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PRODUÇÃO E FORMULAÇÃO DE BIOSSURFACTANTE DE *Pseudomonas cepacia* UCP 6659 PARA APLICAÇÃO NA REMOÇÃO DE POLUENTES AMBIENTAIS GERADOS NA INDÚSTRIA DE PETRÓLEO

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Recife – PE

2017

RITA DE CÁSSIA FREIRE SOARES DA SILVA

Produção e formulação de biossurfactante de *Pseudomonas cepacia* UCP 6659 para aplicação na remoção de poluentes ambientais gerados na indústria de petróleo

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

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"Os esforços que dedicamos às conquistas de hoje,
são investimentos que asseguram o conforto do amanhã"
Rita Freire

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RESUMO

A busca por compostos naturais, biodegradáveis e menos tóxicos tem estimulado o desenvolvimento de produtos alternativos. Os biossurfactantes, moléculas obtidas por via microbiológica, tornaram-se uma estratégia importante na obtenção de compostos mais compatíveis com o meio ambiente. Neste sentido, foram avaliadas a produção, caracterização e aplicação de um biossurfactante obtido de *Pseudomonas cepacia* UCP 6659 na remoção de derivado de petróleo em solos e águas. Inicialmente, as condições de cultivo foram estatisticamente otimizadas através de um delineamento composto central rotacional (CCRD) quanto à influência das variáveis tempo de cultivo, concentração do inóculo e velocidade de rotação. O biossurfactante selecionado foi caracterizado quanto à estabilidade das propriedades tensoativas e toxicológicas. Investigou-se sua produção em biorreator semi-industrial de 50 L. O biossurfactante foi formulado utilizando três métodos de conservação, sendo armazenado durante 120 dias. Posteriormente, o biossurfactante foi testado na descontaminação de derivados de petróleo e em processo de biorremediação. Como resultados, os níveis mais significativos selecionados pelo (DCCR) foram 250 rpm, 60h e inóculo de 1,5 %, alcançando uma tensão superficial de 27 mN/m, um rendimento de 8,0 g/L e uma CMC de 600 mg/L. A caracterização química (FTIR, ^{1}H RMN e GC-MS) revelou sua natureza glicolipídica e aniônica. O biotensoativo apresentou eficiente atividade emulsificante em óleo de motor acima de 90 % e estabilidade frente a condições extremas (120℃, 12 % de NaCl e pH de 2 a 12). O biossurfactante foi atóxico ao microcrustáceo *Artemia salina* e as sementes de repolho (*Brassica oleracea)*. Com o aumento de escala de produção foi alcançado uma concentração de 40,5 g/L em biossurfactante, com uma tensão superficial de 29 mN/m. O biossurfactante apresentou estabilidade em todos os métodos de conservação, demonstrando ser economicamente viável para aplicação em larga escala, devido ao baixo custo de obtenção do produto formulado, estimado em torno de US $ 0,14-0,15/L, podendo ser produzido a um custo reduzido em comparação aos comercialmente disponíveis no mercado mundial. O biossurfactante demonstrou eficácia de até 75 % na recuperação de óleo residual a partir de amostras de areia saturada com óleo, dispersão de 81 % de petroderivado em água do mar e recuperação de 90 % de óleo de motor em superfície sólida. O produto formulado atingiu uma remoção de óleo em solo de 76,55 %, 84,5 % em pedras marinhas, e promoveu uma biodegradação de óleo de 70 % em água do mar. Portanto, o biossurfactante produzido por *P. cepacia* apresenta potencial para aplicação na indústria de petróleo e na descontaminação de petroderivados no ambiente.

Palavras-chave: Biossurfactante, *Pseudomonas cepacia,* Formulação, Descontaminação ambiental, Indústria de petróleo.

ABSTRACT

The search for natural, biodegradable and less toxic compounds has stimulated the development of alternative products. Biosurfactants, microbiologically obtained molecules, have become an important strategy in obtaining compounds more compatible with the environment. In this sense, we evaluated the production, characterization and application of a biosurfactant obtained from *Pseudomonas cepacia* UCP 6659 in the removal of petroleum derivative in soils and waters. Initially, the cultivation conditions were statistically optimized through a central rotational compound design (CCRD) regarding the influence of the variables culture time, inoculum concentration and rotation speed. The selected biosurfactant was characterized for the stability of the tensoactive and toxicological properties. Its production was investigated in a semi-industrial bioreactor of 50 L. The biosurfactant was formulated using three conservation methods and stored for 120 days. Subsequently, the biosurfactant was tested in the decontamination of petroleum derivatives and in bioremediation process. As results, the most significant levels selected by the DCCR were 250 rpm, 60h and inoculum of 1.5 %, reaching a surface tension of 27 mN/m, a yield of 8.0 g / L and a CMC of 600 mg / L. The chemical characterization (FTIR, ^{1}H NMR and GC-MS) revealed its glycolipid and anionic nature. The biotensoativo presented efficient emulsifying activity in motor oil above 90 % and stability against extreme conditions (120°C, 12 % NaCl and pH of 2 to 12). The biosurfactant was non-toxic to microcrack *Artemia salina* and cabbage seeds (*Brassica oleracea*). With the increase of the scale of production, a concentration of 40.5 g / L in biosurfactant was reached, with a surface tension of 29 mN/m. The biosurfactant showed stability in all conservation methods, showing that it is economically feasible for large scale application, due to the low cost of obtaining the formulated product, estimated at around US$ 0.14-0.15 / L, and can be produced At a reduced cost compared to those commercially available on the world market. The biosurfactant demonstrated efficacy of up to 75 % recovery of residual oil from samples of oil-saturated sand, 81 % dispersion of petroderivative in sea water and recovery of 90 % of motor oil on solid surface. The formulated product reached 76.55 % oil removal, 84.5 % in sea stones, and promoted 70 % oil biodegradation in sea water. Therefore, the biosurfactant produced by *P. cepacia* presents potential for application in the petroleum industry and in the decontamination of petroderivatives in the environment.

Keywords: Biosurfactant, *Pseudomonas cepacia*, Formulation, Environmental decontamination, Oil industry.

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^{1}H NMR – Proton Nuclear Magnetic Resonance

ABNT – Associação Brasileira de Normas Técnicas

ANEEL – Agência Nacional de Energia Elétrica

ANOVA – *Analysis Of Variance*

APHA – *American Public Health Association*

CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CCRD – *Central Composite Rotational Design*

CG-MS – *Gas Chromatography and Mass Spectroscopy*

CMC – Concentração Micelar Crítica

CNPq – Conselho Nacional de Desenvolvimento Científico e Tecnológico

COPPE – Instituto Alberto Luiz Coimbra de Pós-graduação e Pesquisa de Engenharia

DCCR – Delineamento Composto Central Rotacional

EOR – *Enhanced Oil Recovery*

FACEPE – Fundação de Amparo à Ciência e Tecnologia de Pernambuco

FTIR – *Fourier Transform Infrared Spectroscopy*

HPAS – Hidrocarbonetos aromáticos policíclicos

IATI – Instituto Avançado de Tecnologia e Inovação

LC_{50} – Lowest concentration that kills 50 % of tested population

MEOR – *Microbial Enhanced Oil Recovery*

MPN – *Most Probable Number*

NPCIAMB – Núcleo de Pesquisas em Ciências Ambientais

OD – *Optical Density*

PD&I – Pesquisa, Desenvolvimento e Inovação

RENORBIO – Rede nordeste de Biotecnologia

Rf – *Retention factor*

RSM – *Response Surface Methodology*

SRB – *Sulfate Reducing Bacteria*

TERMOPE – Termopernambuco

TLC – *Thin Layer Chromatography*

UNICAP – Universidade Católica de Pernambuco

SUMÁRIO

1. INTRODUÇÃO

Surfactantes são compostos anfipáticos com ambas as porções hidrofílicas e hidrofóbicas. A porção apolar é frequentemente uma cadeia de hidrocarbonetos, enquanto que a porção polar pode ser iônica (catiônica ou aniônica), não iônica ou anfotérica, que se distribuem preferencialmente entre interfaces com diferentes graus de polaridade (óleo/água ou ar/água) e pontes de hidrogênio. Estas moléculas reduzem a tensão superficial e interfacial (forças de coesão entre as moléculas), conferindo excelentes propriedades de formação de microemulsões (nas quais óleos podem ser solubilizados em água ou vice – versa), formação de espuma, dispersão e detergência, o que as tornam mais versáteis em processos químicos e industriais (CAMPOS et al., 2013; MAO et al., 2015; SANTOS et al., 2016).

A maioria dos surfactantes são produzidos quimicamente a partir de derivados do petróleo. No entanto, nos últimos anos, os problemas causados por tais agentes tensoativos sintéticos (geralmente tóxicos e difíceis de quebrar por meio da ação microbiana) têm motivado a comunidade científica a buscar surfactantes naturais, tais como os obtidos através da produção microbiana, conhecidos como biossurfactantes. Estas promissoras biomoléculas são alternativas ecologicamente corretas, as quais atendem às novas legislações de controle ambiental e as preocupações relativas ao meio ambiente (SANTOS et al., 2016; VIJAYAKUMAR; SARAVANAN, 2015).

Biossurfactantes são tensoativos de origem biológica que em sua grande parte são produzidos por vegetais, animais e micro-organismos (bactérias, leveduras e fungos), sendo geralmente classificados, de acordo com sua composição química, como glicolipídeos, lipopeptídeos, ácidos graxos, polímeros ou compostos particulados. A maioria dos biossurfactantes apresenta natureza aniônica ou neutra, enquanto que alguns dos que contêm grupos amina são catiônicos. O grupo hidrofóbico geralmente possui ácidos graxos de cadeia longa e a porção hidrofílica pode ser um açúcar, um peptídeo, aminoácido, ácido carboxílico, fosfato ou álcool (LUNA et al., 2013; RUFINO et al., 2014). Esta diversidade estrutural exibida por estas biomoléculas oferece um número de vantagens sobre os surfactantes sintéticos, tais como biodegradabilidade, compatibilidade com o ambiente, baixa toxicidade e, principalmente, atividade sob condições extremas de temperatura, pH e salinidade (KAPADIA; YAGNIK, 2013; SANTOS et al., 2013). Estas características contribuem para a aplicabilidade de biossurfactantes em diferentes setores da

indústria tais como o setor médico, farmacêutico, cosmético, mineração, metalurgia básica, agroquímico, fertilizantes, bebidas, alimentos, produtos químicos e orgânicos e especialmente na indústria de petróleo, onde são encontradas condições adversas em muitos processos (CAMPOS et al., 2013; MOHAN; JENIFER, 2014; VIJAYAKUMAR; SARAVANAN, 2015).

O papel fisiológico dos biossurfactantes ainda permanece incerto, no entanto, sabe-se que sua síntese por micro-organismos é geralmente realizada com a finalidade de aumentar a biodisponibilidade de substratos quase inacessíveis e permitir a sobrevivência em condições de pouca água ou em ambientes que contêm metais pesados e antibióticos (DZIEGIELEWSKA; ADAMCZAK, 2013). As bactérias, juntamente com as arqueobactérias, são os maiores responsáveis pela produção destes compostos tensoativos; bactérias das famílias *Pseudomonacea* e *Bacillacea* são capazes de produzir biossurfactantes eficientes na remoção de petróleo e seus derivados (SANTOS, 2012; SILVA et al., 2014). Várias bactérias do gênero *Pseudomonas*, especialmente as linhagens de *P. aeruginosa*, são amplamente conhecidas como as mais potentes produtoras de biossurfactantes. Outras espécies como *P. putida, P. fluorescens* e mais recentemente *P. cepacia*, têm se destacado como ótimas produtoras de biotensoativos a partir de resíduos industriais, os quais têm sido aplicados com sucesso em diversos segmentos do mercado (SAKTHIPRIYA et al., 2015; SILVA et al., 2013).

Muitas das aplicações dos biossurfactantes já realizadas no mercado dependem de um processo de produção mais econômico. O alto custo de produção de biossurfactantes é um fator limitante que dificulta o seu crescimento no mercado. Além disso, a escolha da fonte de carbono estabelece um papel importante no rendimento e na estrutura de emulsionantes microbianos (HELMY et al., 2011; RUFINO et al., 2014). Neste sentido, biossurfactantes podem ser produzidos a partir de resíduos industriais, tais como milhocina (ROCHA e SILVA et al., 2013), glicerol (SILVA et al., 2010), vinhaça (OLIVEIRA et al., 2013), manipueira (BARROS et al., 2008), óleo soja residual (LUNA et al., 2011), óleo de canola residual (SILVA et al., 2013), gordura animal (SANTOS et al., 2013), gordura vegetal (GUSMÃO et al., 2010) e melaço (SANTOS et al., 2010). A utilização de biossurfactantes na indústria do petróleo é economicamente vantajosa, pois requer baixas especificações de pureza, eliminando as etapas de purificação que representam quase 60% dos custos totais de produção (SARUBBO et al., 2015).

A produção de biossurfactante em larga escala apresenta-se como uma estratégia eficaz para superar a competitividade com os seus homólogos sintéticos (HELMY et al., 2011; RUFINO et al., 2014). As principais empresas de biossurfactantes no mercado mundial são Ecover, Saraya, Soliance, MG Intobio, AGAE Technologies e Jeneil Biotech e os mercados-alvo são América do Norte, Europa e Ásia (SAJNA et al., 2015).

Nos últimos anos, um aumento da consciência ambiental tem levado as indústrias a terem muito mais interesse em substituir alguns ou todos os surfactantes químicos por biossurfactantes sustentáveis (CAMPOS et al., 2013; MARCHANT; BANAT, 2012). De acordo com estudos, a Europa foi o maior mercado consumidor de biossurfactantes, com um consumo de 178,9 mil toneladas em 2013, representando mais de 50% do consumo global. A América do Norte foi o segundo maior consumidor de biossurfactantes no mesmo ano, com uma participação de mais de um quarto, enquanto que a Ásia teve um mercado relativamente pequeno em 2013, mas a previsão é que esta participação aumente de forma significativa ao longo dos próximos seis anos, devido à presença de grandes indústrias na região (GRAND VIEW RESEARCH, 2016).

A indústria de petróleo constitui o maior mercado para os biossurfactantes, os quais, por meio de mecanismos de solubilização, mobilização ou emulsificação, aumentam a área de contato de hidrocarbonetos na fase aquosa. Neste processo, maiores concentrações de biossurfactantes melhoram consideravelmente a solubilidade dos componentes hidrofóbicos, principalmente quando existe a formação de micelas. Estas biomoléculas podem ser aplicadas nas diversas etapas da cadeia de produção do petróleo, tais como na recuperação melhorada de óleo, remoção de óleos incrustados em tanques de estocagem, diminuição da viscosidade do óleo nos dutos de transporte e na biorremediação/dispersão de manchas oleosas, tanto no solo quanto no mar (JOSEPH; JOSEPH, 2009; SARUBBO et al., 2015).

Para a maximização da produção de metabólitos de interesse industrial, o emprego de ferramentas estatísticas representa uma tática eficaz para estabelecer parâmetros envolvidos no desempenho de um determinado processo fermentativo e na redução dos custos (GAO et al., 2013). Além disso, a estabilidade das propriedades do biossurfactante é um fator fundamental para sua produção em larga escala e estocagem em longo prazo, principalmente por se tratar de um produto biotecnológico que demora a ser produzido perante a urgência da aplicação em um

desastre petrolífero. Logo, a durabilidade precisa ser elevada com a finalidade de utilização imediata, uma vez que, se um bioproduto não mantem as propriedades iniciais por muitos dias, será inviável utilizá-lo periodicamente (MARCHANT; BANAT, 2012).

Considerando que o Brasil é um país essencialmente agrícola, a facilidade de acesso aos subprodutos agroindustriais é bastante significativa, motivando as pesquisas nessa área. Neste sentido, o presente trabalho teve por objetivo a produção de um biossurfactante por *Pseudomonas cepacia* CCT6659 utilizando substratos agroindustriais para estudar suas propriedades tensoativas, natureza química, toxicidade e capacidade de emulsificação e dispersão de compostos hidrofóbicos. Avaliou-se o aumento da escala de produção do biotensoativo assim como sua formulação comercial, para aplicação deste produto como agente dispersante na remoção de óleos derramados por indústrias petrolíferas e em processos de biorremediação ambiental.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Produzir e caracterizar um biossurfactante comercial obtido de *Pseudomonas cepacia* em meio de cultura de baixo custo para aplicação na remoção de derivado de petróleo em solos e águas, atendendo aos aspectos econômicos, sociais e de conservação ambiental.

2.2 OBJETIVOS ESPECÍFICOS

- Produzir o biossurfactante de *Pseudomonas cepacia* UCP 6659 em meio de cultivo pré estabelecido utilizando resíduos industriais.
- Utilizar um delineamento composto central rotacional (DCCR) na seleção das melhores condições de cultivo para maximizar a produção do biossurfactante.
- Avaliar a estabilidade do biossurfactante sob condições específicas de pH, temperatura e na presença de sal.
- Isolar o biossurfactante produzido para determinar a concentração e a sua eficiência através da tensão superficial e da Concentração Micelar Crítica (CMC).
- Caracterizar bioquimicamente e determinar a toxicidade do biossurfactante.
- Aplicar o biossurfactante na dispersão de manchas de derivado de petróleo em água do mar.
- Realizar testes de aplicação do biossurfactante na lavagem de superfície sólida contaminada com derivado de petróleo.
- Avaliar um Scale up de produção do biossurfactante em biorreator semi-industrial.
- Determinar a estabilidade de ação do biossurfactante estocado e previamente formulado para comercialização.
- Realizar uma estimativa de custo para produção e comercialização do biossurfactante formulado.
- Determinar a remoção do contaminante hidrofóbico impregnado em areia e solos pelo biossurfactante formulado em frascos e colunas empacotadas.
- Determinar a remoção do poluente hidrofóbico adsorvido em pedras marinhas.
- Avaliar o potencial do biossurfactante como agente de biorremediação.

3. REVISÃO BIBLIOGRÁFICA

3.1 Petróleo e energia

O petróleo é a principal fonte de energia, matéria-prima e insumos químicos da atualidade e tem impulsionado o desenvolvimento do mundo moderno e um intenso crescimento econômico global desde o século passado (OKOLIEGBE; AGARRY, 2012; SILVA et al., 2014). Há uma dependência atual muito grande do petróleo e de seus derivados para o atendimento das necessidades básicas de calor, energia e transporte. A demanda mundial de energia prevista indica um aumento anual de 1,7 % no número de barris de petróleo produzidos entre os anos de 2000 a 2030, enquanto que o consumo de petróleo deverá atingir 15,3 bilhões de toneladas por ano (ELRAIES; TAN, 2012; SILVA et al., 2014).

Não existe atualmente nenhuma fonte de energia disponível que possa substituir ou concorrer com o petróleo, tornando os maiores consumidores de energia dependentes daqueles países detentores das maiores reservas de petróleo (ELRAIES et al., 2012; TAN, 2012). O Departamento de Energia dos Estados Unidos (US Department of Energy) anunciou recentemente que a maior parte das fontes primárias de energia dentro dos EUA (≈83,0 %) vem dos derivados de combustíveis fósseis, da qual 57,0 % são de produtos petrolíferos. Só em 2010, 19,2 milhões de metros cúbicos de petróleo foram consumidos diariamente (SANTOS et al., 2016b).

De acordo com a Agência Internacional de Energia, em países como a China, Canadá, Venezuela, México e os EUA, óleos pesados representam aproximadamente metade de todos os recursos petrolíferos recuperáveis (CERÓN-CAMACHO et al., 2013). Considerando que boa parte deste óleo bruto (que são óleos leves e mais facilmente extraíveis nos poços de produção) estará mais limitada nos próximos anos, duas abordagens principais de exploração de petróleo estão sendo atualmente estudas:

- Maximização da eficiência de todos os estágios de produção e processamento (*upstream* e *downstream*);
- Utilização de óleo mais pesado, betume e outros componentes de areia betuminosa (SANTOS et al., 2016b).

Além disso, esforços têm sido direcionados por meio da criação de leis ao longo das décadas, para minimizar o desperdício de óleo decorrente de acidentes

envolvendo derramamentos de petróleo e subprodutos, visto que, o transporte marítimo com carga de petróleo continuará a existir (FREITAS et al., 2016).

Estima-se que entre aproximadamente 0,4 e 0,8 % de toda a produção mundial de petróleo chegue aos oceanos. Os derramamentos de navios de transporte correspondem a 98 % de todas as perdas de petróleo e seus subprodutos. Os derramamentos acidentais representam os 2 % restantes, o que contribui para a liberação de aproximadamente 400.000 toneladas de petróleo por ano para o meio ambiente. Por isso, tem havido um movimento cada vez mais constante em direção ao desenvolvimento por soluções sustentáveis de tecnologias eficientes para utilização máxima de petróleo e derivados sem desperdícios (FREITAS et al., 2016; HAZRA et al., 2012).

3.2 Biotecnologia relacionada à indústria de petróleo

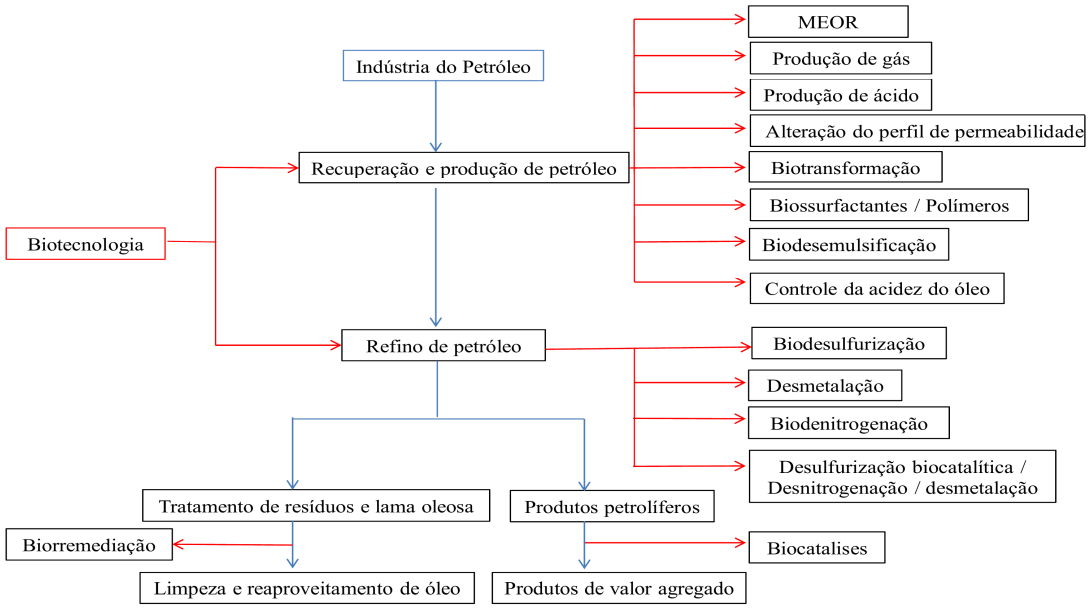
A biotecnologia do petróleo visa à implementação de processos biológicos nas etapas de exploração, produção, transformação e refino de petróleo, bem como na geração de subprodutos de maior valor agregado, além de auxiliar na diminuição e gerenciamento de efluentes industriais do petróleo, ajudando na redução final da poluição (SILVA et al., 2014).

Para resolver a estagnação em reservatórios de petróleo após um processo convencional de recuperação, utiliza-se, principalmente, métodos mecânicos, físicos e químicos. No entanto, a utilização de micro-organismos e seus produtos metabólicos capazes de transformar matérias-primas complexas em condições ambientais adversas, tais como, alta salinidade, temperaturas extremas, valores extremos de pH, pressão e hidrofobicidade para aplicações específicas tem desempenhado um papel significativo no aumento da recuperação de petróleo bruto de reservatórios esgotados (ALMEIDA et al., 2016; BANAT et al., 2010; LUNA et al., 2013).

