarubbo LA. Effect of medium components on the production of a biosurfactant from *Candida tropicalis* applied to the removal of hydrophobic contaminants in soil. Water Environ Res 2010;82:418–425.

[40] Asmer H, Lang S, Wagner F, Wray V. Microbial production, structure elucidation and bioconversion of sophorose lipids. J Am Oil Chem Soc 1998;65:1460-6.

[41] Zhou Q-H, Kosaric N. Utilization of canola oil and lactose to produce biosurfactant with *Candida bombicola.* J Am Chem Soc 1995;72:67–71.

[42] Kim Y-B, Yun HS, Kim E-K. Enhanced sophorolipid production by feeding-rate-controlled fed-batch culture. Bioresour Technol 2009;100:6028–6032.

[43] Rand GM, Wells PG, McCarty LS. Fundamentals of aquatic toxicology: effects, environmental fate, and risk assessment, In: Rand GM, editor. 2nd ed. Washington: Taylor & Francis; 1995.

[44] Saeki H, Sasaki KM, Komatsu O, Miura A, Matsuda H. Oil spill remediation by using the remediation agent JE1058BS that contains a biosurfactant produced by *Gordonia* sp. strain JE-1058. Bioresour Technol 2009;100:572-577.

[45] Franzetti A, Gandolfi I, Raimondi C, Bestetti G, Banat IM, Smyth TJ, Papacchini M, Cavallo M, Fracchia L. Environmental fate, toxicity, characteristics and potential applications of novel bioemulsifiers produced by *Variovorax paradoxus* 7bCT5. Bioresour Technol 2012;108:245–251.

[46] Lima TMS, Procópio LC, Brandão FD, Leão BA, Tótola MR, Borges AC. Evaluation of bacterial surfactant toxicity towards petroleum degrading microorganisms. Bioresour Technol 2011;102:2957–2964.

[47] Aronstein BN, Calvillo YM, Alexander M. Effects of surfactants at low concentrations on the desorption and biodegradation of sorbed aromatic compounds in soils. Environ Sci Technol 1991;25:1728–1731.

[48] Herman DC, Zhang Y, Miller-Maier RM. Rhamnolipid (biosurfactant) effects on cell aggregation and biodegradation of residual hexadecane under saturated flow conditions. Appl Environ Microbiol 1997;63:3622–3627.

CAPITULO 4

Artigo submetido para publicação Periódico International Frontiers in Microbiology

Candida lipolytica UCP0988 Biosurfactant: Potential as a Bioremediation Agent and in Formulating a Commercial Related Product

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Abstract

A biosurfactant produced by *Candida lipolytica* in a bioreactor containing industrial residues was tested in different remediation techniques of organic and inorganic pollutants. In tests carried out with seawater, the crude biosurfactant demonstrated 80% oil spreading efficiency. The dispersion rate was 50% for the biosurfactant at a concentration twice that of the CMC. The biosurfactant removed 70% of motor oil from contaminated cotton cloth in detergency tests. The crude biosurfactant also removed 30 to 40% of Cu and Pb from standard sand, while the isolated biosurfactant removed approximately 30% of the heavy metals. The conductivity of solutions containing Cd and Pbwas sharply reduced after biosurfactants' addition. A product was prepared through adding a preservative and tested over 120 days. The formulated biosurfactant was analysed for emulsification and surface tension under different pH values, temperatures and salt concentrations and tested for toxicity. The results showed that the formulation had no toxicity and did not cause significant changes in the tensoactive capacity of the biomolecule while maintaining activity demonstrating suitability for potential future commercial product formulation.

Keywords: biosurfactant; *Candida lipolytica*; animal fat; corn steep liquor; bioremediation; petroleum; heavy metals

1. Introduction

Surfactants are chemical compounds that preferentially partition at the interface between phases (gas, liquid and solid) with different degrees of polarity and hydrogen bonding. They are therefore amphipathic molecules with hydrophilic and hydrophobic moieties in which the polar portion is either ionic (cationic or anionic), non-ionic or amphoteric and the nonpolar portion is often a hydrocarbon chain [1]. These characteristics allow these compounds to reduce surface and interfacial tensions as well as form micro-emulsions in which hydrocarbons are solubilised in water or vice versa solubilising water in hydrocarbons. Such properties enable a broad spectrum of potential industrial applications involving emulsification, detergency, lubrication, wetting, foaming, dispersions or solubilisation of different phases [2].

Most commercially available surfactants are synthesised from petroleum by-products [2]. However, environmental concerns mostly driven by consumer demands combined with new regulations aimed at managing the environment have led to the

pursuit to find alternative natural surfactants to replace existing products. Various compounds with such tensioactive properties are often synthesised by biological systems such as plants (saponins), microorganisms (glycolipids) and animals (bile salts, skin exudates), which are considered natural surface active compounds [3].

Compounds of a microbial origin that exhibit surfactant properties are mainly metabolic by-products of bacteria, filamentous fungi and yeasts capable of lowering surface tension and exhibiting a high emulsifying capacity are the most predominant type of biosurfactants [4].The main types of chemical structures of biosurfactants are glycolipids, lipopeptides, lipoproteins, phospholipids, fatty acids and polymeric in nature. Biosufactants have numerous advantages over surfactants of a synthetic origin in having lower toxicity, stability under wider ranges of temperature and pH and ability to remain active at high salt concentrations [5].

The oil industry remains the major market for biosurfactants utilisation, where they can be used in processes involved with the removal and mobilisation oil residue, bioremediation hydrocarbon contaminated environment and microbial enhanced oil recovery technology [2]. The bioremediation of soil and water encounters obstacles associated with the biodegradation of petroleum hydrocarbons, as these hydrophobic compounds bond to soil particles and have a low degree of solubility in water, which reduces their bioavailability to microorganisms and consequently limits the transfer of mass for biodegradation [6]. The key in the process of enhancing the bioavailability of contaminating oils is the mobilisation of the hydrophobic pollutant through the aqueous phase. Thus, the use of surfactants develops as an alternative as a mechanism to enhance the solubility of oils through initiating desorption and the consequent mobilisation and solubilisation of hydrocarbons facilitating transport, access and assimilation of these compounds by microbial cells [7].

Besides organic pollutants, heavy metals are also found in soil and are considered the inorganic pollutants with the greatest potential risk to humans. Metals ions can exist as fixed or soluble minerals in rocks, sand and soilor as dissolved ions in water or vapours. Metals can also be attached to inorganic or organic molecules or even attached air particles. Both anthropogenic and natural activities and processes can emit metals into water and air [8].

Surfactants can potentially be used, and have been used, to remediate soils contaminated with metals and oils through desorption, solubilisation and dispersion of contaminants in soil, thereby allowing there removal, collection or reutilisation [9,10].

The necessity to replace synthetic chemical surfactants with natural compounds however, has motivated studies seeking biological alternatives such as surfactin and rhamnolipids, both of which are of a bacterial biosurfactants [11], and sophorolipids derived from yeasts [12-15]. The ionic nature of these agents as well as their low toxicity, biodegradability and excellent surface properties, make them potential candidates for heavy metalsremoval from contaminated soil, sediment and waste water.

Most known biosurfactants are produced on media containing water immiscible substrates such as oil, fats and liquid or solid hydrocarbons, although many have been obtained on readily available soluble carbon substrates [16]. The type of raw material and availability of substrate to produce biosurfactants contribute considerably to the cost of production (estimated to be 10-30% of total cost [9]. On the other hand, millions of tons of waste materials (residual pollutants) are either deliberately discarded or accidentally leaked into the environment worldwide every year. Treatment and mitigation processes to reduce or eliminate such contaminant represent a high cost to local governments and industries.

The aim of the present study was to investigate the potential application of a biosurfactant from *Candida lipolytica,* which has previously been produced and characterised under optimised conditions [17,18], as an adjunct materials to enhance the remediation processes of hydrophobic pollutants and heavy metals generated by the oil industry and propose the formulation of a safe, stable remediation agent.

2. Materials and Methods

2.1. Materials

All chemicals reagent were of analytical grade. The animal fat used was choice white grease from a bovine processing plant located in the city of Recife (Brazil) and was used without any further processing. Corn steep liquor was obtained from Corn Products from Brazil in the city of Cabo de Santo Agostinho (Brazil). According to Akhtar et al. [35] and Cardinal and Hedrick [36], corn steep liquor is 21 to 45% protein, 20 to 26% lactic acid, 8% ash (containing Ca2+, Mg2+, K+), 3% sugar and has a low fat content (0.9 to 1.2%).

Engine lubricating oil (motor oil) was obtained from an automotive maintenance establishment in the city of Recife, Pernambuco, Brazil. Samples of NBR 7214 standard sand [37] were used in the heavy metal removal experiments. The sand had

a particle size on the order of 0.15 to 0.30 mm, 0.2% water, a specific density of 2.620 g/cm^3 and organic matter content of 100 ppm. The sea water used in the removal of motor oil was collected from the municipality of Cabo de Santo Agostinho, state of Pernambuco, Brazil. Water samples were collected and stored in 5-L plastic bottles.

2.2. Microorganism

Candida lipolytica UCP0988 was obtained from the culture collection of the Universidade Católica de Pernambuco (Brazil). The microorganism was maintained at 5 °C on yeast mould agar slants containing (w/v) yeast extract (0.3%), malt extract (0.3%), tryptone (0.5%), D-glucose (1.0%) and agar (5.0%). Transfers were made to fresh agar slants each month to maintain viability.

2.3. Growth conditions

The inoculum of *Candida lipolytica* was prepared by transferring cells grown on a slant to 50 mL of yeast mould broth. The seed culture was incubated for 24 h at 28 °C and agitated at 150 rpm.The yeast was cultivated in a submerged culture in 500-ml flasks containing 100 ml production medium with agitation in a New Bruswick C-24 shaker. The production medium contained 5% animal fat and 2.5% corn steep liquor. The medium was sterilised by autoclaving at 121 °C for 20 min (all components were sterilised together). The final pH of the medium was 5.3. The inoculum (1% v/v) containing approximately 10^4 cells/mL was introduced into chilled yeast medium.

2.4. Biosurfactant Production and Isolation

Biosurfactant production was performed in a 2-l bioreactor (Tec-Bio-Plus, Tecnal Ltda., Brazil) with a working volume of 1.2 l operated in a batch mode, with controlled pH (5.3) and temperature (28°C). The culture medium was inoculated with a 24-h inoculum and fermentation was carried out at 200 rpm in the absence ofaeration for 144 h [17]. The biosurfactant was recovered from the cell-free broth by cold acetone precipitation, as described by Ilori et al. [38].

2.5. Surface Tension

The surface tension of the culture supernatants obtained by centrifuging the cultures at 5000 g for 20 minutes was measured using a Sigma 700 digital surface tensiometer (KSV Instruments LTD - Finland) as described by Santos et al. [18].

2.6. Screening Dispersion Test

A quick comparative test method using small vials (25 ml) was used for the visual determination of the dispersant effectiveness of the biosurfactant. The motor oil sample (100 µl) was carefully added to the surface of seawater (20 ml) and a vortex with a depth of 1 cm was created by slow magnetic stirring. The dispersant mixture (5.0 µl), i.e., crude biosurfactant (cell-free broth after fermentation) or isolated biosurfactant at half the critical micelle concentration (1/2 x CMC), at the full CMC and twice the CMC (2 x CMC) was added to the centre of the vortex. The stirring rate was immediately increased, maintained at a maximum rate of 2000 rpm for 60 s and then stopped. The level of oil dispersion in the water was visually estimated after a one-minute rest. Classification A was attributed to the resulting brown-black mixture when all the oil was dispersed in the water leaving no slick at the surface, whereas Classification E was used to describe a complete lack of dispersion, i.e. all the oil returned to the surface a few seconds after the end of stirring, leaving the aqueous phase nearly transparent. Classification B to D represented intermediate situations. All screen tests were carried out at room temperature [39].

2.7. Swirling Bottle Test

A 1-L cylindrical open bottle (diameter: 10 cm) with an outlet valve at the bottom to take samples was used in the dispersion experiment. Samples of 200 mL of sea water were added to the bottle and 2 mL of oil was gently added to the surface of the water with a pipette. The crude or the isolated biosurfactant solution was dispensed in the centre of the oil slick in the following proportions of biosurfactant-to-oil: 1:1, 1:2, 1:10 and 1:20 (v/v). The isolated biosurfactant was usedat half the CMC, the full CMC and twice the CMC. The bottle was placed on an orbital shaker table at 28 ºC to induce a swirling motion in the water content of the bottle. The shaking speed was 150 rpm for a period of 10 min, followed by 1 to 2 min settling time to allow the larger droplets to return to the surface. Samples were taken after 15 min. The first 2 mL of the sample was removed through the stopcock and discarded and 30 mL of the sample was collected. This sample was extracted three times with hexane, as the biosurfactant is insoluble in hexane. The extract was adjusted. Efficacy was calculated by dividing the concentration of dispersed oil in the water (determined by analysing the hexane extract) by the total concentration of oil, which depended on the total volume of oil added to the flask [40,41].

2.8. Removal of Motor Oil from Contaminated Cotton Cloth

The efficiency of the biosurfactant to remove oil with respect to a commercially available detergent (Soap powder, Asa LTDA, Recife, PE, Brazil) was investigated. The detergent and biosurfactant were individually dissolved in water and their efficiency in removing oil from contaminated cotton cloth was checked individually as well as in combination with the biosurfactant at a 1:1 (v/v) ratio. For such, 3 g of lubricant oil was poured on a 25 x 25 cm cotton cloth and allowed to dry at 40 ºC for 24 h. To test the oil removal capacity, each piece of cloth impregnated with oil was soaked in flasks containing 100 mL of tap water (control), biosurfactant solutions (cell-free broth and isolated biosurfactant at 1/2 the CMC, the full CMC and twice the CMC), detergent (sodium lauril ether sulfate at the CMC) and a biosurfactant/detergent solution (1:1 v/v) at their CMC. The flasks were kept on a shaker at 30 °C and 100 rpm for 60 min. The post-wash water was used to measure the amount of oil removed from the cotton cloth by extracting it with hexane. The extraction process was repeated three times. The hexane was recovered using a rotary evaporator and the residual lubricant oil was measured gravimetrically [40].

2.9. Preparation of Contaminated Sand with Heavy Metals

The standard sand was artificially contaminated in the laboratory with a metal solution ($Cu(NO3)_2$ + $Pb(NO_3)_2$ + $Zn(NO_3)_2$). The salts were separately dissolved in deionised water to achieve a concentration of 1000 mg/l and then added together to the sand without pH adjustment. The sand was left in contact with the solution for three days in a shaker (200 rpm at 25 °C) and then centrifuged at 5000 rpm for 10 min to remove non-adsorbed metals in the solution. The supernatant was discarded and the contaminated sand was dried in an oven at 50 °C for 24 h [42].

2.10. Treatment of Contaminated Sand with Heavy Metals with Biosurfactant

A series of washings was performed using the isolated biosurfactant at 1/2 the CMC, at the full CMC and twice the CMCas well as the crude biosurfactant (cell-free broth). Distilled water was used as the control. A 1% NaOH solution and 0.7% HCl solution as well as combinations of biosurfactant solutions and cell-free broth with 0.7% HCl or 1% NaOH as additives were also tested. 5.0 g of the contaminated sand were transferred to 125-mL Erlenmeyer flasks and 50 mL of the washing solution were added at the different concentrations described above. The samples were incubated on a rotary shaker (200 rpm) for 24 h at 27 °C and then were centrifuged at 5000 g for

10 min. The supernatants were analysed for metal concentration using an atomic absorption spectrophotometer (Perkin Elmer AAnalyst™ 800).

2.11. Biosurfactant Treatment of Synthetic Wastewater Contaminated With Heavy Metals

The ability to remove heavy metals in water by the biosurfactant was determined in a synthetic fluent containing Pb and Cd. Biosurfactant solutions at1/2 the CMC, the full CMC and twice the CMC were then added separately to 500 and 1000 ppm solutions of lead nitrate and cadmium nitrate. The metal-biosurfactant precipitate was removed and conductivity of the resulting solution was measured. The conductivity meter (TEC-4MP, Tecnal Ltda., Brazil) was calibrated with deionised water before measuring the conductivity of each sample. All tests were performed in triplicate [43].

2.12. Formulation of Biosurfactant

After fermentation, the broth was centrifuged at 5000 rpm for 20 min for the removal of the cells. Potassium sorbate (0.2%) was added to the cell-free both with the crude biosurfactant. After the treatment of the crude biosurfactant in accordance with the preservation method, the broth was stored at room temperature (28 to 30°C) for 120 days, with samples withdrawn at 0, 15, 30, 45, 90 and 120 days to determine stability.

2.13. Effect of Environmental Factors on Formulated Biosurfactant Activity

The effects of the addition of different concentrations of NaCl (1, 3 and 5%), different temperatures (40 and 50 ^{o}C) for 60 min and different pH values evaluated after adjustment of the broth pH to 5, 7 and 9 with 6.0 M NaOH or HCl on surface tension and emulsification were evaluated at 0, 15, 30, 45, 90 and 120 days.

2.14. Emulsification Activity

The emulsification index was measured using the method described by Cooper and Goldenberg [44], whereby 2 mL of motor oil obtained from a local automotive manufacturer in the city of Recife, Brazil, or vegetal corn oil was added to 2 mL of the cell-free broth in a graduated screwcap test tube and vortexed at high speed for 2 min. Emulsion stability was determined after 24 h and the emulsification index was calculated by dividing the measured height of the emulsion layer by the total height of the mixture and multiplying by 100.

2.15. Toxicity of Formulated Biosurfactant Against Poecilia vivipara

Acute toxicity tests were performed using the fish *Poecilia vivipara* as an indicator for the determination of the lethal concentration (LC50) of the formulated biosurfactant for 96 h. The specimens were maintained at 26°C in the laboratory and kept in polyethylene aquaria (capacity: 60 l) supplied with fresh tap water. The fish were fed with commercial fish food (Alcon Basic Ltda, Santa Catarina, Brazil). Water temperature, oxygenation and pH in the aquaria were periodically checked throughout the experiment. After a week of acclimation, the fish were exposed to the formulated biosurfactant.

Assays were performed at the Quarantine Laboratory of Sustainable Mariculture of the Fisheries and Aquaculture Department of the Federal Rural University of Pernambuco (UFRPE), Pernambuco, Brazil. Ten specimens were exposed for 96 h without food or water exchange. Experiments were carried out using a static acute experimental methodology. The animals were kept in 2-l fibreglass boxes with 1.5 l of seawater with salinity of 26 and an average temperature of 27°C under constant aeration and a 12h light/12h dark cycle. Three dilutions of the formulated biosurfactant (cell-free broth plus 0.2% potassium sorbate) in seawater were tested with three replicates each: 1:1, 1:2 and 1:5 (v/v). Controls contained seawater alone. The result of the experiment was based on determination of lethal concentration for 50% of specimens (LC50) and expressed in terms of the mean mortality of three replicates for each dilution tested with the biosurfactant.

2.16. Statistical Analysis

All determinations were performed at least three times. Means and standard errors were calculated using the Microsoft Office Excel 2003 (Version 7).

3. Results and Discussion

3.1. Screening Dispersion Test

The results of the screening dispersion test demonstrated that both the crude and isolated biosurfactant dispersed a reasonable amount of oil, with a greater concentration of biosurfactant leading to a greater percentage of dispersion (Table 1). However, it is important to consider that the use of the cell-free broth represents a considerable reduction in production cost of the compound, as described in Santos et al. [1].

In a previous study by our research group [18], we tested motor oil dispersion characteristics of *C. lipolytica* UCP0988 cell-free broth containing biosurfactants and reported high oil spreading efficiency (54% oil displacement).

According to Bai et al. [19], “dispersion is aprocess by which a hydrocarbon is dispersed in the aqueous phase as tiny emulsions. Emulsions are not generally thermodynamically stable, but may remain stable for significant periods of time due tokinetic restrictions. Dispersion is related to interfacial tension and surfactant concentration and differsfrom displacement, which is related only to interfacial tension between the aqueous and hydrophobicphases, with no formation of emulsion”.

Table 1. Motor oil dispersion by biosurfactant from *C. lipolytica* cultivated in distilled water supplemented with 5% animal fat and 2.5% corn steep liquor using beaker washing method.

Biosurfactant solution	Classification
Crude biosurfactant (cell-free broth)	C[1]
Isolated biosurfactant at ½ x CMC	C[1]
Isolated biosurfactant at CMC	B[1]
Isolated biosurfactant at 2 x CMC	A[1]

[1]A: 100% oil dispersion; B: 75% oil dispersion; C: 50% oil dispersion; D: 25% oil dispersion; E: no dispersion

3.2. Swirling Bottle Test

One of the oil spill remediation techniques is the application of dispersants to oil slicks. Dispersants used for this purpose are usually composed of a mixture of surfactants and solvents with some additives designed to enhance the dispersion of oils as well as their removal from contaminated surfaces. Dispersants application reduces the effects of the oil spills as it removes the oil from the surface of water reducing the amount of spilled oil. The dispersion of oil into tiny droplets also increase the surface area of exposure which stimulates biodegradation by indigenous microorganisms [20]. The effect of factors such as oil viscosity, mixing energy and temperature on the efficacy of a dispersant need to be evaluated. The solvent normally contained in dispersants acts as a solution for the surfactant components and serves as a surfactant carrier, enabling penetration into an oil slick.

According to Sorial et al. [21], the “baffled flask test” developed by the Environmental Protection Agency if the USA has been proposed as a replacement

protocol for categorising oil spill dispersants in a “National Contingency Plan Product Schedule”. Therefore, in the present study a similar experiment was performed for the evaluation of the biosurfactant from *C. lipolytica* as an oil spill dispersant measuring the efficacy using motor oil.

To study the effect of the proportion of biosurfactant to motor oil on dispersant efficacy, tests were carried out with different ratios. In this study, the crude and the isolated biosurfactant without the addition of solvents or additives were tested for 15 min after the simulation of an oil spill in a seawater sample (Table 2).

Table 2. Evaluation of biosurfactant from *C. lipolytica*cultivated in distilled water supplemented with 5% animal fat and 2.5% corn steep liquor as oil spill dispersant (data expressed as mean ± standard deviation).

Biosurfactant/oil ratio	Dispersion index (%)			
	Biosurfactant (1/2 x CMC)	**Biosurfactant (CMC)**	**Biosurfactant (2 X CMC)**	**Crude biosurfactant**
1:1	5.01 ± 0.4	15.5 ± 0.6	50.0 ± 0.7	41.0 ± 0.2
1:2	2.06 ± 0.6	6.06 ± 0.5	22.0 ± 0.1	20.0 ± 0.5
1:10	2.02 ± 0.8	3.0 ± 0.1	5.7 ± 0.6	3.5 ± 0.4
1:20	1.0 ± 0.7	2.0 ± 0.3	2.7 ± 0.5	2.4 ± 0.7

The biosurfactant concentration is a critical parameter, since a lower concentration of biosurfactant leads to a smaller amount of dispersion. The dispersant/oil ratio is another critical factor influencing dispersant efficacy. The best dispersion index occurred with a biosurfactant/oil ratio of 1:1 (v/v) with a solution of the biosurfactant twice the CMC (50%), while the crude biosurfactant dispersed approximately 25% of the oil under the same condition. The biosurfactant used below the CMC was inefficient under the tested conditions. It is likely that the increase in agitation speed allowed greater interaction between the biosurfactant and oil, consequently leading to a greater dispersion percentage. In any case, the results of this test demonstrate that the biosurfactant alone has potential for application as a dispersant, but it is likely that additives will increase the efficiency.

3.3.Motor Oil Removal from Contaminated Cotton Cloth

The use of biosurfactant as a detergent was tested on cotton cloth samples (Table 3). The performance of the biosurfactant was excellent, removing 70% oil at twice the

CMC in comparison to oil removal by the commercially available detergent. The biosurfactant at its CMC was also efficient at removing the oil, while the crude biosurfactant was superior to the isolated biosurfactant at half the CMC. On the other hand, the biosurfactant did not exhibit compatibility with the commercial detergent at a ratio of 1:1 (v/v). Commercially available detergents usually contains an anionic surfactant, water softening components, enzymes andbleaching agents that helps enhancing the washing performance [22]. Thus, the addition of these substances could increase the efficiency of the biosurfactant from *C. lipolytica*.

Table 3. Removal of motor oil from contaminated cotton clothby biosurfactant from *C. lipolytica* cultivated in distilled water supplemented with 5% animal fat and 2.5% corn steep liquor using beaker washing method (data expressed as mean ± standard deviation).

Washing solutions	**Removal (%)**
Distilled water (control)	04.10 ± 0.4
Crude biosurfactant (cell-free broth)	36.00 ± 0.5
Isolated biosurfactant at ½ x CMC	30.20 ± 0.7
Isolated biosurfactant at CMC	48.09 ± 0.4
Isolated biosurfactant at 2 x CMC	70.30 ± 0.6
Commercial detergent	28.07 ± 0.3
Isolated biosurfactant at CMC + commercial detergent at CMC (1:1, v/v)	32.45 ± 0.7

3.4. Biosurfactant Treatment Heavy Metals Contaminated Sand

Advances in treatment technologies for heavy metals contaminated soils have increased the interest in finding new washing products, such as anionic biosurfactants capable of bonding to metals and do not pose risks to the environment due to their characteristics of lower toxicity and biodegradability [23]. The mechanisms for heavy metals extraction by biosurfactants include ionic exchange, chelation, dissolution, precipitation and associations with contra-ions. Metals are believed to be removed through complexes formation with a surfactant at soil surfaces which are mobilised due to the reduction in interfacial tension and the consequent association with surfactants' micelles. Anionic surfactants are negatively charged and therefore have a good affinity towards metal cations while enhancing better removal due to their capacity to reduce interfacial tension [9].

It is important for biosurfactants to remain in the aqueous phase and have minimal interactions with the treated soils . However, when large concentrations of biosurfactant are used to ascertain effective heavy metals removal from soil, sorption to soil particles may occur. Hence, its behaviour will inevitably depend on the biosurfactants' molecular characteristics, such as charge, hydrophobicity and soil characteristics [9]. Thus, the low-cost biosurfactant we produced using *C. lipolytica* was tested with regard to the removal of copper, lead and zinc contained in samples of standard sand. The standard sand with organic matter content of 100 ppm was used to minimise the interaction of the biosurfactant with the soil and maximise metal-biosurfactant interactions.

Solutions of the isolated biosurfactant at different concentrations [1/2 x CMC (0.04%), CMC (0.08%) and 2 x CMC (0.16%)] were tested to evaluate the removal of metals with and without the formation of micelles, which are efficient structures for the mobilisation of heavy metals during soil treatment. Metal removal with the cell-free crude biosurfactant was investigated. The likelihood of increasing percentage metal removal was tested using the surfactant with HCl and NaOH as additives. The additives were employed separately while using distilled water as control. The results of the treatment of sand with the biosurfactant solutions are shown in Table 4.

The results demonstrate that, under the herein tested conditions, *C. lipolytica* biosurfactant was more efficient in the removal of copper and lead. The control treatments showed 11 to 17% metals removal from sand while other treatments achieve >80% removal (Table 4). Ochoa-Loza et al. [24], stated that different surfactants have varying affinities to different metals and are invariably affected by type and concentration of biosurfactant, interaction with additives (acids or alkalines) and soil characteristics.

Heavy metals removal was not proportional to the increase in the concentration of biosurfactant, remaining around 30% for copper and lead as well as 7% for zinc at the concentrations used (1/2 x CMC, CMC and 2 x CMC). As seen, zinc was not removed efficiently by the biosurfactant solutions. This metal had an affinity for the acid, which removed 30 to 40% of the metals when combined with the biosurfactant.

Doong et al. [25] reported heavy metals removal increasing linearly with surfactants increase at concentrations below CMC and remained relatively constant at concentrations above CMC and depended the type of surfactant, the metals involved and type of soil. The high concentration necessary in some experiments is most often

related to biosurfactants' sorption or bonding to the components of the soil particles [26].

The acid removed 50 to 60% of the metals adsorbed to the soil when used alone and this removal percentage increased significantly when the acid was combined with the solutions of the isolated biosurfactant and cell-free broth. The base removed approximately 15% of the metals and generally increased the percentage of copper and lead removal by the biosurfactant, although it had no positive effect on the removal of zinc when combined with the biosurfactant solutions. The combination of the base and acid together when used with the biosurfactant was also not favourable to the removal of the heavy metals. The results suggest that neutralisation of the positive effect of the acid occurred when the base was added to the biosurfactant-acid solutions. It should be stressed that treatment with both alkaline and acidic components may reduce soil fertility and change it chemical composition [9]. Hong et al. [27], mentioned that Na^+ may compete with heavy metals binding to the surfactant and forming Na-surfactant complexes. This may also be the case when using biosurfactant, thereby diminishing metal removal when NaOH used compared to the use of acid. However, França et al. [28] reported higher Zn, Cr and Cu removal rates when NaOH was added to a biosurfactant solution derived from *B. subtilis*. A possible explanation for this would be the increase in the solubility of the biosurfactant in the presence of NaOH.

The cell-free crude extract removed 30-40% of lead and copper from the, indicating that the crude biosurfactant could be used in the treatment of heavy metal contaminated soils. This would be considerable advantage, since the downstream process to purify biosurfactant obtained through fermentation could account for 60% of the production cost [29].

The possibility of using biosurfactants for the removal of heavy metals has been shown in laboratory studies [30] A 4% solution of *Torulopsis bombicola* derived sophorolipids removed 3% of the Cu ions from soil samples and did not remove Zn. The addition of 1% of NaOH to the 4% sophorolipid solution, lead to an increase to 36 and 7% Cu and Zn removal, respectively. The highest removal occurred when 0.7% HCl was added to the 4% sophorolipid solution, achieving Cu and Zn removal rates of 37 and 16%, respectively. Rhamnolipid extracts from *Pseudomonas aeruginosa* in comparison removed 35 and 20% Cu and Zn ions, respectively, when used at a concentration of 12%, whereas a concentration of 2% was able to to remove 10 and 5% Cu and Zn, respectively. The addition of 1% NaOH to the 2% surfactant solution

led to a significant increase in copper removal from 10 to 28%, but a reduction in zinc removal from 5 to 3%.

Table 4. Removal of heavy metals contained in contaminated standard sand by washing solutions (data expressed as mean ± standard deviation).

Treatment	Removal (%)		
	Cu	Pb	Zn
Distilled water (control)	17 ± 1.3	11 ± 1.3	15 ± 1.5
1% NaOH solution	11 ± 2.1	16 ± 0.8	15 ± 1.0
0.7% HCl solution	60 ± 1.4	54 ± 1.5	50 ± 1.3
Cell-free broth	40 ± 1.8	30 ± 1.5	7.1 ± 1.5
Cell-free broth + 0.7% HCl	81 ± 1.6	78 ± 1.8	39 ± 1.9
Cell-free broth + 1% NaOH	53 ± 1.4	49 ± 1.5	5.4 ± 1.7
Cell-free broth + 1% NaOH + 0.7% HCl	40 ± 1.2	30 ± 1.4	5.3 ± 1.3
0.04% biosurfactant solution (1/2 x CMC)	30 ± 1.4	35 ± 1.7	7.1 ± 1.1
0.04% biosurfactant solution (1/2 x CMC) + 0.7% HCl	81 ± 2.0	80 ± 1.1	40 ± 1.3
0.04% biosurfactant solution (1/2 x CMC) + 1% NaOH	39 ± 2.0	40 ± 1.3	6.2 ± 1.1
0.04% biosurfactant solution (1/2 x CMC) + 0.7% HCl + 1% NaOH	38 ± 1.7	47 ± 1.4	5.1 ± 1.3
0.08% biosurfactant solution (CMC)	31 ± 1.4	33 ± 1.6	7.6 ± 1.4
0.08% biosurfactant solution (CMC) + 0.7% HCl	81 ± 0.8	82 ± 1.5	30 ± 1.2
0.08% biosurfactant solution (CMC) + 1% NaOH	45 ± 2.1	33 ± 1.4	6.2 ± 1.2
0.08% biosurfactant solution (CMC) + 0.7% HCl + 1% NaOH	49 ± 1.5	31 ± 1.8	5.1 ± 1.1
0.16% biosurfactant solution (2 x CMC)	30 ± 1.5	35 ± 1.5	6.3 ± 1.4
0.16% biosurfactant solution (2 x CMC)+ 0.7% HCl	70 ± 1.6	65 ± 1.5	29 ± 1.2
0.16% biosurfactant solution (2 x CMC) + 1% NaOH	45 ± 1.7	40 ± 1.7	5.1 ± 1.8
0.16% biosurfactant solution (2 x CMC)+ 0.7% HCl + 1% NaOH	50 ± 1.9	45 ± 2.1	6.5 ± 1.5

Mulligan et al. [31] reported an increase in Zn removal when a 2% surfactin solution was used in combination with an alkaline, whereas Cu removal was unaffected by the presence of NaOH. The combination of the base with a 0.5% rhamnolipid solution favoured the removal of both metals (65 and 18%) in comparison to the base

alone. On the other hand, 100% copper and zinc removal were achieved with 0.7% HCl in a 4% sophorolipid solution. Heavy metals removal from soil using a saponin (0.1 to 10%) was reported to be proportional to its concentration [27]. Daharazma and Mulligan [29] also reported heavy metal removal from soil increasing linearly with increased rhamnolipid concentration used and that a 5% solution removed 37% of Cu, 7.5% of Zn and 33.2% of Ni. This is similar to our results which showed metals removal. The addition of a 0.5% rhamnolipid solution to NaOH increased the removal of copper to 28.3% and Ni to 11.5% in comparison to removal rates achieved with the base alone (1% NaOH).

3.5. Biosurfactants' Ability to Bind with Heavy Metals in Aqueous Solution

The biosurfactant treatment of waste water contaminated with heavy metals was tested using conductivity measures. The conductivity of the biosurfactant solution at half the CMC was de 178 µS/cm and increased to 190 and 198 µS/cm at the CMC and twice the CMC, respectively. This increase was due to the anionic nature of the surface active agent. However, conductivity of the solutions containing cadmium (Cd) and lead (Pb) underwent an accentuated reduction upon the addition of the biosurfactant to the metal solutions due to the chelation/precipitation of the positively charged metals, thereby reducing metal ions in solution and consequently reducing its conductivity (Table 5).

Table 5. Conductivity of metal solutions before and after washing with solutions of biosurfactant isolated from *C. lipolytica*.

Heavy metal	Conductivity (µS/cm) of metal solution	Conductivity (µS/cm) after treatment with biosurfactant solutions		
		1/2xCMC	CMC	2xCMC
Cd	510.4	15.40	15.28	12.74
Pb	670.4	21.00	21.32	21.83

The results also demonstrate the efficiency of the biosurfactant at the lowest concentration tested (1/2 x CMC), as only little variation in the conductivity of the metal solutions occurred at the higher concentrations (CMC and 2 x CMC). These results indicate that more micelles led to fewer free ions and conductivity was therefore much less than in the solutions with the absence of biosurfactant.

3.6. Effect of Environmental Factors on Formulated Biosurfactant Activity

To offer a commercial surfactant agent, the biosurfactant was formulated and its properties (surface tension, which allows the breakdown of an oil spill, and emulsification, which allows a blend in the form of droplets, to facilitate biodegradation by microorganisms) were evaluated over a 120-day period, thereby estimating the shelf life of the proposed product. Potassium sorbate, a widely used a preservative that inhibits the growth of mould, was added to the biosurfactant at the same concentration used in foods.

The formulated biosurfactant with potassium sorbate was analysed at different pH values and temperatures as well as different salt concentrations (Tables 6 to 9). The surface tension of the formulated biosurfactant generally exhibited a small, gradual increase throughout the 120 days of storage in the presence of NaCl with variations in pH and temperature. As the change in tension did not surpass 10 units under the conditions tested, one may presume that the formulation with potassium sorbate did not cause significant changes in the tensioactive capacity of the biomolecule, indicating the possibility of using the biosurfactant under specific environmental conditions of pH, temperature and salinity.

Table 6. Surface tension of biosurfactant formulated with potassium sorbate (0.2%) over 120 days with changes in pH and temperature as well as in different concentrations of NaCl (data expressed as mean ± standard deviation).

Time (days)	Surface tension (mN/m)							
	NaCl (%)			pH			Temperature (ºC)	
	1	3	5	5	7	9	40	50
0	26 ± 1.0	27 ± 1.2	27 ± 1.0	27 ± 1.8	28 ± 1.1	28 ± 1.0	26 ± 1.5	27 ± 1.1
15	33 ± 1.1	34 ± 1.0	28 ± 1.2	33 ± 1.3	33 ± 1.4	35 ± 1.3	27 ± 0.9	29 ± 1.3
30	33 ± 1.3	33 ± 1.5	30 ± 1.5	35 ± 1.3	35 ± 1.0	35 ± 1.2	30 ± 1.5	30 ± 0.9
45	33 ± 1.9	33 ± 1.4	33 ± 1.0	35 ± 1.5	35 ± 1.3	37 ± 1.6	32 ± 1.3	29 ± 1.1
90	35 ± 1.0	33 ± 1.0	33 ± 1.3	35 ± 1.4	37 ± 0.9	37 ± 0.5	32 ± 1.1	29 ± 1.2
120	35 ± 1.5	33 ± 1.1	32 ± 1.2	35 ± 1.1	39 ± 1.1	40 ± 1.0	33 ± 1.3	35 ± 1.1

The emulsification index values in the presence of NaCl, showed some improvement with increase storage time especially after 30 days. Higher salt concentrations had no negative effect on the action of the biosurfactant showing ability to use in saline environments.

The emulsification of corn oil remained practically stable with the change in pH values throughout the storage time, whereas the emulsification of motor showed slight increase after 30 days, demonstrating that the interaction between the biosurfactant and oil may be strengthened over time, indicating greater stability of the inter-molecular bonds.

Table 7. Emulsification of motor and corn oil by biosurfactant formulated with potassium sorbate (0.2%) over 120 days with different concentrations of NaCl (data expressed as mean ± standard deviation).

Time (days)	Emulsification (%)					
	1% NaCl		3% NaCl		5% NaCl	
	Motor oil	Corn oil	Motor oil	Corn oil	Motor oil	Corn oil
0	50 ± 3.0	37 ± 2.1	60 ± 1.9	40 ± 3.5	50 ± 2.3	45 ± 2.0
15	60 ± 2.5	38 ± 2.5	80 ± 2.8	44 ± 2.0	76 ± 2.4	46 ± 2.8
30	88 ± 2.0	50 ± 2.4	85 ± 3.0	47 ± 2.1	95 ± 3.2	46 ± 3.0
45	88 ± 2.0	48 ± 2.5	85 ± 2.4	54 ± 2.5	95 ± 3.0	50 ± 2.7
90	88 ± 3.0	48 ± 3.1	85 ± 2.5	54 ± 3.5	95 ± 2.5	50 ± 3.0
120	88 ± 1.5	48 ± 3.0	87 ± 2.0	54 ± 2.5	95 ± 3.0	50 ± 2.5

The findings demonstrated that it was possible to formulate a product that remains free of contamination and maintains stability and can be commercialised as an efficient, low-cost, biodegradable agent for use by different industries. The results regarding the formulation of the biosurfactant are difficult to discuss, as the literature on this topic is scarce. Some studies describe the use of spray drying for the conservation of biosurfactants and later application [32]. Spray drying has also been effective in the recovery and concentration of a biosurfactants while maintaining their surface activity and the dry product maintained its characteristics and activity during storage at room temperature throughout a 120 day evaluation period [33].

Table 8. Emulsification of motor and corn oil by biosurfactant formulated with potassium sorbate (0.2%) over 120 days with different pH values (data expressed as mean ± standard deviation).

Time (days)	Emulsification (%)					
	pH 5		pH 7		pH 9	
	Motor oil	Corn oil	Motor oil	Corn oil	Motor oil	Corn oil
0	80 ± 3.1	45 ± 2.0	50 ± 1.5	50 ± 1.1	50 ± 2.7	45 ± 3.0
15	85 ± 2.1	45 ± 1.8	55 ± 2.5	50 ± 2.5	50 ± 2.3	45 ± 2.3
30	88 ± 2.5	45 ± 2.7	100 ± 1.0	55 ± 2.1	95 ± 2.2	45 ± 1.8
45	88 ± 1.8	55 ± 3.1	100 ± 1.0	55 ± 1.8	95 ± 2.3	45 ± 2.5
90	88 ± 2.3	55 ± 2.2	100 ± 0.5	50 ± 2.3	95 ± 2.1	45 ± 3.0
120	88 ± 1.6	55 ± 1.1	100 ± 1.0	50 ± 3.5	95 ± 1.2	45 ± 3.1

Table 9. Emulsification of motor and corn oil by biosurfactant formulated with potassium sorbate (0.2%) over 120 days with different temperatures (data expressed as mean ± standard deviation).

Time (days)	Emulsification (%)			
	40 ºC		50 ºC	
	Motor oil	Corn oil	Motor oil	Corn oil
0	50 ± 2.0	40 ± 1.7	50 ± 2.7	45 ± 3.0
15	50 ± 2.8	40 ± 1.9	60 ± 2.9	45 ± 2.8
30	55 ± 3.0	40 ± 2.4	95 ± 2.7	45 ± 2.7
45	60 ± 2.7	50 ± 2.0	95 ± 3.2	55 ± 2.0
90	60 ± 2.3	50 ± 2.8	95 ± 3.0	55 ± 1.9
120	60 ± 1.7	50 ± 3.0	95 ± 1.7	55 ± 3.0

3.7. Toxicity of Formulated Biosurfactant

The fish *Poecilia vivípara* belongs to the family *Poecilidae*, which occurs from the United States to Argentina. This species has been used as a bioindicator in the monitoring of aquatic environments due to its sensitivity and response capacity to environmental pollutants [34]. Acute toxicity tests were conducted with this fish to determine the mean lethal concentration (LC_{50}) of the formulated biosurfactant over a 96-hour period. The biosurfactant formulated from *C. lipolytca*was considered to have

low toxicity, as the *P. vivipara* survival rate was respectively 70, 75 and 95% for biosurfactant/seawater dilutions of 1:1, 1:2 and 1:5 (v/v).

4. Conclusions

The present findings demonstrate that industrial waste products can be successfully used in the production of surfactant agents with broad applications in the environmental remediation of organic and inorganic pollutants. Biosurfactant from *C. lipolytica* presented satisfactory results regarding the treatment of sites contaminated with petroleum products and heavy metals. The possibility of commercialising an agent with long-term stability was also demonstrated, making the production process and application of biosurfactants more viable in the current market of chemical surfactants derived from petroleum.

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References

1. Santos, D.K.F.; Rufino, R.D.; Luna, J.M.; Santos, V.A.; Sarubbo, L.A. Biosurfactants: Multifunctional Biomolecules of the 21st Century. *Int. J. Mol. Sci.***2016**, 17, 401.

2. Silva, R.C.F.S.; Almeida, D.G.; Luna, J.M.; Rufino, R.D.; Santos, V.A.; Sarubbo, L.A. Applications ofbiosurfactants in the petroleum industry and the remediation of oil spills. *Int. J. Mol. Sci*. **2014**, 15,12523–12542.
3. Campos, J.M.; Stamford, T.L.M.; Sarubbo, L.A.; Luna, J.M.; Rufino, R.D.; Banat, I.M. Microbial biosurfactantsas additives for food industries. *Biotechnol. Prog*. **2013**, 29, 1097–1108.
4. Marchant, R.; Banat, I.M. Biosurfactants: A sustainable replacement for chemical surfactants? *Biotechnol. Lett*. **2012**, 34, 1597–1605.
5. Banat, I.M.; Satpute, S.K.; Cameotra, S.S.; Patil, R.; Nyayanit, N. Cost effective technologies and renewablesubstrates for biosurfactants' production. *Front. Microbiol*. **2014**, 5, doi:10.3389/fmicb.2014.00697.
6. Souza, E.C.; Vessoni-Penna, T.C.; Souza Oliveira, R.P. Biosurfactant-enhanced hydrocarbon bioremediation:An overview*. Int. Biodeterior. Biodegrad*. **2014**, 89, 88–94.
7. Burghoff, B. Foam fractionation applications. *J. Biotechnol*. **2012**, 161, 126-137.
8. Sarubbo, L.A.; Rocha Júnior, R.B.; Luna, J.M.; Rufino, R.D.; Santos, V.A.; Banat, I.M. Some aspects of heavy metals contamination remediation and role of biosurfactants. *Chem. Ecol*. **2015**, 31, 707–723.
9. Marchant, R.; Banat, I.M. Biosurfactants: A sustainable replacement for chemical surfactants? *Biotechnol. Lett*.**2012**, 34, 1597–1605.
10. Asçi, Y.; Nurbas, M.; Açikel, Y. A comparative study for the sorption of Cd(II) by soils with different claycontents and mineralogy and the recovery of Cd(II) using rhamnolipid biosurfactant. *J. Hazard. Mater*. **2008**,154, 663–673.
11. Barros, F.F.C.; Quadros, C.P.; Maróstica, M.R.; Pastore, G.M. Surfactina: Propriedades químicas, tecnológicas e funcionais para aplicações em alimentos. *Quím. Nova* **2007***,* 30, 1–14.
12. Coimbra, C.D.; Rufino, R.D.; Luna, J.M.; Sarubbo, L.A. Studies of the cell surface properties of *Candida* species and relation with the production of biosurfactants for environmental applications. *Cur. Microbiol*. **2009**, 58, 245-249.
13. Albuquerque, C.F.; Luna-Finkler, C.L.; Rufino, R.D.; Luna, J.M.; Menezes, C.T.B.; Santos, V.A.; Sarubbo, L.A.Evaluation of biosurfactants for removal of heavy metal ions from aqueous effluent using flotation techniques.*Int. Rev. Chem. Eng*. **2012**, 4, 156–161.
14. Menezes, C.T.B.; Barros, E.C.; Rufino, R.D.; Luna, J.M.; Sarubbo, L.A. Replacing synthetic with microbial surfactants as collectors in the treatment of aqueous

effluent produced by acid mine drainage using the dissolved air flotation technique. *Appl. Biochem. Biotechnol*. **2011**, 163, 540–546.

15. Rufino, R.D.; Luna, J.M.; Marinho, P.H.C.; Farias, C.B.B.; Ferreira, S.R.M.; Sarubbo, L.A. Removal of petroleum derivative adsorbed to soil by biosurfactant Rufisan produced by *Candida lipolytica. J. Petr. Sci. Eng.***2013**, 109, 117–122.
16. Pacwa-Plociniczak, M.; Plaza, G.A.; Piotrowska-Seget, Z.; Cameotra, S.S. Environmental applications of biosurfactants: Recent advances. *Int. J. Mol. Sci*. **2011**, 13, 633–654.
17. Santos, D.K.F.; Brandão, Y.B.; Rufino, R.D.; Luna, J.M.; Salgueiro, A.A.; Santos, V.A.; Sarubbo, L.A. Optimization of cultural conditions for biosurfactant production from *Candida lipolytica*. *Biocatal. Agric. Biotechnol*. **2014**, 3, 48–57.
18. Santos, D.K.F.; Rufino, R.D.; Luna, J.M.; Santos, V.A.; Salgueiro, A.A.; Sarubbo, L.A. Synthesis and evaluation of biosurfactant produced by *Candida lipolytica* using animal fat and corn steep liquor. *J. Pet. Sci. Eng*. **2013**, 105, 43–50.
19. Bai, G.; Brusseau, M.L.; Miller, R.M. Biosurfactant-enhanced removal of residual hydrocarbon from soil.*J. Contam. Hydrol*. **1997**, 25, 157–170.
20. National Research Council, Committee on Understanding Oil Spill Dispersants, Oil Spill Dispersants: Efficacy and Effects, Ocean Studies Board, The National AcademiesPress, Washington, DC, 2005.
21. Sorial, G.A.; Venosa, A.D.; Koran, K.M.; Holder, E.; King, D.W. Oil spilldispersant effectiveness protocol: I. Impact of operational variables. *J. Environ.Eng.-ASCE* 2004, 130, 1073–1084.
22. Mukherjee, S.; Das, P.; Sen, R. Towards commercial production of microbial surfactants. *Trends Biotechnol.***2007**, 24, 509-515.
23. Maity, J.P.; Huang, Y.M.; Fan, C.-W.; Chen, C.-C.; Li, C.-Y.; Hsu, C.-M.; Chang, Y.-F.; Wu, C.-I.; Chen, C.-Y.; Jean, J.-S. Evaluation of remediation process with soapberry derived saponin for removal of heavy metals from contaminated soils in Hai-Pu Taiwan*. J. Environ. Sci*. **2013**, 25, 1180–1185.
24. Ochoa-Loza, F.J.; Noordman,W.H.; Jannsen, D.B.; Brusseau, M.L.; Maier, R.M. Effect of clays, metal oxides, and organic matter on rhaminolipid biosurfactant sorpition by soil. *Chemosphere***2007**, 66, 1634–1642.
25. Doong, R.A.; Wu, Y.W.; Lei, W.G. Surfactant enhanced remediation of cadmium contaminated soils. *Water Sci. Technol*. **1998**, 37, 65–71.