A utilização de bioprocessos nesta indústria expandiu-se para a aplicação de técnicas emergentes como biodesulfurização, biodemetalização, biodenitrogenação e biotransformação nas áreas de refino de petróleo associadas à melhoria de combustíveis, produção de produtos químicos finos, controle de acidez durante a produção, complementando as técnicas anteriores de biotecnologia, tais como

recuperação microbiana melhorada de óleo (MEOR) e biorremediação (Figura 1) (BACHMANN et al., 2014; SINGH et al., 2012).

Figura 1 – Potenciais aplicações da biotecnologia na indústria petrolífera. Linhas azuis representam as principais etapas de processamento de petróleo, e as linhas vermelhas representam as aplicações biotecnológicas nas respectivas etapas



Fonte: ALMEIDA et al. (2016)

De todas as biotecnologias propostas acima, as que aplicam os biossurfactantes têm sido as mais promissoras e têm recebido maior atenção, já que a aplicação de biossurfactantes encontra espaço em quase todos os estágios da cadeia de produção de petróleo (ALMEIDA et al., 2016; SILVA et al., 2014).

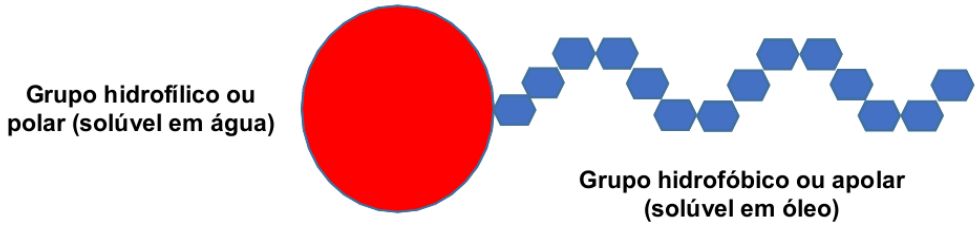
3.3 Biossurfactantes

Os avanços das tecnologias sustentáveis têm impulsionado a busca por compostos naturais e biodegradáveis para tratar locais contaminados por hidrocarbonetos. Isto levou à descoberta de surfactantes de origem natural, obtidos a partir de organismos vivos, tais como, saponinas produzidas por plantas, sais biliares produzidos por animais e glicolipídeos produzidos por micro-organismos. Todos estes

compostos com propriedades surfactantes são denominados biossurfactantes (SOUZA et al., 2014).

Biossurfactantes são moléculas anfipáticas, com porções hidrofóbicas e hidrofílicas, que atuam entre fluidos com diferentes polaridades (óleo/água), permitindo acesso aos substratos hidrofóbicos e causando uma redução da tensão superficial, um aumento na área de contato de compostos insolúveis e uma melhor mobilidade, biodisponibilidade e biodegradação de tais compostos (SILVA et al., 2014). A porção hidrofóbica pode ser uma proteína ou um peptídeo com uma porcentagem elevada de aminoácidos contendo cadeias laterais hidrofóbicas ou uma cadeia de hidrocarboneto de um ácido graxo com 10 a 18 átomos de carbono, embora ácidos graxos com pesos moleculares mais elevados também já tenham sido relatados. A porção hidrofílica, que categoriza o biossurfactante, pode ser um éster, um grupo hidróxi, fosfato, grupo carboxilato ou açúcar (Figura 2) (CAMPOS et al., 2013).

Figura 2 – Molécula de biossurfactante com porções apolares (hidrofóbicas) e polares (hidrofílicas)



Fonte: Autora da tese, 2017

Os biossurfactantes são geralmente classificados como moléculas de baixa massa molecular, mais eficientes na redução das tensões superficiais e interfaciais, e compostos de alto massa molecular, que são mais eficazes como agentes de estabilização de emulsões. As principais classes de biossurfactantes de baixo peso molecular são glicolipídeos, lipopeptídeos e fosfolipídeos, ao passo que os biossurfactantes de alto peso molecular incluem os biossurfactantes poliméricos em partículas (KAPADIA; YAGNIK, 2013).

3.4 Propriedades e principais características dos biossurfactantes

Os biossurfactantes são verdadeiros sabões biológicos que atuam como agentes facilitadores da formação de emulsões devido à capacidade de reduzir a tensão interfacial entre duas fases distintas, estabilizando subsequentemente a emulsão formada. Apesar da diversidade de composição química, várias características e propriedades são comuns à maioria dos biossurfactantes. Muitas destas características singulares oferecem uma série de vantagens sobre os surfactantes químicos, fornecendo novas possibilidades para aplicações industriais e ambientais (SANTOS et al., 2016b) sendo elas:

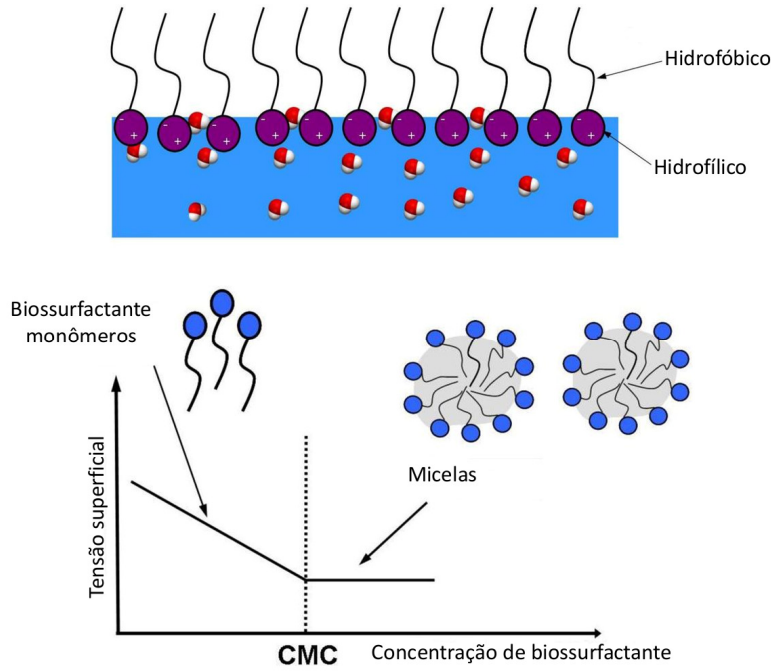
1. Biodegradabilidade: diferentes dos surfactantes químicos, os biossurfactantes são facilmente biodegradáveis na água e no solo, o que os tornam adequados para aplicações como biorremediação e tratamento de resíduos (SANTOS et al., 2016a).

2. Obtenção de biossurfactantes a partir de resíduos industriais: esta é uma das grandes vantagens que tem dois benefícios, um dos quais é a diminuição dos custos de produção e o outro é o reaproveitamento de resíduos que poderiam alcançar o ambiente. Países essencialmente agrícolas como o Brasil, possibilitam o desenvolvimento de tecnologias que incorporem resíduos agroindustriais no processo produtivo de biossurfactantes (SILVA et al., 2014).

3. Atividade superficial e interfacial: os biossurfactantes são mais eficientes e mais efetivos do que os surfactantes convencionais (detergentes aniônicos sulfatados), uma vez que produzem menores tensões superficiais com menores concentrações de biossurfactante (SANTOS et al., 2016b). A tensão superficial é a força de atração existente entre as moléculas dos líquidos. A tensão superficial diminui à medida que a concentração de biossurfactantes no meio aquoso aumenta, até o ponto em que ocorre a formação de micelas, atingindo, neste momento a Concentração Micelar Crítica (CMC), que corresponde à mínima concentração de biossurfactante necessária para que a tensão superficial seja reduzida ao máximo. As moléculas anfipáticas, a partir desta concentração, formam agregados com as porções hidrofílicas posicionadas para a parte externa da molécula e as porções hidrofóbicas para a parte interna (PACWA-PLOCINICZAK et al., 2011). A eficiência é uma

característica básica essencial que determinam se um surfactante é efetivo, a qual é medida através da CMC (Figura 3).

Figura 3 – Relação entre a concentração de biossurfactante, tensão superficial e formação de micelas



Fonte: PACWA-PŁOCINICZAK et al. (2011)

4. Tolerância a condições extremas de temperatura, pH e salinidade: vários biossurfactantes apresentam elevada estabilidade em diferentes valores de temperature e pH, podendo ser utilizados em ambientes adversos. O biossurfactante da levedura *Candida lipolytica* UCP0988 mostrou-se estável após tratamento com temperaturas chegando a 120 ℃, bem como propriedades quase inalteradas a uma faixa de pH entre 2 e 12 (SANTOS et al., 2013). Os biossurfactantes suportam concentrações de NaCl de até 12 %, enquanto que uma concentração salina de 2 – 3 % é suficiente para inativar a maioria dos surfactantes convencionais (CAMPOS et al., 2013).

5. Baixa toxicidade e compatibilidade com o ambiente: os biossurfactantes têm recebido maior atenção também devido à crescente preocupação da população com os efeitos alérgicos dos produtos artificiais; além disto, sua baixa toxicidade permite o

uso na indústria de alimentos, cosméticos e produtos farmacêuticos (CAMPO et al., 2013).

6. Não derivados do petróleo: um fator bastante importante à medida que os preços do petróleo aumentam, por este ser um recurso não renovável (NITSCHKE et al., 2011).

7. Aumento da potencialidade do biossurfactante: a possibilidade de modificação da estrutura química e das propriedades físicas dos biossurfactantes através de manipulações genéticas, biológicas ou químicas permite o desenvolvimento de produtos para necessidades específicas.

8. Elevada seletividade: biosurfactantes são moléculas complexas contendo grupos funcionais característicos, os quais, em muitos casos, permitem a destoxificação específica de diferentes poluentes (KAPADIA; YAGNIK, 2013; SANTOS et al., 2013).

Estas vantagens contribuem para a aplicação de biossurfactantes em diferentes indústrias (SILVA et al., 2014; SANTOS et al., 2016b).

3.5 Micro-organismos produtores de biossurfactantes

Uma grande variedade de micro-organismos alimenta-se de substâncias que são imiscíveis em água, produzindo e utilizando substâncias com atividades de superfície denominadas biossurfactantes (BANAT et al., 2010; SOBRINHO et al., 2013). Estas biomoléculas são secretadas no meio para aumentar a biodisponibilidade de substratos hidrofóbicos imiscíveis facilitando a translocação através das membranas celulares dos micro-organismos e auxiliando no crescimento em condições de baixa umidade (CAMPOS et al., 2013).

Biossurfactantes produzidos por leveduras vêm sendo estudados com mais ênfase na última década, onde as leveduras *C. lipolytica*, *C. sphaerica* e *C. bombicola* são as mais comumente utilizadas para a produção de biossurfactantes (LUNA et al., 2015; SILVA et al., 2014). Dentre as bactérias, *Bacillus subtilis* é outro micro-organismo amplamente estudado com relação à produção de biossurfactantes, bem

conhecido por sua eficiência na produção de um lipopeptídeo com atividade de superfície denominada surfactina (CHANDRAN; DAS, 2011).

As bactérias gram-negativas do gênero *Pseudomonas* são ambientalmente versáteis e excepcionalmente habilidosas para colonizar nichos ecológicos onde há escassez de nutrientes. São conhecidas por sua capacidade de degradar hidrocarbonetos e de metabolizar vários compostos orgânicos complexos de difícil assimilação por outros organismos.

A espécie *Pseudomonas cepacia*, atualmente conhecida como *Burkholderia cepacia*, é um bacilo gram-negativo, comumente encontrado em ambientes úmidos. A cepa foi descrita pela primeira vez em 1950 por Walter Burkholder (BURKHODER, 1950). *B. cepacia* está agora ganhando crescente interesse na agricultura, biotecnologia e medicina. Espécies deste gênero produzem grandes quantidades de ramnolipídeos classificados como glicolipídios que apresentam diversas aplicações biotecnológicas, em especial na área ambiental e na indústria de petróleo, pois são capazes de sintetizar uma grande variedade de enzimas sob as mais diversas condições de cultivo (HEBBAR et al., 1992; MCLOUGHLIN et al., 1992; TOMICH; MOHR, 2004; CHANDRAN; DAS, 2011; SARUBBO et al., 2015).

Os ramnolipídeos estão entre os biossurfactantes mais eficientes e mais estudados, formados por uma ou duas moléculas de ramnose ligadas a uma ou a duas moléculas de ácido *β*-hidroxidecanóico. A produção de glicolipídeos contendo ramnose por *Pseudomonas aeruginosa* foi primeiramente relatada por Jarvis e Johnson (1949). Os principais glicolipídeos produzidos por *P. aeruginosa* são os ramnolipídeos dos tipos L-ramnosil-*β*-hidroxidecanoil-*β*-hidroxidecanoato e L-ramnosil-L-ramnosil-*β*-hidroxidecanoil-*β*-hidroxidecanoato (SILVA et al., 2010; SANTOS et al., 2016b).

Técnicas analíticas sensíveis levaram à descoberta de congêneres e homólogos ramnolipídicos (aproximadamente 60) produzidos em diferentes concentrações por espécies de *Pseudomonas* e bactérias pertencentes a outras famílias, classes ou mesmo filos (CHRZANOWSKI et al., 2012). Por exemplo, várias espécies de *Burkholderia* demonstraram produzir ramnolipídeos que têm cadeias alquilas mais longas do que as produzidas por *P. aeruginosa* (ABDEL-MAWGOUD et al., 2010; NITSCHKE et al., 2011). Os valores de tensões superficiais de 29 mN/m constituem uma característica de tais biossurfactantes, que podem ser produzidos

utilizando diferentes substratos, tais como alcanos, piruvato, citratos, frutose, glicerol, azeite e glucose (ŁAWNICZAK et al., 2013).

Os biossurfactantes microbianos compreendem uma grande variedade de estruturas que explica o porquê deste grupo de moléculas continuar a despertar interesse científico (ŁAWNICZAK et al., 2013; LUNA et al., 2013; MARCHANT; BANAT, 2012). Essas estruturas são conhecidas como lipopeptídeos, complexos proteínas-polissacárideos, fosfolipídeos, glicolipídios e ácidos graxos produzidos principalmente por micro-organismos aeróbicos cultivados em substratos insolúveis (resíduos industriais e hidrocarbonetos) e solúveis (carboidratos) em meio aquoso (FREITAS et al., 2016; SILVA et al., 2014). A Tabela 1 apresenta as principais classes de biossurfactantes e seus respectivos micro-organismos produtores.

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

Classe/Tipo de Biossurfactante	**Micro-organismo**
Glicolipídeos	
Ramnolipídeos	*Pseudomonas aeruginosa*
Soforolipídeos	*Torulopsis bombicola, T. apicola*
Trealolipídeos	*Rhodococcus erythropolis, Mycobacterium sp.*
Lipopeptídeos e lipoproteínas	
Peptídeo-lipídeo	*Bacillus licheniformis*
Viscosina	*Pseudomonas fluorescens*
Serrawetina	*Serratia marcenscens*
Surfactina	*Bacillus subtilis*
Subtilisina	*Bacillus subtilis*
Ácidos graxos, lipídeos neutros e fosfolipídeos	
Ácidos graxos	*Corynebacterium lepus*
Lipídeos neutros	*Nocardia erythropolis*
Fosfolipídeos	*Thiobacillus thiooxidans*
Surfactantes poliméricos	
Emulsan	*Acinetobacter calcoaceticus*
Biodispersan	*Acinetobacter calcoaceticus*
Liposan	*Candida lipolytica*
Carboidrato-lipídeo-proteína	*Pseudomonas fluorescens*
Manana-lipídeo-proteína	*Candida tropicalis*
Surfactantes particulados	
Vesículas	*Acinetobacter calcoaceticus*
Células	Várias bactérias

Fonte: SILVA et al., 2014.

É possível observar que existe um campo aberto para a exploração de novos compostos, com potencial para substituir os produtos derivados do petróleo, não apenas pelo seu apelo ambiental, mas por dispor de propriedades que atendem às especificidades requeridas na formulação de novos produtos em determinadas aplicações.

3.6 Patentes em biossurfactantes aplicadas à indústria do petróleo

Devido às suas diversas aplicações industriais, muitos autores entraram com pedido de patentes sobre biossurfactantes. Na verdade, as patentes foram emitidas para biossurfactantes produzidos por micro-organismos, especialmente *Pseudomonas* spp., *Acinetobacter* spp., *Bacillus* spp. e *Candida* spp., para processos de produção e aplicações industriais (SACHDEV; CAMEOTRA, 2013).

As patentes relacionadas especificamente aos biossurfactantes soforolipídeos, surfactinas e ramnolipídeos, representam 24%, 13% e 12% do número total de patentes, respectivamente. No entanto, estes números podem estar subestimados, uma vez que muitas das patentes não descrevem claramente o micro-organismo produtor, referindo-se, apenas, a uma descrição geral de uma biomolécula com propriedades surfactantes, sem muitos detalhes (RANDHAWA; RAHMAN, 2014; REIS et al., 2013; SHETE et al., 2006). Estas patentes têm sido relacionadas principalmente com utilizações na indústria petrolífera relacionadas às propriedades destas moléculas, incluindo emulsificação, separação de fases, solubilização, formação de espuma, desemulsificação, inibição da corrosão e redução da viscosidade de óleos pesados. As patentes descrevem métodos e composições para facilitar a combustão e o transporte de emulsões altamente viscosas de hidrocarbonetos em água e, em particular, emulsões de hidrocarboneto-água estabilizadas com bioemulsificantes (SHETE et al., 2006).

Outras aplicações patenteadas incluem a utilização de biossurfactantes na separação de hidrocarbonetos de areias betuminosas (ZAJIC; GERSON, 1981), na recuperação do petróleo bruto de reservatórios pelo método de MEOR (SHEEHY, 1992), na limpeza de tanques de estocagem de óleo, no transporte de óleo pesado, na recuperação de óleo de tanques de armazenamento e de borras oleosas, dentre muitas outras aplicações (BACHMANN et al., 2014). A Tabela 2 lista importantes patentes em biossurfactantes e bioemulsificantes com potencial de aplicação na indústria do petróleo.

Tabela 2 – Patentes importantes emitidas nos últimos anos com potencial de aplicação na indústria do petróleo

Bioemulsificantes e biossurfactantes / Micro-organismos	**Título da patente**	**Nº da Patente/ Data de Publicação**	**Detentor da Patente**	**Aplicações**
Glicolipídeos	Método e instalação para inundações de poços de petróleo e areias betuminosas	CA 1119794 A1/ 16 mar. 1982	Fritz Wagner, Peter Rapp, Hans Bock, Walter Lindorfer, Walther Schulz, Wilhelm Gebetsberger	Recuperação de óleo de poço de petróleo ou de areias betuminosas
Mistura de micro-organismos produtores de biossurfactantes	Processos de recuperação avançada de óleo utilizando micro-organismos	US 4450908/ 29 mai. 1984	Donald O. Hitzman	Recuperação avançada de óleo
Micro-organismos endógenos produtores de biossurfactantes	Recuperação de óleo a partir de reservatórios de petróleo	US 5083610/ 28 jan. 1992	Alan Sheehy	Recuperação de óleo
Injeção de nutrientes microbianos para estimular a produção de biossurfactantes	Método de injeção de nutrientes para processos microbianos subterrâneos	US 5083611A/ 28 jan. 1992	James B. Clark, Gary E. Jenneman	Métodos de injeção de nutrientes para recuperação microbiana avançada de óleo (MEOR)
Lipopeptideos	Biosurfactant and enhanced oil recovery	US 4522261A/ 11 jun. 1985	Michael J. McInerney, Gary E. Jenneman, Roy M. Knapp, Donald E. Menzie	Recuperação de óleo
Fluido de tratamento contendo biossurfactantes	Sistema e processo para tratamento de tanque com borra oleosa para a recuperação de hidrocarbonetos e adição na separação de materiais	US 6069002/ 30 mai. 2000	John E. Powell, Jr.	Recuperação de hidrocarbonetos

Fonte: ALMEIDA et al., 2016.

3.7 Economia de produção e comercialização de biossurfactantes

A economia da produção de biossurfactantes merece uma atenção particular, considerando que o aumento da consciência ambiental tem sido o principal motivador na procura de um substituto para os surfactantes químicos. Muitas indústrias estão atualmente tentando substituir alguns ou todos os surfactantes químicos por biossurfactantes sustentáveis (MARCHANT; BANAT, 2012). Renomados produtores

de surfactantes químicos, tais como a BASF-Cognis e Ecover, estão também investindo no mercado de biossurfactantes (SAJNA et al., 2015).

De acordo com estudos recentes, o mercado global para as assim chamadas “alternativas verdes" aos surfactantes sintéticos atingiu US $ 1.735,5 milhões em 2011. Em 2013, a produção mundial estimada de biossurfactantes foi de aproximadamente 344 mil toneladas. E as projeções para esse mercado são ainda mais encorajadoras, uma vez que a estimativa até 2020 é atingir a cifra de US $ 2.308,8 milhões, quando o mercado mundial deverá alcançar uma produção total de biossurfactantes em torno de 462 mil toneladas. A taxa média anual de crescimento prevista para este mercado é de 4,3 % até o ano de 2020 (GRAND VIEW RESEARCH, 2016; GUDIÑA et al., 2015; SEKHON et al., 2012).

Atualmente, o custo médio de um biossurfactante comercializado varia de acordo com a sua aplicação e com o seu grau de pureza. Por exemplo, para a surfactina pura (98 % de pureza), utilizada na pesquisa médica, o custo varia de cerca de US$ 10/mg; Já para as fórmulas de surfactina propostas para a limpeza de tanques de estocagem de óleo ou para a recuperação avançada de petróleo, o custo esteve em torno de US$ 24/kg. O preço atual de soforolipídeos, um outro tipo de glicolipídeo produzido pela espécie de levedura *Candida bombicola*, oferecidos por empresas como a SophoronTM na "Saraya" (Japão) e a "Soliance" (França), varia aproximadamente entre US$ 2,5–6,3/kg (FREITAS et al., 2016). A Jeneil Biosurfactant Co. (Saukville, Wisconsin) foi a empresa mais bem-sucedida atualmente na produção de biossurfactantes em escala industrial, tendo desenvolvido com sucesso um processo de produção de biossurfactantes ramnolipídeos com capacidade para realizar fermentações em lotes de até 20.000 litros (HELMY et al., 2011; RUFINO et al., 2014).

Embora a indústria de biossurfactantes tenha demonstrado um crescimento notável nas últimas décadas, a produção em grande escala destas biomoléculas continua sendo um desafio do ponto de vista econômico. Isso se deve principalmente à enorme diferença entre o investimento financeiro necessário e a produção industrial (CHIKERE et al., 2012; LUNA et al., 2015). Assim, para que a produção de biossurfactantes se torne verdadeiramente viável, os principais critérios que devem ser considerados são: tipo de matérias-primas, tipos de micro-organismos, concepção adequada dos biorreatores industriais, investimentos financeiros, mercado-alvo, processos de purificação, propriedades de biossurfactante, condições de produção,

especialmente o tempo necessário para a fermentação, rendimentos de produção adequados (SANTOS et al., 2016b). Essas parecem ser estratégias eficazes para superar a competitividade com os seus correspondentes sintéticos. A Tabela 3 sumariza os principais fabricantes comerciais de biossurfactantes com uso potencial na indústria do petróleo.

Tabela 3 – Empresas produtoras de biossurfactantes comercialmente disponíveis e potenciais aplicações na indústria de petróleo

Empresa	Biossurfactantes	Aplicações
AGAE Technologies – USA	Ramnolipídeos	Recuperação melhorada de óleo (EOR, do inglês)
Jeneil Biosurfactant– USA	Ramnolipídeos	EOR
Rhamnolipid Companies– USA	Ramnolipídeos	EOR
Synthezyme – USA	Soforolipídeos	Emulsificação de óleo bruto
BioFuture–Ireland	Ramnolipídeos	Lavagem de tanques de óleo combustível
Logos Technologies - USA	Ramnolipídeos	EOR
TensioGreen – USA	Ramnolipídeos	Indústria do petróleo, EOR
Synthezyme – USA	Soforolipídeos	Petróleo e gás
EcoChem Organics Company – Canada	Ramnolipídeos	Agente dispersivo de hidrocarbonetos insolúveis em água
EnzymeTechnologies– USA	Biossurfactante bacteriano (desconhecido)	Remoção de óleo; Recuperação e processamento de óleo, EOR

Fonte: ALMEIDA et al., 2016.