26. Wang, S.; Mulligan, C.N. Rhamnolipid biosurfactant – enhanced soil flushing for the removal of arsenic and heavy metals from mine tailings. *Process Biochem*. **2009**, 44, 296-301.
27. Hong, K.J.; Tokunaga, S.; Kajiuchi, T. Evaluation of remediation process with plant-derived biosurfactant for recovery of heavy metals from contaminated soils. *Chemosphere***2002**, 49, 379-387.
28. França, Í.W.L.; Lima, A.P.; Lemos J.A.M.; Lemos, C.G.F.; Melo, V.M.M.; Sant'ana, H.B.; Goncalves, L.R.B. Production of a biosurfactant by *Bacillus subtilis* ICA56 aiming bioremediation of impacted soils. *Catal. Today***2015**, 255, 10-15.
29. Dahrazma, B.; Mulligan, C.N. Investigation of the removal of heavy metals from sediments using rhamnolipid in a continuous flow configuration. *Chemosphere***2007**, 69, 705-711.
30. Mulligan, C.N.; Yong, R.N.; Gibbs, B.F. An evolution of technologies for the heavy metal remediation of dredged sediments. *J. Haz. Mat*. **2001**, 85, 145-163.
31. Mulligan, C.N.; Yong, R.N.; Gibbs, B.F. Metal removal from contaminated soil and sediments by the biosurfactant surfactin. *Environ. Sci. Technol*. **1999**, 33, 3812–3820.
32. Saeki, H.; Sasaki, K.M.; Komatsu, O.; Miura, A.; Matsuda, H. Oil spill remediation by using the remediation agent JE1058BS that contains a biosurfactant produced by *Gordonia* sp strain JE-1058. *Bioresor. Technol.* **2009***,* 100, 572-577.
33. Barcelos, G.S.; Dias, L.C.; Fernandes, P.L.; Fernandes, R.C.R.; Borges, A.C.; Kalks, K.H.M.; Tótola, M.R. Spray drying as a strategy for biosurfactant recovery, concentration and storage. *Springer Plus* **2014**, 3, 49. doi: 10.1186/2193-1801-3-49
34. Breseghelo, L.; Cardoso, MP.; Borges-Deoliveira, R.; Costa, MF.; Barreto JCB.; Sabóia-Morais, SMT.; Yamada, AT. Effects of sodium fluoride in gill epithelium of Guppy fish (*Poecilia vivipara*). *Braz. J. Veter. Res. Animal Sci.* **2004**, 41, 274-280.
35. Akhtar, M.; Lentz, M.J.; Banchette, R.A.; Kirk, T.K. Corn steep liquor lowers the amount of inoculum for biopulping. *TAPPI J.***1997**, 80, 161–164.
36. Cardinal, E.V.; Hedrick, L.R. Microbiological assay of corn steep liquor for amino acid content. *J. Biol. Chem.***1948**, 172, 609–612.
37. Associacão Brasileira de Normas Técnicas (ABNT) Brazilian Association of Technical Standards, NBR8492: Tijolo maciço de solo-cimento: Determinação da Resistência à Compressão e da Absorção de Água, Método de Ensaio, Rio de Janeiro, Brasil, 1982.

38. Ilori, M.O.; Amobi, C.J.; Odocha, A.C.; Factors affecting biosurfactant production by oil degrading Aeromonas spp. isolated from a tropical environment. *Chemosphere***2005**, 61, 985–992.
39. Brochu, C.; Polletier, E.; Caron, G.; Desnoyers, J.E. Dispersion of crude oil in seawater: The role of synthetic surfactants. *Oil Chem. Pol.***1986/87**,3, 257-279.
40. Jain, R.M.; Mody, K.; Mishra, A.; Jha, B. Physicochemical characterization of biosurfactant and its potential to remove oil from soil and cotton cloth, *Carbohyd. Pol.***2012**, 89, 1110-1116. doi:10.1016/j.carbpol.2012.03.077.
41. Holakoo, L. On the capability of Rhamnolipids for oil spill control of surface water. Master in Applied Science, Concordia University, Montreal, Canada, 2001.
42. Juwarkar, A.A.; Nair, A.; Dubey, K.V.; Singh, S.; Devotta, S. Biosurfactant technology for remediation of cadmium and lead contaminated soils. *Chemosphere***2007**, 68, 1996-2002.
43. Das, P.; Mukherjee, S.; Sen, R. Antiadhesive action of a marine microbial surfactant. *Coll. Surf. B Biointerfaces***2009**, 71, 183–186.
44. Cooper, D.G.; Goldenberg, B.G. Surface active agents from two *Bacillus* species. *Appl. Environ. Microbiol*. **1987**, 53, 224-229.

CONCLUSÕES

Os resultados obtidos nessa pesquisa permitem as seguintes conclusões:

- A maximização da produção do biossurfactante de *C. lipolytica* em biorreator foi efetiva com a utilização do planejamento fatorial, possibilitando a aplicação industrial do tensoativo na redução da contaminação ambiental provocada por petróleo e derivados.
- A biomolécula foi produzida na fase estacionária de crescimento após 96 horas de cultivo.
- O *scale up* permitiu o aumento de quatro vezes na produção do biossurfactante em reator semi-industrial, com maior redução da tensão superficial e em intervalo de tempo um pouco superior.
- A biomassa produzida durante a produção do biossurfactante pode ser usada como complemento nutricional para uso como ração animal.
- A caracterização estrutural do biossurfactante sugere sua natureza glicolipídica.
- A biomolécula testada na forma bruta é atóxica frente a sementes de vegetais, aos indicadores marinhos *Artemia salina* e *Anomalocardia brasiliana* e à microbiota da água do mar.
- O biossurfactante apresenta potencial de aplicação como agente de biorremediação de derrames no mar, estimulando a degradação de poluentes orgânicos pela microbiota autóctone.
- O biossurfactante apresenta potencial como agente dispersante de manchas de óleos na água do mar na forma bruta e isolado.
- O biossurfactante demonstra eficiência como detergente na remoção de óleo em água.
- O biossurfactante apresenta capacidade de remoção de metais pesados adsorvidos em solos.
- O biossurfactante apresenta capacidade de complexar cátions metálicos em soluções aquosas, sugerindo o potencial desse agente como coadjuvante dos processos de remedição de efluentes poluídos por metais pesados.
- A formulação aumentou a vida de prateleira do biossurfactante com manutenção de suas propriedades tensoativas e emulsificantes produto
- O biossurfactante formulado mantém suas propriedades tensoativas e emulsificantes sob diferentes condições ambientais de pH, temperatura e presença de sal.

- O biossurfactante formulado é atóxico frente ao indicador ambiental *Poecilia vivipara.*
- A possibilidade de comercialização de um agente estável a longo prazo foi demonstrada, tornando o processo de produção e aplicação dos biossurfactantes mais viável frente ao mercado dos surfactantes químicos derivados de petróleo.

ANEXOS

CAPÍTULO 2

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Optimization of cultural conditions for biosurfactant production from *Candida lipolytica*

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ABSTRACT

The maximisation of the production of metabolites of industrial interest from fermentative processes, such as biosurfactants, requires standardisation of the medium and the cultivation conditions. In this sense, experiments were conducted to maximise the production of the biosurfactant from *Candida lipolytica* UCP0988 cultivated on 5% animal fat and 2.5% corn steep liquor using a 2^3 full factorial design. The effects and interactions of the agitation speed (200, 300 and 400 rpm), the variables aeration (0, 1 and 2 vvm) and time of cultivation (48, 96 and 144 h) on the surface tension, yield and biomass were evaluated. The results showed that the variable time of cultivation had positive influence on the production of biosurfactant, while the increase of the variables aeration and agitation showed a negative effect. These results indicate that the biosurfactant has a strong potential to be applied as a clean-up of oil spills at sea and on shorelines.

1. Introduction

Interest in microbially produced biosurfactants has increased recently, mainly due to their potential as agents in enhanced oil recovery. A variety of microbes and their products have been assessed for their surface-active properties, and it has been suggested that biosurfactants may prove useful in a broad spectrum of potential applications which presently utilize synthetic surfactants (Banat et al., 2010). Biosurfactants can be as effective as some widely-used synthetics and have conceivable advantages, often being more biodegradable than many synthetics, reducing pollutant loads, and sometimes being less sensitive to extremes of temperature, pH, or salinity (Pacwa-Plociniczak et al., 2011). In economic terms there are prospects of their being cheaper than the synthetics, if production by fermentation technology from cheap renewable substrates is compared with production by petrochemical routes from a diminishing hydrocarbon resource (Marchant and Banat, 2012). One of the strategies to improve production is to optimise the growth media and cultivation conditions in order to get maximum production (Mukherjee et al., 2006). Statistical experimental designs such as Response Surface Methodology (RSM) and Contour Curve (CC), factorial and Taguchi designs have been used to increase the product yields (Sen, 1997; Rodrigues et al., 2006; Rivera et al., 2007; Whei et al., 2007). These statistical optimization procedures minimise the number of experiments saving time and labour.

Statistical techniques commonly referred to as Response Surface Methodology and Contour Curve are powerful experimental design tools that have been used to optimise and evaluate the performance of complex systems (Aleboyeh et al., 2008; Barros Neto et al., 2010; Chaves, 2008).

In the present study, the production of the biosurfactant from the yeast *Candida lipolytica* UCP0988 using low-cost fermentative medium based on animal fat and corn steep liquor was improved by using a bioreactor and a full factorial design. One method of modelling, *i.e.*, RSM/CC, applying a 2^3 full-factorial, was used to determine the relationship and influence between input and output variables on the process efficiency. The input variables were agitation, aeration and time of the process, and the output variables were the surface tension, yield and biomass. The importance of each input variable on the variation of the output

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(Sarubbo et al., 2006). Therefore, the use of statistical modelling is an important tool that can be used to explain the influence not only more relevant, but also the interaction parameters involved in fermentation performance of a given process (Rufino et al., 2007; Luna et al., 2011). According to Davila et al. (1992), is an efficient way to generate information from a few experimental tests, thus reducing the costs and time required for development of experimental procedures.

In this sense, tests performed to optimise the production of the surfactant were evaluated by means of a 2^3 full-factorial design, in order to analyse the main effects and interactions of variables: aeration, agitation speed and time of cultivation on the response variables surface tension, biosurfactant yield and biomass concentration.

3.1. Study of the surface tension

The following response equation was used to correlate the dependent and independent variables given by Eq. (1)

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3, \quad (1)$$

where Y is the response variable or surface tension efficiency; b_0 is a constant; b_1, b_2 and b_3 are regression coefficients for the linear effects and b_{12}, b_{13} and b_{23} are interaction coefficients.

To analyse the mathematical model, adjustments to the points were made by nonlinear regression methods. Table 2 shows the adjustments made between the observed (experimental) and predicted values (simulated) of the model to obtain the best response in relation the lower surface tension (<20 mN/m). At this rate, the experimental points varied from 11 to 55% and predicted points varied from 9.5 to 53.28% (Fig. 1).

The regression coefficient values, standard deviation, t_{exp} and significance level are presented in Table 3. It can be seen that b_2 and b_3, as well as the interaction coefficients b_{12}, b_{13} and b_{23}, are significant. Therefore, the linear effect of the variables (the coefficients b_2 and b_3) and the interaction of the agitation with aeration (coefficients b_{12}), agitation with time (coefficients b_{13}) and aeration with time (coefficients b_{23}) are the most influential parameters. The significance of these interaction effects between variables would have been lost if the experiments were conducted using conventional methods.

The application of RSM offers, on the basis of parameter estimation (Table 3), the following empirical relationship (Eq. (2)) between the surface tension (Y) and independent variables studied

$$Y = (47.730) + (-0.028)x_1 + (21.975)x_2 + (-0.376)x_3 + (-0.027)x_1x_2 + (0.001)x_1x_3 + (-0.149)x_2x_3\ , \quad (2)$$

Table 2
2^3 full-factorial with three replicates of the centre point for the surface tension efficiency.

Run	Agitation (rpm)	Aeration (vvm)	Time (h)	Surface tension (mN/m)	
				Experimental	Predicted
1	−1	−1	−1	36.09	34.58
2	+1	−1	−1	41.20	39.48
3	−1	+1	−1	55.00	53.28
4	+1	+1	−1	48.77	47.26
5	−1	−1	+1	21.20	19.48
6	+1	−1	+1	46.87	45.36
7	−1	+1	+1	11.01	9.50
8	+1	+1	+1	26.19	24.47
9	0	0	0	30.64	34.18
10	0	0	0	30.00	34.17
11	0	0	0	29.00	34.18

The results predicted by Eq. (3) indicate good agreement between the experimental and predicted values for the process efficiency. An analysis of variance (ANOVA) showed that the predictive model represented the experimental data at a level of approximately 95.39% because the significance level calculated from the ratio of mean squares obtained from the regression was 0.68. Additionally, the regression model had a high coefficient of determination ($R(2)=0.890$).

This implies that 89% of the variation in the process efficiency is explained by the independent variables and also that only approximately 11% of the variation was not explained by the model. The model in Eq. (3) was then optimised. The optimal values of the process parameters were obtained in coded units, converted to uncoded units by using Eq. (3) and then experimentally validated, as shown in Table 4.

Silva et al. (2013) optimised the production of a biosurfactant by *Pseudomonas cepacia* CCT6659 with aid of a combination of a Rotational Central Composite Design (RCCD) and Response Surface Methodology (RSM). The empirical forecast model developed through RSM regarding effective nutritional factors was adequate for explaining 89% of the variation observed in biosurfactant

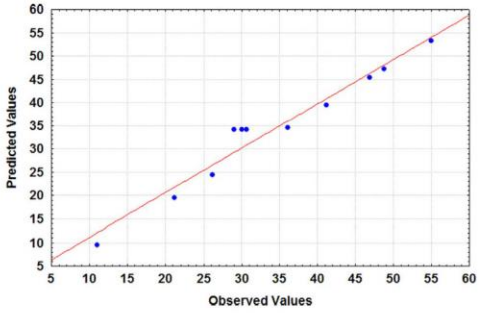

Fig. 1. Observed values *versus* predicted values by model for the answer surface tension.

Table 3
Estimated regression coefficients and corresponding t_{exp} as well as significance levels for the surface tension.

Coefficient	Value	Standard deviation	t_{exp}	Significance level (%)
b_0	47.730	2.333	20.460	<0.01
b_1	−0.028	0.007	−3.908	0.06
b_2	21.975	1.093	20.097	<0.01
b_3	−0.376	0.020	−18.617	<0.01
b_{12}	−0.027	0.003	−9.338	0.011
b_{13}	0.001	0.000	17.952	<0.01
b_{23}	−0.149	0.006	−24.531	<0.01

Table 4
Optimum values of the process parameters for the maximum process efficiency.

Parameter	Optimum value	
	Predicted	Experimental
Time (h)	144	150
Aeration (vvm)	0	0
Agitation (rpm)	200	200
Surface tension (mN/m)	19.48	18.53

production. Maximal reduction in surface tension of 26 mN m^{-1} was obtained under the optimal conditions.

Figs. 2–4 show the response surfaces and contour curves determined. In these experiments, the surface tension was assessed varying the time between 48 and 144 h, the aeration between 0 and 2 vvm, and the agitation between 200 and 400 rpm.

Fig. 2 shows that an agitation below than 200 rpm and an aeration equal to 0 vvm, a lower surface tension is obtained (< 20 mN/m). However, when the agitation is greater than 320 rpm and the aeration is below 1 vvm, the highest surface tension values are obtained (> 40 mN/m).

Fig. 3 shows that an agitation below than 200 rpm and a time of the process higher than 120 h, a lower surface tension is obtained (< 20 mN/m). However, when the agitation is greater than 380 rpm to a time of the process higher than 120 h, the highest surface tension values are obtained (> 50 mN/m).

Oliveira et al. (2006) observed, for *Pseudomonas aeruginosa*, the decrease of the surface tension to 35 mN/m at 150 rpm, while the velocities of 50,100 and 200 rpm increased the surface tension to 55.39 and 51 mN/m. Cunha et al. (2004) observed that agitation had a negative effect on surface tension reduction by the biosurfactant produced by *Serratia* sp. SVGG16, and that best results were obtained with the lowest value, of 100 rpm, when compared to 200 and 300 rpm.

Fig. 4 shows that an aeration greater than 1 vvm and a time of the process higher than 120 h, a lower surface tension values is obtained (< 10 mN/m). When the aeration is equal to 0 vvm and a time higher than 120 h, the surface tension is below (< 20 mN/m). However, an aeration greater than 1.4 vvm and a time than 40 h, the highest surface tension values are obtained (> 50 mN/m).

The reduction in surface tension is used as a primary criterion for selecting microorganisms producing biosurfactants, although emulsifiers and dispersants do not necessarily have ability to reduce surface tension (Youssef et al., 2004).

According to literature, the bacterial biosurfactants are more effective in reducing surface tension. In particular, the bacterium *P. aeruginosa* have been the most studied microorganism to produce potent biosurfactants. Most biosurfactant produced by this bacterium has demonstrated the ability to reduce surface tension to values around 28–27 mN/m (Santa Anna et al., 2001; Silva et al., 2010). Although biosurfactants produced by yeasts described in the literature in the past decades have demonstrated ability to reduce surface tension to values around 35 mN/m (Kitamoto et al., 2002), more recent research has revealed values consistent with bacterial biosurfactants, as the result obtained in this work for the biosurfactant from *C. lipolytica* UCP 0988. Rufino et al. (2008) noted that the biosurfactant produced by the yeast *C. lipolytica* grown on industrial waste as substrate reduced the

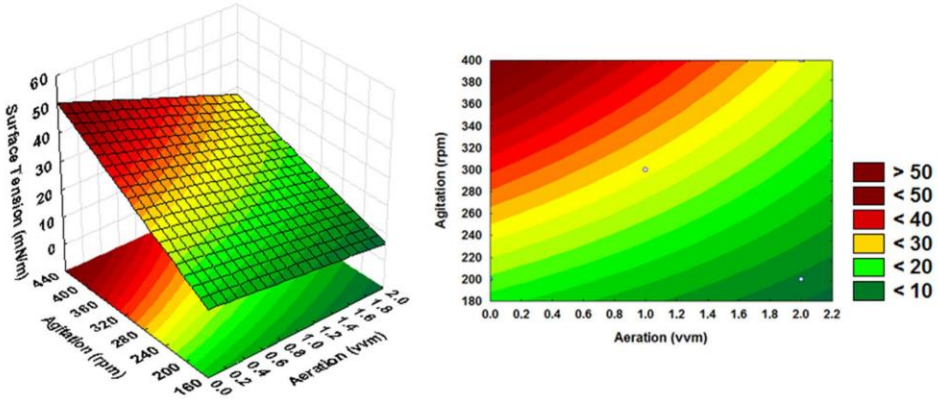

Fig. 2. Response surface and contour curves for the surface tension. (Time) = 144 h.

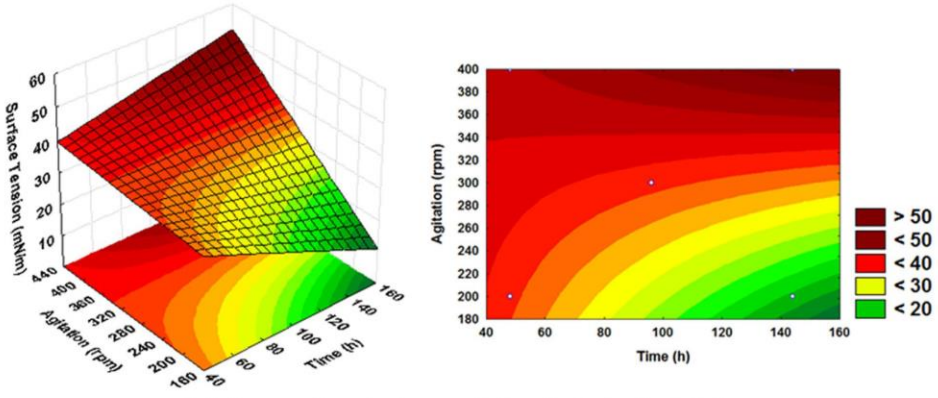

Fig. 3. Response surface and contour curves for the surface tension. (Aeration) = 0 vvm.

This implies that 93% of the variation in the process efficiency is explained by the independent variables and also that only approximately 7% of the variation was not explained by the model. The model in Eq. (5) was then optimised. The optimal values of the process parameters were obtained in coded units, converted to uncoded units by using Eq. (5) and then experimentally validated, as shown in Table 7.

Figs. 6–8 show the response surfaces and contour curves determined. In these experiments, the biomass was assessed varying the time between 48 and 144 h, the aeration between 0 and 2 vvm, and the agitation between 200 and 400 rpm.

Fig. 6 shows that an agitation until 270 rpm and an aeration equal to 0 vvm, a lower biomass values is obtained (<8 g/L). However, when the aeration is greater than 1 vvm and the agitation is below than 200 rpm, the highest biomass values are obtained (>9 g/L).

Fig. 7 shows that independent of the agitation (varying between 200 and 400 rpm) to a time of the process lower than 70 h, a lower biomass is obtained (<2 g/L). However, when the agitation is lower than 200 rpm to a time of the process higher than 120 h, the highest biomass values are obtained (<8 g/L).

Fig. 8 shows that an aeration equal to 0 vvm and a time of the process higher than 120 h, the biomass values is observed (<8 g/L), being efficient for the process. However, an aeration below than 1 vvm and a time lower than 70 h, the lower biomass values are obtained (<2 g/L).

The results obtained by Konishi et al. (2011) can explain the ones obtained in our work. Agitation speeds seemed to negligibly affect the cell growth of *Pseudozyma hubeinensis* SY62 cultivated in glucose. On the other hand, agitation speed markedly affected mannosylerythritol lipid (MEL) biosurfactant production. Agitation speed of less than 150 rpm resulted in MEL production one-half of those at higher speeds. Therefore, aeration with high agitation speeds seemed to stimulate the MEL production. However, excess of agitation, *i.e.*, 250 rpm, seemed to stimulate the growth on the wall of flasks, and resulted in a slight decrease in MEL production. The aggregation on wall would limit the material transfer between the medium and cells,

Table 7
Optimum values of the process parameters for the maximum process efficiency.

Parameter	Optimum value	
	Predicted	Experimental
Time (h)	144	150
Aeration (vvm)	0	0
Agitation (rpm)	200	200
Biomass (g/L)	7.50	7.90

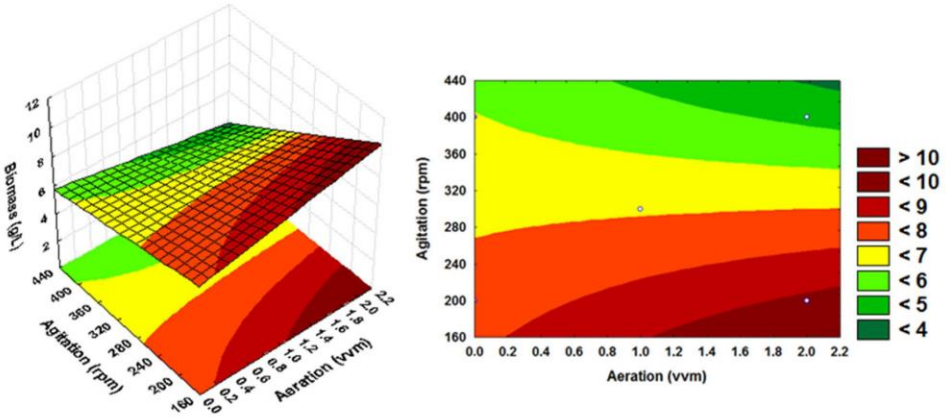

Fig. 6. Response surface and contour curves for the biomass. (Time)=144 h.