O mercado para a exploração de biossurfactantes no Brasil é bastante promissor, dado a inexistência de empresas especializadas na produção destes produtos. De fato, ainda não há registro de uma empresa especializada no país que desenvolva tais produtos, embora esforços já tenham sido empregados para este fim. É o caso da primeira unidade-piloto do país para a produção de biossurfactantes por via microbiana, inaugurada em 2009 no Instituto Alberto Luiz Coimbra de Pós-graduação e Pesquisa de Engenharia (COPPE), com financiamento da Petrobras, cuja produção máxima alcançada foi de 200 litros por semana.

O mercado alvo é de fundamental importância para a implantação de um projeto industrial de produção de biossurfactantes. Para produtos cosméticos,

medicinais e alimentares, a produção só é viável em pequena escala, visto que os métodos de cromatografia em coluna empregados no processo de purificação para separar moléculas não são econômicos em larga escala (SANTOS et al., 2016b). Por outro lado, a utilização de caldos de fermentação brutos pode ser uma solução viável, especialmente se a aplicação estiver num contexto ambiental, uma vez que os biossurfactantes, nestes casos, não necessitam ser puros, podendo ser sintetizados utilizando uma mistura de fontes de carbono baratas, o que permitiria a criação de uma tecnologia economicamente viável para os processos de biorremediação (SANTOS et al., 2016b; SILVA et al., 2014).

Um fator chave que regula o sucesso da produção de biotensoativos é o desenvolvimento de um processo econômico que utilize materiais de baixo custo e que ofereça um rendimento elevado. De fato, o alto custo de produção é uma grande desvantagem que compromete à aceitação do produto no mercado. Nesse sentido, uma das grandes vantagens de empresas do setor de biotecnologia frente à concorrência, além da natureza biodegradável e atóxica de seus produtos, é a utilização de substratos de baixo custo como parte de seu processo produtivo. Sendo assim, a escolha de resíduos industriais é importante, visto que o substrato é responsável por até 50% do custo de produção final (ALMEIDA et al., 2016; SILVA et al., 2014). Diante deste cenário, espera-se que o mercado de biossurfactantes ultrapasse e supere o mercado de surfactantes sintéticos (SILVA et al., 2014).

3.8 Resíduos industriais para produção de biossurfactantes

A sociedade atual é caracterizada por ser altamente consumista, aumentando a necessidade de reutilizar materiais devido às preocupações ambientais. Consequentemente, foi dada maior ênfase à recuperação, reciclagem e reutilização. A indústria passou a utilizar toda e qualquer matéria-prima para o beneficiamento de bens de consumo que tragam, em curto prazo, produtos que venham acoplados com sinônimo de desenvolvimento, contribuindo para a competitividade do mercado global (BANAT et al., 2014; MORAIS et al., 2012).

A produção acelerada traz consigo a geração de resíduos agroindustriais, os quais prejudicam enormemente o meio ambiente por conta do seu armazenamento ou descarte inadequado. A partir da constatação do dano ecológico, o grande desafio é equilibrar a produção de bens e serviços e crescimento econômico com

sustentabilidade ambiental, reduzindo o descarte de resíduos agroindustriais, uma vez que o descarte inadequado representa a perda de nutrients de alto valor energético, provocando elevados custos ao governo e às indústrias para controlar os danos decorrentes da poluição (MORAIS et al., 2012).

Os resíduos industriais têm dispertado o interesse dos pesquisadores para a produção de biossurfactantes. A seleção dos resíduos deve garantir o equilíbrio adequado dos nutrientes para permitir o crescimento microbiano e consequente produção de biosurfactantes. Os resíduos industriais com elevados teores de carboidratos ou de lípidos são ideais para utilização como substratos (MAKKAR; CAMEOTRA, 2002; SANTOS et al., 2016b).

A utilização de resíduos agroindustriais tem possibilitado a implementação de uma produção viável de biossurfactante em escala industrial, empregando recursos renováveis como óleos vegetais, resíduos de destilaria e resíduos lácteos, para a qual é necessária a otimização das diferentes variáveis de processo envolvidas (BARROS et al., 2007; SILVA et al., 2013). A Tabela 4 lista os resíduos e subprodutos estudados por pesquisadores brasileiros para ajudar a reduzir o custo de produção de biossurfactante.

Tabela 4 – Resíduos e subprodutos utilizados para a produção de biossurfactantes e seus respectivos micro-organismos produtores

Resíduos / subproduto	**Biossurfactantes/micro-organismos produtores**
Óleo de canola residual de fritura e milhocina	*Pseudomonas cepacia* CCT6659
Glicerol	*Pseudomonas aeruginosa* UCP0992
Suco de caju clarificado	*Bacillus subtilis* LAMI005
Vinhaça e óleo de fritura	*Bacillus pumilus*
Manipueira	*Bacillus subtilis* LB5a
Resíduo de refinaria de óleo de soja e milhocina	*Candida sphaerica* UCP0995
Resíduo de refinaria de óleo de amendoim e milhocina	*Candida sphaerica* UCP0995
Gordura animal e milhocina	*Candida lipolytica* UCP0988
Gordura vegetal	*Candida glabrata* UCP1002
Óleo residual de fritura	*Candida tropicalis* UCP0996
Melaço	*Pseudomonas aeruginosa* (P.A.)

Fonte: Silva et al., 2014.

3.8.1 Óleos de fritura

Os óleos vegetais são uma fonte de carbono lipídico, constituídos principalmente por ácidos graxos saturados ou insaturados com cadeias de 16 a 18

átomos de carbono (MAKKAR et al., 2011). Os óleos vegetais residuais de fritura são gerados diariamente, e destinados principalmente, à fabricação de sabões ou rações animais. O problema é que seu descarte ocorre de modo inadequado na rede de esgotos, o que é danoso ao meio ambiente pela baixa solubilidade desses óleos em água (MORAIS et al., 2012).

Dentre os principais óleos de fritura, os óleos de soja, canola, azeite de oliva, óleo de amendoim, girassol, milho, óleo ou gordura de coco de babaçu, óleo ou gordura de coco e o azeite de dendê tornaram-se uma alternativa atrativa de fonte de carbono de baixo custo e de grande disponibilidade para a produção de biossurfactantes e biodiesel (CARA, 2009; EMBRAPA, 2015).

O óleo de canola vem sendo foco de vários estudos por apresentar um alto potencial para a produção de biodiesel. Na Europa, o óleo de canola é a principal matéria-prima de obtenção deste combustível, tornando-se um destaque mundial. A produtividade, situada entre 350 e 400 kg de óleo por hectare, tem sido considerada satisfatória para as condições européias (EMBRAPA, 2007).

No Brasil, o maior estado produtor da canola com 69 % da área cultivada é o Rio Grande do Sul. Nas demais regiões brasileiras, a canola avança ainda em fase experimental, distribuída nos estados do Pará (22,9 %), Minas Gerais (3,7 %), Santa Catarina (2,8 %), Goiás (0,6 %), Mato Grosso (0,5 %), São Paulo (0,3 %) e Mato Grosso do Sul (0,2 %). Esses números devem crescer ainda mais nos próximos anos. O agronegócio da canola possui grande valor sócio-econômico por oportunizar a produção de óleos vegetais no inverno, vindo somar à produção de soja no verão, e assim, contribuir para a otimização dos meios de produção (terra, equipamentos e pessoas) disponíveis. No Rio Grande do Sul, segundo a Emater/RS (Referência de Qualidade em Extensão Rural), o mercado na safra de 2015 ofereceu preços equivalentes ao da soja. Portanto, o cultivo de canola poderá permitir a expansão da produção de óleo para utilização como biodiesel, além de expandir o emprego desse óleo para o consumo humano e contribuir decisivamente para tornar o Brasil um importante exportador desse produto (EMBRAPA, 2015; TOMM, 2005).

Diferentes óleos são substratos adequados para produção de biossurfactantes. O óleo de babaçu (5 % v/v) combinado com glicose (a 1 % p/v) como fontes de carbono constituem um bom meio para a produção de biossurfactante por *Candida lipolytica* (1055 e 1120) produzindo biossurfactante ao final da fase de crescimento exponencial e início da fase estacionária (SARUBBO et al., 1999). Os óleos de

girassol e de azeite de oliva provaram serem fontes de energia e de carbono adequadas para a produção de biossurfactantes. As linhagens de *P. aeruginosa* produzem biossurfactantes em resíduos de milho, soja e de canola (RAZA et al., 2007). O óleo residual de canola mais nitrato de sódio tem sido relatado como adequado para o crescimento microbiano e para a produção de até 8,5 g/L de ramnolipídeos. A combinação de glicose e óleo de canola tem sido utilizada para a produção bem sucedida de um biossurfactante por *C. lipolytica* (SANTOS et al., 2016b; SARUBBO et al., 2007).

3.8.2 Milhocina

A milhocina é um subproduto do beneficiamento do milho, que contém grande quantidade de nitrogênio, aminoácidos, entre outros nutrientes, e 40 % de matéria sólida. Este resíduo consiste em 21 % a 45 % de proteínas, 20 % a 26 % de ácido láctico, aproximadamente 8 % de cinzas (contendo Ca^{2+}, Mg^{2+}, K^{+}, etc.), aproximadamente 3 % de carboidratos e um baixo teor de gordura (0,9 % a 1,2 %). Este subproduto é utilizado principalmente como alimento complementar na fabricação de ração para aves e ruminantes e fonte de nutrientes para o processo de fermentação industrial (SOBRINHO, 2008). Por ser um resíduo industrial, possui composição variável, que pode depender da condição e qualidade do grão de milho e do processamento. Na análise de sua composição química, pode-se observar que há uma alta concentração de matéria orgânica que quando disposta de forma inadequada, se torna uma fonte de contaminação para o meio ambiente, podendo causar a morte de animais no ecossistema aquático (HELMY et al., 2011; SANTOS et al., 2016b).

De acordo com a literatura, resíduos de refinaria de óleo de noz e milhocina são fontes de nutrientes de baixo custo para a produção de glicolípidos por *Candida sphaerica* (UCP 0995). O biossurfactante desta linhagem mobiliza e remove até 95 % de óleo de motor em areia, tornando-o útil para biorremediação (LUNA et al., 2013; LUNA et al., 2015). Rocha e Silva et al. (2014) também relataram a produção de um biossurfactante de *Pseudomonas cepacia* crescida em meio mineral suplementado com 2,0 % milhocina e 2,0 % de óleo soja residual de fritura.

3.9 Aplicações industriais dos biossurfactantes

Os biossurfactantes têm uma vasta gama de aplicações biotecnológicas nas indústrias de alimentos, bebidas, cosméticos, detergentes, têxteis, tintas, mineração, celulose, farmacêutica, agrícola, ambiental e nanotecnologia (NITSCHKE, 2011; SANTOS et al., 2016b). Atualmente, o principal mercado é a indústria do petróleo, na qual os biossurfactantes podem ser utilizados para a exploração e produção de petróleo, em operações como transporte, refino, recuperação de resíduos de óleo de tanques de armazenamento ou em processos de limpeza de derramamentos de óleo e de biorremediação de solo e água. Portanto, as atividades citadas podem ser otimizadas através da utilização de algum tipo de biossurfactante (ALMEIDA et al., 2016; SILVA et al., 2014; SOBRINHO et al., 2013). A Tabela 5 apresenta um resumo das principais aplicações biotecnológicas dos biossurfactantes em diferentes indústrias.

Tabela 5 – Aplicações de biossurfactantes para usos industriais e ambientais

Indústrias	Aplicação	Papel dos biossurfactantes
Ambiental	Biorremediação; Operações de limpeza de derramamento de óleo; Reabilitação e limpeza do solo	Emulsificação de óleos, redução da tensão interfacial, dispersão de óleos, solubilização de óleos, umedecimento, espalhamento, detergência, formação de espuma, inibição de corrosão em equipamentos, limpeza do solo.
Petróleo	Recuperação avançada de óleo; Desemulsificação	Emulsificação de óleos, redução da tensão interfacial, desemulsificação de óleos, solubilização de óleos, redução da viscosidade, dispersão de óleos, umedecimento de superfícies sólidas, espalhamento, detergência, formação de espuma, inibição de corrosão em equipamentos.
Mineração	Limpeza de metais pesados; Reabilitação do solo; Flotação	Molhabilidade, coletores e espumantes, remoção de íons metálicos de soluções aquosas, solo e sedimentos, adsorção de metais pesados, dispersão, inibição de corrosão.
Alimentos	Emulsificação e desemulsificação; Ingrediente funcional	Solubilização de óleos aromatizados, controle de consistência, emulsificação, lubrificante, dispersão, detergência, formação de espuma, espessante.
Medicina	Microbiológia; Farmacêutica e terapêutica	Agentes anti-adesivos, agentes antifúngicos, agentes antibacterianos, agentes antivirais, vacinas, terapia genética, moléculas imunomoduladoras.
Agricultura	Fertilizantes; Biocontrole	Molhabilidade, dispersão, suspensão de pesticidas e fertilizantes em pó, emulsificação de soluções pesticidas, facilitação de mecanismos de biocontrole de micróbios,

		eliminação de patógenos de plantas e aumento da biodisponibilidade de nutrientes para micróbios benéficos associados a plantas.
Cosméticos	Saúde e beleza	Emulsificação, agentes espumantes, solubilização, agentes molhantes, limpadores, agentes antimicrobianos, mediadores de ação enzimática.
Limpeza	Detergentes para lavagem	Detergentes e desinfectantes para lavagem, molhagem, espalhamento, inibição da corrosão.
Têxtil	Preparação de fibras; Tingimento e impressão; Acabamento de têxteis	Molhagem, penetração, solubilização, emulsificação, detergência e dispersão, humidificação e emulsificação em formulações de acabamento, amaciamento.

Fonte: SANTOS et al., 2016b.

3.10 Aplicação dos biossurfactantes na descontaminação ambiental de derramamentos de óleo

A liberação de contaminantes como o petróleo e seus derivados para o meio ambiente é uma das principais causas da poluição global e tornou-se um foco de grande preocupação, tanto em países industrializados como em desenvolvimento, devido à ampla distribuição ambiental no solo, águas subterrâneas e no ar (ALMEIDA et al., 2017; LUNA et al., 2013). As fontes de contaminação são diversas: acidentes durante o transporte de combustível por navios e caminhões; vazamento dos tanques de armazenamento subterrâneos que estão sujeitos à corrosão, tais como postos de gasolina; operações de extração e processamento de petróleo; e liberação inadequada de resíduos gerados pelas indústrias que utilizam derivados de petróleo em seus processos de produção, tais como as indústrias de plásticos e de solventes (EPA, 2016; ŁAWNICZAK et al., 2013; LIN et al., 2010).

Metade da produção de petróleo do mundo (cerca de três bilhões de toneladas/ano) é transportada por navios, o que têm aumentado os níveis de contaminação por hidrocarbonetos em diferentes ecossistemas marinhos devido a acidentes. A principal fonte de hidrocarbonetos nos oceanos vem de operações de rotina de lavagem de navios, vazamento de óleo natural no fundo do mar e acidentes durante a exploração de petróleo e transporte (SOUZA et al., 2014).

A mídia tem consistentemente relatado a fuga de milhares de toneladas de petróleo que contaminam a água do mar (OESP). Um dos derramamentos de maior impacto ocorreu em novembro de 2011 na plataforma de petróleo Sedco 706 operada pela Chevron Brasil na Baia de Campos (Rio de Janeiro, Brasil). Um total de 5.943

milhões de litros vazou, cobrindo 163 km^2 (SOUZA et al., 2014). Outro grande derrame de petróleo de maior repercursão no mundo ocorreu no Golfo do México, em 2010, após a explosão de uma plataforma de petróleo ao longo da costa dos estados da Louisiana e Mississippi (EUA). Após o afundamento da plataforma, os dutos abertos na área de perfuração (profundidade de 1,5 km) continuaram lançando petróleo no mar por um período de três meses antes de finalmente serem obstruídos. Relatórios oficiais indicaram a liberação de mil barris de petróleo por dia, com um total estimado de três a quatro milhões de barris de óleo derramados, tornando este o maior desastre ambiental da história dos Estados Unidos (ROCHA e SILVA et al., 2013). Em julho de 2010, um derramamento de 1.500 toneladas de óleo bruto causou sérios problemas ambientais no mar da costa de Dalian, China (ZHANG et al., 2011). Em janeiro de 2000, mais de 1,3 milhão de litros de óleo pesado vazaram de um oleoduto da refinaria da Baía de Guanabara, no Rio de Janeiro, causando grandes danos às áreas de mangue preservadas (ALMEIDA et al., 2017).

A *US Environmental Protection Agency* propõe diferentes tecnologias físicas, químicas e biológicas para o tratamento de ambientes contaminados. Dentre as abordagens biológicas, uma das mais estudadas é a biorremediação (SILVA et al., 2014). Este conjunto de tecnologias utiliza a capacidade de degradação natural de plantas e de micro-organismos para a conversão parcial de contaminantes em compostos menos tóxicos ou para a conversão completa destas substâncias em dióxido de carbono e água.

Populações de micro-organismos degradadores conduzem os processos de biorremediação de maneira mais rápida e eficiente. Portanto, esta técnica pode ser conduzida por meio da bioestimulação, que consiste em estimular o crescimento de micro-organismos presentes no local contaminado. O processo envolve a introdução de receptores de elétrons específicos, oxigênio e nutrientes para a degradação do contaminante, bem como substâncias para corrigir o pH. A biorremediação também pode ser realizada através da bioaumentação, na qual os micro-organismos autóctones são adicionados ao ambiente contaminado para acelerar e completar a degradação do poluente (SANTOS et al., 2016a).

Embora a biorremediação seja um método eficaz e ambientalmente amigável, o tempo e os custos envolvidos tornam esse processo inviável para o tratamento de grandes quantidades de contaminantes (SHAVANDI et al., 2011). Assim, os biossurfactantes surgem como uma alternativa segura para melhorar a solubilidade

de compostos hidrofóbicos permitindo a dessorção de hidrocarbonetos na fase aquosa, aumentando a biodisponibilidade destes substratos hidrofóbicos facilitando a assimilação por células microbianas, com a remoção subsequente de tais poluentes por biodegradação (APARNA et al., 2011; OLKOWSKA et al., 2012). Portanto, os biossurfactants têm sido aplicados com sucesso nos últimos anos como agentes de remediação no solo e ambientes aquáticos (MAO et al., 2015). No que se refere à sua capacidade de solubilizar hidrocarbonetos, os biossurfactantes são utilizados em diferentes processos de remediação originados da indústria petrolífera (BANAT et al., 2010).

Numerosos exemplos confirmam o potencial de aplicação de biossurfactantes na descontaminação ambiental. Estudos realizados por Urum et al. (2003) demonstraram que a mobilização ou solubilização de compostos hidrofóbicos por surfactantes pode ou não variar dependendo da concentração utilizada. Alguns surfactantes de origem vegetal não foram capazes de aumentar a solubilização de compostos hidrofóbicos em concentrações acima da CMC. Contudo, quando se utilizaram ramnolipídeos, a solubilidade do óleo aumentou com o aumento da concentração de surfactante. Em outro estudo dos mesmos autores, foi demonstrado que um aumento na concentração de ramnolipídeos de 0,004 % para 0,55 % melhorou o processo de biorremediação de solos contaminados com óleo. Gusmão et al. (2010) investigaram a aplicação de um biotensoativo bruto produzido por *Candida glabrata* UCP 1002 em um sistema de solo-água contendo contaminantes hidrofóbicos e encontraram uma taxa de remoção muito elevada, de 92,6 %. Luna et al. (2011) avaliaram um novo biotensoativo, denominado Lunasan, produzido por *Candida sphaerica* UCP 0995. Este biotensoativo removeu 95% de óleo de motor adsorvido em areia, demonstrando um considerável potencial para uso em processos de biorremediação. Rufino et al. (2013) testaram um biossurfactante Rufisan produzido por *Candida lipolytica* para a remoção de óleo de motor em diferentes tipos de solos e encontraram taxas de remoção entre 30 % e 98 % para o biossurfactante isolado. Santos et al. (2016a) investigaram o biossurfactante de *Candida lipolytica* UCP 0988 produzido em meio contendo gordura animal e milhocina como agente de biorremediação, e comprovaram que o biotensoativo estimulou a degradação do óleo de motor pelos micro-organismos autóctones (bactérias e fungos) da água do mar.

3.10.1 Avaliação da toxicidade de biossurfactantes em processos de biorremediação

Grandes quantidades de petróleo bruto que entram no ambiente marinho, nas águas subterrâneas e no solo podem causar danos significativos para os organismos residentes (LIN et al., 2014). O petróleo é um hidrocarboneto hidrofóbico com efeitos negativos sobre as propriedades estruturais e funcionais das membranas celulares de organismos vivos, oferecendo considerável risco de contaminação em ambos os ecossistemas marinho e terrestre (SOBRINHO et al., 2013). Quando em contato com a água, o petróleo e seus derivados distribuídos formam uma camada fina sobre a superfície que impede a troca de gases entre o ar e a água, bloqueando a passagem de luz solar e o processo de respiração e fotossíntese. Portanto, os impactos de resíduos de hidrocarbonetos nas comunidades de fitoplânctons causam um colapso fundamental na cadeia alimentar (ASIMIEA; SAM-WOBO, 2011; SOUZA et al., 2014). Os danos para a saúde humana decorrente de hidrocarbonetos estão ligados às propriedades físicas e químicas destes compostos, que são absorvidos pela pele e rapidamente se espalha pelo organismo se ingerido ou inalado (BACHMANN et al., 2014; COSTA et al., 2012).

Um dos principais obstáculos à expansão do mercado de surfactantes para a remediação *in situ* é a falta de conhecimento sobre seus efeitos no meio ambiente e a toxicidade dessas substâncias. A presença de surfactantes sintéticos no ambiente aquático nos últimos 30 anos resultou em grandes danos a microbiota marinha. Como consequência, foi reunida ao longo dos anos uma extensa base de dados de testes laboratoriais de toxicidade de vários surfactantes comerciais. Por outro lado, a toxicidade de biossurfactantes no ambiente não é bem conhecida (SANTOS et al., 2016a). Edwards et al. (2003), em uma comparação da toxicidade entre três surfactantes sintéticos e três surfactantes microbianos, concluiu que os biossurfactantes foram menos tóxicos do que os surfactantes sintéticos para algumas espécies de invertebrados (SANTOS et al., 2016a).

Os riscos ambientais decorrentes de biossurfactantes, avaliados através de análises de composição das comunidades microbianas, não são suficientemente elucidados. Enquanto a toxicidade microbiana de biotensoativos é uma causa possível da inibição da biorremediação, muitos biossurfactantes não são tóxicos para os micro-organismos em concentrações perto dos seus valores de CMC (SILVA et al., 2014).

A menor toxicidade e maior biodegradabilidade dos tensoativos biológicos em comparação com os seus homólogos químicos é a principal razão para a sua elevada aceitabilidade. No entanto, essas características são muitas vezes assumidas como única consequência direta da sua origem natural. Por estas razões, as características ambientais de novos biossurfactantes devem ser cuidadosamente consideradas e investigadas antes de sua liberação no meio ambiente (FRANZETTI et al., 2012).

3.11 Biossurfactantes para extração de petróleo em reservatórios

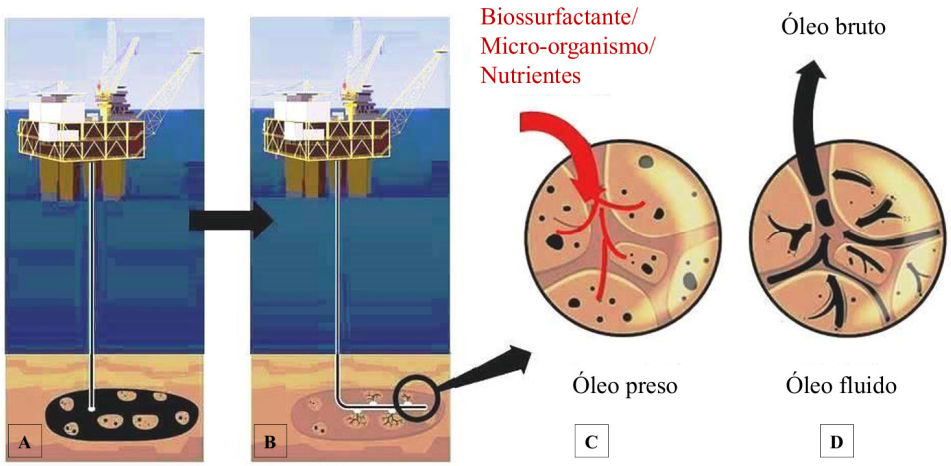
Atualmente, são utilizados no mundo vários processos de recuperação de petróleo: térmico, químico, físico, etc. (AL-SULAIMANI et al., 2011). No entanto, estes processos são muito caros e prejudiciais para o ambiente. Assim, a busca por alternativas de baixo custo e sustentáveis aos métodos químicos e térmicos de recuperação avançada de petróleo é necessária. Vários processos biotecnológicos têm sido propostos para aumentar a produção de petróleo na atual escassez de energia (SILVA et al., 2014; SUN et al., 2011). Dentre estes, os que utilizam biossurfactantes possuem potenciais aplicações, uma vez que estes compostos naturais melhoram a mobilização, aumentando a área de contato de hidrocarbonetos, otimizando a recuperação de petróleo bruto a partir de reservatórios, em um processo denominado recuperação microbiana melhorada de óleo (MEOR) (ALMEIDA et al., 2016).

MEOR consiste na recuperação terciária de óleo na qual os micro-organismos ou seus produtos metabólicos são utilizados para recuperar o óleo residual. Este processo é menos caro em comparação com a recuperação de óleo realizada por métodos químicos, uma vez que os micro-organismos podem produzir produtos eficientes como polímeros e biossurfactantes a partir de matérias-primas ou substratos de baixo custo (SARAFZADEH et al., 2014). Existem três estratégias principais para o uso de biossurfactantes em MEOR (Figura 4C):

1. Produção *ex situ* de biossurfactantes em reatores (cultura em batelada ou contínua) em condições industriais seguido de injeção subsequente no reservatório juntamente com a inundação de água.
2. Injeção de micro-organismos que produzem biossurfactantes na interface célula / óleo dentro do reservatório, implicando a penetração de células metabolicamente ativas no reservatório (espalhamento in situ).

3. Injecção de nutrientes essenciais e (inibidores de crescimento microbiano indesejado) no reservatório para estimular o crescimento de micro-organismos autóctones desejados produtores de biossurfactantes. Em condições ambientais favoráveis, a população microbiana cresce exponencialmente e seus produtos metabólicos mobilizam o óleo residual dentro do poço de petróleo (AL-BAHRY et al., 2013; BACHMANN et al., 2014).

Figura 4 – Recuperação microbiana de petróleo bruto utilizando biossurfactante. A) Extração de óleo utilizando a pressão natural do reservatório. (B) Diminuição da pressão do poço de óleo. (C) Principais estratégias de uso dos biosurfactante para a liberação de óleo. (D) Pressão do poço de óleo restaurada facilitando a extração do óleo.



Fonte: ALMEIDA et al. (2016)

Todas as estratégias acima aumentam os rendimentos de petróleo de um reservatório empobrecido por meio da redução da tensão superficial e interfacial de óleo a e consequente diminuição das forças capilares que impedem o movimento do óleo através dos poros das rochas. Os biossurfactantes também ajudam na formação de emulsões de água-óleo estáveis e quebram de filme do óleo na rocha, o que é importante para uma extração máxima de óleo, estendendo-se, portanto, a vida útil do reservatório (AL-BAHRY et al., 2013; BACHMANN et al., 2014).

3.12 Transporte de petróleo bruto por oleodutos

O petróleo bruto muitas vezes precisa ser transportado por longas distâncias a partir dos campos de extração até às refinarias. O transporte de petróleo bruto pesado e extra-pesado acarreta dificuldades operacionais que limitam a sua

viabilidade econômica. A baixa fluidez devido ao elevado grau de viscosidade e teor de asfalteno no petróleo bruto pesado está entre os principais problemas, os quais levam a inconvenientes, tais como a deposição de asfaltenos e/ou parafinas, bem como uma queda na pressão, que pode causar problemas com entupimentos nos gasodutos (CERÓN-CAMACHO et al., 2013; PERFUMO et al., 2010). A precipitação de asfaltenos em tubulações de metal na presença de íons férricos, combinados com condições ácidas, forma um sólido denominado "lama asfáltica", que se deposita no oleoduto e obstrui o fluxo livre de petróleo bruto. Solventes, tais como tolueno e xileno são aplicados para solubilizar este tipo de lama, o que aumenta o custo do processo e gera resíduos altamente tóxicos (ASSADI; TABATABAEE, 2010).

Uma promissora tecnologia foi desenvolvida recentemente envolvendo a produção de uma emulsão estável de óleo-em-água que facilita a mobilidade de óleo. Emulsionantes baseados em biossurfactantes (bioemulsificantes) são particularmente adequados para esta aplicação. Bioemulsificantes são tensoativos de elevado peso molecular com propriedades diferentes das dos glicolípideos e lipopéptideos. Estes produtos não são efica zes na redução das tensões interfaciais, mas têm uma excelente capacidade para estabilizar emulsões óleo-em-água. Devido ao elevado número de grupos reativos na molécula, os bioemulsificantes se ligam fortemente às gotículas de óleo e formam uma barreira eficaz que evita a coalescência (PERFUMO et al., 2010). Emulsan é o mais poderoso bioemulsificante e tem potenciais aplicações na indústria de petróleo, incluindo a formação de emulsões água-óleo pesado para a redução da viscosidade do óleo bruto durante o transporte em gasodutos (ASSADI e TABATABAEE, 2010; PERFUMO et al., 2010). Emulsan e seus análogos, como alasan e biodispersan, têm sido extensivamente estudados e são certamente os mais poderosos entre os bioemulsificantes sintetizados por diferentes linhagens de *Acinetobacer* (MULLIGAN et al., 2014). Estima-se que em condições ótimas, a emulsão pode ser transportada por aproximadamente 41.850 Km. Uma vez transportados para a refinaria, os hidrocarbonetos podem ser desemulsionados ou utilizados diretamente sem desidratação ou tratados com enzimas específicas denominadas emulsanos para despolimerizar o bioemulsificantes, rompendo assim a emulsão antes do uso (ALMEIDA et al., 2016). Amani e Kariminezhad (2016) investigaram a remoção de óleo bruto de um tubo de aço inoxidável usando um biossurfactante tipo emulsan, produzido por *Acinetobacter calcoaceticus* PTCC1318,

demonstrando sua utilidade para a limpeza do tubo com porcentagens de remoção de 100% à temperatura ambiente, sugerindo seu uso para transporte em gasodutos.

3.13 Limpeza de óleo em tanques de estocagem

Grandes quantidades de petróleo bruto são movidos por dia, distribuídos para as refinarias e colocados em tanques de armazenamento. A manutenção destes tanques requer lavagem periódica. No entanto, os resíduos e as frações pesadas do petróleo que se acumulam no fundo e nas paredes dos tanques de armazenamento são altamente viscosos e se tornam depósitos sólidos que não podem ser removidos com bombeamento convencional. A remoção deste material requer lavagem com solventes, limpeza manual e mão de obra intensiva, procedimentos caros, perigosos e demorados, como operações de limpeza que incluem a pulverização de água quente, solubilização por solventes, e subsequente eliminação de resíduos (MATSUI et al., 2012; PERFUMO et al., 2010).

O uso de biossurfactantes microbianos é um procedimento alternativo de limpeza para diminuir a viscosidade de depósitos de borra oleosa através da formação de uma emulsão de óleo em água que facilita o bombeamento dos resíduos. Além disso, este processo permite a recuperação do petróleo bruto quando a emulsão é quebrada (SILVA et al., 2014).

A aplicação de biossurfactantes para a limpeza de tanques foi proposta pela primeira vez em 1981, como uma alternativa aos métodos tradicionais. Dez anos mais tarde, Banat et al. (1991) descreveram a aplicação de biossurfactantes microbianos para a limpeza de tanques de armazenamento de óleo (GALABOVA et al., 2014; MULLIGAN et al., 2014). Um ensaio de campo realizado na Kuwait Oil Company demonstrou que os biossurfactantes podem efetivamente conduzir a atividade de limpeza do tanque de armazenamento. A adição contínua de duas toneladas de caldo de cultura contendo biossurfactantes ramnolipídeos durante 5 dias a uma temperatura local de 40℃ a 50℃ foi efetiva na mobilização e solubilização da borra oleosa do fundo do tanque, formando uma emulsão. O tratamento recuperou 91 % de hidrocarbonetos da borra oleosa e o valor obtido do óleo bruto recuperado cobriu o custo da operação de limpeza (GALABOVA et al., 2014; MULLIGAN et al., 2014). O hidrocarboneto recuperado apresentou excelentes propriedades e poderia ser vendido depois de ser misturado com o óleo bruto fresco (ALMEIDA et al., 2016).

Uma forma melhorada desta tecnologia foi patenteada em 2004 por Idrabel Italia (Itália) e Jeneil Biosurfactant Company (Estados Unidos). Como resultado da implementação do processo proposto, a recuperação de petróleo foi maior que 90 % e a redução do material a ser descartado foi menor que 5 % (GALABOVA et al., 2014).

Matsui et al. (2012) investigaram o tratamento de borra oleosa de fundo de tanque com um novo biossurfactante JE1058BS, produzido pelo actinomiceto *Gordonia* sp. A atividade de dispersão foi maior do que o obtido com um tensoativo derivado de planta ou produto químico e o bissurfactante manteve-se estável durante pelo menos três semanas. Diab e El Din (2013) avaliaram o efeito do sobrenadante de *P. aeruginosa* SH 29 aplicado à limpeza de recipientes contaminados com óleo. O óleo foi recuperado a partir do fundo e paredes dos recipientes em apenas 15 minutos e flutuou no sobrenadante como uma fase distinta. Segundo os autores, isso indica que o biossurfactante no sobrenadante esterilizado de *P. aeruginosa* SH 29 pode ser usado diretamente para a limpeza de tanques de armazenamento e outros recipientes utilizados para o transporte e armazenamento de petróleo bruto. Rocha e Silva et al. (2013) testaram o potencial de um biossurfactante isolado a partir de *Pseudomonas cepacia* CCT6659 para a limpeza de paredes de recipientes contaminados com uma camada de óleo, e encontraram uma taxa de remoção de 80 %, o que sugere a aplicabilidade deste biotensoativo na limpeza de tanques de armazenamento.

3.14 Biossurfactantes como anti-corrosivos na indústria petrolífera

A corrosão representa um dos maiores problemas na indústria do petróleo. De fato, os equipamentos e peças metálicas utilizadas nos campos de extração de petróleo, nas refinarias e nas plantas petroquímicas estão suscetíveis à corrosão, com consequentes efeitos negativos sobre o investimento do setor de petróleo (ABBASOV et al., 2015; NOOR EL-DIN et al., 2016). A corrosão mais comumente observada na indústria petrolífera começa com a adsorção de prótons em superfícies metálicas, seguida de uma reação eletroquímica irreversível entre os prótons e os átomos metálicos. Os cátions metálicos podem dissolver-se na fase aquosa ou reagir com ânions tais como enxofre, expondo ainda mais a superfície metálica para ataques subsequentes. Além disso, os problemas de corrosão nas operações de refino de petróleo associados à presença de constituintes do ácido naftênico e de

compostos de enxofre nos óleos brutos foram reconhecidos há muitos anos (ALMEIDA et al., 2016).

O controle da corrosão tem sido o foco de pesquisas por séculos e o uso de inibidores é um dos métodos mais práticos para a proteção de materiais contra a corrosão. A inibição da corrosão em processos petrolíferos é mais complicada e requer a utilização de inibidores especiais dependendo da área de aplicação, como: refinarias, poços, unidades de recuperação, dutos de transporte, etc. Os inibidores são geralmente de natureza inorgânica, orgânica, inibidores tensoativos e inibidores mistos (MALIK; AHMED, 2012; SAJI, 2010).

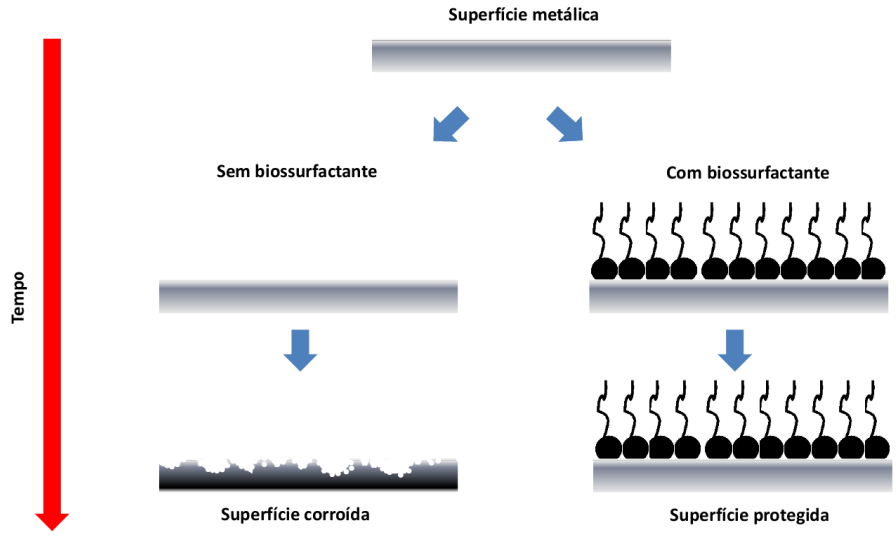
Os surfactantes sintéticos têm sido muito utilizados para controlar a corrosão. Isto é atribuído à sua grande capacidade para influenciar as propriedades das superfícies e interfaces. A ação de inibição mais importante é a adsorção do grupo funcional do surfactante sobre a superfície do metal, uma vez que a adsorção é crítica para a inibição da corrosão. No entanto, o uso dessas substâncias em processos industriais pode causar riscos para a saúde humana e ambiental. Uma alternativa é a aplicação de biossurfactantes para substituir os compostos surfactantes sintetizados quimicamente (ALMEIDA et al., 2016; KORENBLUM et al., 2012).

A maioria dos biossurfactantes exibem propriedades anti-corrosivas. Existe um grande potencial de utilização de biossurfactantes como inibidores através do condicionamento de superfícies metálicas, para retardar o processo de corrosão (ARAUJO; FREIRE, 2013; KORENBLUM et al., 2012). O processo de corrosão do metal resulta na formação de produtos de corrosão e libertação de energia, isto é, as superfícies mais protegidas contra a corrosão são aquelas com valores mais baixos de energia livre na superfície total. Por interação com íons H^+, a superfície tende a se tornar mais hidrofílica, o que pode ser a indicação da ocorrência de um processo corrosivo. No entanto, as superfícies condicionadas por biossurfactantes apresentam formação de uma película dessas moléculas na superfície, orientando a cauda hidrofóbica para o ambiente externo, e a cabeça hidrofílica para a superfície metálica, mantendo a superfície protegida da interação com os íons O_2 e H^+, evitando a corrosão (ARAUJO; FREIRE, 2013).

Num estudo do comportamento da corrosão de superfície metálica feito por Dagbert et al. (2006), foi relatado que a presença de biossurfactante obtido de *Pseudomonas fluorescens* (Pf495), uma bactéria gram negativa, retardou a corrosão da superfície de aço inoxidável AISI 304 (o aço é o material mais frequentemente

utilizado). Estes testes de corrosão foram avaliados em vários estados de oxidação superficial e mostraram que o biossurfactante produzido por *P. fluorescens* tem um bom potencial como inibidor da corrosão. A Figura 5 ilustra uma representação esquemática geral para o processo de adsorção de moléculas de biossurfactante sobre a superfície do metal.

Figura 5 – Efeito inibidor da corrosão através do condicionamento superficial pelo biossurfactante



Fonte: Autora da tese, 2017

Os avanços da indústria do petróleo estão se tornando cada vez mais evidentes nos últimos anos. Devido à eficiência comprovada da aplicação de biossurfactantes em processos relacionados à cadeia produtiva do petróleo e na remoção de contaminantes hidrofóbicos do meio ambiente, estas biomoléculas promissoras ganharam espaço e tornaram-se valiosas ferramentas versáteis, passando a desempenhar papéis essenciais, o que vem transformando e modernizando a biotecnologia do petróleo.

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5. ARTIGOS DERIVADOS DA TESE

5.1 CAPÍTULO 1

Artigo de revisão intitulado "*Applications of Biosurfactants in the Petroleum Industry and the Remediation of Oil Spills*", publicado pela revista *International Journal of Molecular Sciences.*

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Review

Applications of Biosurfactants in the Petroleum Industry and the Remediation of Oil Spills

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Abstract: Petroleum hydrocarbons are important energy resources. However, petroleum is also a major pollutant of the environment. Contamination by oil and oil products has caused serious harm, and increasing attention has been paid to the development and implementation of innovative technologies for the removal of these contaminants. Biosurfactants have been extensively used in the remediation of water and soil, as well as in the main stages of the oil production chain, such as extraction, transportation, and storage. This diversity of applications is mainly due to advantages such as biodegradability, low toxicity and better functionality under extreme conditions in comparison to synthetic counterparts. Moreover, biosurfactants can be obtained with the use of agro-industrial waste as substrate, which helps reduce overall production costs. The present review describes the potential applications of biosurfactants in the oil industry and the remediation of environmental pollution caused by oil spills.

Keywords: surface tension; industrial wastes; patents; sustainable technologies

1. Introduction

Petroleum is one of the most important energy resources and a raw material of the chemical industry. The world depends on oil and the use of oil as fuel has contributed to intensive economic development. Although petrochemical plants and oil refineries are beneficial to society, they produce a large amount of hazardous waste. Moreover, oil spills during exploration, transportation, and refining, have caused serious environmental problems [1–6].

The remediation of contaminated sites can be achieved by physicochemical or biological methods. Conventional physicochemical methods can rapidly remove the majority of spilled oil, but, in most cases, removal simply transfers contaminants from one environmental medium to another and can even produce toxic byproducts. Moreover, crude oil cannot be completely cleaned up with physicochemical methods. Thus, more attention is being given to biological alternatives [7,8]. Biosurfactants play an important role in remediation processes due to their efficacy as dispersion and remediation agents as well as their environmentally friendly characteristics, such as low toxicity and high biodegradability [9]. Indeed, biosurfactants have applications in different industrial processes as well as possible novel uses in the future and are expected to become known as multifunctional materials of the 21st century [5]. Currently, the major market for biosurfactants is the petroleum industry, in which these compounds can be used in the cleanup of oils spills, the removal of oil residue from storage tanks, microbial-enhanced oil recovery, and the bioremediation of soil and water [3].

The diverse structures of biosurfactants have useful properties with industrial potential. The production of surfactants by microorganisms is performed to enhance both the bioavailability of nearly inaccessible substrates and survival under conditions of low moisture. Biosurfactant production generally requires a hydrophobic and hydrophilic carbon source in the culture medium. The process is economically and environmentally attractive when waste products are used [10,11].

This review addresses the potential roles and applications of biosurfactants, focusing on the petroleum industry and bioremediation processes. The key features of the microbial biosynthesis of biosurfactants, their physicochemical and bioactive properties and the use of industrial wastes as cost-effective alternatives for biosurfactant production are also summarized.

2. Biosurfactants

The advance of sustainable technologies has driven the search for natural, biodegradable compounds to remediate sites contaminated by hydrocarbons. This has led to the discovery of surfactants of a natural origin. Most of these surfactants are synthesized by living organisms, such as, saponins produced by plants, glycolipids produced by microorganisms, and bile salts produced by animals. Compounds with surfactant properties produced by microorganisms are denominated biosurfactants [2,12].

Biosurfactants are mainly produced by aerobic microorganisms in aqueous media with a carbon source feedstock, such as carbohydrates, hydrocarbons, fats, and oils. It is believed that biosurfactants are secreted into the culture medium to assist in the growth of the microorganism by facilitating the translocation of insoluble substrates across cell membranes [13]. These compounds have amphipathic molecules with hydrophobic and hydrophilic portions that act between fluids of different polarities

(oil/water and water/oil), allowing access to hydrophobic substrates and causing a reduction in surface tension, an increase in the area of contact of insoluble compounds (such as hydrocarbons) and the enhancement of the mobility, bioavailability, and biodegradation of such compounds [12]. The lipophilic moiety can be a protein or peptide with a high proportion of hydrophobic side chains or a hydrocarbon chain of a fatty acid with 10 to 18 carbon atoms, although fatty acids with a higher molecular weight have been reported. The hydrophilic moiety can be an ester, hydroxy, phosphate, carboxylate group, or sugar [13]. Biosurfactants are generally classified into low molecular-mass molecules, which efficiently lower surface and interfacial tensions, and high molecular-mass polymers, which are more effective as emulsion-stabilizing agents. The major classes of low-mass surfactants are glycolipids, lipopeptides, and phospholipids, whereas high-mass surfactants include polymeric and particulate surfactants [14].

Biosurfactants offer a number of advantages over chemical surfactants, such as biodegradability due to their simple chemical structure, environmental compatibility, low toxicity, which allows use in the cosmetic, pharmaceutical and food industry, high selectivity due to presence of specific functional groups, allowing specificity in the detoxification of specific pollutants, and activity under conditions of extreme temperatures, pH and salinity [14,15]. These traits contribute to the applicability of biosurfactants in different industries [16,17].

The fact that biosurfactants are characterized by a vast structural diversity and display a broad range of properties may explain why this group of molecules continues to pique scientific interest [5,16,18]. Due to their diverse industrial applications, many authors have filed for patents on biosurfactants. Indeed, patents have been issued for biosurfactant producing microbes, especially *Pseudomonas* spp., *Acinetobacter* spp., *Bacillus* spp., and *Candida* spp., types of biosurfactant, the production process and industrial applications [19]. Table 1, adapted from Sachdev and Cameotra [19], lists some of the important patents issued in recent years.

The economics of biosurfactant production merit particular attention. The total production of surfactants in 2012 was ~12 million tons, only 3.5 million tons of which were biosurfactants. Moreover, revenues from the bio-based portion of the market were US$ 6.588 million [13]. However, the focus on sustainability and new environmental legislation has led to the search for natural surfactants as alternatives to existing products [13]. Industries are currently seeking to replace some or all chemical surfactants with sustainable biosurfactants [5], but the high production cost is a major drawback. A key factor governing the success of biosurfactant production is the development of an economical process that uses low-cost materials and offers high yield. Indeed, the choice of a low-cost substrate is important to the overall economics, as the substrate accounts for up to 50% of the final production cost. Fortunately, biosurfactants can be produced with economical, renewable resources, such as vegetable oils, distillery waste, and dairy waste [20].

Essentially agricultural countries, such as Brazil, have easy access to agro-industrial byproducts. Table 2 lists wastes and byproducts studied by Brazilian researchers to help reduce the cost of biosurfactant production.

Table 1. Patents on biosurfactants produced by microorganisms.