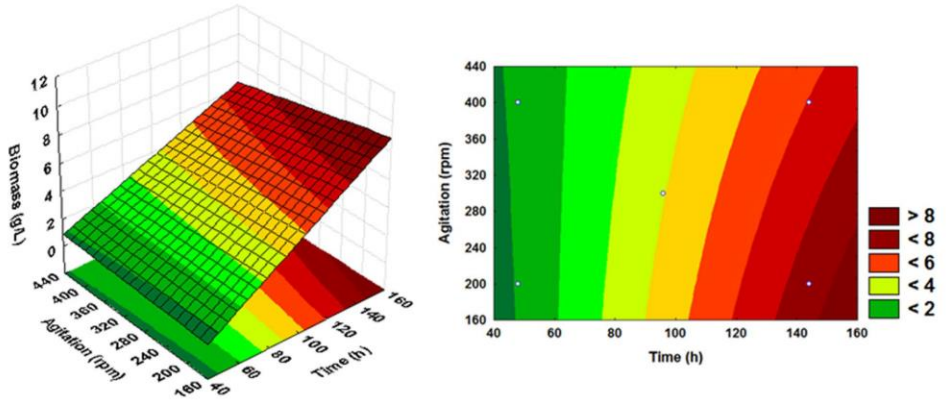

Fig. 7. Response surface and contour curves for the biomass. (Aeration)=0 vvm.

model. The model in Eq. (6) was then optimised. The optimal values of the process parameters were obtained in coded units, converted to uncoded units by using Eq. (7) and then experimentally validated, as shown in Table 10.

Figs. 10–12 show the response surfaces and contour curves determined. In these experiments, the yield was assessed varying the time between 48 and 144 h, the aeration between 0 and 2 vvm, and the agitation between 200 and 400 rpm.

Fig. 10 shows that an agitation below than 200 rpm and an aeration equal to 0 vvm, a highest yield is obtained (> 6.5 g/L). However, when the agitation is greater than 360 rpm, independent of the aeration (varying between 0 and 2 vvm), the lower yield values are obtained (< 2.5 g/L).

Fig. 11 shows that a time of the process higher than 120 h and an agitation below than 200 rpm, the highest yield is obtained (> 8 g/L). However, when the agitation is greater than 320 rpm and the time is below than 60 h, the lower yield are observed (< 2 g/L).

Fig. 12 shows that an aeration equal to 0 vvm and a time of the process higher than 120 h, the highest yield is observed (> 7 g/L). However, when the time is below than 80 h, the lower yield are obtained (< 4 g/L), independent of the aeration (varying between 0 and 2 vvm).

An analysis of variance ANOVA showed that the model predicted represented the experimental data at levels ranging from approximately 95.39%, 97.27% and 99.94% for surface tension, yield and biomass, respectively.

The importance of oxygenation for biosurfactant production has been well documented (Silva et al., 2010). In previous studies, the agitation speed of the medium is also a determining factor in the oxygen mass transfer into the cultures using agitated flasks (Oliveira et al., 2009; Cunha et al. (2004); Silva et al., 2010).

Syldatk and Wagner (1987) described a decrease in surfactant production by *Nocardia erythropolis* with the increase of agitation

Table 10
Optimum values of the process parameters for the maximum process efficiency.

Parameter	Optimum value	
	Predicted	Experimental
Time (h)	144	150
Aeration (vvm)	0	0
Agitation (rpm)	200	200
Yield (g/L)	7.08	7.27

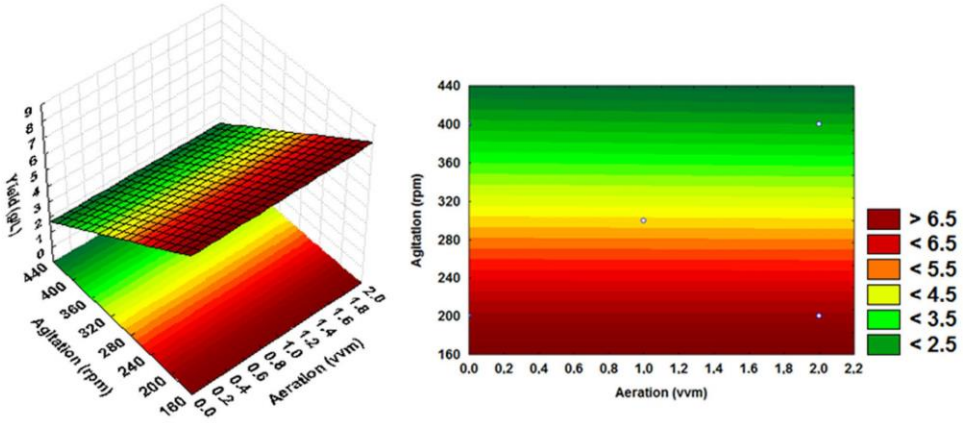

Fig. 10. Response surface and contour curves for the yield. (Time) = 144 h.

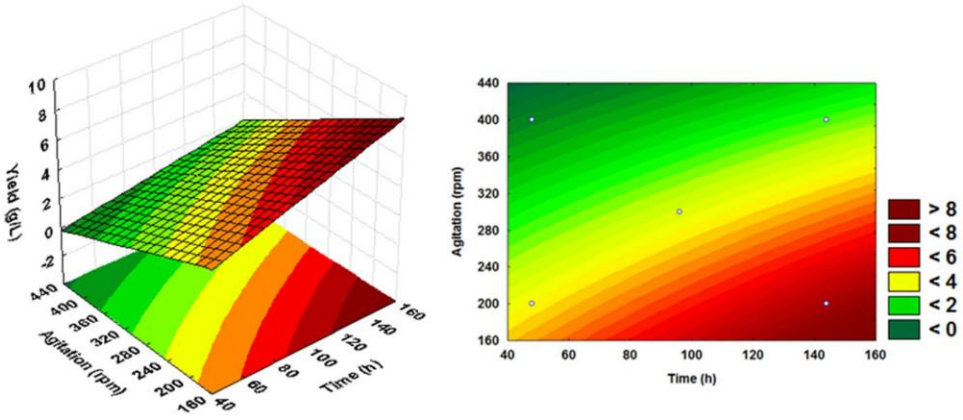

Fig. 11. Response surface and contour curves for the yield. (Aeration) = 0 vvm.

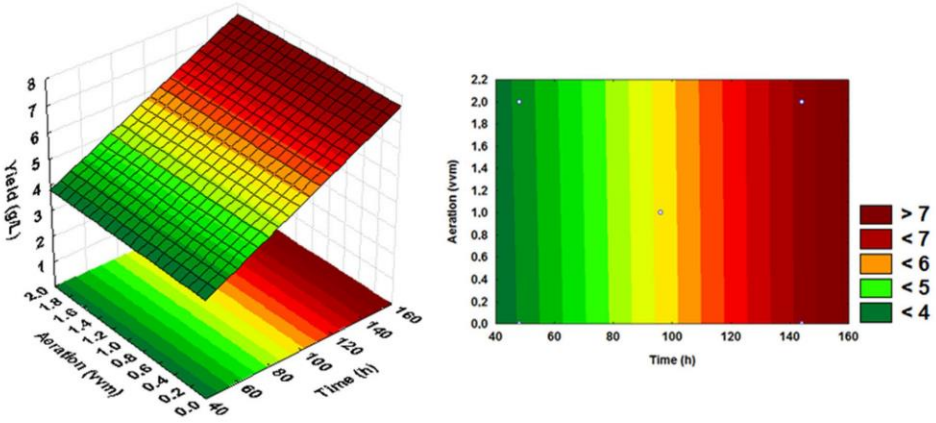

Fig. 12. Response surface and contour curves for the yield. (Agitation)=200 rpm.

speed from 250 to 500 rpm due to a shear rate effect on the growth kinetics of the microorganism. Results obtained from agitation speed tests indicated that this factor had no significant effect on the maximum biosurfactant produced by a microbial consorptium (Darvishi et al., 2011).

Chen et al. (2012) found that the yield of biosurfactant production from *Bacillus licheniformis* TKU004 is significantly correlated with oxygen content. Under anaerobic conditions, *B. licheniformis* TKU004 cannot produce biosurfactant.

Oliveira et al. (2006), on the other hand, describes that the increase in rotary velocity had a positive effect on the biosurfactant production from *Pseudomonas alcaligenes*. According to these authors, *P. alcaligenes* is an aerobic bacterium, and as such, higher rotary velocities may increase the oxygen mass transfer to the aqueous medium yielding the best conditions for microbial growth and biosurfactant production, once the biosurfactant is produced during the exponential growth phase. As previously presented in our results, the biosurfactant from *C. lipolytica* is mainly produced in the stationary growth phase. Thus, less oxygen must be required for surfactant biosynthesis, explaining the negative effect of this cultural parameter in the biosurfactant production.

4. Conclusions

Sensitivity analysis was performed on the matematical parameters to determine those that are most influential in the reduction of the surface tension and biomass as well as the increase in the yield of the production biosurfactant. As expected, the most influential parameters were optimised involving these studied conditions. Parameter estimation was performed to best fit the experimental data and the correlation coefficient obtained was 0.95 for surface tension, 0.97 for biomass and yield 0.99.

The present developments include the integrated optimization of the process, as well as the extension of the model to address the cultural conditions for biosurfactant production from *C. lipolytica*.

After analysis of the results obtained in complete factorial planning, the selected condition and optimised was the smallest value of agitation (200 rpm), the greater time of the process (144 h) and an aeration equal to 0 vvm, since under these conditions the production of biosurfactant showed that the variables established favoured the reduction of the surface tension and biomass, in turn enabled the increase in the yield of the production biosurfactant by obtaining a biopolymer promising for applications in bioremediation processes.

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References

Aleboyeh, A., Kasiri, M.B., Olya, M.E., Aleboyeh, H., 2008. Prediction of azo dye decolorization by UV/H_2O_2 using artificial neural networks. Dyes Pigm. 288, 277–294.

Barros Neto, B., Scarminio, I.S., Bruns, R.E., 2010. Como Fazer Experimentos, 4th ed. Bookman, Porto Alegre.

Banat, I.M., Franzetti, A., Gandolfi, I., Bestetti, G., Martinotti, M.G., Fracchia, L., Smyth, T.J., Marchant, R., 2010. Microbial biosurfactants production, applications. Appl. Microbiol. Biotechnol. 87, 427–444.

Calado, V., Montgomery, D., 2003. Planejamento de Experimentos Usando O Statistica, 1st ed. E-Papers Serviços Editoriais, Rio de Janeiro.

Chaves, A.T.C.A., 2008. Otimização do processo de biodiesel etílico do óleo de girassol (*Hellianthus annus*) aplicando um delineamento composto central rotacional (DCCR) (Master dissertation). João Pessoa-PB.

Chen, Y.-C., Chiang, T.J., Liang, T.W., Wang, I.L., Wang, S.L., 2012. Reclamation of squid pen by Bacillus licheniformis TKU004 for the production of thermally stable and antimicrobial biosurfactant. Biocatal. Agric. Biotechnol. 1, 62–69.

Cunha, C.D., et al., 2004. Serratia sp. SVGG16: a promising biosurfactant producer isolated from tropical soil during growth with ethanol-blended gasoline. Process Biochem. 39, 2277–2282.

Davila, A.M., Marchal, R., Vandecasteele, J.P., 1992. Kinetics and balance of a fermentation free from product inhibition: sophorose lipid production by *Candida bombicola*. Appl. Microbiol. Biotechnol. 38, 6–11.

Darvishi, P., Ayatollahi, S., Mowlaa, D., Niazi, A., 2011. Biosurfactant production under extreme environmental conditions by an efficient microbial consortium, ERCPPI-2. Coll. Surf. B: Biointerfaces 84, 292–300.

Felse, P.A., Shah, V., Chan, J., Rao, K.J., Gross, R.A., 2007. Sophorolipid biosynthesis by *Candida bombicola* from industrial fatty acid residues. Enzyme Microb. Technol. 40 (2), 316–323.

Ilori, M.O., Amobi, C.J., Odocha, A.C., 2005. Factors affecting biosurfactant production by oil degrading Aeromonas spp. Isolated from a tropical environment. Chemosphere 61, 985–992.

Kitamoto, D., Isoda, H., Nakahara, T., 2002. Functions and potential applications of glycolipid biosurfactants: from energy saving materials to gene delivery carriers. J. Biosci. Bioeng. 94, 187–201.

Konishi, M., Nagahama, T., Fukuoka, T., Morita, T., Imura, T., Kitamoto, D., Hatada, Y., 2011. Yeast extract stimulates production of glycolipid biosurfactants, mannosylerythritol lipids, by *Pseudozyma hubeiensis* SY62. J. Biosci. Bioeng. 111 (6), 702–705.

Luna, J.M., Rufino, R.D., Albuquerque, C.D.C., Sarubbo, L.A., Campos-Takaki, G.M., 2011. Economic optimised medium for tenso-active agent production by

Candida sphaerica UCP0995 and application in the removal of hydrophobic contaminant from sand. Int. J. Mol. Sci. 12, 2463–2476.
Luna, J.M., Sarubbo, L., de Campos-Takaki, G.M., 2009. A new biosurfactant produced by *Candida glabrata* UCP 1002: characteristics of stability and application in oil recovery. Braz. Arch. Biol. Technol. 52, 785–793.
Marchant, R., Banat, I., 2012. Microbial biosurfactants: challenges and opportunities for future exploitation. Trends Biotechnol. 30, 558–565.
Myers, R.H., Montgomery, D.C., 2002. Response surface methodology: process and product optimization using designed experiments, 2nd ed. John Wiley and Sons, New York.
Mukherjee, S., Das, P., Sen, R., 2006. Towards commercial production of microbial surfactants. Trends Biotechnol. 24, 509–515.
Oliveira, F.J.S., Vazquez, L., Campos, N.P.,X, França, F.P., 2009. Production of rhamnolipids by a *Pseudomonas alcaligenes* strain. Process Biochem. 44, 383–389.
Oliveira, F.J.S., Vazquez, L., Campos, N.P., França, F.P., 2006. Biosurfactants production by *Pseudomonas aeruginosa* FR using palm oil. Appl. Biochem. Biotechnol. 131, 727–737.
Pacwa-Plociniczak, M., Plaza, G.A., Piotrowska-Seget, Z., Cameotra, S.S., 2011. Environmental applications of biosurfactants: recent advances. Int. J. Mol. Sci. 13, 633–654.
Rivera, O.M.P., Moldes, A.B., Torrado, A.M., Domínguez, J.M., 2007. Lactic acid and biosurfactants production from hydrolyzed distilled grape marc. Process Biochem. 42, 1010–1020.
Rodrigues, M.I., Lemma, A.F., 2009. Planejamento de Experimentos and Otimização de Processos, 2nd ed. Casa do Espírito Amigo Fraternidade Fé e Amor, Campinas, SP.
Rodrigues, L., Teixeira, J., Oliveira, R., Mei, H.C.V., 2006. Response surface optimization of the medium components for the production of biosurfactants by probiotic bacteria. Process Biochem. 41, 1–10.
Rufino, R.D., Sarubbo, L.A., Campos-Takaki, G.M., 2007. Enhancement of stability of biosurfactant produced by *Candida lipolytica* using industrial residue as substrate. World J. Microbiol. Biotechnol. 23, 729–734.
Rufino, R.D., Sarubbo, L.A., Benicio, B.N., Campos-Takaki, G.M., 2008. Experimental design for the production of tensio-active agent by *Candida lipolytica*. J. Ind. Microbiol. Biotechnol. 35, 907–914.
Sarubbo, L.A., Luna, J.M., Campos-Takaki, G.M., 2006. Production and stability studies of the bioemulsier obtained from a new strain of *Candida glabrata* UCP1002. Electron. J. Biotechnol. 9, 400–406.
Santa Anna, I.M., Sebastian, G.V., Pereira , N., Alves, T.L.M., Menezes, E.P., Freire, D.M.G., 2001. Production of biosurfactant from a new and promissing strain of *Pseudomonas aeruginosa* P. A. 1. Appl. Biochem. Biotechnol. 91–93, 459–467.
Sen, R., 1997. Response surface optimization of the critical media components for the production of surfactin. J. Chem. Technol. Biotechnol. 68, 263–270.
Silva, R.C.F.S., Rufino, R.D., Farias, C.B.B., Filho, H.J.B., Santos, V.A., Sarubbo, L.A., 2013. Enchancement of biosurfactant production from *Pseudomonas cepacia* CCT6659 through optimisation of nutricional parameters using response surface methodology. Tenside Surfactans Deterg. 50, 137–142.
Silva, S.N.R.L., Farias, C.B.B., Rufino, R.D., Luna, J.M., Sarubbo, L.A., 2010. Glycerol as subtrate for the production of biosurfactant by *Pseudomonas aeruginosas* UCP 0992. Coll. Surf. B: Biointerfaces 79, 174–183.
Syldatk, C., Wagner, F., 1987. Production of biosurfactants. In: Kosaric, N., Cairns, W.L., Gray, N.C.C. (Eds.), Biosurfactants and Biotechnology. Marcel Decker, New York, pp. 89–120.
Whei, Y.H., Lai, C.C., Chang, J.S., 2007. Using taguchi experimental designmethods to optimise trace element composition for enhanced surfactin production by *Bacillus subtilis* ATCC 21332. Process Biochem. 42, 40–45.
Youssef, N.H., Duncan, K.E., Nagle, D.P., Savage, K.N., Hnapp, R.M., Mcinerney, M.J., 2004. Comparison of methods to detect biosurfactants production by diverse microorganisms. J. Microbiol. Methods 56, 339–347.

CAPÍTULO 3

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Biosurfactant production from *Candida lipolytica* in bioreactor and evaluation of its toxicity for application as a bioremediation agent

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ABSTRACT

This large-scale production, toxicity, characterization and economic analysis of the biosurfactant from *Candida lipolytica* UCP 0988 produced in the low-medium formulated with animal fat and corn steep liquor was investigated. The biosurfactant was produced in the stationary phase under 200 rpm in the absence of aeration and reduced the surface tension of the medium from 50 to 28 mN/m after 96 h, yielding 10.0 g/L of isolated biosurfactant in a 2 L bioreactor. The production was maximized in a 50 L bioreactor, reaching 40 g/L biosurfactant and 25 mN/m. The cell biomass was quantified and characterized for use in animal nutrition. Chemical structures of the biosurfactant were identified using FTIR and NMR. The crude biosurfactant was not toxic to the bivalve *Anomalocardia brasiliana*, to the microcrustacean *Artemia salina*, or three species of vegetables seeds. The biosurfactant stimulated the degradation of motor oil by the seawater indigenous microorganisms. The results obtained indicate that the biosurfactant produced has great potential to be applied as a bioremediation agent for cleaning oil spills.

1. Introduction

Biosurfactants have been successfully applied in recent years as remediation agents in soil and aquatic environments [1,2]. Regarding their ability to solubilize hydrocarbons by partitioning them into the surfactant micelles above the CMC, biosurfactants are used in different remediation processes originated form the oil industry [3]. One of the main obstacles to the expansion of the surfactant market for in situ remediation is the lack of knowledge about its effects on the environment and the toxicity of these substances [4]. The presence of synthetic surfactants in the aquatic environment in the last 30 years has resulted in great toxicity. As a consequence, an extensive database of laboratory toxicity tests of various commercial surfactants has been constructed over the years. On the other hand, the toxicity of biosurfactants in the environment has been little studied. Edwards et al. [5] in a comparison of the toxicity of three synthetic surfactants and three biosurfactants, concluded that biosurfactants were less toxic than synthetic surfactants against some invertebrate species [6].

Many *Candida* biosurfactants with potential to be used industrially have been produced in our laboratories [7–11]. The biosurfactant from the yeast *Candida lipolytica* UCP0988 described here was previously produced in shake flasks and properties of the produced biosurfactant based on its emulsification activity and its stability with different oils and effect of environmental factors on the emulsification activity and stability were reported [12]. The optimization of cultural conditions for the biosurfactant production using surface response methodology (SRM) was also described [13].

In this work, the production of the biosurfactant from *Candida lipolytica* UCP0988 cultivated in a low-cost fermentative medium based on animal fat and corn steep liquor was evaluated in flasks scale and bioreactors. The characterization and the toxicity of the biomolecule against vegetables and marine indicators and its application as a bioremediation agent are reported. Finally, an economic analysis of the biosurfactant produced in bioreactor was described.

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2. Materials and methods

2.1. Materials

All chemicals were of reagent grade. Growth media were purchased from Difco Laboratories, USA. The animal fat used was white choice grease from a bovine processing plant located in the city of Recife (Brazil) and was used without any further processing. The animal fat is composed by the following fatty acids: stearic (43.41%), palmitic (26.40%), oleic (24.16%) and miristic (6.03%) [12].

Corn steep liquor was obtained from Corn Products do Brasil in the city of Cabo de Santo Agostinho (Brazil). According to Akhtar et al. [14] and Cardinal and Hedrick [15] corn steep liquor is 21–45% protein, 20–26% lactic acid, 8% ash (containing Ca^{2+}, Mg^{2+}, K^{+}), 3% sugar and has low fat content (0.9–1.2%).

2.2. Microorganism

Candida lipolytica UCP0988 was obtained from the culture collection of the *Universidade Católica de Pernambuco* (Brazil). The microorganism was maintained in the asexual phase at 5 °C on yeast mould agar slants containing (w/v) yeast extract (0.3%), malt extract (0.3%), tryptone (0.5%), D-glucose (1.0%) and agar (5.0%). Transfers were made to fresh agar slants each month to maintain viability.

2.3. Growth conditions

The inoculum of *Candida lipolytica* was prepared by transferring cells grown on a slant to 50 mL of yeast mould broth. The seed culture was incubated for 24 h at 28 °C and agitated at 150 rpm. The inoculum (1% v/v) was introduced in the amount of 10^4 cells/mL to the cool production medium. The production medium contained 5% animal fat and 2.5% corn steep liquor. The medium was sterilised by autoclaving at 121 °C for 20 min (all components were sterilised together). The final pH of the medium was 5.3 and its surface tension was 50 mN/m.

2.4. Biosurfactant production

Biosurfactant production was first performed in a 2 L bioreactor (Tec-Bio-Plus, Tecnal Ltda., Brazil) with a working volume of 1.2 L operated in a batch mode, with controlled pH (5.3) and temperature (28 °C). The reactor was aerated by a sparger. The culture medium was inoculated with a 24 h inoculum at 200 rpm in the absence of aeration during 144 h. For larger-scale production, i.e., production maximization, a 50 L bioreactor (MA502, Marconi Ltda., Brazil) was used. All assays were carried out in triplicate and did not vary more than 5%. The Growth and biosurfactant production were monitored along fermentation. At regular intervals, different process parameters such as growth, pH, surface tension and biosurfactant concentration were evaluated.

2.5. Biomass estimation

Biomass was monitored by estimating the cell dry weight after removal of the hydrophobic substrates at 10000 g for 15 min [16]. Briefly, a 10 mL aliquot of the fermentation broth was centrifuged to obtain a cell pellet. The supernatant was discarded and the cell pellet was washed twice with distilled water to remove the medium components. The pellet was then washed with 10 mL of ethyl acetate to remove hydrophobic substances. The washed cells were centrifuged to obtain a pellet, which was dried in vacuum to a constant weight (indicated by a variation of less than ±0.2 g). Suitable calculations were made to express cell mass in terms of g/L.

2.6. Biomass composition

To determine protein, carbohydrate and lipid, cells obtained by centrifuging one volume of whole broth were resuspended in 1 vol each of saline and ethyl acetate, centrifuged again, resuspended in saline, then analysed. Protein concentration was estimated using a total protein test kit (Labtest Diagnostica S.A., Brazil). Total carbohydrate content was estimated using the phenol-sulphuric acid method [17]. Lipid content was determined based on the method described by Manocha et al. [18].