Microorganism/Type of Biosurfactant	Patent Holder	Title of Patent	Publication No.	Publication Date
Sophorolipid producer	Borzeix F	Sophorolipids as stimulating agent of dermal fibroblast metabolism	US 6057302 A	2 May 2000
Sophorolipid producer	Borzeix F, Concaix	Use of sophorolipids comprising diacetyl lactones as agent for stimulating skin fibroblast metabolism	US 6596265 B1	22 July 2003
New strains of hydrocarbon-degrading bacteria capable of producing biosurfactants	Robin L. Brigmon, Sandra Story, Denis Altman, Christopher J. Berry	Surfactant biocatalyst for remediation of recalcitrant organics and heavy metals	PI 0519962-0 A2	28 June 2005
Sophorolipid producer	Gross RA, Shah V, Doncel GF	Spermicidal and virucidal properties of various forms of sophorolipids	WO 2005089522 A2	29 September 2005
C. albicans, C. rugosa, C. tropicalis, C. lipolytica, C. torulopsis	Awada S, Spendlove R, Awada M	Microbial biosurfactants as agents for controlling pests	US 20050266036 A1	1 December 2005
Pseudomonas aeruginosa	Silvanito Alves Barbosa, Roberto Rodrigues De Souza	Biosurfactant production for development of biodegradable detergent	PI 1102592-1 A2	16 May 2011
Sophorolipid producer	Cox TF, Crawford RJ, Gregory LG, Hosking SL, Kotsakis	Mild to skin, foaming detergent composition	WO2011120776 A1	6 October 2011
Streptomyces sp.	Ana LF Porto, Eduardo F Santos, Leonie A Sarubbo	Biosurfactant and production process	PI 1105951-6 A2	28 November 2011

Table 1. *Cont.*

Microorganism/Type of Biosurfactant	Patent Holder	Title of Patent	Publication No.	Publication Date
Candida guilliermondii	Leonie A Sarubbo, Valdemir A Santos, Raquel D Rufino, Juliana M Luna	Production process of biosurfactant produced by *Candida guilliermondii* using agro-industrial waste	BR102012023115	13 September 2012
Candida bombicola ATCC 2214	Soetaert W, De MS, Saerens K, Roelants S, Van BI	Modified sophorolipid production by yeast strains and uses	EP 2580321 A1	17 April 2013
Lipopeptide producer	X. Vecino, R. Dvesa-Rey, J.M. Cruz, A.B. Moldes	Method for separating the surfactants present in the washing liquors of corn and uses	WO2014044876 A1	27 March 2014

Table 2. Waste and byproducts used for biosurfactant production and respective producing microorganisms.

Waste/by-Product	Biosurfactant-Producing Microorganism	Reference
Canola waste frying oil and corn steep liquor	*Pseudomonas cepacia* CCT6659	[21]
Glycerol	*Pseudomonas aeruginosa* UCP0992	[22]
Clarified cashew apple juice	*Bacillus subtilis* LAMI005	[23]
Vinasse and waste frying oil	*Bacillus pumilus*	[24]
Cassava wastewater	*Bacillus subtilis* LB5a	[25]
Soybean oil refinery residue and corn steep liquor	*Candida sphaerica* UCP0995	[26]
Ground-nut oil refinery residue and corn steep liquor	*Candida sphaerica* UCP0995	[27]
Animal fat and corn steep liquor	*Candida lipolytica* UCP0988	[15]
Vegetable fat	*Candida glabrata* UCP1002	[28]
Waste frying oil	*Candida tropicalis* UCP0996	[29]
Molasses	*Pseudomonas aeruginosa* (P.A.)	[30]

3. Biosurfactant-Producing Microorganisms

A number of microorganisms, such as fungi, yeasts, and bacteria, feed on substances that are immiscible in water, producing and using a surface-active substance (biosurfactant) [3,17]. Among bacteria, the genus *Pseudomonas* is known for its capacity to produce extensive quantities of glycolipids. These biosurfactants are classified as rhamnolipids. *Bacillus subtilis* is

another microorganism widely studied for biosurfactant production and is known for its efficiency in producing a lipopeptide with surface activity denominated surfactin or subtilisin [2,12,31–33]. *Candida bombicola* and *Candida lipolytica* are among the most commonly studied yeasts for the production of biosurfactants [13]. Table 3 offers a list microorganisms that produce biosurfactants [3,34].

Table 3. Main classes of biosurfactants and respective producer microorganisms.

Class/Type of Biosurfactant	Microorganisms
Glycolipids	
Rhamnolipids	*Pseudomonas aeruginosa*
Sophorolipids	*Torulopsis bombicola, T. apicola*
Trehalolipids	*Rhodococcus erythropolis, Mycobacterium* sp.
Lipopeptides and lipoproteins	
Peptide-lipid	*Bacillus licheniformis*
Viscosin	*Pseudomonas fluorescens*
Serrawettin	*Serratia marcenscens*
Surfactin	*Bacillus subtilis*
Subtilisin	*Bacillus subtilis*
Gramicidin	*Bacillus brevis*
Polymyxin	*Bacillus polymyxia*
Fatty acids, neutral lipids and phospholipids	
Fatty acid	*Corynebacterium lepus*
Neutral lipids	*Nocardia erythropolis*
Phospholipids	*Thiobacillus thiooxidans*
Polymeric surfactants	
Emulsan	*Acinetobacter calcoaceticus*
Biodispersan	*Acinetobacter calcoaceticus*
Liposan	*Candida lipolytica*
Carbohydrate-lipid-protein	*Pseudomonas fluorescens*
Mannan-lipid-protein	*Candida tropicalis*
Particulate surfactant	
Vesicles	*Acinetobacter calcoaceticus*

4. Environmental Contamination by Oil Spills and Biosurfactant-Enhanced Remediation

The release of contaminants, such as petroleum and petroleum byproducts, into the environment is one of the main causes of global pollution and has become a focus of great concern both in industrialized and developing countries due to the broad environmental distribution in soil, groundwater, and air [18,35]. The contamination sources are diverse: accidents during fuel transportation by ships and trucks; leakage from underground storage tanks that are subject to corrosion, such as gas stations; oil extraction and processing operations; and inadequate release of waste generated by industries that use oil byproducts in the production of plastics, solvents, pharmaceuticals, and cosmetics [16,36–38]. Half the world's oil production (around three billion tons/year) is transported by ship and hydrocarbon contamination levels in different marine ecosystems have increased due to accidents. The major hydrocarbon source in oceans comes from routine operations of ship washing, natural oil leakage on the sea bed, and accidents during oil exploration and transportation [2].

The media has consistently reported the leakage of thousands of tons of oil that contaminate seawater [39]. One of the most impacting spills occurred in November of 2011 on the Sedco 706 oil rig, operated by Chevron Brazil in Campos Bay (Rio de Janeiro, Brazil). A total of 5943 L leaked, covering 163 km^2 [2]. Another of the largest oil spills in the world occurred in the Gulf of Mexico in 2010, following the explosion of an oil rig off the coast of the states of Louisiana and Mississippi (USA). After the sinking of the rig, the open ducts in the drilling area (depth of 1.5 kms) continued spewing oil into the sea for a three-month period before finally being capped. Official reports indicate the release of a thousand barrels of oil per day, with an estimated total of three to four million barrels of oil spilled, making this the largest environmental disaster in the history of the United States [6]. In July 2010, an oil spill of 1500 tons of crude oil caused serious environmental problems to the ocean and coast in Dalian, China [1]. In January 2000, more than 1.3 million L of heavy oil leaked from a refinery pipeline into Guanabara Bay in Rio de Janeiro, Brazil, causing extensive damage to preserved mangrove areas [40].

Over ten events of oil tankers with important wastes have occurred in Europe since 1967. The *Prestige* oil spill may be considered as one of the worst. The oil tanker *Prestige*, loaded with a cargo of 77,000 tons of heavy bunker oil ran into problems off the Galician coast (NW Spain) on 13 November 2002. After several days following an erratic path and spilling 19,000 tons, the tanker finally sank 130 miles west off the southern coast [41].

Large amounts of crude oil entering the marine environment, groundwater and soil can cause significant harm to resident organisms [8]. Petroleum is a hydrophobic hydrocarbon with negative effects on the structural and functional properties of cell membranes in living organisms, offering considerable risk of contamination in both marine and terrestrial ecosystems [3]. When in contact with water, oil and its byproducts spread and form a thin layer on the surface that prevents gas exchange between the air and water, blocking the passage of sunlight and impeding the respiration and photosynthesis process. Thus, hydrocarbon waste impacts phytoplankton communities, causing a fundamental breakdown of the food chain [42,43]. The potential threat to human health posed by hydrocarbons is linked to the physical and chemical properties of these compounds, which are absorbed by the skin and quickly spread through the organism if ingested or inhaled [44,45].

The most common role of biosurfactants is to enhance the dispersal of contaminants in the aqueous phase and increase the bioavailability of the hydrophobic substrate to microorganisms, with subsequent removal of such pollutants through biodegradation [12,46]. Numerous examples demonstrate the potential application of biosurfactants in environmental decontamination. Sobrinho *et al*. [27] tested a biosurfactant produced by *Candida sphaerica* for the removal of motor oil from soil and seawater and found removal rates of 75% and 92% from clay and silty soil, respectively; in tests carried out with seawater, the biosurfactant exhibited an oil spreading efficiency of 75%, demonstrating its potential for application as an adjuvant in biotechnological processes of environmental decontamination. Batista *et al*. [29] investigated the application of a biosurfactant produced by *Candida tropicalis* for removing motor oil from sand and found removal rates of 78% to 97%, demonstrating considerable potential with regard to soil bioremediation. Gusmão *et al*. [28] investigated the application of a crude biosurfactant produced by Candida glabrata UCP1002 in a soil-water-hydrophobic contaminant system and found a removal rate as high as 92.6%. Luna *et al*. [26] evaluated a new biosurfactant, denominated Lunasan, produced by *Candida sphaerica* UCP 0995. This biosurfactant removed 95%

of motor oil adsorbed to sand, demonstrating considerable potential for use in bioremediation processes. The remediation of hydrocarbons contaminated soils using a cell-bound biosurfactant from *Lactobacillus pentosus* has been also described [47,48]. Table 4 offers a list of different types of biosurfactants and their producing microorganisms with potential applications in the bioremediation of oil-polluted environments [10,49].

Table 4. Biosurfactants, producing microorganisms and uses in the bioremediation of oil-contaminated environments.

Microorganisms	Type of Biosurfactant	Applications
Rhodococcus erythropolis 3C-9	Glucolipid and trehalose lipid	Oil spill cleanup operations
Pseudomonas aeruginosa S2	Rhamnolipid	Bioremediation of oil-contaminated sites
Rhodococcus sp. TW53	Lipopeptide	Bioremediation of marine oil pollution.
R. wratislaviensis BN38	Glycolipid	Bioremediation applications
Bacillus subtilis BS5	Lipopeptide	Bioremediation of hydrocarbon-contaminated sites
Azotobacter chroococcum	Lipopeptide	Environmental applications.
Pseudomonas aeruginosa BS20	Rhamnolipid	Bioremediation of hydrocarbon-contaminated sites
Micrococcus luteus BN56	Trehalose tetraester	Bioremediation of oil-contaminated environments
Nocardiopsis alba MSA10	Lipopeptide	Bioremediation
Pseudoxanthomonas sp. PNK-04	Rhamnolipid	Environmental applications
Pseudomonas alcaligenes	Rhamnolipid	Environmental applications
Nocardiopsis lucentensis MSA04	Glycolipid	Bioremediation of marine environment
Calyptogena soyoae	Mannosylerythritol lipid	Bioremediation of marine environment
Pseudozyma hubeiensis	Glycolipid	Bioremediation of marine oil pollution
Pseudomonas cepacia CCT6659	Rhamnolipid	Bioremediation of marine and soil environments
Candida bombicola	Sophorolipids	Environmental applications
C. glabrata UCP1002	Protein-carboydrate-lipid complex	Oil recovery from sand
C. lipolytica UCP0988	Sophorolipids	Oil recovery
C. lipolytica UCP0988	Sophorolipids	Oil removal
C. sphaerica UCP0995	Protein-carboydrate-lipid complex	Removal of oil from sand
C. lipolytica UCP0988	Sophorolipids	Control of environmental oil pollution
C. sphaerica UCP0995	Protein-carboydrate-lipid complex	Bioremediation processes
C. glabrata UCP1002	Protein-carboydrate-lipid complex	Oil removal
C. guilliermondii UCP0992	Glycolipid complex	Removal of petroleum derivate motor oil from sand
C. tropicalis UCP0996	Protein-carboydrate-lipid complex	Removal of petroleum and motor oil adsorbed to sand
C. lipolytica UCP0988	Sophorolipids	Removal of petroleum and motor oil adsorbed to sand
C. sphaerica UCP0995	Protein-carboydrate-lipid complex	Oil removal

5. Application of Biosurfactants in the Petroleum Industry

Petroleum is one of the major energy sources. The energy demand in the world indicates a 1.7% increase in the number of barrels of oil produced per year, between 2000 and 2030, while consumption is expected to reach 15.3 billion tons of oil per year. Oil reserves allow meeting the world's demand for approximately 40 years if current levels of consumption are maintained [45,50,51]. It is therefore important to develop technologies that allow the efficient use of this resource. According to the International Energy Agency, petroleum production is steadily moving toward unconventional crude oils, such as heavy and extra-heavy oils rather than medium and light oils. Heavy and extra-heavy crude oils represent at least one half of recoverable oil resources in countries such as Canada, China, Mexico, Venezuela, and the USA [52].

In the petroleum industry, biosurfactants have been applied effectively for the exploration of heavy oil, offering advantages over their synthetic counterparts throughout the entire petroleum processing chain (extraction, transportation and storage). Biosurfactants are used in microbial-enhanced oil recovery, the cleaning of contaminated vessels and to facilitate the transportation of heavy crude oil by pipeline [53,54]. Table 5 offers a list of biosurfactant applications in the oil industry [53].

Table 5. Common applications of biosurfactants in the petroleum industry.

Step in Petroleum Production Chain	Applications
Extraction	Reservoir wettability modification
	Oil viscosity reduction
	Drilling mud
	Paraffin/asphalt deposition control
	Enhanced oil displacement
	Oil viscosity reduction
Transportation	Oil viscosity reduction
	Oil emulsion stabilization
	Paraffin/asphalt deposition
Oil tank/container cleaning	Oil viscosity reduction
	Oily sludge emulsification
	Hydrocarbon dispersion

5.1. Extraction of Crude Oil from Reservoirs

Several enhanced oil recovery processes are currently employed worldwide: thermal, chemical, physical, *etc.* [55]. However, these processes are very expensive as well as environmentally harmful. Thus, the search for alternative, cost-effective, eco-friendly alternatives to the chemical and thermal enhanced oil recovery methods is necessary. A number of biotechnology-based processes have been proposed to increase oil production in the current energy shortage [56]. Biosurfactants have applications in this realm, as these natural compounds improve the mobilization of hydrocarbons, thereby enhancing the recovery of crude oil from reservoirs in a process denominated microbial-enhanced oil recovery (MEOR) [57].

MEOR consists of the tertiary recovery of oil in which microorganisms or their metabolic products are used to recover residual oil. Microorganisms produce polymers and biosurfactants, which reduce

oil-rock surface tension by diminishing the capillary forces that impede the movement of oil through the pores of rock. Biosurfactants also aid in the emulsification and breakdown of oil film in rock. MEOR involves different strategies, such as the injection of microorganisms that produce biosurfactants into the reservoir and subsequent spread in situ, the injection of nutrients into the reservoir to stimulate the growth of wild microorganisms that produce biosurfactants or the further production of biosurfactants in reactors and subsequent injection into the reservoir [31]. These processes enhance oil recovery from a depleted reservoir, thereby extending the life of the reservoir. MEOR is less-expensive in comparison to chemically-enhanced oil recovery, as microorganisms produce efficient products out of low-cost substrates or raw materials [58].

5.2. Transport of Crude Oil by Pipelines

Crude oil often needs to be transported over long distances from the extraction fields to refineries. The transportation of heavy and extra-heavy crude oil entails operational difficulties that limit its economic viability. The major problems are low flowability due to the high degree of viscosity and asphaltene content in heavy crude oil, which leads to inconveniences, such as the deposition of asphaltenes and/or paraffins as well as a drop in pressure that can cause plugging problems in the pipeline [52,57]. Asphaltenes precipitate in metal pipelines and in presence of ferric ions combined with acidic conditions, forming a solid denominated "asphaltene mud", which deposits in the pipeline and obstructs the free flow of crude oil. Solvents, such as toluene and xylene, are applied to dissolve this type of mud, which increases the production cost and generates highly toxic waste residue [53].

A promising technology involving the production of a stable oil-in-water emulsion that facilitates oil mobility has been recently developed. Biosurfactant-based emulsifiers (bioemulsifiers) are particularly suitable for this application. Bioemulsifiers are high-molecular weight surfactants with different properties in comparison to glycolipids and lipopeptides. These products are not effective at reducing interfacial tensions, but have an excellent capacity to stabilize oil-in-water emulsions. Due to the high number of reactive groups in the molecule, bioemulsifiers bind tightly to oil droplets and form an effective barrier that prevents drop coalescence [57]. Emulsan is the most powerful bioemulsifier and has potential applications in the petroleum industry, including the formation of heavy oil-water emulsions for viscosity reduction during pipeline transport [53,57].

5.3. Oil Storage Tank Cleaning

Large amounts of crude oil are moved daily, distributed to refineries and placed in storage tanks. The maintenance of these tanks requires periodic washing. However, waste and heavy oil fractions that build up at the bottom and on the walls of storage tanks are highly viscous and become solid deposits that cannot be removed with conventional pumping. The removal of this material requires washing with solvents and manual cleaning, which is a hazardous, time-consuming, labor intensive, expensive procedure, as cleaning operations may include hot water spraying, solvent liquefaction and subsequent land waste disposal [57,59].

The use of microbial biosurfactants is an alternative cleaning procedure to decrease the viscosity of sludge and oil deposits through the formation of an oil-in-water emulsion that facilitates the pumping of waste. Moreover, this process allows the recovery of crude oil when the emulsion is broken.

Matsui *et al*. [59] investigated the treatment of oil tank bottom sludge with a novel biosurfactant, JE1058BS, produced by the actinomycete *Gordonia* sp. Dispersion activity was greater than that achieved with a chemical or plant-derived surfactant and the biosurfactant remained stable for at least three weeks. Diab and El Din [60] evaluated the effect of the supernatant from *P.aeruginosa* SH 29 applied to the cleaning of oil-contaminated vessels. The oil was recovered from the bottom and walls of the vessels in just fifteen minutes and floated on the supernatant as a distinct phase. According to the authors, this indicates that the biosurfactant in the sterilized supernatant of *P.aeruginosa* SH 29 can be used directly for cleaning oil storage tanks and other vessels used for the transportation and storage of crude oil. Rocha and Silva *et al*. [6] tested the potential of an isolated biosurfactant from *Pseudomonas cepacia* CCT6659 for cleaning beaker walls contaminated with a layer of oil and found a removal rate of 80%, which suggests the applicability of this biosurfactant in the cleaning of storage tanks.

6. Toxicity of (Bio)Surfactants and Dispersants on Organisms in the Bioremediation Process

The toxicity of biosurfactants in the environment is not well known. Edwards *et al*. [61], in a comparison of toxicity of three synthetic surfactants and three microbial surfactants, concluded that the biosurfactants were less toxic than the synthetic surfactants to some invertebrate species. However, the environmental risks posed by biosurfactants, evaluated through microbial community composition analysis have not been sufficiently evaluated [62].

The lower toxicity and higher biodegradability of biological surfactants compared to their chemical counterparts is the main reason for their high acceptability. However, these features are often assumed as only direct consequence of their natural origin. For these reasons, the environmental features of novel biosurfactants should be carefully considered and investigated before their release into the environment [63].

While the microbial toxicity of (bio)surfactants is a possible cause of bioremediation inhibition, many (bio)surfactants are not toxic to microorganisms at concentrations near their CMC values [64]. Another possible cause of a reduced rate of bioremediation in the presence of (bio)surfactant is due to increased toxicity of the hydrophobic contaminant due to its increased (pseudo)solubility. (Bio)surfactants increase the apparent aqueous solubility of hydrophobic substrates. In addition, some (bio)surfactants or pseudosolubilized contaminants may exhibit selective toxicity toward specific pure cultures but may have a limited inhibitory impact in a remediation system involving a diverse indigenous microbial population [65].

Regarding the use of dispersants, several classes of these chemical agents are employed for environmental mitigation and cleanup; however, these industrial chemicals may present risks to aquatic organisms individually and when mixed with oil [66,67].

The U.S. Clean Water Act and Oil Pollution Act of 1990 requires the maintenance of a National Oil and Hazardous Substances Pollution Contingency Plan (NCP) for response to oil spills that identifies specific commercial products used for control of oil discharges and the quantities and water bodies in which the products may be used [68]. These products consist of dispersants, surface washing agents, surface collecting agents, bioremediation agents, and other miscellaneous oil spill control agents. Under the NCP, the U.S. Environmental Protection Agency has statutory responsibility for obtaining

toxicity and efficacy information from the manufacturers before placing a dispersant on the National Product Schedule [67,68]. Fourteen dispersants are listed on the U.S. Environmental Protection Agency (U.S. EPA). Although the exact compositions of most commercially available oil dispersants are proprietary, they typically contain a high percentage of one or more uncharged or charged anionic surfactants of different solubility [68].

Probabilistic hazard assessment approaches including Chemical Toxicity Distributions (CTDs) may be useful as an initial step toward prioritizing environmental hazards from the use of dispersants. The CTD approach to two acute toxicity datasets (NCP—the contingency plan dataset and DHOS—a subset of NCP listed dispersants reevaluated subsequent to the Deepwater Horizon oil spill) contain median lethal concentration (LC50) values for dispersants alone and dispersant: oil mixtures, in two standard marine test species, *Menidia beryllina* and *Mysidopsis bahia*. These CTDs suggest that dispersants alone are generally less toxic than oil. In contrast, most dispersant: oil mixtures are more toxic than oil alone [66].

Rigorous toxicological comparison of untreated and dispersant-treated oil is complicated by the fact that when oil, seawater, and dispersants are mixed, a complex multiphase system results. In this complex system, aquatic organisms can be exposed to many toxicants, in many forms, which can have several modes of action. Moreover, chemical dispersion of oil can yield: (1) dissolved petroleum hydrocarbons; (2) dissolved dispersant surfactants; (3) mixed droplets of bulk oil and surfactants (often in micellar form); and (4) nonmicellar, particulate bulk oil [67].

A second important issue for determining the effects of dispersants, is the separate and combined toxicity of the dispersant and the crude oil droplets. Toxicity became an important issue in the late 1960s and early 1970s when application of toxic products resulted in substantial loss of sea life [67]. Since that time, dispersants have been formulated to minimize toxicity to aquatic organisms. For example, the LC50 values of dispersants used in the early 1970s ranged from about 5 to 50 mg/L to the rainbow trout in 96 h exposures. In contrast, LC50s for dispersants available today vary from 200 to 500 mg/L and contain a mixture of surfactants and a less toxic solvent. The U.S. EPA uses a five-step scale of toxicity categories to classify pesticides based on their acute toxicity to aquatic organisms [69]. Nonetheless, use of oil dispersants remains a controversial countermeasure to minimize the impact of oil spills [67,68].

Because the purpose of a dispersant is to facilitate the acceleration of natural attenuation and dilution of spilled oil, the aquatic toxicity of the dispersant: oil mixture is an important consideration. This further complicates a comparative toxicity evaluation, because the course of toxicity in a mixture may be unknown and potentially different for each dispersant. Therefore, although the presence of polycyclic aromatic hydrocarbons does not represent the only factor in determining oil toxicity, evidence links the increased presence of polycyclic aromatic hydrocarbons in chemically dispersed oils to increased toxicity to aquatic organisms [68,70].

The general proposal of toxicity tests is to establish the potential impact of chemicals on the biota of a given environment. The information acquired can be used to regulate use of chemical substances and evaluate the necessity for treatment after their release to the environment [71,72]. An understanding of the factors that contribute to the toxicity of surfactants is necessary to interpret results of toxicity tests of this class of compounds [72]. The most important factor to consider is chemical structure. Since several years, all studies have emphasized the benefits of using rapid, sensitive, reproducible and

cost-effective bacterial assays for toxicity screening and assessment [73]. Microorganisms are useful in ecotoxicity testing because they may be evaluated over a short time and they occupy trophic levels in which bioaccumulation and/or bioconcentration are potential problems [74]. Bacterial toxicity tests measure a wide variety of endpoints including mutagenicity tests [75], population growth [76], CO_2 production [77], enzyme biosynthesis [78], glucose mineralization [79], and bioluminescence inhibition [80].

Several tests have been used to evaluate the toxicity of chemical and biological surfactants on various organisms. The lethal concentration (LC_{50}) is a method that evaluates the rate of population mortality of a species and indicates that the higher the concentration, the lower the toxicity of the surfactant [61,81]. The germination index (GI) it is another method which combines measures of relative vegetable seed germination and relative root elongation to evaluate the toxicity of biosurfactants. The GI value of 80% has been used as an indicator of the disappearance of phytotoxicity [6,18,20,22].

Table 6 lists toxicity values of (bio)surfactants, dispersants, crude oils and dispersant/crude oil mixtures to vegetables and organisms collected from the literature.

Table 6. Results of toxicity tests of (bio)surfactants, dispersants, crude oils, and dispersant/crude oil mixtures to vegetables and organisms.

Test Compound	Organisms/Vegetables Test	Toxicity	References
Biosurfactants			
Emulsan	*Mysidopsis bahia*	LC_{50} (200 mg/L)	[61]
Emulsan	*Menidia beryllina*	LC_{50} (300 mg/L)	[61]
Candida sphaerica UCP 0995 biosurfactant	*Brassica oleracea*	86% GI	[81]
Candida sphaerica UCP 0995 biosurfactant	*Artemia salina*	LC_{50} (600 mg/L)	[81]
Candida sphaerica UCP 0995 biosurfactant	*Brassica oleracea*	no toxicity	[18]
Candida sphaerica UCP 0995 biosurfactant	*Artemia salina*	no toxicity	[18]
Candida lipolytica UCP 0988 biosurfactant	*Brassica oleracea*	no toxicity	[20]
Candida lipolytica UCP 0988 biosurfactant	*Artemia salina*	no toxicity	[20]
Pseudomonas aeruginosa UCP 0992 biosurfactant	*Brassica oleracea*	80% GI	[22]
Pseudomonas aeruginosa UCP 0992 biosurfactant	*Artemia salina*	LC_{50} (525 mg/L)	[22]

Table 6. *Cont.*

Test Compound	Organisms/Vegetables Test	Toxicity	References
Emulsifiers/Dispersing agents			
Dodecylbenzene sulfonate/LAS	*Dugesia japonica*	LC_{50} (1.45 mg/L)	[82]
Lauryl sulfate/SDS	*Dugesia japonica*	LC_{50} (0.36 mg/L)	[82]
Triton X-100	*Mysidopsis bahia*	LC_{50} (3.3 mg/L)	[61]
Triton X-100	*Menidia beryllina*	LC_{50} (2.5 mg/L)	[61]
Lauryl sulfate/SDS	*Americamysis bahia*	LC_{50} (18–23 mg/L)	[68]
Lauryl sulfate/SDS	*Menidia beryllina*	LC_{50} (10 mg/L)	[68]
Oil spill dispersants			
Corexit 9500	*Mysidopsis bahia*	LC_{50} (13.4 mg/L)	[61]
Corexit 9500	*Menidia beryllina*	LC_{50} (75.7 mg/L)	[61]
Corexit 9500	*Porites astreoides*	13% surviving	[83]
Corexit 9500	*Montastraea faveolata*	0% surviving	[83]
Corexit 9500	*Americamysis bahia*	42 (mg/L)	[68]
Corexit 9500	*Menidia beryllina*	130 (mg/L)	[68]
Corexit 9500	*Brachionus plicatilis*	LC_{50} (0.447 mg/L)	[67]
Corexit 9500	*Brachionus manjavacas*	LC_{50} (14.2 mg/L)	[67]
Crude oils			
BP Horizon source oil	*Porites astreoides*	67% surviving	[83]
BP Horizon source oil	*Montastraea faveolata*	27% surviving	[83]
Louisiana sweet crude oil	*Americamysis bahia*	LC_{50} (2.7 mg/L)	[68]
Louisiana sweet crude oil	*Menidia beryllina*	LC_{50} (3.5 mg/L)	[68]
Macondo sweet crude oil	*Brachionus plicatilis*	LC_{50} (2.47 mg/L)	[67]
Macondo sweet crude oil	*Brachionus* sp.	LC_{50} (19.3 mg/L)	[67]
Dispersant/oil mixtures			
Corexit 9500/BP Horizon source oil	*Porites astreoides*	67% surviving	[83]
Corexit 9500/BP Horizon source oil	*Montastraea faveolata*	20% surviving	[83]
Corexit 9500/Louisiana sweet crude oil	*Americamysis bahia*	LC_{50} (5.4 mg/L)	[68]
Corexit 9500/Louisiana sweet crude oil	*Menidia beryllina*	LC_{50} (7.6 mg/L)	[68]
1:10 Corexit 9500/Macondo sweet crude oil	*Brachionus manjavacas*	0.21 (mg/L)	[67]
1:50 Corexit 9500/Macondo sweet crude oil	*Brachionus manjavacas*	0.23 (mg/L)	[67]

GI: germination index; LC_{50}: concentration lethal to 50% of the test species.

7. Conclusions

This review provided information on the application of biosurfactants as a promising alternative in the petroleum industry and the bioremediation of oil spills. Since biosurfactants are not yet competitive with chemical surfactants from the economic standpoint, a more thorough investigation of biosurfactant production from agro-industrial waste is needed to reduce the production cost and allow the large-scale

production of these natural compounds. The versatility and efficiency demonstrated in the application of biosurfactants in the oil production chain and the removal of hydrophobic contaminants make these compounds promising biomolecules.

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Author Contributions

Proposed the theoretical frame: Leonie Asfora Sarubbo. Wrote the paper: Rita de Cássia F. S. Silva, Darne G. Almeida, Raquel D. Rufino, Juliana M. Luna, Valdemir A. Santos and Leonie Asfora Sarubbo.

Conflicts of Interest

The authors declare no conflict of interest.

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5.2 CAPÍTULO 2

Artigo intitulado "*Enhanced production of a bacterial surfactant on statiscal screening of operational parameters*", publicado pela revista *International Review of Chemical Engineering.*

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Enhanced Production of a Bacterial Surfactant on Statistical Screening of Operational Parameters

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Abstract – *Biosurfactants have gained attention because they exhibit some advantages such as biodegradability, low toxicity, ecological acceptability and ability to be produced from renewable and cheaper substrates. However, the high cost of production is the limiting factor for widespread industrial applications. Thus, optimization of operational parameters for biosurfactant production by P. cepacia CCT6659 grown in a low-cost medium formulated with industrial wastes was carried out using response-surface methodology. The application of a central composite rotatable design (CCRD) led to the identification of agitation speed, time and inoculum size as significant variables affecting the fermentation process. The optimal levels of the aforementioned variables were 250 rpm agitation speed, 60h of cultivation time and 1.5% inoculum size. The experimental verifications substantiated the model predictions by showing a maximum relative surface tension reduction of 27 mN/m, which was found to be equivalent to about 8.0 g/l isolated biosurfactant as estimated gravimetrically, thereby resulting in an improved production.*

Keywords: *Biosurfactant, Optimization, Pseudomonas, Oil, Response Surface Methodology, Surface Tension*

I. Introduction

Pollution caused by petroleum hydrocarbons in aquatic and terrestrial environment is a common phenomenon that causes significant ecological and social problems. Physical and chemical cleaning processes used to decontaminate the oil polluted areas have been limited in their application [1]. Oil spills are often treated using synthetic surfactants to disperse oil and accelerate its mineralization.

Desorption and/or solubilization are rate limiting steps of hydrophobic petroleum hydrocarbon mineralization in soil and water [2]. Surfactants increase the surface area of hydrophobic contaminants in soil or water and thus increase their aqueous solubility and consequently their microbial degradation [3].

Synthetic surfactants used to increase contaminant solubility are often toxic, representing an additional source of contamination [2]. Microbially produced surface-active compounds have similar properties but are less toxic, biodegradable and can be produced in situ, at the contaminated site [4].

Hydrocarbon-degrading bacteria and yeasts release surfactants/ emulsifiers in order to facilitate assimilation of these insoluble substrates [5]. Surface-active compounds produced by microorganisms are of two main types, those that reduce surface tension at the air–water interface (biosurfactants-BS) and those that reduce the interfacial tension between immiscible liquids, or at the solid–liquid interface (bioemulsifiers).

Biosurfactants usually exhibit emulsifying capacity but bioemulsifiers do not necessarily reduce surface tension [3]. Biosurfactants and bioemulsifiers are not only of interest for bioremediation processes in the petroleum industry.

These compounds can be used to enhance oil recovery from wells, reduce the heavy oil viscosity, clean oil storage tanks, increase flow though pipelines and stabilize fuel water–oil emulsions [2]-[6].

At the beginning when BS were discovered, they mainly attracted attention as "alternatives to chemical surfactants" due to their higher biodegradability and safety. During the last decade, unique properties of BS, like versatile self-assembling and biochemical properties, which are not observed at all in conventional chemical surfactants, have one after another been found [7]. In addition to these advantages, the production efficiency of biosurfactants using microorganisms has been improved along with the progress of biotechnology [8].

Production economy is the major setback in biosurfactant production, as in the case with most biotechnological processes. Often the amount and type of a raw material can contribute considerably to the production cost; it is estimated that raw materials account for 10–30% of the total production cost in most biotechnological processes [9].

The identification and optimization of the cultivation conditions that affect the surfactant production represent key points for the development of a cost-competitive process [10].

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There are a number of operating parameters controlling biosurfactant production, which are required to be maintained within a certain range in operating conditions whereby the activity of bacteria with the resultant of maximum production of biosurfactant can be optimized. In this regard, medium composition, agitation speed, inoculum size and cultivation time are of great importance for control and optimization of biosurfactant production.

The amount of biosurfactant synthesis depends greatly on the availability of carbon sources and on the balance between carbon and other limiting nutrients [11]. Factors affecting surfactant biosynthesis have been studied extensively, but there is dirt of information about optimal conditions for biosurfactant production. Biosurfactant producers can only be effective if they are maintained at their optimal ambient conditions required for growth and activity.

The application of statistical experimental design techniques in bioprocess development and optimization can result in enhanced product yields, closer conformance of the process output or response to target requirements and reduced process variability, development time and cost.

Response surface methodology is a statistical technique employed for multiple regression analysis by using quantitative data obtained from properly designed experiments to solve multivariate equations simultaneously [12]-[13].

The objective of this work was to develop more effective operational conditions for high-yield production of the biosurfactant from *Pseudomonas cepacia* CCT6659 to stimulate commercial application of this biomolecule.

II. Materials and Methods

II.1. Materials

All chemicals were of reagent grade. Growth media were purchased from Difco Laboratories, USA. Canola waste frying oil was received from a local restaurant in Recife-PE, Brazil and was stored according to supplier's recommendations and used without any further processing.

Corn steep liquor was obtained from the factory Corn Products do Brasil, Cabo de Santo Agostinho-PE, Brazil.

II.2. Bacterial Strain and Preparation of Seed Culture

A strain of *P. cepacia* CCT6659 was provided from the culture collection of the Fundação André Tosello de Pesquisa e Tecnologia, Campinas city, São Paulo, Brazil.

The cultures were maintained in nutrient agar slants at 4°C. For pre-culture, the strain from a 24-hour culture on nutrient agar was transferred into 50 ml nutrient broth to prepare the seed culture. The cultivation condition for the seed culture was 28°C, 150 rpm, and 10-14 h of incubation time.

II.3. Fermentation Media

The components of the production medium were dissolved in a mineral medium containing 0.2% $NaNO_3$, 0.05% KH_2PO_4, 0.1% K_2HPO_4, 0.05% $MgSO_4.7H_2O$, 0.01% KCl and 0.001% $FeSO_4.7H_2O$. This mineral medium was then supplemented with the low-cost substrates canola waste frying oil (3%) and corn steep liquor (2%). The medium pH was adjusted to 7.0 by 1.0M HCl.

Aliquots of the cell suspension of 0.7 OD (optical density) at 600 nm, corresponding to an inoculum of 10^7 C.F.U./ml, were used to inoculate 500 ml Erlenmeyer flasks, containing 100 ml of sterile production medium.

Cultivation was carried out at 27°C with shaking in a New Brunswick C-24 shaker (New Brunswick Scientific, NJ, USA). Agitation speed, cultivation time and inoculum size were determined according to the factorial design.

There was no adjustment of pH during cultivation. At the end of fermentation, samples were taken from the liquid culture to determine the surface tension.

II.4. Optimization of Culture Conditions by RSM

The biosurfactant production was evaluated using an experimental design. A Central Composite Rotatable Design (CCRD) was carried out to verify the effects and interactions of cultivation conditions on the production of the biosurfactant. Surface tension was considered as the response variable.

Agitation speed, cultivation time and inoculum size were considered as independent variables. In these designs, a set of 20 experiments, with six replicates at the central points, were performed. The range and levels of the components (factors or independent variables) under study are given in Table I. Each factor in the design was studied at five levels (-1.68, -1, 0 , +1 and +1.68).

In these designs, a set of all the variables were taken at the central coded value considered as zero. The values of the levels were based on results obtained in preliminary experiments. According to the factorial design matrix the surface tension was studied at various combinations of the cultivation conditions. The optimum values from the two CCRD were obtained by solving the regression equation and also by analyzing the response surface contour plots [14]-[15].

To determine the significance of effects, the analysis of variance (ANOVA) with 95% confidence limits was used.

TABLE I
EXPERIMENTAL RANGE AND LEVELS OF THE INDEPENDENT VARIABLES STUDIED IN THE CENTRAL COMPOSITE ROTATABLE DESIGN (CCRD)

Test variables	Range and levels				
	1.68	-1	0	+1	+1.68
Agitation speed (W_1), rpm	116	150	200	250	284
Time (W_2), hours	52	60	72	84	92
Inoculum size (W_3), %	1,2	1.5	2.0	2.5	2.8

The effects and significance of the variables were graphically illustrated using Pareto's charts. A Pareto's chart consists of bars with a length proportional to the absolute value of the estimated effects, divided by the standard error. In the Pareto's chart analysis of variance effect estimates are sorted from the largest absolute value to the smallest absolute value.

The chart includes a vertical line at the critical t-value for an alpha of 0.05. Effects for which the bars are smaller than the critical t-value are considered as not significant and not affecting the response variables.

Effects may be positive or negative. Variance analysis, regressions coefficients determination and graphs were performed using Statistica software version 7.0 (Statsoft.Inc, USA).

II.5. Surface Tension Determination

Surface tension changes were carried out on the cell-free broth obtained by centrifuging the cultures at 5000 g for 20 minutes by the ring method using a Sigma 70 Tensiometer (KSV Instruments LTD - Finland) at room temperature. Tensiometers determine the surface tension with the help of an optimally wettable ring suspended from a precision balance.

In the Ring method the liquid is raised until contact with the surface is registered. The sample is then lowered again so that the liquid film produced beneath the liquid is stretched. As the film is stretched a maximum force is experienced, the force is measured and used to calculate the surface tension.

The instrument was calibrated against Mill-Q-4 ultrapure distilled water (Millipore, Illinois, USA). Prior to use the platinum plate and all the glassware were sequentially washed with chromic acid, deionised water, acetone and finally flamed with a Bunsen burner.

II.6. Biosurfactant Isolation

The biosurfactants was extracted from culture media after cell removal by centrifugation at 5 000g for 30 min.

The supernatant pH was adjusted to 2.0 with 6.0 M HCl, and an equal volume of $CHCl_3$/CH_3OH (2:1) was added.

The mixture was vigorously shaken for 15 min and allowed to set until phase separation. The organic phase was removed and the operation was repeated twice again.

The product was concentrated from the pooled organic phases using a rotary evaporator. The viscous yellowish product obtained was dissolved in methanol and concentrated again by evaporation of the solvent at 45°C [16].

III. Results and Discussion

Culture conditions and other growth factors are strongly influenced cell growth and the accumulation of metabolic products, thus the optimization of these parameters can improve the bacterial efficiency.

As mentioned, RSM can be an excellent approach to study a process response and to figure out the best correlation among the parameters of a process.

This is done via developed models based on the statistical methods to investigate the relation between the inputs and outputs of any process. With the help of the RSM, we can execute the statistical models and to evaluate the effect of parameters of a particular process as well as to optimize the conditions for desirable responses. The RSM is utilized as a statistical design to model the reduction in surface tension (biosurfactant production) process and to determine the significance of operational parameters and their interactions [14].

The factors affecting the biosurfactant production have been extensively studied in recent years, but few of these studies used proper statistical tools for experimental design. The classical method of medium optimization consists in changing one variable at time and keeping the others at fixed level. However in this study we have optimized the culture conditions of the *P. cepacia* strain by the help of RSM for designing the experiments with aim to achieve highest rate of biosurfactant production. Due to the complex nature of biological processes, it is very difficult to predict distinctively the effects of all parameters, which may have multiple interactions. Therefore, RSM was applied to build up an empirical model for modeling biosurfactant production in terms of the operational parameters of agitation speed, time and inoculum size.

Thus, the initial results focused on the experimental design and the effects of the studied variables on biosurfactant production.

Table II shows the experimental results of surface tension together with the process variables that were studied in the Central Composite Rotatable Design (DCCR).

TABLE II
EXPERIMENTAL DESIGN MATRIX OF BIOSURFACTANT PRODUCTION BY *P. CEPACIA* CCT6659 ACCORDING TO THE CCRD

Run Number	Agitation speed (W_1),rpm	Time (W_2), h	Inoculum size (W_3), %	Surface tension (Y_1), (mN/m)
1	-1(150)	-1(60)	-1(1.5)	27.71
2	−1(250)	-1(60)	-1(1.5)	27.60
3	-1(250)	-1(60)	-1(1.5)	27,60
4	−1(250)	+1(840	-1(1.5)	27.42
5	-1(150)	-1(60)	+1(2.5)	29.12
6	−1(250)	-1(60)	+1(2.5)	29.00
7	-1(150)	+1(84)	+1(2.5)	29.53
8	−1(250)	+1(84)	+1(2.5)	29.26
9	-1.68(116)	0(72)	0(2.0)	28.19
10	+1.68(284)	0(72)	0(2.0)	31.14
11	0 (200)	-1.68(52)	0(2.0)	28.08
12	0(200)	+1.68(92)	0(2.0)	29.02
13	0(200)	0(72)	-1.68(1.2)	28.40
14	0(200)	0(72)	+1.68(2.8)	28.70
15	0(200)	0(72)	0(2.0)	28.55
16	0(200)	0(72)	0(2.0)	28.52
17	0(200)	0(72)	0(2.0)	28.55
18	0(200)	0(72)	0(2.0)	28.55
19	0(200)	0(72)	0(2.0)	28.54
20	0(2000	0(72)	0(2.0)	28.54

Central point repetition was done aiming to provide the opportunity of detection of measurement errors and to subsequently use the deviation in the calculation of surface tension to obtain the variance [17].

It can be observed from this table that the desired values for surface tension were in the central ranges of values of the factors applied, indicating a good choice of these ranges for the experiments. The summary of the analysis of variance (ANOVA) representing the results of the quadratic response surface model fitting is shown in Table III. A ANOVA table is essential to test the significance and adequacy of the model. The *p*-value and *F*-value (with 95% confidence interval) were used as tools to check the significance of each studied variable and their interactions. This can be observed by the Pure Error. These parameters reached values much larger than 4. According to [18], Fisher variance ratio must be large enough to justify a very high degree of adequacy of the model and also to indicate that the treatment combinations are highly significant. The squared regression statistic (R^2), the determination coefficient, a measure of the goodness of fit of the model, was very much significant at the level of 92.37%, meaning that the model was unable to explain only 8% of the total variations. The adjusted R value (85.5%) also indicates the significance of the model. The analysis of the components relative to the regression of the prediction model indicates that all terms were statistically significant. The analysis of the residue components (Lack of fit and Pure error) indicates the existence of a lack of fit for the values of F and p. In contrast, the Pure error, with values less than 1.0%, indicates an excellent domain for these experiments. Thus, the results of these analyses can then recommend the adoption of the proposed prediction model resulting discussed using the ANOVA. The Pareto's chart (Fig. 1) clearly shows that all the factors and interactions were significant at the 95% level, confirming the analysis of the ANOVA table (Table III). The stirring speed is the single highest level of statistical significance in the prediction model, followed by time and inoculum size, which showed practically the same level of significance.

TABLE III
ANALYSIS OF VARIANCE (ANOVA) FOR THE QUADRATIC MODEL

Factor	Square sum (SS)	Degree of freedom	Mean square (MS)	*F*-value	*p*-value
W_1 (L)	11.50763	1	11.50763	84202.18	0.000000
W_1 (Q)	0.48677	1	0.48677	3561.70	0.000000
W_2 (L)	0.37429	1	0.37429	2738.70	0.000000
W_2 (Q)	0.07958	1	0.07958	582.28	0.000002
W_3 (L)	0.01655	1	0.01655	121.12	0.000108
W_3 (Q)	0.07958	1	0.07958	582.28	0.000002
W_1 by W_2	0.05445	1	0.05445	398.41	0.000006
W_1 by $_3$	0.00500	1	0.00500	36.59	0.001781
W_2 by W_3	0.03380	1	0.03380	247.32	0.000019
Lack of Fit	1.04928	5	0.20986	1535.53	0.000000
Pure Error	0.00068	5	0.00014	-	-
Total SS	13.7572	19	-	-	-
Some important statistics:			R^2 (%) = 92.37; Adj., 85.50		

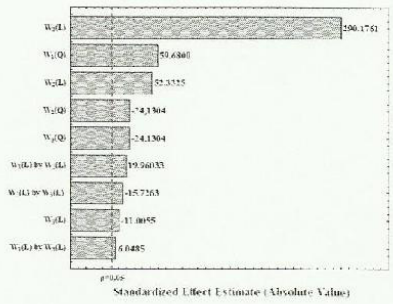


Fig. 1. CCRD - Pareto's Chart of standardized effects for (W_1) agitation speed, (W_2) time and (W_3) inoculum size using surface tension (Y_1) as response variable. The point at which the effect estimates were statistically significant ($p = 0.050$) is indicated by dashed line

The addition of negative signals appearing on some terms indicate that an increase in the value of these terms imply, in general, in a reduction in the value of the dependent variable, the surface tension of the biosurfactant. The terms associated with interactions are marked by lower levels of statistical significance of the prediction model. This means that predictions can be made to obtain different levels of surface tension based on isolated modifications of any of the factors involved.

The following regression Eq. (1) shows the relative surface tension value (Y_1) as a function of the test variables (W) in coded units:

$$Y_1 = 28.55 + 0.92W_1 + 0.18W_1^2 + 0.16W_2 + \\ -0.07W_2^2 - 0.04W_3 - 0.07X_3^2 + ... \\ +0.08W_1W_2 + 0.03W_1W_3 - 0.07W_2W_3 \quad (1)$$

where Y_1 is the response, W_1, W_2 and W_3 are the coded values of the agitation speed, time of cultivation and inoculum size, respectively. The coefficients of Eq. (1) were calculated using RSM and their values were listed along with the parameter estimates in Table III. The predicted versus experimental plot for surface tension showed that actual values were distributed near to the straight line (Fig. 2), which indicated that actual values were very close to the predicted ones (R^2 =92.37%).

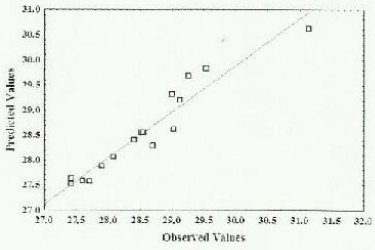


Fig. 2. Experimental reduction in surface tension vs. predicted reduction in surface tension for the CCRD

Thus, it was a suitable model to predict the biosurfactant production efficiency using aforementioned experimental conditions.

Figs. 3 show response surface for the effects of factors on the surface tension of the biosurfactant produced. Fig. 3(A) shows that there is a simultaneous effect of the factors cultivation time and agitation speed in reducing the surface tension.

Fig. 3(B) repeats a behavior similar to Fig. 3(A), influenced by the inoculum size and by the agitation speed.

The shapes of these surfaces change from concave to convex when the handled factors are the inoculum size and time of cultivation.