2.7. Surface tension

The surface tension of the culture supernatants obtained by centrifuging the cultures at 5000 g for 20 min was measured using a Sigma 700 digital surface tensiometer (KSV Instruments LTD – Finland) working on the principle of the Du Nuoy ring method. Ten millilitres of each sample were transferred to a clean 20 mL beaker and placed on the tensiometer platform. A platinum wire ring was submerged in the solution and then slowly pulled through the liquid-air interface to measure surface tension (mN/m). Between each measurement, the platinum wire ring was rinsed with chromic acid, deionised water and acetone and was then flamed and allowed to dry. Calibration was performed using Mill-Q-4 ultrapure distilled water (surface tension = 71.5 mN/m ± 0.5) before taking measurements of the samples.

2.8. Biosurfactant isolation

The biosurfactant was recovered from the cell-free broth by cold acetone precipitation, as described by Ilori et al. [19]. Three volumes of chilled acetone were added and allowed to stand for 10 h at 4 °C. The precipitate was collected by centrifugation and evaporated to dryness to remove residual acetone. The precipitate was then redissolved in sterile water. The yield in isolated biosurfactant was expressed in g/L.

2.9. Fourier transform infrared spectroscopy

The solid biosurfactant extract recovered from the supernatants of *C. lipolytica* was subjected to Fourier Transform Infra Red spectrometry (FT-IR) to elucidate the chemical nature by identifying the types of functional groups and chemical bonds. One-milligram biosurfactant was ground with 100 mg KBr pellet and pressed with 7500 kg for 30 s to obtain translucent pellet. The FT-IR spectrum was recorded in 4000–400 cm^{-1} region on a GX-FTIR system (Perkin Elmer, USA) with a spectral resolution and wave number accuracy of 4 and 0.01 cm^{-1}, respectively. All the measurements consisted of 500 scans and a KBr pellet was used as background reference.

2.10. Nuclear magnetic resonance spectroscopy

The extracted biosurfactant was re-dissolved in deuterated chloroform ($CDCl_3$) and the respective ^1H and 13C NMR spectra were recorded at 25 °C using an Agilent 300Mz spectrometer operating at 300.13 MHz. Chemical shifts (δ) are given on the ppm scale relative to tetramethylsilane (TMS).

2.11. Phytotoxicity assay

The phytotoxicity of the biosurfactant was evaluated in a static test based on seed germination and root elongation of the vegetables *Brassica oleracea var. botrytis* L., *Lactuca sativa* L. and *Brassica oleracea* var. capitata L. following the methods described by Tiquia et al. [20]. Solutions of the isolated biosurfactant were prepared with distilled water at concentrations of ½ the CMC (0.04%), the

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CMC (0.08%) and 2× the CMC (1.6%). Toxicity was determined in sterilised Petri dishes (1 × 10 cm) containing Whatman No. 1 filter paper. The seeds were pre-treated with sodium hypochlorite. Ten seeds were inoculated in each Petri dish with 5 mL of the test solution at 27 °C. After five days of incubation in the dark, seed germination, root elongation (≥5 mm) and the germination index (a factor of relative seed germination and relative root elongation) were determined as follows:

$$\text{Relative seed germination (\%)} = (\text{number of seeds germinated in the extract/number of seeds germinated in the control}) \times 100$$

$$\text{Relative root length(\%)} = (\text{mean root length in the extract/mean root length in the control}) \times 100$$

$$\text{Germination index} = [(\text{\% of seed germination}) \times (\text{\% of root growth})]/100\%$$

2.12. Artemia assay

The toxicity assay was performed using brine shrimp (*Artemia salina*) as the toxicity indicator. Brine shrimp eggs were obtained from a local store. Larvae were used within one day of hatching. Following dilutions of a biosurfactant solution at the CMC (0.08%) with saline water (33 g/L) to give concentrations of 0.02, 0.04, 0.06 and 0.08%, the assays were conducted in 10 mL penicillin tubes containing 10 brine shrimp larvae in 5 mL of saline water per tube. The brine shrimp larvae in each tube were tested using 5 mL of each concentration of the biosurfactant solution. The samples were observed for 24 h for the calculation of the mortality rate [21]. The toxicity threshold concentration, expressed as biosurfactant concentration per 100 mL of saline water, was defined as the lowest concentration that killed all brine shrimp within 24 h. Each test was run in triplicate and saline water was used as the control.

2.13. Toxicity against Anomalocardia brasiliana

Bioassays were performed using the native bivalve mollusc *Anomalocardia brasiliana* according to the method mentioned in the Standard Methods for the Examination of Water and Wasterwater [22]. The species is widely distributed along the Brazilian coast and inhabits areas protected from the action of waves and currents [23,24].

Assays were performed at room Quarantine Laboratory of Sustainable Mariculture of the Fisheries and Aquaculture Department from the Federal Rural University of Pernambuco (UFRPE), Pernambuco, Brazil. The animals used in the tests were collected at Mangue Seco beach, in the city of Igarassu, Pernambuco, transported to the laboratory and kept in Boxes glass fiber with a volume of 400 L in seawater with salinity of 26 and an average temperature of 26 ± 0.5 °C under constant aeration and fed with a mixture of algal *Chaetoceros gracilis* and *Navicula*, at a concentration of 10^4 cells/ml for 10 days prior to the experiment. Before performing each test, the specimens were transferred to a 100 L container, acclimated and kept without food for 24 h. For the tests of acute toxicity during 96 h, 2 L glass beakers were filled with 1.0–2.0 L of test solution, with variable dilutions employing water with salinity of 26, maintained with constant aeration, in a room with an average temperature of 26 ± 0.5 °C, and natural photoperiod of approximately 12/12 h. During the duration of the experiments, the animals were not fed. Five dilutions of the crude biosurfactant (cell-free broth) were tested: surfactant:brine (1:10, 1:20, 1:40, 1:160 and 1:80, v/v) with three replicates each. Controls contained just seawater. The result of the experiment was based on determination of lethal concentration for 50% of subjects employed (LC50), expressed in terms of mortality mean of three replicates for each dilution tested with the biosurfactant.

2.14. Bioremediation test

Bioremediation tests were performed according to the method mentioned in the Standard Methods for the Examination of Water and Wasterwater [22]. In brief, 250-ml Erlenmeyer flasks were filled with 100 mL fresh seawater obtained from the Suape Petrochemical Complex, Pernambuco State, Brazil, 1% of motor oil, and biosurfactant solutions at concentrations of ½ the CMC (0.04%), the CMC (0.08%) and 2× the CMC (1.6%). Control flasks contained seawater and motor oil. The flasks were incubated at 28 °C on an orbital shaker rotating at 150 rpm. Triplicate shake flasks were sacrificed on days 1, 5, 7, 21 and 30 of incubation and then analysed for the number of microorganisms by using the most probable number (MPN).

2.15. Statistical analysis

The determination of all surface tensions, biosurfactant and biomass concentrations was performed at least three times. Mean and standard error values were calculated using the Microsoft Office Excel 2003 (Version 7).

3. Results and discussion

3.1. Biosurfactant production

Industrial wastes have attracted great interest from researchers as alternative for the provision of low cost substrates for the production of biosurfactants [25]. Distillery waste [26], whey [27], molasses [28] and wastewater of olive oil [29], among others, have been described as substrates for the production of biosurfactants. Thus, based on the promising results previously obtained for the cultivation of the yeast *C. lipolytica* in media containing animal fat [12,13], we have studied the production of the biosurfactant obtained in a low-cost medium composed of 5% animal fat and 2.5% corn steep liquor in bioreactors in order to maximize its production.

Growth and biosurfactant production by *C. lipolytica* on medium containing 5% animal fat and 2.5% corn steep liquor substrate were followed for 6 days in a 2 L bioreactor. The time course of this process is shown in Fig. 1. The maximum biosurfactant production (10.0 g/L) was reached in the stationary growth phase at 96 h. This observation suggests that biosurfactant production by *C. lipolytica* is a secondary microbial metabolic process. The initial pH after inoculation was adjusted to 5.5. Thereafter it did not change along cultivation, showing a stable profile along 144 h. Growth started without a lag phase and cells reached a maximum concentration (7.8 g/L) at 144 h. Other biosurfactants from yeasts are produced in the stationary growth phase, as described by Amézcua-Vega et al. [30], Desphande and Daniels [31] and Luna et al. [10] for cultivations of *C. igens*, *C. bombicola* and *C. sphaerica*, respectively.

Biosurfactant production was markedly higher than previously reported [12], i.e., 2.0 g/L in shake flasks after 144 h against 10.0 g/L in a 2 L bioreactor after 96 h. Benincasa et al. [32] improved the production of rhamnolipids from soapstock as the sole carbon source to 15.9 g/L using a 2 L bioreactor. The production of another rhamnolipid from waste frying oil reached over 20 g/L in a 50 L bioreactor against 12.47 g/L in shake flasks [33]. Marti et al. [34] produced two biosurfactants from genetically-modified strains of *Bacillus subtilis*

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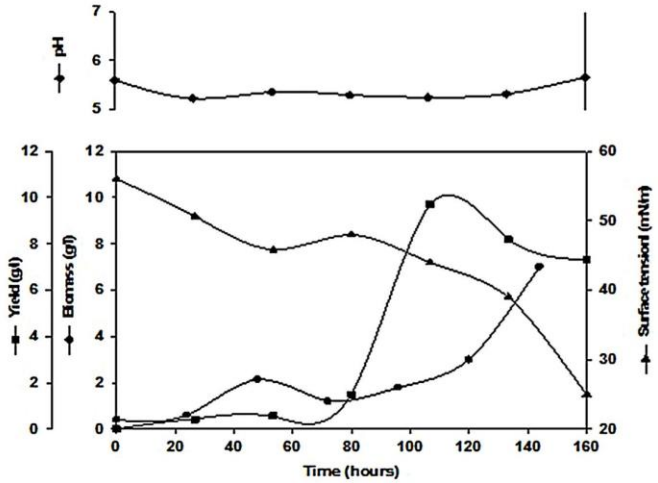

Fig. 1. Growth, pH, surface tension and biosurfactant yield profiles from *C. lipolytica* cultivated in distilled water supplemented with 5.0% animal fat and 2.5% corn steep liquor during 144 h at 200 rpm and 28 °C.

and observed that biosurfactant yields were higher in shake flasks than in bioreactors.

The production profiles for the biosurfactant from *C. lipolytica* were very similar in both 2 and 50 L bioreactors, although the biosurfactant yield and surface tension had reached 40 g/L and 25 mN/m in the 50 L bioreactor. Marti et al. [34] compared the production of biosurfactants in 5 and 15 L bioreactors. They observed that the final concentration of biosurfactants were lower in residual broth of larger fermentation vessels, as most of the surfactant was removed with the foam and concentrated in this fraction. Since there was no foam resulting from biosurfactant production in the conditions of this work, the production was higher in the larger vessel.

3.2. Chemical analysis of cells

In fermentation processes, the biomass obtained is generally sold as a component of animal nutrition. In this sense, the nutritional characteristics of the cells from *C. lipolytica* were analysed after production of the biosurfactant from animal fat and corn steep liquor. The content of proteins, carbohydrates and lipids represented 25, 15 and 20% dry weight, respectively. This content is higher in lipids when compared to the yeasts that grow in the presence of carbohydrates (10.5%) but not as high as to oleaginous yeasts, for which are reported values around 32–72% [31]. Considering FAO (Food and Agriculture Organization of the United Nations) guidelines for high-content protein, the results obtained suggest the possibility of applying the biomass of *C. lipolytica* for animal nutrition.

3.3. Biosurfactant characterization

The biosurfactant isolated from *C. lipolytica* was characterized by FT-IR and NMR analyses. The IR spectrum (Fig. 2) showed a stretch region between the ranges of 3000–35000 cm^{-1}, indicating the presence of hydroxyl groups in a carboxylic acid junction. The carbonyl group (C=O) was detected in 1711.29 cm^{-1} and aliphatic carbon bonds in 2855–3010 cm^{-1} regions.

The H^1NMR (Fig. 3) suggested the presence of hydrogen closed to the carboxylic acid groups in 10–11 ppm, close to double bonds in 5–6 ppm and aliphatic carbons in 1–3 ppm range. The C^{13} NMR (Fig. 4) confirms previous results showing a characteristic pick of carboxylic acid in 180 ppm, double bonds picks between 120 and 140 ppm and aliphatic carbons in 10–40 ppm region. This result suggests that the biomolecule of surfactant is a kind of metabolized carboxylic acid probably linked to carbohydrates, as described for other biosurfactants produced by yeasts, which are glycolipids.

Our results are similar to the ones obtained for the characterization of biosurfactants isolated from yeast species cultivated in diesel oil [35]. The broad band observed for the biosurfactants from *C. rugosa* and *Rhodotorula muciliginosa* was at 3410–3434 cm^{-1} corresponding to the O—H stretch. The asymmetrical stretching (V_{as} CH_2) of methylene occurred at 2920–2926 cm^{-1} and the band 1620–1627 cm^{-1} was from stretching of unsaturated C=C bonds.

3.4. Comparison of biosurfactant production by Candida species in bioreactors

The limiting factor for the commercialization of fermented products, including biosurfactants, is the economics of large-scale production, although the economy of biosurfactant production has not been detailed in the literature [36]. For the commercial success of biosurfactants, they must compete with synthetic surfactants, which are typically sold at lower prices to $ 2/kg [2]. Several approaches can be established to achieve this goal. The economic strategies extensively described include the selection of low cost substrates, increased product yields and reducing total processing time, which includes the preparation of materials, fermentation, extraction and purification. All this translates into material costs, capital and labour reduced and hence reduced production costs.

Many researchers have studied the preparation of culture media for production of biosurfactants [31,37,38]. Several researches describe media containing glucose, yeast extract, urea and a hydrophobic compound such as a vegetable oil or a fatty acid or a long chain alkane [7,39]. The components used in this work are industrially attractive: corn steep liquor, which has a greatly

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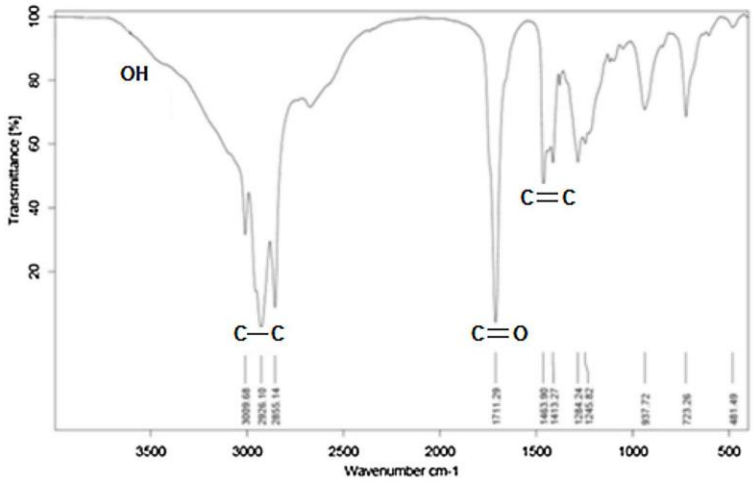

Fig. 2. Transmission FT-IR spectrum of solid surfactant obtained after purification and sample preparation in KBr tablets from 400 to 4000 cm^{-1}.

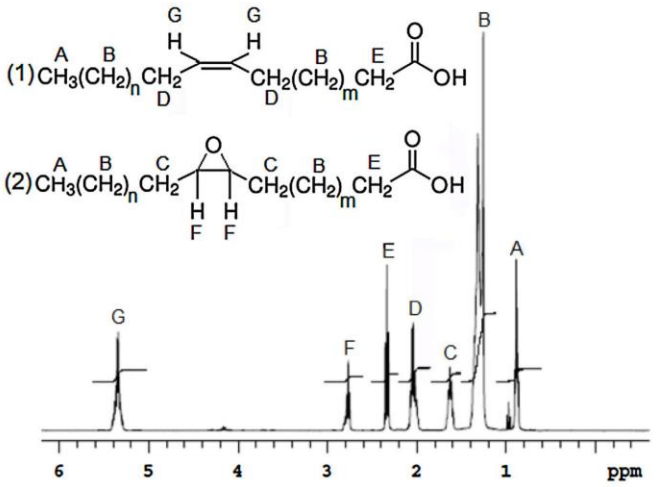

Fig. 3. ^{1}H NMR spectrum for biosurfactant extract recorded in $CDCl_3$.

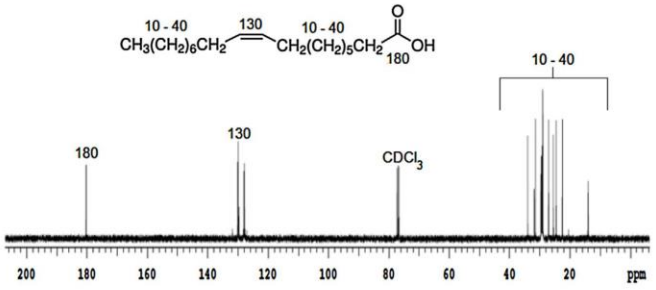

Fig. 4. ^{13}C NMR spectrum for biosurfactant extract recorded in $CDCl_3$.

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Table 1
Comparison of biosurfactant production from *Candida* species in bioreactors described in the literature.

Media components (%)	Time (h) to reach max. level	Production rate (g/L/h)	Max. yield (g/L)	Surface tension (mN/m)	Reference
5% animal fat, 2.5% corn steep liquor (2 L bioreactor)	96	0.1	10	28	This work
5% animal fat, 2.5% corn steep liquor (50 L bioreactor)	120	0.3	40	25	This work
10% glucose, 3.6% oleic acid, 1% yeast extract (30 L bioreactor)	120	0.5	34	ND	[40]
13.6% oleic acid, 0.1% yeast extract (30 L bioreactor)	160	1.0	74	ND	[40]
10.0% glucose, 9.5% sunflower oil, 0.5% yeast extract (7 L bioreactor)	130	1.0	28	37	[37]
10% glucose, 10.5% canola oil, 0.4% yeast extract (1 L bioreactor)	192	1.1	160	34	[41]
10.0% glucose, 10.0% animal fat, 0.4% corn steep liquor (15 L bioreactor)	60	1.7	97	ND	[31]
10.0% glucose, 4% tallow fatty acid residue, 0.1% yeast extract, 0.01% urea (fed batch and flasks)	240	0.5	120	ND	[42]
3–4% glucose, rapeseed oil (fed batch and 2.5 L bioreactor)	192	1.9	365	ND	[33]

ND: Not described.

reduced cost compared to yeast extract, and animal fat compared to pure fatty acids or vegetable oils.

In this sense, the influence of culture conditions including agitation, aeration and time of cultivation were evaluated in the production of biosurfactant from a culture medium with composition previously established [12]. The temperature was maintained at 28 °C, which is favourable industrially since the costs associated with cooling are larger, and hinder the mixing of fat in half. The maintenance of pH during the cultivation also helps reduce the cost of maintenance thereof, as well as a lower cell lysis. The absence of aeration contributes decisively to a further reduction in process costs.

Table 1 describes the data obtained over the years to biosurfactants produced by *Candida* species in bioreactors. Comparing our results with those detailed in this Table, it is observed that the maximum yield and rate of production obtained in this study are inferior, while the cultivation time is within the ranges stated. Moreover, the surface tension is favourable when compared to the literature, indicating more efficient production of a surfactant when compared to the others. Importantly, also, is that the ability of bioreactors and agitation speeds differ greatly from those used in this work, and that the media described include glucose, yeast extract, vegetable oils and fatty acids, which inevitably will favour the results, although an extremely cost up compared to the medium formulated with only two residues and distilled water described in our work. The inclusion of molasses as a low-cost substrate would be interesting to increase yields.

Biosurfactant production is still not considered competitive against detergents like sodium lauryl sulphates and alkyl benzene sulfonates, but it is likely that microbial surfactants will compete, in a near future, with the synthetic surfactants used in many industries [2]. Although the investments necessary to research, development, and capital expenditures are high, there is the possibility of creating an industry based on biosurfactants produced from animal fat and corn steep liquor.

3.5. Toxicity of the biosurfactant

Toxicity can be defined as the capacity of a substance to cause a harmful effect to a living organism. It depends on the concentration and properties of the chemical to which the organism is exposed and on the exposure time [43].

The toxicity of the biosurfactant from *C. lipolytica* on three vegetable species is shown in Table 2. The results indicated that the seeds germination occurred even in the presence of high concentrations of the isolated biosurfactant. The crude biosurfactant also did not show inhibitory effects to the vegetable species (data not shown). This result shows the possibility of using the biosurfactant in soil remediation. The results obtained also indicated that increasing the concentration of the surfactant reduced the percentage of seed germination.

According to the protocols described by the APHA [22] for testing oil spill dispersants, acute toxicity bioassay studies were conducted using brine shrimp and a bivalve.

The results obtained for the brine shrimp larvae *Artemia salina* and the bivalve *Anomalocardia brasiliana* subjected to different dilutions of the crude biosurfactant for 24 and 96 h, respectively, showed 100% survival, demonstrating the potential of the crude biosurfactant for use in aquatic environments. No mortality of the *Artemia salina* was observed when the brine shrimp was treated with the isolated biosurfactant solutions from 0.02 to 0.06%, while the biosurfactant solution at 0.08% killed 100% of the larvae.

Acute toxicity bioassay studies were conducted by Saeki et al. [44] with the surfactant JE1058BS produced by the bacterium *Gordonia* sp. Two marine larval species (*Mysidopsis bahia* and *Menidia beryllina*) were tested. The shrimp larvae were exposed to the samples for 48 h, while the fish larvae were exposed for 96 h. The results showed that JE1058BS had a considerably low toxicity. Franzetti et al. [45] also conducted ecotoxicity tests by the bioemulsifiers from *V. paradoxus* 7bCT5. Acute toxicity test on crustaceans showed a 100% survival of the organisms. The Germination Indexes calculated from seed germination and root elongation tests did not significantly differ from their controls, showing that the

Table 2
Phytotoxicity of the isolated biosurfactant from *C. lipolytica* UCP0988 cultivated in medium formulated with 5% animal fat and 2.5% corn steep liquor on three vegetable species seeds.

Vegetable Seeds	Germination index (GI) (%)		
	Isolated biosurfactant at 0.04%	Isolated biosurfactant at 0.08%	Isolated biosurfactant at 1.6%
Brassica oleracea var. botrytis L.	60 ± 0.25	55 ± 0.13	40 ± 0.11
Brassica oleracea var.capitata	75 ± 0.19	56 ± 0.29	41 ± 0.09
Lactuca sativa L.	75 ± 0.30	60 ± 0.15	40 ± 0.21

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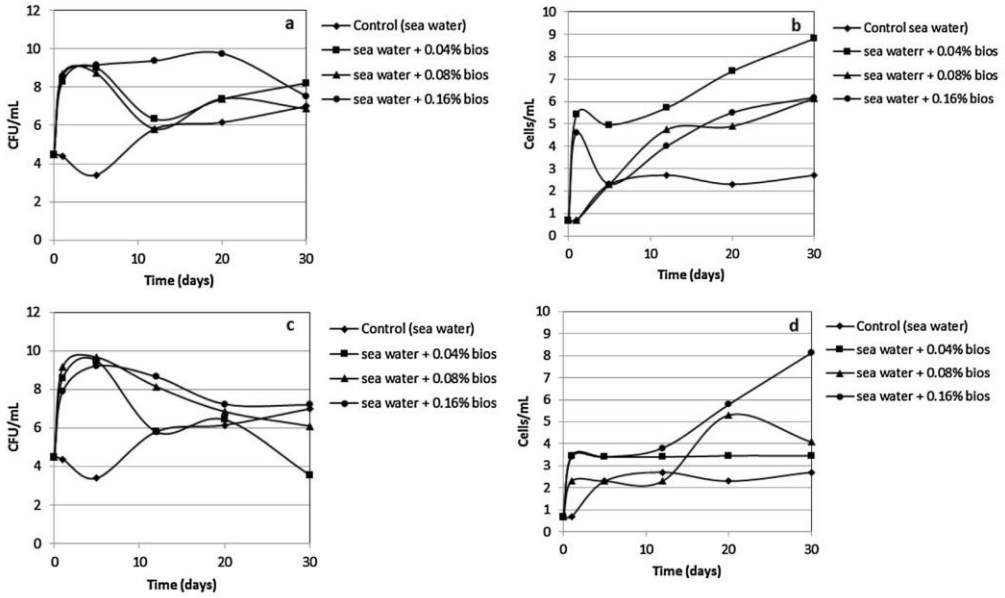

Fig. 5. Influence of the isolated biosurfactant (0.04, 0.08 and 0.16%) from *C. lipolytica* UCP0988 on the growth of indigenous microorganisms. (a) bacteria in seawater; (b) fungi in seawater; (c) bacteria in seawater supplemented with motor oil; (d) fungi in seawater supplemented with motor oil.

bioemulsifiers were not toxic to any tested organisms (crustaceans, bacteria or plants). The same solution was used for contact tests on earthworms, showing 100% of survival.

3.6. Bioremediation test

The effect of the biosurfactant on the biodegradation of motor oil via the activity of indigenous marine bacteria and fungi was evaluated during 30 days. The presence of the biosurfactant from *C. lipolytica* favoured the growth of microorganisms in seawater at the concentrations evaluated (½ the CMC, CMC and 2× CMC) (Fig. 5a,b). It was observed increased growth of bacteria in the presence of the highest concentration of the biosurfactant, while the solution of biosurfactant in the lower concentration stimulated fungi growth. Moreover, in the presence of petroleum-based motor oil, higher concentrations of the biosurfactant favoured the growth of all microorganisms in sea water in the conditions studied in this work (Fig. 5c,d). It could be also observed that the growth of the indigenous fungi in the control formulated with sea water and petroleum was poor in the absence of the biosurfactant. The stimulation of oil degradation by the biosurfactant could be attributed to the dispersion of oil and the increase in the number of cells that utilized the biosurfactant as well as the nutrients derived from the culture broth.

Saeki et al. [44], in a similar experiment, found that the degradation of the total saturated hydrocarbons and total target PAHs (polycyclic aromatic hydrocarbons) was stimulated by the biosurfactant from *Gordonia* sp. in sea water samples.

Lima et al. [46] evaluated the effects of different concentrations (2× CMC, 4× CMC and 8× CMC) of bacterial surfactants on the growth of pure cultures of bacteria in mineral medium supplemented with glucose. They observed that the influence of the biosurfactants at twice the CMC in the growth of the bacterial isolates was not significant. However, an inhibition of the bacterial isolates growth occurred when the biosurfactants were tested at 8× CMC, showing the toxicity of these biosurfactants at high concentrations. The literature describes the higher effectiveness of surfactants at concentrations slightly above their CMC than at concentrations well above the CMC values [47,48].

4. Conclusions

In the present work the production of the biosurfactant by *C. lipolytica* cultivated in a low-cost medium have been described in bioreactors. Good surface tension reduction and biosurfactant yield were obtained. The biosurfactant not only has low toxicity but also a significant potential to be applied as an oil spill remediation agent in marine environments. The remediation experiments showed that the biosurfactant can be used as an oil spill dispersant. The biosurfactant could also stimulate the degradation of the spilled oil by the native microorganisms. The economic analysis shows that the biosurfactant and the biomass produced from inexpensive raw materials have potential to be used not only in the petroleum industry but also in other industries.

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References

[1] I.M. Banat, S.K. Satpute, S.S. Cameotra, R. Patil, N. Nyayanit, Cost effective technologies and renewable substrates for biosurfactants' production, Front. Microbiol. 5 (2014) 1–18.
[2] D.K.F. Santos, R.D. Rufino, J.M. Luna, V.A. Santos, L.A. Sarubbo, Biosurfactants: multifunctional biomolecules of the 21 st Century, Int. J. Mol. Sci. 17 (2016) 401.
[3] L.A. Sarubbo, R.B. Rocha Júnior, J.M. Luna, R.D. Rufino, V.A. Santos, I.M. Banat, Some aspects of heavy metals contamination remediation and role of biosurfactants, Chem. Ecol. 31 (2015) 707–723.
[4] A. Franzetti, P. Di Gennaro, A. Bevilacqua, M. Papacchini, G. Bestetti, Environmental features of two commercial surfactants widely used in soil remediation, Chemosphere 62 (2006) 1474–1480.
[5] K.R. Edwards, J.E. Lepo, M.A. Lewis, Toxicity comparison of biosurfactants and synthetic surfactants used in oil spill remediation to two estuarine species, Mar. Poll. Bull. 46 (2003) 1309–1316.
[6] R.C.F.S. Silva, D.G. Almeida, J.M. Luna, R.D. Rufino, V.A. Santos, L.A. Sarubbo, Applications of biosurfactants in the petroleum industry and the remediation of oil spills, Int. J. Mol. Sci. 15 (2014) 12523–12542.
[7] C.D. Coimbra, R.D. Rufino, J.M. Luna, L.A. Sarubbo, Studies of the cell surface properties of *Candida* Species and relation with the production of biosurfactants for environmental applications, Curr. Microbiol. 58 (2009) 245–251.
[8] C.A.B. Gusmão, R.D. Rufino, L.A. Sarubbo, Laboratory production and characterization of a new biosurfactant from *Candida glabrata* UCP1002 cultivated in vegetable fat waste applied to the removal of hydrophobic contaminant, World J. Microbiol. Biotechnol. 26 (2010) 1683–1692.
[9] J.M. Luna, L.A. Sarubbo, G.M. Campos-Takaki, A new biosurfactant produced by Candida glabrata UCP 1002: characteristics of stability and application in oil recovery, Braz. Arch. Biol. Technol. 52 (2009) 785–793.
[10] J.M. Luna, R.D. Rufino, L.A. Sarubbo, G.M. Campos-Takaki, Characterization, surface properties and biological activity of a biosurfactant produced from industrial waste by *Candida sphaerica* UCP0995 for application in the petroleum industry, Colloids Surf. B. 102 (2012) 202–209.
[11] R.D. Rufino, G.I.B. Rodrigues, G.M. Campos-Takaki, L.A. Sarubbo, S.R.M. Ferreira, Application of a yeast biosurfactant in the removal of heavy metals and hydrophobic contaminant in a soil used as slurry barrier, Appl. Environ. Soil Sci. (2011) 1–7.
[12] D.K.F. Santos, R.D. Rufino, J.M. Luna, V.A. Santos, A.A. Salgueiro, L.A. Sarubbo, Synthesis and evaluation of biosurfactant produced by *Candida lipolytica* using animal fat and corn steep liquor, J. Pet. Sci. Eng. 105 (2013) 43–50.
[13] D.K.F. Santos, Y.B. Brandão, R.D. Rufino, J.M. Luna, A.A. Salgueiro, V.A. Santos, L.A. Sarubbo, Optimization of cultural conditions for biosurfactant production from *Candida lipolytica*, Biocatal. Agric. Biotechnol. 3 (2014) 48–57.
[14] M. Akhtar, M.J. Lentz, R.A. Blanchette, T.K. Kir, Corn steep liquor lowers the amount of inoculum for biopulping, TAPPI J 80 (1997) 161–164.
[15] E.V. Cardinal, L.R. Hedrick, Microbiological assay of corn steep liquor for amino acid content, J. Biol. Chem. 172 (1984) 609–612.
[16] P.A. Felse, V. Shah, J. Chan, K.J. Rao, R.A. Gross, Sophorolipid biosynthesis by *Candida bombicola* from industrial fatty acid residues, Enzyme Microb. Tech. 40 (2007) 316–323.
[17] M. Dubois, K.A. Gilles, J.K. Hamilton, Colorimetric method for determination of sugar and related substances, Anal. Chem. 28 (1956) 350–356.
[18] M.S. Manocha, G. San-Blas, S. Centeno, Lipid composition of *Paracoccidioides brasilienses*: possible correlation with virulence of different strains, J. Gen. Microbiol. 117 (1980) 147–154.
[19] M.O. Ilori, C.J. Amobi, A.C. Odocha, Factor's affecting biosurfactant production by oil degrading *Aeromonas* spp. isolated from a tropical environment, Chemosphere 61 (2005) 985–992.
[20] S.M. Tiquia, N.F.Y. Tam, I.J. Hodgkiss, Effects of composting on phytotocicity of spent pig-manure sawdust litter, Environ. Pollut. 93 (1996) 249–256.
[21] T.M. Kajs, C.V. Vanderzant, Batch-scale utilization studies of tallow by food yeasts, Dev. Ind. Microbiol. 21 (1980) 481–488.
[22] APHA, Standard Methods for the Examination of Water and Wastewater, 21 st ed., American Public Health Association, Washington, 2005.
[23] G. Boehs, A.R.M. Magalhães, Simbiontes associados com *Anomalocardia brasiliana* (Gmelin) (Mollusca, Bivalvia, Veneridae) na Ilha de Santa Catarina e região continental adjacente, Santa Catarina, Brasil, Rev. Bras. Zool. 21 (2004) 865–869.
[24] D.B. Quayle, G.F. Newkirk, Farming Bivalve Molluscs: Methods for Study and Development, The World Aquaculture Society, Louisiana, 1989, pp. 285.
[25] I.N.A. Van Bogaert, J. Zhang, W. Soetaert, Microbial synthesis of sophorolipids, Process Biochem. 46 (2011) 821–833.
[26] K. Dubey, A. Juwarkar, Determination of genetic basis for biosurfactant production in distillery and curd whey wastes utilizing *Pseudomonas aeruginosa* strain BS2, Indian J. Biotechnol. 3 (2004) 74–81.
[27] A.K. Koch, J. Reiser, O. Kappeli, A. Fiechter, Genetic construction of lactose-utilizing strains of *Pseudomonas aeruginosa* and their application in biosurfactant production, Biotechnolology 51 (1998) 1335–1339.
[28] P. Patel, A. Desai, Biosurfactant production by *Pseudomonas aeruginosa* GS3 from molasses, Lett. Appl. Microbiol. 25 (1997) 91–96.
[29] M.E. Mercade, M.A. Manresa, The use of agro industrial by products for biosurfactant production, J. Am. Oil Chem. Soc. 1 (1994) 61–64.
[30] C. Amézcua-Veja, H.M. Poggi-Varaldo, F. Esparza-Garcia, E. Rios-Leal, R. Rodriguez-Vazquez, Effect of culture conditions on fatty acids composition of a biosurfactant produced by *Candida ingens* and changes of surface tension of cultura media, Bioresour. Technol. 98 (2007) 237–240.
[31] M. Deshpand, L. Daniels, Evaluation of sophorolipid biosurfactant production by *Candida bombicola* using animal fat, Bioresour. Technol. 54 (1995) 143–150.
[32] M. Benincasa, J. Contiero, M.A. Manresa, I.O. Moraes, Rhamnolipid production by *Pseudomonas aeruginosa* LBI growing on soapstock as the sole carbon source, J. Food Eng. 54 (2002) 283–288.
[33] Y. Zhu, J.-J. Gan, G.-L. Zhang, B. Yau, W.-J. Zhu, Q. Meng, Reuse of waste frying oil for production of rhamnolipids using *Pseudomonas aeruginosa* zju.u1M, J. Zhejiang Univ. Sci. A 8 (2007) 1514–1520.
[34] M.E. Marti, W.J. Colonna, P. Patra, H. Zhang, C. Greene, G. Reznik, M. Pynn, K. Jarrell, J.A. Nyman, P. Somasundaran, C.E. Glatz, B.P. Lamsal, Production and characterization of microbial biosurfactants forpotential use in oil-spill remediation, Enzyme Microb. Tech. 55 (2014) 31–39.
[35] P. Chandran, N. Das, Characterization of sophorolipid biosurfactant produced by yeast species grown on diesel oil, Int. J. Sci. Nat. 2 (2011) 63–71.
[36] K. Muthusamy, S. Gopalakrishnan, T.K. Ravi, P. Sivachidambaram, Biosurfactants: properties, commercial production and application, Curr. Sci. 94 (2008) 736–747.
[37] D.G. Cooper, D.A. Paddock, Production of a biosurfactant from *Torulopsis bombicola*, Appl. Environ. Microbiol. 47 (1984) 173–176.
[38] L.A. Sarubbo, C.B.B. Farias, G.M. Campos-Takaki, Co-utilization of canola oil and glucose on the production of a surfactant by *Candida lipolytica*, Curr. Microbiol. 54 (2007) 68–73.
[39] R.M. Batista, R.D. Rufino, J.M. Luna, J.E.G. Souza, L.A. Sarubbo, Effect of medium components on the production of a biosurfactant from *Candida tropicalis* applied to the removal of hydrophobic contaminants in soil, Water Environ. Res. 82 (2010) 418–425.
[40] H. Asmer, S. Lang, F. Wagner, V. Wray, Microbial production, structure elucidation and bioconversion of sophorose lipids, J. Am. Oil Chem. Soc. 65 (1998) 1460–1466.
[41] Q.-H. Zhou, N. Kosaric, Utilization of canola oil and lactose to produce biosurfactant with *Candida bombicola*, J. Am. Chem. Soc. 72 (1995) 67–71.
[42] Y.-B. Kim, H.S. Yun, E.-K. Kim, Enhanced sophorolipid production by feeding-rate-controlled fed-batch culture, Bioresour. Technol. 100 (2009) 6028–6032.
[43] G.M. Rand, P.G. Wells, L.S. McCarty, in: G.M. Rand (Ed.), Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment, 2nd ed., Taylor & Francis, Washington, 1995.
[44] H. Saeki, K.M. Sasaki, O. Komatsu, A. Miura, H. Matsuda, Oil spill remediation by using the remediation agent JE1058BS that contains a biosurfactant produced by *Gordonia* sp strain JE-1058, Bioresour. Technol. 100 (2009) 572–577.
[45] A. Franzetti, I. Gandolfi, C. Raimondi, G. Bestetti, I.M. Banat, T.J. Smyth, M. Papacchini, M. Cavallo, L. Fracchia, Environmental fate, toxicity, characteristics and potential applications of novel bioemulsifiers produced by *Variovorax paradoxus* 7bCT5, Bioresour. Technol. 108 (2012) 245–251.
[46] T.M.S. Lima, L.C. Procópio, F.D. Brandão, B.A. Leão, M.R. Tótola, A.C. Borges, Evaluation of bacterial surfactant toxicity towards petroleum degrading microorganisms, Bioresour. Technol. 102 (2011) 2957–2964.
[47] B.N. Aronstein, Y.M. Calvillo, M. Alexander, Effects of surfactants at low concentrations on the desorption and biodegradation of sorbed aromatic compounds in soils, Environ. Sci. Technol. 25 (1991) 1728–1731.
[48] D.C. Herman, Y. Zhang, R.M. Miller-Maier, Rhamnolipid (biosurfactant) effects on cell aggregation and biodegradation of residual hexadecane under saturated flow conditions, Appl. Environ. Microbiol. 63 (1997) 3622–3627.

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CAPITULO 4

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frontiers

Candida lipolytica UCP0988 Biosurfactant: Potential as a Bioremediation Agent and in Formulating a Commercial Related Product

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Author contribution statement

All authors contributed in this work. Leonie Sarubbo conceived and designed the experiments; Danyelle Kadhydja Santos, Ana Helena M. Resende, Darne G. Almeida and Rita de Cássia F. Soares da Silva performed the experiments; Ibrahim M. Banat, Raquel D. Rufino and Juliana M. Luna analyzed the data and contributed to analysis tools; Leonie A. Sarubbo and Ibrahim M. Banat wrote the paper.

Keywords

Candida lipolytica, Animal fat, Corn steep liquor, bioremediation, Petroleum, heavy metals

Abstract

Word count: 175

A biosurfactant produced by Candida lipolytica in a bioreactor containing industrial residues was tested in different remediation techniques of organic and inorganic pollutants. In tests carried out with seawater, the crude biosurfactant demonstrated 80% oil spreading efficiency. The dispersion rate was 50% for the biosurfactant at a concentration twice that of the CMC. The biosurfactant removed 70% of motor oil from contaminated cotton cloth in detergency tests. The crude biosurfactant also removed 30 to 40% of Cu and Pb from standard sand, while the isolated biosurfactant removed approximately 30% of the heavy metals. The conductivity of solutions containing Cd and Pb was sharply reduced after biosurfactants' addition. A product was prepared through adding a preservative and tested over 120 days. The formulated biosurfactant was analysed for emulsification and surface tension under different pH values, temperatures and salt concentrations and tested for toxicity. The results showed that the formulation had no toxicity and did not cause significant changes in the tensoactive capacity of the biomolecule while maintaining activity demonstrating suitability for potential future commercial product formulation.

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Ethics statements

(Authors are required to state the ethical considerations of their study in the manuscript, including for cases where the study was exempt from ethical approval procedures)

Does the study presented in the manuscript involve human or animal subjects: No

frontiers

Candida lipolytica UCP0988 Biosurfactant: Potential as a Bioremediation Agent and in Formulating a Commercial Related Product

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Keywords: *Candida lipolytica*, animal fat, corn steep liquor, bioremediation, petroleum, heavy metals.

Abstract

A biosurfactant produced by *Candida lipolytica* in a bioreactor containing industrial residues was tested in different remediation techniques of organic and inorganic pollutants. In tests carried out with seawater, the crude biosurfactant demonstrated 80% oil spreading efficiency. The dispersion rate was 50% for the biosurfactant at a concentration twice that of the CMC. The biosurfactant removed 70% of motor oil from contaminated cotton cloth in detergency tests. The crude biosurfactant also removed 30 to 40% of Cu and Pb from standard sand, while the isolated biosurfactant removed approximately 30% of the heavy metals. The conductivity of solutions containing Cd and Pb was sharply reduced after biosurfactants' addition. A product was prepared through adding a preservative and tested over 120 days. The formulated biosurfactant was analysed for emulsification and surface tension under different pH values, temperatures and salt concentrations and tested for toxicity. The results showed that the formulation had no toxicity and did not cause significant changes in the tensoactive capacity of the biomolecule while maintaining activity demonstrating suitability for potential future commercial product formulation.

1 Introduction

Surfactants are chemical compounds that preferentially partition at the interface between phases (gas, liquid and solid) with different degrees of polarity and hydrogen bonding. They are therefore amphipathic molecules with hydrophilic and hydrophobic moieties in which the polar portion is either ionic (cationic or anionic), non-ionic or amphoteric and the nonpolar portion is often a hydrocarbon chain (Santos et al., 2016). These characteristics allow these compounds to reduce surface and

interfacial tensions as well as form micro-emulsions in which hydrocarbons are solubilised in water or vice versa solubilising water in hydrocarbons. Such properties enable a broad spectrum of potential industrial applications involving emulsification, detergency, lubrication, wetting, foaming, dispersions or solubilisation of different phases (Silva et al., 2014).

Most commercially available surfactants are synthesised from petroleum by-products (Silva et al., 2014). However, environmental concerns mostly driven by consumer demands combined with new regulations aimed at managing the environment have led to the pursuit to find alternative natural surfactants to replace existing products. Various compounds with such tensioactive properties are often synthesised by biological systems such as plants (saponins), microorganisms (glycolipids) and animals (bile salts, skin exudates), which are considered natural surface active compounds (Campos et al., 2013).

Compounds of a microbial origin that exhibit surfactant properties are mainly metabolic by-products of bacteria, filamentous fungi and yeasts capable of lowering surface tension and exhibiting a high emulsifying capacity are the most predominant type of biosurfactants (Marchant and Banat, 2012). The main types of chemical structures of biosurfactants are glycolipids, lipopeptides, lipoproteins, phospholipids, fatty acids and polymeric in nature. Biosufactants have numerous advantages over surfactants of a synthetic origin in having lower toxicity, stability under wider ranges of temperature and pH and ability to remain active at high salt concentrations (Banat et al., 2014).

The oil industry remains the major market for biosurfactants utilisation, where they can be used in processes involved with the removal and mobilisation oil residue, bioremediation hydrocarbon contaminated environment and microbial enhanced oil recovery technology (Silva et al., 2014). The bioremediation of soil and water encounters obstacles associated with the biodegradation of petroleum hydrocarbons, as these hydrophobic compounds bond to soil particles and have a low degree of solubility in water, which reduces their bioavailability to microorganisms and consequently limits the transfer of mass for biodegradation (Souza et al., 2014). The key in the process of enhancing the bioavailability of contaminating oils is the mobilisation of the hydrophobic pollutant through the aqueous phase. Thus, the use of surfactants develops as an alternative as a mechanism to enhance the solubility of oils through initiating desorption and the consequent mobilisation and solubilisation of hydrocarbons facilitating transport, access and assimilation of these compounds by microbial cells (Burghoff, 2012).

Besides organic pollutants, heavy metals are also found in soil and are considered the inorganic pollutants with the greatest potential risk to humans. Metals ions can exist as fixed or soluble minerals in rocks, sand and soil or as dissolved ions in water or vapours. Metals can also be attached to inorganic or organic molecules or even attached air particles. Both anthropogenic and natural activities and processes can emit metals into water and air (Sarubbo et al., 2015).

Surfactants can potentially be used, and have been used, to remediate soils contaminated with metals and oils through desorption, solubilisation and dispersion of contaminants in soil, thereby allowing the removal, collection or reutilisation (Asçi et al., 2012). The necessity to replace synthetic chemical surfactants with natural compounds however, has motivated studies seeking biological alternatives such as surfactin and rhamnolipids, both of which are of a bacterial biosurfactants (Barros et al., 2007), and sophorolipids derived from yeasts (Coimbra et al., 2009; Menezes et al., 2011; Albuquerque et al., 2012; Rufino et al., 2013). The ionic nature of these agents as well as their low toxicity, biodegradability and excellent surface properties, make them potential candidates for heavy metals removal from contaminated soil, sediment and waste water (Sarubbo et al., 2015).

Most known biosurfactants are produced on media containing water immiscible substrates such as oil, fats and liquid or solid hydrocarbons, although many have been obtained on readily available soluble carbon substrates (Pacwa-Plociniczak et al., 2011). The type of raw material and availability of substrate to produce biosurfactants contribute considerably to the cost of production (estimated to be 10-30% of total cost (Marchant and Banat, 2012). On the other hand, millions of tons of waste

materials (residual pollutants) are either deliberately discarded or accidentally leaked into the environment worldwide every year. Treatment and mitigation processes to reduce or eliminate such contaminant represent a high cost to local governments and industries.

The aim of the present study was to investigate the potential application of a biosurfactant from *Candida lipolytica,* which has previously been produced and characterised under optimised conditions (Santos et al., 2013; 2014), as an adjunct materials to enhance the remediation processes of hydrophobic pollutants and heavy metals generated by the oil industry and propose the formulation of a safe, stable remediation agent.

2 Materials and Methods

2.1 Materials

All chemicals reagent were of analytical grade. The animal fat used was choice white grease from a bovine processing plant located in the city of Recife (Brazil) and was used without any further processing. Corn steep liquor was obtained from Corn Products from Brazil in the city of Cabo de Santo Agostinho (Brazil). According to Akhtar et al. (1997) and Cardinal and Hedrick (1948), corn steep liquor is 21 to 45% protein, 20 to 26% lactic acid, 8% ash (containing Ca^{2+}, Mg^{2+}, K^{+}), 3% sugar and has a low fat content (0.9 to 1.2%).
Engine lubricating oil (motor oil) was obtained from an automotive maintenance establishment in the city of Recife, Pernambuco, Brazil. Samples of NBR 7214 standard sand (1982) were used in the heavy metal removal experiments. The sand had a particle size on the order of 0.15 to 0.30 mm, 0.2% water, a specific density of 2.620 g/cm^3 and organic matter content of 100 ppm. The sea water used in the removal of motor oil was collected from the municipality of Cabo de Santo Agostinho, state of Pernambuco, Brazil. Water samples were collected and stored in 5-L plastic bottles.

2.2 Microorganism

Candida lipolytica UCP0988 was obtained from the culture collection of the Universidade Católica de Pernambuco (Brazil). The microorganism was maintained at 5 °C on yeast mould agar slants containing (w/v) yeast extract (0.3%), malt extract (0.3%), tryptone (0.5%), D-glucose (1.0%) and agar (5.0%). Transfers were made to fresh agar slants each month to maintain viability.

2.3 Growth Conditions

The inoculum of *Candida lipolytica* was prepared by transferring cells grown on a slant to 50 mL of yeast mould broth. The seed culture was incubated for 24 h at 28 °C and agitated at 150 rpm.The yeast was cultivated in a submerged culture in 500-ml flasks containing 100 ml production medium with agitation in a New Bruswick C-24 shaker. The production medium contained 5% animal fat and 2.5% corn steep liquor. The medium was sterilised by autoclaving at 121 °C for 20 min (all components were sterilised together). The final pH of the medium was 5.3. The inoculum (1% v/v) containing approximately 10^4 cells/mL was introduced into chilled yeast medium.