Thus, the increase of these factors causes reductions in surface tension for a simultaneous increase of these factors involved.

Fig. 3(C) shows the interactive effects of agitation speed and inoculum size on surface tension reduction. It can be observed that the surface tension decreases by decreasing both time and inoculum size.

In almost all cases the parallelism of the level curves of the factors involved, illustrated by the corresponding graphics, confirm the predictions of the low degree of interaction between these factors.

The results obtained from the contours plots indicated that the best condition for biosurfactant production was obtained for an agitation speed of 150 rpm during 60 hours with an inoculum size of 1.5%.

However, considering the importance of the yields in isolated biosurfactant for industrial production, we had observed that the speed of 250 rpm favored the accumulation in biosurfactant (8.0g/L against 6 g/L for agitation speed of 150 rpm), allowing still maintaining the medium surface tension around 27 mN/m. Then, the speed of 250 rpm was chosen for further biosurfactant production.

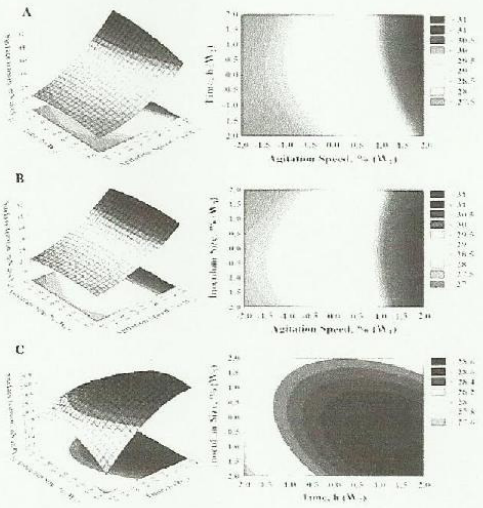
Sen [19] optimized a fermentation medium to maximize the surfactin production by *B. subtilis* DSM 3256, where a 2^4 full-factorial central composite experimental design was followed by multi-stage Monte Carlo optimization.

Jacques et al. [20] reported optimization of biosurfactant lipopeptide production by *B. subtilis* S499 using a Plackett–Burman design. The amount of biosurfactant lipopeptide in the supernatant of a culture carried out in the optimized medium was about five times higher than that obtained in a non-optimized rich medium.

Wei et al. [21], using Taguchi experimental design methods to optimize trace element composition achieved 3.34 g/l surfactin production by *B. subtilis* ATCC 21332 which represented two fold increase from the original control.

The effect of agitation speed on biosurfactant production has been studied. Silva et al. [22] studied the production of a biosurfactant from *P. aeruginosa* UCP0992 at 150 and 200 rpm.

The results obtained that the velocity of 200 rpm favoured the accumulation of biosurfactant (6.5 g/L) against the concentration of 5.0g/l obtained at 150 rpm, although the surface tension (27.5 mN/m) remained unaltered for both velocities tested.



Figs. 3. Three dimensional plots for the minimum surface tension (maximum biosurfactants production). RSM plots were generated using the data shown in Table III. Inputs were the 20 experimental runs carried out under the conditions established by the CCD design. (A) Reduction in surface tension as a function of agitation speed and time of cultivation. (B) Reduction in surface tension as a function of agitation speed and inoculum size. (C) Reduction in surface tension as a function of time of cultivation and inoculum size

The effect of the variation in the agitation speed from cultures of 50-200 rpm has been studied by Oliveira et al. [23] for *P. alcaligenes* cultivated in 0.5% palm oil. They observed that the increase of the rotation velocity from 260 to 300 favoured the reduction of the surface tension of the cell-free broth to 27.6 mN/m. In a previous work, however, Oliveira et al. [24] observed, for *P. aeruginosa* cultivated in palm oil, 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, respectively. Darvishi et al. [25] observed that the agitation speed had no significant effect on the maximum biosurfactant produced by the consortium ERCPPI-2 cultivated in olive oil during 48 h.

Cunha et al. [26] observed that agitation had a negative effect on surface tension reduction by the biosurfactant produced by *Serratia* sp. SVGG16 cultivated in hydrocarbons, and that best results were obtained with the lowest value, of 100 rpm, when compared to 200 and 300 rpm.

The agitation speed of the medium is a determining factor in the mixture of the aqueous and oily phase as well as in the oxygen mass transfer into the cultures using agitated flasks. Higher rotary velocities may increase the oxygen mass transfer to the aqueous medium yielding the best conditions for microbial growth.

Biosurfactants are mainly produced in the exponential growth phase, as will be described below. Thus, the appropriate oxygen amount that is required for microbial growth and, consequently for surfactant biosynthesis may be achieved in the upper level of 250 rpm of experimental design.

The inoculum size influences the production of biosurfactants. However, no systematic studies to elucidate the effects of the inoculum size on surfactant production have been reported [27]. In our case, the best inoculum size was obtained in the downer level of the CCRD.

The cultivation time of 60 h, selected according to the second factorial design against the period of 100h before optimization, was very important as this parameter is determinant for the large scale production of biosurfactant molecules.

IV. Conclusion

The present study aims at optimizing the agitation speed, time and inoculum size to maximize the *P. cepacia* biosurfactant yield and analyzing the individual, cumulative and interactive effects of these three critical parameters on biosurfactant production. Thus, surface response modeling based on central composite rotating design (CCRD) was done using batch fermentation flasks studies. The optimal agitation speed, time and inoculum size are 250, 60 and 1.5%, respectively, at which the model predicts a maximum surface reduction of 27 mN/m and a biosurfactant yield of 8 g/l, as estimated gravimetrically. The interaction between all the three parameters largely contributed towards enhancing the yield of the biosurfactant, showing that these variables have considerable influence on the fermentative production of biosurfactants. The statistical modeling based on response surface methodology was effectively used to develop an optimal seeding strategy to improve biosurfactant production.

Acknowledgements

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5.3 CAPÍTULO 3

Artigo intitulado "*Production and characterization of a new biosurfactant from Pseudomonas cepacia grown in low-cost fermentative medium and its application in the oil industry"*, submetido à revista *Biocatalysis and Agricultural Biotechnology*.
E-mail de confirmação de submissão encontra-se ao final deste manuscrito.

Manuscript Details

Manuscript number	BAB_2016_224
Title	Production and characterization of a new biosurfactant from Pseudomonas cepacia grown in low-cost fermentative medium and its application in the oil industry
Article type	Research Paper

Abstract

The production of a biosurfactant by Pseudomonas cepacia CCT6659 was studied in a low-cost medium formulated with 2% waste frying oil, 3% corn steep liquor and 0.2% NaNO3 for 60 hs at 28 ºC and 250 rpm. Biosurfactant production was growth associated, as indicated by the growth and biosurfactant production kinetics. Surface tension was reduced to less than 25.5 mN/m. The properties of the biosurfactant that was separated by acid precipitation and organic solvent extraction were investigated and its critical micelle concentration was determined as 600 mg/L. Preliminary chemical characterisation revealed the anionic nature of the biosurfactant. The biosurfactant produced by the isolate was characterised by Fourier transform infrared spectroscopy (FTIR), proton nuclear magnetic resonance (1 H NMR) and gas chromatography and mass spectroscopy (CG-MS). Biosurfactant demonstrated good surface tension reduction capacity and emulsifying activity with motor oil (up to 90%). The biosurfactant also demonstrated stability during exposure to high temperatures (up to 120°C for 15 min), high salinity (12% NaCl) and a wide pH range (2-12). The crude biosurfactant was not toxic to the microcrustacean Artemia salina or two varieties of cabbage. The crude biosurfactant was effective at recovering up to 75% of the residual oil from oil-saturated sand samples, at displacing oil (81%) and recovering up to 90% of motor oil from the walls of beakers. These results indicate the potential value of this biosurfactant for application in the oil industry, especially in enhanced oil recovery, tank cleaning and the bioremediation of spills at sea and in soil.

Keywords	Biosurfactant; Pseudomonas; Environment; Oil; Surface tension; Toxicity.
Manuscript category	Environmental Biotechnology
Corresponding Author	Leonie Sarubbo
Corresponding Author's Institution	Catholic University of Pernambuco
Order of Authors	Rita de Cássia Soares da Silva, Darne Germano, Hugo Meira, Elias Silva, Charles Bronzo Farias, Raquel Rufino, Juliana Luna, Leonie Sarubbo
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Semptember 08, 2016

Biocatalysis and Agricultural Biotechnology

Editor-in-Chief: Dr. Ching T. Hou

U.S. Department of Agriculture (USDA), Agricultural Research Service (ARS), Peoria, Illinois, USA

Dear Editor,

We would like to submit for appreciation the manuscript **"Production and characterization of a new biosurfactant from *Pseudomonas cepacia* grown in low-cost fermentative medium and its application in the oil industry".**

This is a new contribution and the subject of the article is also within the scope of the journal. The aim of the present study was to describe the production, characterization and applications of a biosurfactant using a optimized mineral low-cost medium supplemented with waste frying oil and corn steep liquor as substrates. This study also describes the kintetics of biosurfactant production, surface active properties, emulsifying capacity, strusctural characterization and toxicity. The successfully application of the biosurfactant in the environment was also investigated.

I inform that all authors agree to submit the work and that the present manuscript is not being submitted to another journal.

Suggestions of possible reviewers are listed below.

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Yours sincerely,

Prof. Dr. Leonie A. Sarubbo

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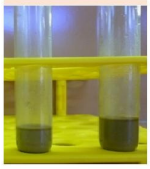
Production of biosurfactant by *Pseudomonas cepacia* grown in 2% waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$

Cell free broth

Surface tension: 27mN/m

Emulsification activity with motor oil: 90%



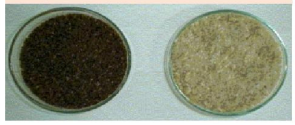
Nontoxic against microcrustacean and cabbage species:

Brassica oleracea

Artemia salina

Applications of the biosurfactant

Recovery of motor oil in sand samples: 75%



Removal of oily surface: 90%

Characterization of the biosurfactant

FTIR, ^1H NMR and CG-MS

Production and characterization of a new biosurfactant from *Pseudomonas cepacia* grown in low-cost fermentative medium and its application in the oil industry

Rita de Cássia F. Soares da Silva[a,c], Darne G. Almeida [a,c], Hugo M. Meira[b,c], Elias J. Silva[b,c], Charles B. B. Farias[b,c], Raquel D. Rufino[b,c], Juliana M. Luna[b,c], Leonie A. Sarubbo[a,b,c,*]

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Abstract

The production of a biosurfactant by *Pseudomonas cepacia* CCT6659 was studied in a low-cost medium formulated with 2% waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$ for 60 hs at 28 ºC and 250 rpm. Biosurfactant production was associated growth of the microrganisms, as indicated by the curve growth and biosurfactant production. The surface tension of the medium was reduced from 65 mN / m to less than 25.5 mN / m. The biosurfactant that was isolated by acid precipitation and organic solvent extraction and their properties were investigated and its critical micelle concentration was determined as 600 mg/L. Preliminary chemical characterisation revealed the anionic nature of the biosurfactant. The biosurfactant produced by the isolate was characterised by Fourier transform infrared spectroscopy (FTIR), proton nuclear magnetic resonance (^{1}H NMR) and gas chromatography and mass spectroscopy (CG-MS). Biosurfactant demonstrated good surface tension reduction capacity and emulsifying activity with motor oil (up to 90%). The biosurfactant also demonstrated stability during exposure to high temperatures (up to 120°C for 15 min), high salinity (12% NaCl) and a wide pH range (2-12). The crude biosurfactant was not toxic to the microcrustacean *Artemia salina* or two *Brassica oleracea* plant species. The crude biosurfactant was effective at recovering up to 75% of the residual oil from oil-saturated sand samples, at displacing oil (81%) and recovering up to 90% of motor oil from the walls of beakers. These results indicate the potential value of this biosurfactant for application in the oil industry, especially in enhanced oil recovery, tank cleaning and the bioremediation of spills at sea and in soil.

Keywords: Biosurfactant; *Pseudomonas*; Environment; Oil; Surface tension; Toxicity.

1. Introduction

Oil is one of the most important resources of energy in the modern industrial world. As long as oil is explored, transported, stored and used there will be the risk of a spillage. Oil spills impose a major problem on the environment (Silva et al., 2014a).Various processes have been developed to remove oil from contaminated areas. Among them mechanical recovery of oil by oil sorbents is one of the most promising countermeasures. This process includes the transfer of oil from the contaminated area to some transportable form of temporary storage with the help of oil sorbents (Choi et al., 1993). However, in this process most of the used sorbents end up in landfills and incineration, which either produces another source of pollution or increase the oil recovery cost. There is an increased interest in promoting environmental responsibility through cleaning products that have traditionally been discarded after a single use.

Biosurfactants, biologically produced, have been increasingly used in soil washing and oil removal from contaminated areas (Mulligan et al., 2001; Wei et al., 2005). Biosurfactants are produced naturally by many microorganisms such as bacteria, yeasts, and fungi. Biosurfactants consist of common cell material (e.g., glycolipids, lipopeptides, and fatty acids). Biosurfactants are widely used in different industries such as cosmetics, special chemicals, food, pharmaceutics, agriculture, cleansers and petroleum (Sarubbo et al., 2015; Pacwa-Plociniczak et al., 2011).

The most important advantage of biosurfactants over chemical surfactants is probably their ecological acceptability. Biosurfactants are biodegradable and thus problems of toxicity and accumulation in natural ecosystems are avoided. In the environmental sector, biosurfactants have potential applications in bioremediation and waste treatment because of their inherent degradability (Pacwa-Plociniczak et al., 2011; Santos et al., 2016).

A major obstacle on the way of wide-scale industrial application of biosurfactant is the high production cost coupled with less production rate as compared to commercially available synthetic surfactants. Therefore, if the production cost becomes competitive with the synthetic surfactants, and as the commercial availability of biosurfactant increases, the industrial use of biosurfactant can be expected to grow tremendously in the coming decade. To achieve this goal, during the recent years, efforts have been directed to explore the means to reduce the biosurfactant production costs through improving the yield, and the use of either cost-free or low-cost feed stocks or agricultural byproducts as substrate(s) for biosurfactant production. Many of the cheaper byproducts such as peat hydrolysate (Sheppard and Mulligan, 1987), olive-oil mill effluent (Mercade and Manersa, 1994), soapstock and waste-water from sunflower oil (Benincasa et al., 2002), de-proteinized whey (Daniel et al., 1998), vegetable oil refinery residue and corn steep liquor (Luna et al., 2012; Rufino et al., 2008), waste frying oil (Batista et al., 2010; Raza et al., 2009), vegetable fat waste (Gusmão et al., 2010), wheat bran and okara (Ohno et al., 1993; 1996), molasses (Makkar and Cameotra, 1997) and potato effluent (Noah et al., 2002) have been targeted as sole source of carbon for biosurfactant production by microbes in submerged fermentation.

Most commercially available surfactants are derived from petroleum products. However, recent environmental control legislation has driven the development of natural surfactants as alternatives to existing products (Silva et al., 2014a).

Apart from the industrial applications of biosurfactants envisage, their application in the oil industry is one of the potential uses which requires lower purity specifications so that whole cell broth could be used, eliminating the purification steps that represent almost 60% of the total production costs (Sarubbo et al., 2015).

Various studies on the production and characterization of rhamnolipids produced by *Pseudomonas* genus using low-cost and renewable raw material have been reported in literature (Sarubbo et al., 2015; Santos et al., 2016). However, to our knowledge, no reports

have been published on biosurfactant production from industrial residues by the *P. cepacia* strain.

Thus, environmental and economic issues have motivated the completion of this study that presents biosurfactant production by a *P. cepacia* strain, coded as *P. cepacia* CCT6659, using a previously optimized mineral low-cost medium supplemented with waste frying oil and corn steep liquor as substrates (Silva et al., 2013a,b; Rocha e Silva et al., 2014). This study also describes the curve of biosurfactant production, its characterization, surface active properties, emulsifying and hydrophobic compounds removal capacity, and toxicity. The application of the biosurfactant in the environment was also investigated.

2. Materials and Methods

2.1. Materials

All chemicals were of reagent grade. Growth media were purchased from Difco Laboratories, USA. Canola waste frying oil was received from a local restaurant in Recife-PE, Brazil and was stored according to supplier's recommendations and used without any further processing. Corn steep liquor was obtained from the factory Corn Products do Brasil, Cabo de Santo Agostinho-PE, Brazil.

2.2. Bacterial strain and preparation of seed culture

A strain of *P. cepacia* CCT6659 was provided from the culture collection of the Fundação André Tosello de Pesquisa e Tecnologia, Campinas city, São Paulo, Brazil. The cultures were maintained in nutrient agar slants at 4 ºC. For pre-culture, the strain from a 24-h culture on nutrient agar was transferred into 50 ml nutrient broth to prepare the seed culture. The cultivation condition for the seed culture was 28 ºC, 150 rpm, and 10-14h of incubation time.

2.3. Fermentation media

Production media that was used for liquid submerged fermentation have the following composition (%): canola waste frying oil (2), corn steep liquor (3), $NaNO_3$ (0.2), KH_2PO_4, (0.05), K_2HPO_4 (0.1), $MgSO_4.7H_2O$ (0.05), KCl (0.01) and $FeSO_4.7H_2O$ (0.001) and the pH was adjusted to 7.0 by 1.0M HCl. The media were sterilized by autoclaving at 121ºC for 15 min. Fermentation was carried out in 500 ml Erlenmeyer flasks with a 100 ml working volume. For inoculation, the flasks were allowed to cool down to room temperature (27°C) before transferring 2% (v/v) primary inocula of the cell suspension of 0.7 OD (optical density) at 600 nm, corresponding to an inoculum of 10^7 C.F.U./ml into the production media. The cultures were incubated in a rotary New Brunswick C-24 shaker (New Brunswick Scientific, NJ, USA) for 60 h at 250 rpm. There was no adjustment of pH during cultivation. The initial surface tension of the production media prior to inoculation was 55 mN/m. All experiments were carried out in triplicate. The curve of microorganism 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.

2.4. Biomass determination

For biomass determination, 10 ml samples were centrifuged at 5 000 g during 30 min and the cell pellet dried in an oven at 105°C for 24 h.

2.5. Emulsifying activity with different hydrophobic compounds

Emulsification index (EI) was measured using the method described by Cooper and Goldenberg (1987), whereby 2 ml of a liquid hydrophobic compound (motor oil, lubricating oil, diesel, kerosene, n-hexadecane and vegetables oils) was added to 2 ml of the culture broth free of cells in a graduated screwcap test tube, and vortexed at high speed for 2 min. The emulsion stability was determined after 24 h and the emulsification index was calculated by

dividing the measured height of the emulsion layer by the mixture's total height and multiplying by 100.

2.6. Surface tension and CMC determination

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 milliliters volume of each sample was transferred into a clean 20 ml beaker and placed onto the tensiometer platform. A platinum wire ring was submerged into the solution and then slowly pulled through the liquid–air interface, to measure the surface tension (mN/m). Between each measurement, the platinum wire ring was rinsed with chromic acid, deionised water, acetone and finally flamed and was allowed to dry. The calibration was done using Mill-Q-4 ultrapure distilled water (surface tension =71.5 mN/m ±0.5) before taking samples measurement.

The critical micelle concentration (CMC) was determined using the same equipment, by measuring the surface tensions of dilutions of isolated biosurfactant in distilled water up to a constant value of surface tension. Stabilization was allowed to occur until standard deviation of 10 successive measurements was less than 0.4 mN/m. Each result was the average of 10 determinations after stabilization. The value of CMC was obtained from the plot of surface tension against surfactant concentration.

2.7. Effect of environmental factors on biosurfactant activity

The effect of addition of different concentrations of NaCl on the activity of the biosurfactant was investigated in the cell-free broth. A specific concentration of NaCl (2-12%, w/v) was added and surface tension and emulsification activity were determined as previously stated. The cell-free broth was also maintained at a constant temperature (0, 5, 28, 70, 100 and 120°C) for 60 min and used for surface tension and emulsification measurements. The effect of

pH on surface tension and emulsification was evaluated after adjustment of the broth pH to 2, 4, 6, 8, 10 and 12 with 6.0 M NaOH or HCl.

2.8. Biosurfactant isolation

The biosurfactants was extracted from culture media after cell removal by centrifugation at 5000g for 30 min. The supernatant pH was adjusted to 2.0 with 6.0 M HCl, and an equal volume of $CHCl_3$/CH_3OH (2:1, v/v) was added. The mixture was vigorously shaken for 15 min and allowed to set until phase separation. The organic phase was removed and the operation was repeated twice. The product was concentrated from the pooled organic phases using a rotary evaporator. The viscous yellowish product obtained was dissolved in methanol and concentrated again by evaporation of the solvent at 45°C (Rocha e Silva et al., 2014). After extraction, the product was treated with a base and crystallized for maximum removal of impurities.

2.9. Biosurfactant characterization by thin-layer chromatography

After isolating the biosurfactant, a sample of 0.1g was dissolved in methanol and analysed by thin layer chromatography (TLC) on silica gel plates (G60; Merck, Germany) to calculate the retention factor, i.e., R_f values. Chromatograms were developed with chloroform:methanol:acetic acid (65:15:2, v/v) and the detection was done by the following methods: (1) exposure to iodine vapours for lipid stains, (2) exposure to the Molish reagent for sugar detection and (3) exposure to 1% ninhydrin solution for free amino groups. The reagents were sprayed, and the plates were heated for 30 to 40 min at 110°C until the appearance of the respective colour (Deshpande and Daniels 1995; Santos et al., 2002).

2.10. Determination of biosurfactant ionic character

The ionic charge of the biosurfactant was determined using the agar double diffusion technique (Meylheuc et al., 2001). Two regularly spaced rows of wells were made in an agar of low hardness (1% agar). Wells of one row were filled with the biosurfactant solution and wells of the other were filled a pure compound of known ionic charge. The anionic substance chosen was sodium dodecyl sulphate (SDS) 20 Mm and the cationic one was barium chloride, 50 mM. The appearance of precipitation lines between the wells, indicative of the ionic character of the biosurfactant, was monitored over a 48-h period at ambient temperature.

2.11. Nuclear magnetic resonance spectroscopy

The extracted biosurfactant was re-dissolved in deuterated chloroform (CDCl3) and the respective ^{1}H NMR spectra were recorded at 25°C using a Agilent 300Mz spectrometer operating at 300.13 MHz. Chemical shifts (δ) are given on the ppm scale relative to tetramethylsilane (TMS).

2.12. Fourier transform infrared spectroscopy

The biosurfactant extract recovered from the supernatant of the *P. cepacia* CCT6659 isolate was characterized by Fourier transform infrared spectroscopy (FTIR). The FTIR spectrum 400 Perkin Elmer, with a resolution of 4 cm^{-1}, were collected from 400 to 4000 wavenumbers (cm^{-1}).

2.13. Gas chromatography and mass spectroscopy (GC-MS)

The fatty acids sample (hydrophobic moiety) of the biosurfactant was analysed on gas chromatograph-mass spectrometer system (Thermo Scientific Trace 1300 - ISQ Single Quadrupole) equipped with a TGMS-5 column (30m x 0.25mm. 0.25 um film thickness). Initial column temperature was 60°C for 3 min, then ramped at 10°C min^{-1} to 300°C and held

for 15 min. 1µL sample was injected. Helium was used as carrier gas. The injector and detector temperatures were maintained at 300 and 280°C, respectively.

2.14. Toxicity against Artemia saline as indicator

The toxicity assay was performed with the biosurfactant using brine shrimp (the microcrustacean *Artemia salina*) as the toxicity indicator. Brine shrimp eggs were obtained in a local story. Larvae were used within 1 day of hatching. The assays were conducted in penicillin tubes of 10 ml capacity containing 10 brine shrimp larvae in 5 ml of seawater per tube and solutions of cell-free broth and the isolated biosurfactant to give concentrations based on the CMC (600 mg/L) until LC_{50} (lowest concentration that kills 50% of tested brine shrimp). They were observed for 24 h to calculate mortality (Meyer et al., 1982). Each test was run in triplicate, and seawater was used as the control.