2.4 Biosurfactant Production and Isolation

Biosurfactant production was performed in a 2-l bioreactor (Tec-Bio-Plus, Tecnal Ltda., Brazil) with a working volume of 1.2 l operated in a batch mode, with controlled pH (5.3) and temperature (28°C). The culture medium was inoculated with a 24-h inoculum and fermentation was carried out at 200 rpm in the absence of aeration for 144 h (Santos et al., 2014). The biosurfactant was recovered from the cell-free broth by cold acetone precipitation, as described by Ilori et al. (2005).

2.5 Surface Tension

The surface tension of the culture supernatants obtained by centrifuging the cultures at 5000 g for 20 minutes was measured using a Sigma 700 digital surface tensiometer (KSV Instruments LTD - Finland) as described by Santos et al. (2013).

2.6 Screening Dispersion Test

A quick comparative test method using small vials (25 ml) was used for the visual determination of the dispersant effectiveness of the biosurfactant. The motor oil sample (100 µl) was carefully added to the surface of seawater (20 ml) and a vortex with a depth of 1 cm was created by slow magnetic stirring. The dispersant mixture (5.0 µl), i.e., crude biosurfactant (cell-free broth after fermentation) or isolated biosurfactant at half the critical micelle concentration (1/2 x CMC), at the full CMC and twice the CMC (2 x CMC) was added to the centre of the vortex. The stirring rate was immediately increased, maintained at a maximum rate of 2000 rpm for 60 s and then stopped. The level of oil dispersion in the water was visually estimated after a one-minute rest. Classification A was attributed to the resulting brown-black mixture when all the oil was dispersed in the water leaving no slick at the surface, whereas Classification E was used to describe a complete lack of dispersion, i.e. all the oil returned to the surface a few seconds after the end of stirring, leaving the aqueous phase nearly transparent. Classification B to D represented intermediate situations. All screen tests were carried out at room temperature (Brochu et al., 1986).

2.7 Swirling Bottle Test

A 1-L cylindrical open bottle (diameter: 10 cm) with an outlet valve at the bottom to take samples was used in the dispersion experiment. Samples of 200 mL of sea water were added to the bottle and 2 mL of oil was gently added to the surface of the water with a pipette. The crude or the isolated biosurfactant solution was dispensed in the centre of the oil slick in the following proportions of biosurfactant-to-oil: 1:1, 1:2, 1:10 and 1:20 (v/v). The isolated biosurfactant was used at half the CMC, the full CMC and twice the CMC. The bottle was placed on an orbital shaker table at 28 ºC to induce a swirling motion in the water content of the bottle. The shaking speed was 150 rpm for a period of 10 min, followed by 1 to 2 min settling time to allow the larger droplets to return to the surface. Samples were taken after 15 min. The first 2 mL of the sample was removed through the stopcock and discarded and 30 mL of the sample was collected. This sample was extracted three times with hexane, as the biosurfactant is insoluble in hexane. The extract was adjusted. Efficacy was calculated by dividing the concentration of dispersed oil in the water (determined by analysing the hexane extract) by the total concentration of oil, which depended on the total volume of oil added to the flask (Holakoo, 2001; Jain et al., 2012).

2.8 Removal of Motor Oil from Contaminated Cotton Cloth

The efficiency of the biosurfactant to remove oil with respect to a commercially available detergent (Soap powder, Asa LTDA, Recife, PE, Brazil) was investigated. The detergent and biosurfactant were individually dissolved in water and their efficiency in removing oil from contaminated cotton cloth was checked individually as well as in combination with the biosurfactant at a 1:1 (v/v) ratio. For such, 3 g of lubricant oil was poured on a 25 x 25 cm cotton cloth and allowed to dry at 40 ºC for 24 h. To test the oil removal capacity, each piece of cloth impregnated with oil was soaked in flasks containing 100 mL of tap water (control), biosurfactant solutions (cell-free broth and isolated biosurfactant at 1/2 the CMC, the full CMC and twice the CMC), detergent (sodium lauril ether sulfate at the CMC) and a biosurfactant/detergent solution (1:1 v/v) at their CMC. The flasks were

kept on a shaker at 30 °C and 100 rpm for 60 min. The post-wash water was used to measure the amount of oil removed from the cotton cloth by extracting it with hexane. The extraction process was repeated three times. The hexane was recovered using a rotary evaporator and the residual lubricant oil was measured gravimetrically (Jain et al., 2012).

2.9 Preparation of Contaminated Sand with Heavy Metals

The standard sand was artificially contaminated in the laboratory with a metal solution ($Cu(NO3)_2$ + $Pb(NO_3)_2$ + $Zn(NO_3)_2$). The salts were separately dissolved in deionised water to achieve a concentration of 1000 mg/l and then added together to the sand without pH adjustment. The sand was left in contact with the solution for three days in a shaker (200 rpm at 25 °C) and then centrifuged at 5000 rpm for 10 min to remove non-adsorbed metals in the solution. The supernatant was discarded and the contaminated sand was dried in an oven at 50 °C for 24 h (Juwarkar et al., 2007).

2.10 Treatment of Contaminated Sand with Heavy Metals with Biosurfactant

A series of washings was performed using the isolated biosurfactant at 1/2 the CMC, at the full CMC and twice the CMC as well as the crude biosurfactant (cell-free broth). Distilled water was used as the control. A 1% NaOH solution and 0.7% HCl solution as well as combinations of biosurfactant solutions and cell-free broth with 0.7% HCl or 1% NaOH as additives were also tested. 5.0 g of the contaminated sand were transferred to 125-mL Erlenmeyer flasks and 50 mL of the washing solution were added at the different concentrations described above. The samples were incubated on a rotary shaker (200 rpm) for 24 h at 27 °C and then were centrifuged at 5000 g for 10 min. The supernatants were analysed for metal concentration using an atomic absorption spectrophotometer (Perkin Elmer AAnalyst™ 800).

2.11 Biosurfactant Treatment of Synthetic Wastewater Contaminated With Heavy Metals

The ability to remove heavy metals in water by the biosurfactant was determined in a synthetic fluent containing Pb and Cd. Biosurfactant solutions at 1/2 the CMC, the full CMC and twice the CMC were then added separately to 500 and 1000 ppm solutions of lead nitrate and cadmium nitrate. The metal-biosurfactant precipitate was removed and conductivity of the resulting solution was measured. The conductivity meter (TEC-4MP, Tecnal Ltda., Brazil) was calibrated with deionised water before measuring the conductivity of each sample. All tests were performed in triplicate (Das et al., 2009).

2.12 Formulation of Biosurfactant

After fermentation, the broth was centrifuged at 5000 rpm for 20 min for the removal of the cells. Potassium sorbate (0.2%) was added to the cell-free both with the crude biosurfactant. After the treatment of the crude biosurfactant in accordance with the preservation method, the broth was stored at room temperature (28 to 30°C) for 120 days, with samples withdrawn at 0, 15, 30, 45, 90 and 120 days to determine stability (Freitas et al., 2016).

2.13 Effect of Environmental Factors on Formulated Biosurfactant Activity

The effects of the addition of different concentrations of NaCl (1, 3 and 5%), different temperatures (40 and 50 °C) for 60 min and different pH values evaluated after adjustment of the broth pH to 5, 7 and 9 with 6.0 M NaOH or HCl on surface tension and emulsification were evaluated at 0, 15, 30, 45, 90 and 120 days.

2.14 Emulsification Activity

The emulsification index was measured using the method described by Cooper and Goldenberg (1987), whereby 2 mL of motor oil obtained from a local automotive manufacturer in the city of Recife, Brazil, or vegetal corn oil was added to 2 mL of the cell-free broth in a graduated screwcap test tube and vortexed at high speed for 2 min. Emulsion stability was determined after 24 h and the emulsification index was calculated by dividing the measured height of the emulsion layer by the total height of the mixture and multiplying by 100.

2.15 Toxicity of Formulated Biosurfactant Against *Poecilia vivipara*

Acute toxicity tests were performed using the fish *Poecilia vivipara* as an indicator for the determination of the lethal concentration (LC50) of the formulated biosurfactant for 96 h. The specimens were maintained at 26°C in the laboratory and kept in polyethylene aquaria (capacity: 60 l) supplied with fresh tap water. The fish were fed with commercial fish food (Alcon Basic Ltda, Santa Catarina, Brazil). Water temperature, oxygenation and pH in the aquaria were periodically checked throughout the experiment. After a week of acclimation, the fish were exposed to the formulated biosurfactant.

Assays were performed at the Quarantine Laboratory of Sustainable Mariculture of the Fisheries and Aquaculture Department of the Federal Rural University of Pernambuco (UFRPE), Pernambuco, Brazil. Ten specimens were exposed for 96 h without food or water exchange. Experiments were carried out using a static acute experimental methodology. The animals were kept in 2-l fibreglass boxes with 1.5 l of seawater with salinity of 26 and an average temperature of 27°C under constant aeration and a 12h light/12h dark cycle. Three dilutions of the formulated biosurfactant (cell-free broth plus 0.2% potassium sorbate) in seawater were tested with three replicates each: 1:1, 1:2 and 1:5 (v/v). Controls contained seawater alone. The result of the experiment was based on determination of lethal concentration for 50% of specimens (LC50) and expressed in terms of the mean mortality of three replicates for each dilution tested with the biosurfactant.

2.16 Statistical Analysis

All determinations were performed at least three times. Means and standard errors were calculated using the Microsoft Office Excel 2003 (Version 7).

3 Results and Discussion

3.1 Screening Dispersion Test

The results of the screening dispersion test demonstrated that both the crude and isolated biosurfactant dispersed a reasonable amount of oil, with a greater concentration of biosurfactant leading to a greater percentage of dispersion (Table 1). However, it is important to consider that the use of the cell-free broth represents a considerable reduction in production cost of the compound, as described in Santos et al. (2016).

In a previous study by our research group (Santos et al., 2013), we tested motor oil dispersion characteristics of *C. lipolytica* UCP0988 cell-free broth containing biosurfactants and reported high oil spreading efficiency (54% oil displacement).

According to Bai et al. (1997), "dispersion is a process by which a hydrocarbon is dispersed in the aqueous phase as tiny emulsions. Emulsions are not generally thermodynamically stable, but may remain stable for significant periods of time due to kinetic restrictions. Dispersion is related to interfacial tension and surfactant concentration and differs from displacement, which is related only to interfacial tension between the aqueous and hydrophobic phases, with no formation of emulsion".

3.2 Swirling Bottle Test

One of the oil spill remediation techniques is the application of dispersants to oil slicks. Dispersants used for this purpose are usually composed of a mixture of surfactants and solvents with some additives designed to enhance the dispersion of oils as well as their removal from contaminated surfaces. Dispersants application reduces the effects of the oil spills as it removes the oil from the surface of water reducing the amount of spilled oil. The dispersion of oil into tiny droplets also increase the surface area of exposure which stimulates biodegradation by indigenous microorganisms (NRC, 2005). The effect of factors such as oil viscosity, mixing energy and temperature on the efficacy of a dispersant need to be evaluated. The solvent normally contained in dispersants acts as a solution for the surfactant components and serves as a surfactant carrier, enabling penetration into an oil slick.

According to Sorial et al. (2004), the "baffled flask test" developed by the Environmental Protection Agency if the USA has been proposed as a replacement protocol for categorising oil spill dispersants in a "National Contingency Plan Product Schedule". Therefore, in the present study a similar experiment was performed for the evaluation of the biosurfactant from *C. lipolytica* as an oil spill dispersant measuring the efficacy using motor oil.

To study the effect of the proportion of biosurfactant to motor oil on dispersant efficacy, tests were carried out with different ratios. In this study, the crude and the isolated biosurfactant without the addition of solvents or additives were tested for 15 min after the simulation of an oil spill in a seawater sample (Table 2).

The biosurfactant concentration is a critical parameter, since a lower concentration of biosurfactant leads to a smaller amount of dispersion. The dispersant/oil ratio is another critical factor influencing dispersant efficacy. The best dispersion index occurred with a biosurfactant/oil ratio of 1:1 (v/v) with a solution of the biosurfactant twice the CMC (50%), while the crude biosurfactant dispersed approximately 25% of the oil under the same condition. The biosurfactant used below the CMC was inefficient under the tested conditions. It is likely that the increase in agitation speed allowed greater interaction between the biosurfactant and oil, consequently leading to a greater dispersion percentage. In any case, the results of this test demonstrate that the biosurfactant alone has potential for application as a dispersant, but it is likely that additives will increase the efficiency.

3.3 Motor Oil Removal from Contaminated Cotton Cloth

The use of biosurfactant as a detergent was tested on cotton cloth samples (Table 3). The performance of the biosurfactant was excellent, removing 70% oil at twice the CMC in comparison to oil removal by the commercially available detergent. The biosurfactant at its CMC was also efficient at removing the oil, while the crude biosurfactant was superior to the isolated biosurfactant at half the CMC. On the other hand, the biosurfactant did not exhibit compatibility with the commercial detergent at a ratio of 1:1 (v/v). Commercially available detergents usually contains an anionic surfactant, water softening components, enzymes and bleaching agents that helps enhancing the washing performance (Mukherjee et al., 2007). Thus, the addition of these substances could increase the efficiency of the biosurfactant from *C. lipolytica*.

3.4 Treatment Heavy Metals Contaminated Sand

Advances in treatment technologies for heavy metals contaminated soils have increased the interest in finding new washing products, such as anionic biosurfactants capable of bonding to metals and do not pose risks to the environment due to their characteristics of lower toxicity and biodegradability (Maity et al., 2013). The mechanisms for heavy metals extraction by biosurfactants include ionic exchange, chelation, dissolution, precipitation and associations with contra-ions. Metals are believed to be

298 removed through complexes formation with a surfactant at soil surfaces which are mobilised due to the
299 reduction in interfacial tension and the consequent association with surfactants' micelles. Anionic
300 surfactants are negatively charged and therefore have a good affinity towards metal cations while
301 enhancing better removal due to their capacity to reduce interfacial tension (Marchant and Banat,
302 2012).
303 It is important for biosurfactants to remain in the aqueous phase and have minimal interactions with
304 the treated soils. However, when large concentrations of biosurfactant are used to ascertain effective
305 heavy metals removal from soil, sorption to soil particles may occur. Hence, its behaviour will
306 inevitably depend on the biosurfactants' molecular characteristics, such as charge, hydrophobicity and
307 soil characteristics (Sarubbo et al., 2015). Thus, the low-cost biosurfactant we produced using *C.*
308 *lipolytica* was tested with regard to the removal of copper, lead and zinc contained in samples of
309 standard sand. The standard sand with organic matter content of 100 ppm was used to minimise the
310 interaction of the biosurfactant with the soil and maximise metal-biosurfactant interactions.
311 Solutions of the isolated biosurfactant at different concentrations [1/2 x CMC (0.04%), CMC
312 (0.08%) and 2 x CMC (0.16%)] were tested to evaluate the removal of metals with and without the
313 formation of micelles, which are efficient structures for the mobilisation of heavy metals during soil
314 treatment. Metal removal with the cell-free crude biosurfactant was investigated. The likelihood of
315 increasing percentage metal removal was tested using the surfactant with HCl and NaOH as additives.
316 The additives were employed separately while using distilled water as control. The results of the
317 treatment of sand with the biosurfactant solutions are shown in Table 4.
318 The results demonstrate that, under the herein tested conditions, *C. lipolytica* biosurfactant was
319 more efficient in the removal of copper and lead. The control treatments showed 11 to 17% metals
320 removal from sand while other treatments achieve >80% removal (Table 4). Ochoa-Loza et al. (2007),
321 stated that different surfactants have varying affinities to different metals and are invariably affected
322 by type and concentration of biosurfactant, interaction with additives (acids or alkalines) and soil
323 characteristics.
324 Heavy metals removal was not proportional to the increase in the concentration of biosurfactant,
325 remaining around 30% for copper and lead as well as 7% for zinc at the concentrations used (1/2 x
326 CMC, CMC and 2 x CMC). As seen, zinc was not removed efficiently by the biosurfactant solutions.
327 This metal had an affinity for the acid, which removed 30 to 40% of the metals when combined with
328 the biosurfactant.
329 Doong et al. (1998) reported heavy metals removal increasing linearly with surfactants increase at
330 concentrations below CMC and remained relatively constant at concentrations above CMC and
331 depended the type of surfactant, the metals involved and type of soil. The high concentration necessary
332 in some experiments is most often related to biosurfactants' sorption or bonding to the components of
333 the soil particles (Wang and Mulligan, 2009). The acid removed 50 to 60% of the metals adsorbed to
334 the soil when used alone and this removal percentage increased significantly when the acid was
335 combined with the solutions of the isolated biosurfactant and cell-free broth. The base removed
336 approximately 15% of the metals and generally increased the percentage of copper and lead removal
337 by the biosurfactant, although it had no positive effect on the removal of zinc when combined with the
338 biosurfactant solutions. The combination of the base and acid together when used with the biosurfactant
339 was also not favourable to the removal of the heavy metals. The results suggest that neutralisation of
340 the positive effect of the acid occurred when the base was added to the biosurfactant-acid solutions. It
341 should be stressed that treatment with both alkaline and acidic components may reduce soil fertility
342 and change it chemical composition (Sarubbo et al., 2015). Hong et al. (2002) mentioned that Na^+ may
343 compete with heavy metals binding to the surfactant and forming Na-surfactant complexes. This may
344 also be the case when using biosurfactant, thereby diminishing metal removal when NaOH used
345 compared to the use of acid. However, França et al. (2015) reported higher Zn, Cr and Cu removal

rates when NaOH was added to a biosurfactant solution derived from *B. subtilis*. A possible explanation for this would be the increase in the solubility of the biosurfactant in the presence of NaOH.

The cell-free crude extract removed 30-40% of lead and copper from the, indicating that the crude biosurfactant could be used in the treatment of heavy metal contaminated soils. This would be considerable advantage, since the downstream process to purify biosurfactant obtained through fermentation could account for 60% of the production cost (Dahrazma and Mulligan, 2007).

The possibility of using biosurfactants for the removal of heavy metals has been shown in laboratory studies (Mulligan et al., 2001). A 4% solution of *Torulopsis bombicola* derived sophorolipids removed 3% of the Cu ions from soil samples and did not remove Zn. The addition of 1% of NaOH to the 4% sophorolipid solution, lead to an increase to 36 and 7% Cu and Zn removal, respectively. The highest removal occurred when 0.7% HCl was added to the 4% sophorolipid solution, achieving Cu and Zn removal rates of 37 and 16%, respectively. Rhamnolipid extracts from *Pseudomonas aeruginosa* in comparison removed 35 and 20% Cu and Zn ions, respectively, when used at a concentration of 12%, whereas a concentration of 2% was able to to remove 10 and 5% Cu and Zn, respectively. The addition of 1% NaOH to the 2% surfactant solution led to a significant increase in copper removal from 10 to 28%, but a reduction in zinc removal from 5 to 3%. Mulligan et al. (1999) reported an increase in Zn removal when a 2% surfactin solution was used in combination with an alkaline, whereas Cu removal was unaffected by the presence of NaOH. The combination of the base with a 0.5% rhamnolipid solution favoured the removal of both metals (65 and 18%) in comparison to the base alone. On the other hand, 100% copper and zinc removal were achieved with 0.7% HCl in a 4% sophorolipid solution. Heavy metals removal from soil using a saponin (0.1 to 10%) was reported to be proportional to its concentration (Hong et al., 2002). Daharazma and Mulligan (2007) also reported heavy metal removal from soil increasing linearly with increased rhamnolipid concentration used and that a 5% solution removed 37% of Cu, 7.5% of Zn and 33.2% of Ni. This is similar to our results which showed metals removal. The addition of a 0.5% rhamnolipid solution to NaOH increased the removal of copper to 28.3% and Ni to 11.5% in comparison to removal rates achieved with the base alone (1% NaOH). Recently, the biosurfactant from *C. sphaerica* showed removal rates of 95, 90 and 79% for Fe, Zn and Pb, respectively, from soil samples collected from an automotive battery industry. The addition of HCl increased the metal removal rate when used with biosurfactant solutions at 0.1 and 0.25% (Luna et al., 2016).

3.5 Biosurfactants' Ability to Bind with Heavy Metals in Aqueous Solution

The biosurfactant treatment of waste water contaminated with heavy metals was tested using conductivity measures. The conductivity of the biosurfactant solution at half the CMC was de 178 µS/cm and increased to 190 and 198 µS/cm at the CMC and twice the CMC, respectively. This increase was due to the anionic nature of the surface active agent. However, conductivity of the solutions containing cadmium (Cd) and lead (Pb) underwent an accentuated reduction upon the addition of the biosurfactant to the metal solutions due to the chelation/precipitation of the positively charged metals, thereby reducing metal ions in solution and consequently reducing its conductivity (Table 5).

The results also demonstrate the efficiency of the biosurfactant at the lowest concentration tested (1/2 x CMC), as only little variation in the conductivity of the metal solutions occurred at the higher concentrations (CMC and 2 x CMC). These results indicate that more micelles led to fewer free ions and conductivity was therefore much less than in the solutions with the absence of biosurfactant.

3.6 Effect of Environmental Factors on Formulated Biosurfactant Activity

To offer a commercial surfactant agent, the biosurfactant was formulated and its properties (surface tension, which allows the breakdown of an oil spill, and emulsification, which allows a blend in the

form of droplets, to facilitate biodegradation by microorganisms) were evaluated over a 120-day period, thereby estimating the shelf life of the proposed product. Potassium sorbate, a widely used a preservative that inhibits the growth of mould, was added to the biosurfactant at the same concentration used in foods.

The formulated biosurfactant with potassium sorbate was analysed at different pH values and temperatures as well as different salt concentrations (Tables 6 to 9). The surface tension of the formulated biosurfactant generally exhibited a small, gradual increase throughout the 120 days of storage in the presence of NaCl with variations in pH and temperature. As the change in tension did not surpass 10 units under the conditions tested, one may presume that the formulation with potassium sorbate did not cause significant changes in the tensioactive capacity of the biomolecule, indicating the possibility of using the biosurfactant under specific environmental conditions of pH, temperature and salinity.

The emulsification index values in the presence of NaCl, showed some improvement with increase storage time especially after 30 days. Higher salt concentrations had no negative effect on the action of the biosurfactant showing ability to use in saline environments.

The emulsification of corn oil remained practically stable with the change in pH values throughout the storage time, whereas the emulsification of motor showed slight increase after 30 days, demonstrating that the interaction between the biosurfactant and oil may be strengthened over time, indicating greater stability of the inter-molecular bonds.

The findings demonstrated that it was possible to formulate a product that remains free of contamination and maintains stability and can be commercialised as an efficient, low-cost, biodegradable agent for use by different industries. The results regarding the formulation of the biosurfactant are difficult to discuss, as the literature on this topic is scarce. Another biosurfactant from *C. bombicola* produced and formulated in our laboratories showed similar results regarding stability (Freitas et al., 2016). Some studies describe the use of spray drying for the conservation of biosurfactants and later application (Saeki et al., 2009). Spray drying has also been effective in the recovery and concentration of a biosurfactants while maintaining their surface activity and the dry product maintained its characteristics and activity during storage at room temperature throughout a 120 day evaluation period (Barcelos et. al., 2014).

3.7 Toxicity of Formulated Biosurfactant

The fish *Poecilia vivipara* belongs to the family *Poecilidae*, which occurs from the United States to Argentina. This species has been used as a bioindicator in the monitoring of aquatic environments due to its sensitivity and response capacity to environmental pollutants (Breseghelo et al., 2004). Acute toxicity tests were conducted with this fish to determine the mean lethal concentration (LC_{50}) of the formulated biosurfactant over a 96-hour period. The biosurfactant formulated from *C. lipolytca* was considered to have low toxicity, as the *P. vivipara* survival rate was respectively 70, 75 and 95% for biosurfactant/seawater dilutions of 1:1, 1:2 and 1:5 (v/v).

4 Conclusion

The present findings demonstrate that industrial waste products can be successfully used in the production of surfactant agents with broad applications in the environmental remediation of organic and inorganic pollutants. Biosurfactant from *C. lipolytica* presented satisfactory results regarding the treatment of sites contaminated with petroleum products and heavy metals. The possibility of commercialising an agent with long-term stability was also demonstrated, making the production process and application of biosurfactants more viable in the current market of chemical surfactants derived from petroleum.

436 **Author Contributions statement**

437 All authors contributed in this work. Leonie Sarubbo conceived and designed the experiments;
438 Danyelle Kadhydja Santos, Ana Helena M. Resende, Darne G. Almeida and Rita de Cássia F. Soares
439 da Silva performed the experiments; Ibrahim M. Banat, Raquel D. Rufino and Juliana M. Luna
440 analyzed the data and contributed to analysis tools; Leonie A. Sarubbo and Ibrahim M. Banat wrote
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451 **Declaration of interest**

452 The authors declare that the research was conducted in the absence of any commercial or financial
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454 **References**

455 Akhtar, M., Lentz, M.J., Banchette, R.A., and Kirk, T.K. (1997). Corn steep liquor lowers the amount
456 of inoculum for biopulping. *TAPPI J.*, 80, 161–164.

457 Albuquerque, C.F., Luna-Finkler, C.L., Rufino, R.D., Luna, J.M., Menezes, C.T.B., Santos, V.A.,
458 and Sarubbo, L.A. (2012). Evaluation of biosurfactants for removal of heavy metal ions from
459 aqueous effluent using flotation techniques. *Int. Rev. Chem. Eng.* 4, 156–161.

460 Asçi, Y., Nurbas, M., and Açikel, Y. (2008). A comparative study for the sorption of Cd(II) by soils
461 with different clay contents and mineralogy and the recovery of Cd(II) using rhamnolipid
462 biosurfactant. *J. Hazard. Mater.* 154, 663–673. http://dx.doi.org/10.1016/j.jhazmat.2007.10.078

463 Associacão Brasileira de Normas Técnicas (ABNT) Brazilian Association of Technical Standards,
464 NBR8492: Tijolo maciço de solo-cimento: Determinação da Resistência à Compressão e da
465 Absorção de Água, Método de Ensaio, Rio de Janeiro, Brasil, 1982.

466 Bai, G., Brusseau, M.L., and Miller, R.M. (1997). Biosurfactant-enhanced removal of residual
467 hydrocarbon from soil. *J. Contam. Hydrol.* 25, 157–170. http://dx.doi.org/10.1016/S0169-
468 7722(96)00034-4

469 Banat, I.M., Satpute, S.K., Cameotra, S.S., Patil, R., and Nyayanit, N. (2014). Cost effective
470 technologies and renewable substrates for biosurfactants' production. *Front. Microbiol.* 5.
471 http://dx.doi.org/10.3389/fmicb.2014.00697.

472 Barcelos, G.S., Dias, L.C., Fernandes, P.L., Fernandes, R.C.R., Borges, A.C., Kalks, K.H.M., and
473 Tótola, M.R. (2014). Spray drying as a strategy for biosurfactant recovery, concentration and
474 storage. *Springer Plus,* 3, 49. http://dx.doi.org/10.1186/2193-1801-3-49

475 Barros, F.F.C., Quadros, C.P., Marostica, M.R., and Pastore, G.M. (2007). Surfactina: Propriedades
476 químicas, tecnológicas e funcionais para aplicações em alimentos. *Quím. Nova,* 30, 1–14.
477 http://dx.doi.org/10.1590/S0100-40422007000200031

478 Breseghelo, L., Cardoso, MP., Borges-De-Oliveira, R., Costa, MF., Barreto JCB., Saboïa-Morais,
479 SMT., and Yamada, AT. (2004). Effects of sodium fluoride in gill epithelium of Guppy fish
480 (*Poecilia vivipara*). *Brazilian J. Veter. Res. Animal Sci.* 41, 274-280.

481 Brochu, C., Polletier, E., Caron, G., and Desnoyers, J.E. (1986/87). Dispersion of crude oil in
482 seawater: The role of synthetic surfactants. *Oil Chem. Pol.* 3, 257-279.

483 Burghoff, B. (2012). Foam fractionation applications. *J. Biotechnol.* 161, 126-137.
484 http://dx.doi.org/10.1016/j.jbiotec.2012.03.008

485 Campos, J.M., Stamford, T.L.M., Sarubbo, L.A., Luna, J.M., Rufino, R.D., and Banat, I.M. (2013).
486 Microbial biosurfactants as additives for food industries. *Biotechnol. Prog.* 29, 1097-1108.
487 http://dx.doi.org/10.1002/btpr.1796

488 Cardinal, E.V., and Hedrick, L.R. (1948). Microbiological assay of corn steep liquor for amino acid
489 content. *J. Biol. Chem.* 172, 609–612.

490 Coimbra, C.D., Rufino, R.D., Luna, J.M., and Sarubbo, L.A. (2009). Studies of the cell surface
491 properties of *Candida* species and relation with the production of biosurfactants for environmental
492 applications. *Cur. Microbiol.* 58, 245-249. http://dx.doi.org/10.1007/s00284-008-9315-5

493 Cooper, D.G., and Goldenberg, B.G. (1987). Surface active agents from two *Bacillus* species. *Appl.*
494 *Environ. Microbiol.* 53, 224-229.

495 Dahrazma, B., and Mulligan, C.N. (2007). Investigation of the removal of heavy metals from
496 sediments using rhamnolipid in a continuous flow configuration. *Chemosphere*, 69, 705-711.
497 http://dx.doi.org/10.1016/j.chemosphere.2007.05.037.

498 Das, P., Mukherjee, S., and Sen, R. (2009). Antiadhesive action of a marine microbial surfactant.
499 *Coll. Surf. B Biointerfaces*, 71, 183–186. http://dx.doi.org/10.1016/j.colsurfb.2009.02.004

500 Doong, R.A., Wu, Y.W., and Lei, W.G. (1998). Surfactant enhanced remediation of cadmium
501 contaminated soils. *Water Sci. Technol.* 37, 65–71. http://dx.doi.org/10.1016/S0273-
502 1223(98)00235-2

503 França, Í.W.L., Lima, A.P., Lemos J.A.M., Lemos, C.G.F., Melo, V.M.M., Sant'ana, H.B., and
504 Goncalves, L.R.B. (2015). Production of a biosurfactant by *Bacillus subtilis* ICA56 aiming
505 bioremediation of impacted soils. *Catal. Today*, 255, 10-15.
506 http://dx.doi.org/10.1016/j.cattod.2015.01.046.

507 Freitas, B.G., Brito J.G.M., Brasileiro, P.P.F., Rufino, R.D., Luna, J.M., Santos, V.A., and Sarubbo,
508 L.A. (2016). Formulation of a commercial biosurfactant for application as a dispersant of

petroleum and by-products spilled in oceans. *Frontiers Microbiol.* http://dx.doi.org/10.3389/fmicb.2016.01646

Holakoo, L. (2001). On the capability of Rhamnolipids for oil spill control of surface water. Master in Applied Science, Concordia University, Montreal, Canada.

Hong, K.J., Tokunaga, S., and Kajiuchi, T. (2002). Evaluation of remediation process with plant-derived biosurfactant for recovery of heavy metals from contaminated soils. *Chemosphere*, 49, 379-387. http://dx.doi.org/10.1016/S0045-6535(02)00321-1.

Ilori, M.O., Amobi, C.J., and Odocha, A.C. (2005). Factors affecting biosurfactant production by oil degrading *Aeromonas* spp. isolated from a tropical environment. *Chemosphere*, 61, 985–992. http://dx.doi.org/10.1016/j.chemosphere.2005.03.066

Jain, R.M., Mody, K., Mishra, A., and Jha, B. (2012). Physicochemical characterization of biosurfactant and its potential to remove oil from soil and cotton cloth, *Carbohyd. Pol.* 89, 1110-1116. http://dx.doi.org/10.1016/j.carbpol.2012.03.077.

Juwarkar, A.A., Nair, A., Dubey, K.V., Singh, S., and Devotta, S. (2007). Biosurfactant technology for remediation of cadmium and lead contaminated soils. *Chemosphere*, 68, 1996-2002. http://dx.doi.org/10.1016/10.1016/j.chemosphere.2007.02.027

Luna, J.M., Santos Filho, A.S., Rufino, R.D., and Sarubbo, L.A., (2016). Production of biosurfactant from *Candida bombicola* URM 3718 for environmental applications. *Chem. Eng. Transactions*, 49, 583-588. http://dx.doi.org/10.3303/CET1649098.

Maity, J.P., Huang, Y.M., Fan, C.-W., Chen, C.-C., Li, C.-Y., Hsu, C.-M., Chang, Y.-F., Wu, C.-I., Chen, C.-Y., and Jean, J.-S. (2013**).** Evaluation of remediation process with soapberry derived saponin for removal of heavy metals from contaminated soils in Hai-Pu Taiwan. *J. Environ. Sci.*, 25, 1180–1185. http://dx.doi.org/10.1016/S1001-0742(12)60162-4

Marchant, R., and Banat, I.M. (2012). Biosurfactants: a sustainable replacement for chemical surfactants? *Biotechnol. Lett.* 34, 1597-605. http://dx.doi.org/10.1007/s10529-012-0956-x

Menezes, C.T.B., Barros, E.C., Rufino, R.D., Luna, J.M., and Sarubbo, L.A. (2011). Replacing synthetic with microbial surfactants as collectors in the treatment of aqueous effluent produced by acid mine drainage using the dissolved air flotation technique. *Appl. Biochem. Biotechnol.* 163, 540–546. http://dx.doi.org/10.1007/s12010-010-9060-7

Mukherjee, S., Das, P., and Sen, R. (2007). Towards commercial production of microbial surfactants. *Trends Biotechnol.* 24, 509-515. http://dx.doi.org/10.1016/j.tibtech.2006.09.005

Mulligan, C.N., Yong, R.N., and Gibbs, B.F. (2001). An evolution of technologies for the heavy metal remediation of dredged sediments. *J. Haz. Mat.* 85, 145-163. http://dx.doi.org/10.1016/S0304-3894(01)00226-6.

Mulligan, C.N., Yong, R.N., and Gibbs, B.F. (1999). Metal removal from contaminated soil and sediments by the biosurfactant surfactin. *Environ. Sci. Technol.* 33, 3812–3820. http://dx.doi.org/10.1021/es9813055

National Research Council, Committee on Understanding Oil Spill Dispersants, Oil Spill Dispersants: Efficacy and Effects, Ocean Studies Board, The National Academies Press, Washington, DC, 2005.

Ochoa-Loza, F.J., Noordman,W.H., Jannsen, D.B., Brusseau, M.L., and Maier, R.M. (2007). Effect of clays, metal oxides, and organic matter on rhaminolipid biosurfactant sorpition by soil. *Chemosphere*, 66, 1634–1642. http://dx.doi.org/10.1016/j.chemosphere.2006.07.068

Pacwa-Plociniczak, M., Plaza, G.A., Piotrowska-Seget, Z., and Cameotra, S.S. (2011). Environmental applications of biosurfactants: Recent advances. *Int. J. Mol. Sci.* 13, 633–654. http://dx.doi.org/1422-0067/12/1/633

Rufino, R.D., Luna, J.M., Marinho, P.H.C., Farias, C.B.B., Ferreira, S.R.M., and Sarubbo, L.A. (2013). Removal of petroleum derivative adsorbed to soil by biosurfactant Rufisan produced by *Candida lipolytica. J. Petr. Sci. Eng.*109, 117–122. http://dx.doi.org/10.1016/j.petrol.2013.08.014

Saeki, H., Sasaki, K.M., Komatsu, O., Miura, A. and Matsuda, H. (2009). Oil spill remediation by using the remediation agent JE1058BS that contains a biosurfactant produced by *Gordonia* sp. strain JE-1058. *Biores. Technol.* 100, 572-577. http://dx.doi.org/10.1016/j.biortech.2008.06.046.

Santos, D.K.F., Brandao, Y.B., Rufino, R.D., Luna, J.M., Salgueiro, A.A., Santos, V.A., and Sarubbo, L.A. (2014). Optimization of cultural conditions for biosurfactant production from *Candida lipolytica. Biocatal. Agr. Biotechnol.* 3, 48-57. http://dx.doi.org/10.1016/j.bcab.2014.02.004

Santos, D.K.F., Rufino, R.D., Luna, J.M., Santos, V.A., Salgueiro, A.A., Sarubbo, L.A. (2013). Synthesis and evaluation of biosurfactant produced by *Candida lipolytica* using animal fat and corn steep liquor. *J. Pet. Sci. En.* 105, 43–50. http://dx.doi.org/10.1016/j.petrol.2013.03.028

Santos, D.K.F., Rufino, R.D., Luna, J.M., Santos, V.A., and Sarubbo, L.A. (2016). Biosurfactants: Multifunctional Biomolecules of the 21st Century. *Int. J. Mol. Sci.* 17, 401. http://dx.doi.org/10.3390/ijms17030401

Sarubbo, L.A., Rocha Júnior, R.B., Luna, J.M., Rufino, R.D., Santos, V.A., and Banat, I.M. (2015). Some aspects of heavy metals contamination remediation and role of biosurfactants. *Chem. Ecol.* 31, 707–723. http://dx.doi.org/10.1080/02757540.2015.1095293

Silva, R.C.F.S., Almeida, D.G., Luna, J.M., Rufino, R.D., Santos, V.A., and Sarubbo, L.A. (2014). Applications of biosurfactants in the petroleum industry and the remediation of oil spills. *Int. J. Mol. Sci.* 15, 12523–12542. http://dx.doi.org/10.3390/ijms150712523

Sorial, G.A., Venosa, A.D., Koran, K.M., Holder, E., and King, D.W. (2004). Oil spill dispersant effectiveness protocol: I. Impact of operational variables. *J. Environ. Eng.-ASCE*, 130, 1073–1084. http://dx.doi.org/10.1061/(ASCE)0733-9372(2004)130:10(1073)

Souza, E.C., Vessoni-Penna, T.C., and Oliveira, R.P.S. (2014). Biosurfactant-enhanced hydrocarbon bioremediation: An overview. *Int. Biodet. Biodegr.* 89, 88-94. http://dx.doi.org/10.1016/j.ibiod.2014.01.007

583 Wang, S., and Mulligan, C.N. (2009). Rhamnolipid biosurfactant – enhanced soil flushing for the
584 removal of arsenic and heavy metals from mine tailings. *Process Biochem.* 44, 296-301.
585 http://dx.doi.org/10.1016/j.procbio.2008.11.006

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Table 1. Motor oil dispersion by biosurfactant from *C. lipolytica* cultivated in distilled water supplemented with 5% animal fat and 2.5% corn steep liquor using beaker-washing method.

Biosurfactant solution	Classification
Crude biosurfactant (cell-free broth)	C[1]
Isolated biosurfactant at ½ x CMC	C[1]
Isolated biosurfactant at CMC	B[1]
Isolated biosurfactant at 2 x CMC	A[1]

[1]A: 100% oil dispersion; B: 75% oil dispersion; C: 50% oil dispersion; D: 25% oil dispersion; E: no dispersion

Table 2. Evaluation of biosurfactant from *C. lipolytica* cultivated in distilled water supplemented with 5% animal fat and 2.5% corn steep liquor as oil spill dispersant (data expressed as mean ± standard deviation).

Biosurfactant/oil ratio	Dispersion index (%)			
	Biosurfactant (1/2 x CMC)	Biosurfactant (CMC)	Biosurfactant (2 X CMC)	Crude biosurfactant
1:1	5.01 ± 0.4	15.5 ± 0.6	50.0 ± 0.7	41.0 ± 0.2
1:2	2.06 ± 0.6	6.06 ± 0.5	22.0 ± 0.1	20.0 ± 0.5
1:10	2.02 ± 0.8	3.00 ± 0.1	5.70 ± 0.6	3.50 ± 0.4
1:20	1.00 ± 0.7	2.00 ± 0.3	2.70 ± 0.5	2.40 ± 0.7

Table 3. Removal of motor oil from contaminated cotton cloth by biosurfactant from *C. lipolytica* cultivated in distilled water supplemented with 5% animal fat and 2.5% corn steep liquor using beaker washing method (data expressed as mean ± standard deviation).

Washing solutions	**Removal (%)**
Distilled water (control)	04.10 ± 0.4
Crude biosurfactant (cell-free broth)	36.00 ± 0.5
Isolated biosurfactant at ½ x CMC	30.20 ± 0.7
Isolated biosurfactant at CMC	48.09 ± 0.4
Isolated biosurfactant at 2 x CMC	70.30 ± 0.6
Commercial detergent	28.07 ± 0.3
Isolated biosurfactant at CMC + commercial detergent at CMC (1:1, v/v)	32.45 ± 0.7

Table 4. Removal of heavy metals contained in contaminated standard sand by washing solutions (data expressed as mean ± standard deviation).

Treatment	**Removal (%)**		
	Cu	**Pb**	**Zn**
Distilled water (control)	17 ± 1.3	11 ± 1.3	15 ± 1.5
1% NaOH solution	11 ± 2.1	16 ± 0.8	15 ± 1.0
0.7% HCl solution	60 ± 1.4	54 ± 1.5	50 ± 1.3
Cell-free broth	40 ± 1.8	30 ± 1.5	7.1 ± 1.5
Cell-free broth + 0.7% HCl	81 ± 1.6	78 ± 1.8	39 ± 1.9
Cell-free broth + 1% NaOH	53 ± 1.4	49 ± 1.5	5.4 ± 1.7
Cell-free broth + 1% NaOH + 0.7% HCl	40 ± 1.2	30 ± 1.4	5.3 ± 1.3
0.04% biosurfactant solution (1/2 x CMC)	30 ± 1.4	35 ± 1.7	7.1 ± 1.1
0.04% biosurfactant solution (1/2 x CMC) + 0.7% HCl	81 ± 2.0	80 ± 1.1	40 ± 1.3
0.04% biosurfactant solution (1/2 x CMC) + 1% NaOH	39 ± 2.0	40 ± 1.3	6.2 ± 1.1
0.04% biosurfactant solution (1/2 x CMC) + 0.7% HCl + 1% NaOH	38 ± 1.7	47 ± 1.4	5.1 ± 1.3
0.08% biosurfactant solution (CMC)	31 ± 1.4	33 ± 1.6	7.6 ± 1.4
0.08% biosurfactant solution (CMC) + 0.7% HCl	81 ± 0.8	82 ± 1.5	30 ± 1.2
0.08% biosurfactant solution (CMC) + 1% NaOH	45 ± 2.1	33 ± 1.4	6.2 ± 1.2
0.08% biosurfactant solution (CMC) + 0.7% HCl + 1% NaOH	49 ± 1.5	31 ± 1.8	5.1 ± 1.1
0.16% biosurfactant solution (2 x CMC)	30 ± 1.5	35 ± 1.5	6.3 ± 1.4
0.16% biosurfactant solution (2 x CMC)+ 0.7% HCl	70 ± 1.6	65 ± 1.5	29 ± 1.2
0.16% biosurfactant solution (2 x CMC) + 1% NaOH	45 ± 1.7	40 ± 1.7	5.1 ± 1.8
0.16% biosurfactant solution (2 x CMC)+ 0.7% HCl + 1% NaOH	50 ± 1.9	45 ± 2.1	6.5 ± 1.5

776 Table 5. Conductivity of metal solutions before and after washing with solutions of biosurfactant
777 isolated from *C. lipolytica.*
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Heavy metal	Conductivity (µS/cm) of metal solution	Conductivity (µS/cm) after treatment with biosurfactant solutions		
		1/2xCMC	CMC	2xCMC
Cd	510.4	15.40	15.28	12.74
Pb	670.4	21.00	21.32	21.83

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Table 6. Surface tension of biosurfactant formulated with potassium sorbate (0.2%) over 120 days with changes in pH and temperature as well as in different concentrations of NaCl (data expressed as mean ± standard deviation).

Time (days)	Surface tension (mN/m)							
	NaCl (%)			pH			Temperature (ºC)	
	1	3	5	5	7	9	40	50
0	26 ± 1.0	27 ± 1.2	27 ± 1.0	27 ± 1.8	28 ± 1.1	28 ± 1.0	26 ± 1.5	27 ± 1.1
15	33 ± 1.1	34 ± 1.0	28 ± 1.2	33 ± 1.3	33 ± 1.4	35 ± 1.3	27 ± 0.9	29 ± 1.3
30	33 ± 1.3	33 ± 1.5	30 ± 1.5	35 ± 1.3	35 ± 1.0	35 ± 1.2	30 ± 1.5	30 ± 0.9
45	33 ± 1.9	33 ± 1.4	33 ± 1.0	35 ± 1.5	35 ± 1.3	37 ± 1.6	32 ± 1.3	29 ± 1.1
90	35 ± 1.0	33 ± 1.0	33 ± 1.3	35 ± 1.4	37 ± 0.9	37 ± 0.5	32 ± 1.1	29 ± 1.2
120	35 ± 1.5	33 ± 1.1	32 ± 1.2	35 ± 1.1	39 ± 1.1	40 ± 1.0	33 ± 1.3	35 ± 1.1

Table 7. Emulsification of motor and corn oil by biosurfactant formulated with potassium sorbate (0.2%) over 120 days with different concentrations of NaCl (data expressed as mean ± standard deviation).

Time (days)	Emulsification (%)					
	1% NaCl		3% NaCl		5% NaCl	
	Motor oil	Corn oil	Motor oil	Corn oil	Motor oil	Corn oil
0	50 ± 3.0	37 ± 2.1	60 ± 1.9	40 ± 3.5	50 ± 2.3	45 ± 2.0
15	60 ± 2.5	38 ± 2.5	80 ± 2.8	44 ± 2.0	76 ± 2.4	46 ± 2.8
30	88 ± 2.0	50 ± 2.4	85 ± 3.0	47 ± 2.1	95 ± 3.2	46 ± 3.0
45	88 ± 2.0	48 ± 2.5	85 ± 2.4	54 ± 2.5	95 ± 3.0	50 ± 2.7
90	88 ± 3.0	48 ± 3.1	85 ± 2.5	54 ± 3.5	95 ± 2.5	50 ± 3.0
120	88 ± 1.5	48 ± 3.0	87 ± 2.0	54 ± 2.5	95 ± 3.0	50 ± 2.5

Table 8. Emulsification of motor and corn oil by biosurfactant formulated with potassium sorbate (0.2%) over 120 days with different pH values (data expressed as mean ± standard deviation).

Time (days)	Emulsification (%)					
	pH 5		pH 7		pH 9	
	Motor oil	Corn oil	Motor oil	Corn oil	Motor oil	Corn oil
0	80 ± 3.1	45 ± 2.0	50 ± 1.5	50 ± 1.1	50 ± 2.7	45 ± 3.0
15	85 ± 2.1	45 ± 1.8	55 ± 2.5	50 ± 2.5	50 ± 2.3	45 ± 2.3
30	88 ± 2.5	45 ± 2.7	100 ± 1.0	55 ± 2.1	95 ± 2.2	45 ± 1.8
45	88 ± 1.8	55 ± 3.1	100 ± 1.0	55 ± 1.8	95 ± 2.3	45 ± 2.5
90	88 ± 2.3	55 ± 2.2	100 ± 0.5	50 ± 2.3	95 ± 2.1	45 ± 3.0
120	88 ± 1.6	55 ± 1.1	100 ± 1.0	50 ± 3.5	95 ± 1.2	45 ± 3.1

Table 9. Emulsification of motor and corn oil by biosurfactant formulated with potassium sorbate (0.2%) over 120 days with different temperatures (data expressed as mean ± standard deviation).

Time (days)	Emulsification (%)			
	40 ºC		50 ºC	
	Motor oil	Corn oil	Motor oil	Corn oil
0	50 ± 2.0	40 ± 1.7	50 ± 2.7	45 ± 3.0
15	50 ± 2.8	40 ± 1.9	60 ± 2.9	45 ± 2.8
30	55 ± 3.0	40 ± 2.4	95 ± 2.7	45 ± 2.7
45	60 ± 2.7	50 ± 2.0	95 ± 3.2	55 ± 2.0
90	60 ± 2.3	50 ± 2.8	95 ± 3.0	55 ± 1.9
120	60 ± 1.7	50 ± 3.0	95 ± 1.7	55 ± 3.0