2.15. Phytotoxicity assay

The phytotoxicity of the biosurfactant was evaluated in static test by seed germination and root elongation of two cabbages species (*Brassica oleracea* var. *botrytis* L. *and Brassica oleracea* var. *capitata*) according to Tiquia et al. (1996). Solutions of the isolated biosurfactant were prepared with distilled water in concentrations at ½xCMC (300 mg/L), at the full CMC (600 mg/L) and twice the CMC (1200 mg/L). The toxicity was determined in sterilized Petri dishes (1x10cm) containing Whatman Nº 1 filter paper. The seeds were pre-treated with Sodium hypochlorite and 10 seeds were inoculated in each Petri dish which was inoculated with 5 ml of the test solution at 27ºC. After five days of incubation in the dark, the seed germination, root elongation (≥ 5 mm) and germination index (GI, 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 control) x 100

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

GI = [(% seed germination) x (%root growth)] / 100%

Controls were prepared with distilled water to replace the biosurfactant solutions. The mean and standard deviation of triplicate samples from each concentration were calculated.

2.16. Application of the biosurfactant in hydrophobic contaminant removal from sand

Biosurfactant suitability for enhanced oil recovery was carried out using artificially contaminated sand with 10% of motor oil as described by Luna et al. (2011). Samples of 50 g of 40/50 mesh (0.3-0.42 mm) and 20/30 mesh (0.6-0.85 mm) fractions of the contaminated Brazilian standard sand NBR 7214 (1982) were transferred to 250-ml Erlenmeyer flasks, which were submitted to the following treatments: addition of 50 ml distilled water (control) or 50 ml of the cell-free broth or 50 ml of a solution of the isolated biosurfactant at ½xCMC (300 mg/L), at the full CMC (600 mg/L) and twice the CMC (1200 mg/L). The samples were incubated on a rotary shaker (150 rpm) for 24 h at 27°C and then were centrifuged at 5 000 g for 20 minutes for separation of the laundering solution and the sand. The pH of the samples was also measured before and after the treatment. The amount of oil residing in the sand after the impact of biosurfactant was gravimetrically determined as the amount of material extracted from the sand by hexane.

2.17. Application of the biosurfactant in hydrophobic contaminant spreading

The oil displacement test was carried out slowly by dropping of 15 µl of motor oil onto the surface of 40 ml of distilled water layer contained in a Petri dish (15 cm in diameter) that spread all over the water surface area. This was followed with the addition of 10 µl of the cell-free broth or aqueous solutions containing the isolated surfactant at ½xCMC (300 mg/L), at the full CMC (600 mg/L) and twice the CMC (1200 mg/L) onto the surface of the oil layer. The

average value of the diameters of the clear zones of triplicate experiments was measured and recorded then calculated as percentage of the Petri dish diameter (Ohno et al., 1996).

2.18. Application of the biosurfactant in hydrophobic contaminant cleaning test

As a means to check the cleaning ability of the biosurfactant, the inner walls of a set of beakers were coated with motor oil. To remove the adhered oil, 50 ml of the cell-free broth or wash solutions containing aqueous solution of the isolated biosurfactant at ½xCMC (300 mg/L), at the full CMC (600 mg/L) and twice the CMC (1200 mg/L) was added to each beaker, vortexed for 1.0 min, and allowed to stand for 6 h (Pruthi and Cameotra, 2000).

2.19. Statistical analysis

All surface tensions, biosurfactant concentrations and emulsification activities 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. Biosurfactant production and curve of microorganism growth

The bacterium *P. cepacia* was able to produce biosurfactant during growth on industrial wastes as growth substrates, indicating its ability to use a wide spectrum of carbon sources ranging from water soluble carbohydrates to water immiscible hydrocarbons.

Fig. 1 shows the pattern of biosurfactant formation and cell growth of *P. cepacia* CCT6659 in the mineral previously optimized medium (Silva et al., 2013a) containing 2% waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$ at 28ºC during 60 h under agitation speed of 250 rpm. *P cepacia* started to produce biosurfactant soon after inoculation along with cell growth. Surface tension measurements were used as an indirect measure of surfactant

production and to evaluate the efficiency of the produced biosurfactant. The culture broth surface tension reached the minimum value of 27 mN/m after 12 h, while the accumulation of biosurfactant was gradually increasing. The maximum biosurfactant production (8 g/l) occurred during the stationary phase of the culture (48-60h). At this point, it was observed not only the maximum biosurfactant accumulation, but also the highest biomass concentration (about 15.0 g/l dry weight). The rapid increase of biomass during the stationary phase is related to the diauxic growth phenomenon, which typically occurs when less complex nutrients sources present in the medium are depleted, forcing the microorganism to the consumption of the complex sources. The surface tension, on the other hand, remained constant because the CMC had been reached. Biosurfactant production by *P. cepacia* was growth-associated since there was an almost parallel relationship between biosurfactant production, cell growth and surface tension reduction.

For the cultivation of *P. cepacia* CCT6659, the pH showed small variations between 6.0 and 7.0 especially during the first 24 h, related to increased metabolic activity and production of organic acids, remaining more stable after 36 h around 7.0 until the end of cultivation.

Different curve profiles for biosurfactant production can be observed as described in the literature. Cha et al. (2008), for example, observed that the production of the biosurfactant from *P. aeruginosa* cultivated in an optimized medium containing 2% acidified soybean oil was found to be a function of cell growth. Biosurfactant was produced at a concentration of 5.0 g/l, with a cell concentration of 25 g/l. George and Jayachandran (2009), on the other hand, observed that the production of 9.2 g/l rhamnolipid biosurfactants using orange fruit peelings from *P aeruginosa* MTCC2297 was growth independent. A surface tension reduction up to 31.1 mN/m was obtained. The rhamnolipid production by *Pseudomonas aeruginosa* cultivated in minimal media provided with n-heptadecane as sole carbon source under shake-flask conditions started at 48 h of incubation and lasted till the end of incubation period (7 days).

The surface tension of distilled water was reduced from 72.0 to 30.0 mN/m (Raza et al., 2009).

The carbon source preference for biosurfactant production seems to be strain dependent. Some reports show that vegetable oils are more efficient substrates in biosurfactant production from *P. aeruginosa* strains, while others show lower rhamnolipid yield from oils than that from glucose and glycerol (Silve et al., 2010).

Wu et al. (2008) described the production of 3.7 and 2.6 g/l of biosurfactant for olive oil and soybean oil, respectively, from *P. aeruginosa* EM1, while Sousa et al. (2011) obtained 1.2 g/l biosurfactant from *P. aeruginosa* MSIC02 grown in hydrolyzed glycerin for a surface tension value of 29.3 mN/m. Oliveira et al. (2009) used experimental design tools to study the effects of process conditions on surfactant production during batch tests conducted using a strain of *P. alcaligenes* growing on palm oil. The authors obtained 2.3 g/l biosurfactant after 48 h of bioprocess, with a surface tension of 31 mN/m. The use of sequencing batch reactors for biosurfactant production from *P. aeruginosa* SP4 growing in palm oil and glucose during 48 h showed a surface tension reduction to 28-30 mN/m (Pansiripat et al., 2010).

Rocha e Silva (2014) also utilizing *P.cepacia* CCT6659 described the production of 5.2g/L of biosurfactant with a surface tension of 27.57 mN/m after 144h of cultivation at 250 rpm utilising 2.0% soybean waste frying oil as the carbon source. As can be seen, the change on the carbon source influences the different results obtained from the same microorganism. This shows that greater importance should be given in the choice of the substrate for greater efficiency in the production of a biosurfactant.

3.2. Biosurfactant emulsification capacity

A practical measurement of a surface-active compound utility is its ability to turn immiscible liquids into stable emulsions. Table 1 presents the hydrophobic substrates tested for emulsification by the cell-free broth containing the biosurfactant from *P. cepacia* CCT6659. Motor oil of car engine was the best substrate while n-hexadecane and soybean vegetable oil

were the poorest. The cell-free broth containing the biosurfactant obtained after 60 h of cultivation was able to emulsify 90% motor oil. The water-oil emulsions showed to be compact and remained stable for more than six months at room temperature, suggesting that the addition of such biosurfactant into a remediation process may enhance the availability of the recalcitrant hydrocarbon. The vegetable oils were particularly not good substrates for emulsification by the biosurfactant from *P. cepacia* (data not shown).

The ability to form and stabilize emulsions is one of the most important features to be considered. The ability in emulsifying hydrocarbons depends on the hydrophobic compound since biosurfactants are substrate specific. The emulsification capacity is related to the compatibility between the biosurfactant conformational structure and the hydrocarbon, which will allows the stabilization or not of the microscopic droplets (Silva et al., 2014a).

3.3. Biosurfactant stability related to surface tension and emulsification

Several factors influence the effectiveness of biosurfactants including temperatures and pH. Therefore, it is important to study the influence of these parameters when considering applications of these metabolites in bioremediation.

The results of stability of the cell-free broth containing the produced biosurfactant (crude biosurfactant) from *P. cepacia* CCT6659 with respect to temperature, pH, salinity and time of heating are shown in Table 2.

As described in material and methods section, various amounts of NaCl were added to the cell-free broth and mixed completely and then surface tension was measured. As seen, the biosurfactant maintained the capacity of reducing the surface tension up to 12 % NaCl, while 80-90% of the original emulsifying activity of both hydrocarbons was retained at concentrations up to 12%. These results could be interpreted as a good salt resistance of the biosurfactant produced by *P. cepacia* under conditions of this work. Since the sea salinity in

the world is around 3%, the biosurfactant from *P. cepacia* CCT6659 could be applied in saline environments.

When the temperature was varied from 0°C to 120°C, the surface tension of the biosurfactant solution showed little variation and remained nearly constant at around 27 mN/m, indicating the usefulness of the biosurfactant in industries where heating to achieve sterility is of paramount importance. The emulsification indexes of the motor oil were also thermally stable, while the emulsification of lubricating oil showed a little increase with the increase of the temperature.

The surface tension of the biosurfactant remained relatively stable to pH changes between pH 5.0 and 12.0 around 28-29 mN/m, whereas below pH 6.0 surface tension showed a little increase, reaching 32 mN/m at pH 2.0. The emulsification of motor oil by the cell-free broth containing the biosurfactant increased with the pH increase, especially at pH 10.0 and 12.0, for which an emulsification index of 100% was obtained, while emulsification indexes of 100% were obtained with lubricating oil in the pH range of 2-6.

In the case of the biosurfactant from *P. cepacia*, it is likely that the increase in temperature has allowed greater interaction between the biosurfactant and the lubricating oil. On the other hand, this effect was not observed for the emulsification of motor oil probably due to its high viscosity compared to the lubricating oil. Regarding the change in pH, it is possible that there has been some alteration in the biosurfactant structure, allowing greater or lesser interaction with each type of oil, which will depend on the composition and structure of the oil being emulsified.

The findings suggest that the robust characteristics of the crude biosurfactant are very beneficial for applications under extreme conditions of salinity, temperature and pH, such as in oil recovery and in the bioremediation of a polluted marine environment.

Considering that the purification accounts for up to 60% of the total production cost of biosurfactants and the economic considerations in the oil industry, most biosurfactants

would require either whole-cell culture broths or crude preparations (Santos et al., 2016). Therefore, the use of the biosurfactant from *P. cepacia* CCT6659 in its crude form can be considered another advantage of this new biomolecule in the petroleum market.

3.4. Surface tension and critical micelle concentration (CMC) of the biosurfactant

The biosurfactant produced by *P. cepacia* CCT6659 is able to reduce the surface tension of supernatant significantly. As seen in Fig. 1, the surface tension of supernatant in all cultures has been drastically decreased from 70 to about 27 mN/m. It has been happened even by the early taken samples that show the production of biosurfactant has taken place at early stage of culture. For further investigation we determined CMC values for the biosurfactant. The presence of the biosurfactant reduced the surface tension, which was proportional to biosurfactant concentration in solutions, until it reached the CMC concentration. The surface tension of water decreased gradually with increasing biosurfactant concentration from 70 mN/m to 25.5 mN/m, with a biosurfactant concentration of 0.06% (600 mg/l), and then remained constant.

3.5. Biosurfactant characterization

The crude extract form *P. cepacia* CCT6659 is a viscous sticky oily residue with brown colour (Fig. 2a). After partial purification of the crude extract, it was observed a floc formation (Fig. 2b) and at the end of the purification process, it was obtained an off-white powder (Fig. 2c). The isolated biosurfactant was soluble in aqueous solution and in organic solvents.

The agar double diffusion method showed the anionic nature of the biosurfactant. A similar result had been observed for the biosurfactant from *P. aeruginosa* UCP0992 (Silva et al., 2010) and *P. fluorescens* 495 (Meylheuc et al., 2001), both submitted to the same test. The biosurfactant isolated from *P. cepacia* was characterized by TLC. The TLC analysis revealed the R*f* (retention factor) value of 0.75 (Fig. 2c). The spot showed positive reactions for sugars

with Molish reagents and for lipids with iodine vapours, but negative reactions for amino groups with ninhydrin, suggesting its glycolipid nature.

Glycolipids rhamnolipids are produced by *Pseudomonas* strains as mixtures of different congeners, being the most common L-rhamnosyl-bhydroxydecanoyl- b-hydroxydecanoate (Rha-C10-C10) and Lrhamnosyl- L-rhamnosyl-b-hydroxydecanoyl-b-hydroxydecanoate (Rha-Rha-C10-C10). Other congeners frequently found include mono- and di-rhamnolipids with acyl chains containing 8, 10, 12 or 14 carbons, mostly saturated, and, less often, containing one or two double bonds, as well as with only one b-hydroxy fatty acid. Exceptionally, acyl chains with a higher (C18, C22 and C24) or an odd number of carbons can be found (Gudiña et al., 2015). The composition and distribution of rhamnolipid congeners vary according to the bacterial strain, the culture conditions and the media composition. Even using the same strain, the culture medium used can influence the composition of the rhamnolipid mixture (Raza et al., 2009). Another possibility can be the presence of impurities such as extracted non-metabolized fatty acids from the culture broth that could influence the surface-active properties.

3.6. ^{1}H NMR spectroscopy

The characterization of biosurfactants produced by *P. cepacia* strains using NMR spectroscopy has been described in the literature (Silva et al., 2014b). Therefore, the composition of biosurfactants obtained from isolate CCT6659 was probed by ^{1}H NMR analysis (Fig. 3). ^{1}H NMR spectrum of the biosurfactant from *P. cepacia*, demonstrated three well-defined regions. The signals between δ 0.75 and 2.5 ppm suggests the presence of aliphatic and methyl groups in biosurfactant; that between δ 5.25 and 5.5 ppm indicate the presence of double bounds and those between δ 9.5 and 10.0 ppm corresponds to the hydrogen bonded to the carboxylic acid. The signals at δ 2.75 and 7.25 ppm were attributed to the residual signal of the solvent (chloroform).

3.7. Fourier transform infrared spectroscopy

In the Fig. 4, the spectra obtained for the biosurfactant presented absorbance band of hydroxyl groups at 3350 cm^{-1}; between 2966 and 2863 cm^{-1} it is clear the presence of aliphatic chains; at ~ 1700 cm^{-1} the presence of C=O group is evidenced; and at ~ 1400 cm^{-1} the spectra show double bonds in the structure of the biosurfactant (–C=C–).

3.8. GC-MS analysis of fatty acids

The fatty acid composition of the biosurfactant was analyzed by GC-MS and compared with the library data. It was found that the biosurfactant is mainly comprised of long chain fatty acids, mainly C-18 long fatty acids (Fig. 5). The major fatty acid found was C-18 Octadecanoic acid (89.49%). Octadecanoic acid was also previously found as the main fatty acid in various studies of purified glycolipids. Saravanakumari and Mani (2010) have isolated a biosurfactant from *Lactococcus lactis* which also contains octadecanoic acid as a fatty acid chain linked to the sugar moiety. Octadecanoic acid was also found as the major fatty acid type in the cell bound biosurfactant produced by *Lactobacillus pentosus* (Vecino et al., 2015). Based on the results obtained by ^{1}H NMR, FTIR spectroscopy, TLC and GC-MS analysis, it is possible to say that the biosurfactant studied shows a glycolipidic nature.

3.9. Biosurfactant toxicity

The use of biosurfactant also depends on their properties. The absence of toxicity is of fundamental importance for application in the environmental realm. Eco-toxicity bioassays are analytical methods that allow characterizing the toxicity of chemical substances.

The biosurfactant from *P. cepacia* was tested for its toxicity in a short term bioassay using brine shrimp, as shown in Table 3. The isolated biosurfactant did not show toxicity to larvae with increasing biosurfactant concentration and not even the cell-free broth after 24 hs. The acute toxicity tests of the surfactant JE1058BS produced by the bacterium *Gordonia* sp.

against two species of marine larvae, *Mysidopsis bahia* (shrimp) and *Menidia beryllina* (fish), also showed the low toxicity of this biosurfactant (Saeki et al., 2009). Based on the *Artemia salina* toxicity test, *P. cepacia* biosurfactant proved to be innocuous (Table 3), as expected for a biologically derived surface-active agent (Camacho-Chab et al., 2013).

The biosurfactant produced by *P. cepacia* was tested for its toxicity using seeds of two cabbages species (*Brassica oleracea*). The results of relative seed germination, relative root growth and germination index (GI) are shown in Table 4. Since the GI value of 80% was used as an indicator of the disappearance of phytotoxicity (Meylheuc et al., 2001), the results showed that the crude biosurfactant (cell-free broth containing the biosurfactant) did not show inhibitory effects on the seed germination and root elongation of cabbage, while increasing the concentration of the surfactant reduced the percentage of seed germination. Nalini and Parthasarathi (2014) reported similar results by the biosurfactant from *Serratia rubidaea* SNAU02 demonstrating 86% germination at a concentration of 500 mg/ml when compared with the control with regard to the vegetal species.

3.10. Application of the biosurfactant in hydrophobic contaminant removal

The *P. cepacia* CCT6659 biosurfactant potential for bioremediation was verified through soil washing of motor oil-contaminated sand. The cell-free broth containing the surfactant and solutions of the isolated surfactant under, at and above the CMC were tested, as shown in Table 5.

Removals in excess of 70% were observed for all solutions tested, with a maximum removal of 96% at 2xCMC. This result can be considered good when compared to the results obtained by Rocha e Silva et al. (2014) also using a biosurfactant from *P. cepacia* (93%). Thus, the biosurfactant produced was efficient in the removal of hydrophobic compounds under kinetic conditions of agitation. It was also observed that the particle size of the sands did not exercise great influence on the percentage removal of the pollutant, neither the biosurfactant

concentration, suggesting the ability of the biosurfactant to be applied in many kinds of soils and in its crude form. The possibility of using the cell-free broth can contribute to increase the use of biosurfactants in applications such as enhanced oil recovery or bioremediation, as their purification constitutes a relevant portion of the overall production costs.

3.11. Application of the biosurfactant in hydrophobic contaminant spreading and hydrophobic contaminant recovery

The drop collapse method depends on the principle that a drop of liquid containing a biosurfactant collapses and spreads over the oily surface. There is a direct relationship between the diameter of the sample and concentration of the biosurfactant (Satpute et al., 2010).

The cell-free broth containing the biosurfactant produced by *P. cepacia* CCT6659 gave a high oil spreading efficiency. This was more effective than the aqueous solutions of the isolated biosurfactant at ½xCMC (0.03%), at the full CMC (0.06%) and twice the CMC (0.12%), as shown in Table 6. According to Sitohy et al. (2010), the biosurfactant produced by *B. subtilis* NRRL B-94*C* (0.1%) gave an oil spreading efficiency of 57% while the well-known industrial surfactant Triton X-100 displaced 80% of the oil at the same concentration.

Of the several envisioned industrial applications of the biosurfactants, one of greatest potential use is in the storage tank cleaning. The cell-free broth from *P. cepacia* CCT6659 was effective in recovery of up to 90% oil from the walls of the beakers, while the aqueous solutions of the isolated biosurfactant at ½ x CMC, at the full CMC and twice the CMC recovered around 80% of the motor oil (Table 6). These results suggest the suitability of the biosurfactant from *P. cepacia* to remove the sticky crude oil from the walls of containers.

Conclusions

Apart from the industrial application of biosurfactant envisaged, their application in oil industry is one of the potentials where lower purity biosurfactant preparations or whole cell

broth can be used, eliminating purification costs. However, the biosurfactants need to be stable under the extreme environmental conditions encountered in the oil reservoir such as high temperature, pressure and salinity. The biosurfactant produced by *P. cepacia* showed stability under extreme conditions of pH, temperature and salinity. The crude biosurfactant could reduce the surface tension of the medium to 27mN/m and with isolated biosurfactant in a CMC decreased to 25.5 mN/m. Our preliminary lab scale results showed that besides the potent surface activity, the crude biosurfactant has high emulsifying activities, capacity to remove hydrophobic contaminants and did not show toxicity. In conclusion, the biosurfactant produced by *P. cepacia* was a kind of preferable surface-active substance, having potential application in bioremediation of hydrocarbons contamination.

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Table 1

Emulsification index (EI) of hydrophobic substrates by the cell-free broth containing the biosurfactant from *P. cepacia* CCT6659 cultivated in mineral medium supplemented with 2% waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$ during 60 h at 250 rpm and 28ºC

Substrate	EI (%)
Motor oil	90.0±4.35
Lubricating oil	79.2±2.78
Diesel	51.2±3.40
Kerosene	10.5±3.02
n-Hexadecane	6.02±1.35
Soybean oil	6.02±2.50

Table 2

Influences of salt concentration, temperature and pH on the surface tension reducing activity and on the emulsifying activity of the cell-free broth containing the biosurfactant from *P. cepacia* CCT6659 cultivated in mineral medium supplemented with 2% waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$ during 60 h at 250 rpm and 28ºC

NaCl (%)	Surface tension (mN/m)	EI (%)[a]	EI (%)[b]
2.0	26.0±0.25	67.7±3.12	71.3±4.22
4.0	26.0±0.15	70.0±3.45	64.5±3.15
6.0	25.8±0.10	68.5±4.11	61.3±2.94
8.0	27.7±0.50	67.0±4.10	67.7±3.47
10.0	25.7±0.30	75.5±3.35	65.0±2.88
12.0	26.7±0.25	79.6±4.30	60.0±2.09
Temperature (°C)	**Surface tension (mN/m)**	**EI (%)[a]**	**EI (%)[b]**
0	27.0±0.14	92.2±2.19	76.1±2.19
5	28.0±0.34	88.4±4.12	75.5±3.88
28	26.3±0.12	90.0±4.35	79.2±2.78
70	26.7±0.22	88.5±5.02	80.5±3.45
100	26.7±0.25	84.0±4.03	83.2±5.15
120	27.0±0.21	86.7±3.10	90.0±5.10
pH	**Surface tension (mN/m)**	**EI (%)[a]**	**EI (%)[b]**
2	32.1±0.11	73.0±2.97	100.0±2.12
4	31.5±0.20	75.3±3.08	100.0±2.10
6	28.8±0.31	76.5±4.09	100.0±1.15
8	26.2±0.31	86.5±4.03	90.75±2.45
10	29.4±0.12	100.0±2.21	90.0±3.27
12	28.3±0.15	100.0±3.05	83.5±2.43

[a] Emulsification index of motor oil

[b] Emulsification index of lubricating oil

Table 3

Toxicity of the biosurfactant from *P. cepacia* CCT6659 cultivated in mineral medium supplemented with 2% waste frying oil, 3% corn steep liquor and 0.2% on brine shrimp larvae

Biosurfactant concentration in saline water	**Mortality of brine shrimp larvae (%)**
Cell-free broth	No mortality
½ x CMC (300 mg/l)	No mortality
CMC (600 mg/l)	No mortality
2 x CMC (1200 mg/l)	No mortality
5 x CMC (3000 mg/l)	10.0±0.11
12 x CMC (LC_{50} =7200 mg/l)	50.0±0.21

Table 4

Phytotoxicity of the biosurfactant from *P. cepacia* CCT6659 cultivated in mineral medium supplemented with 2% waste frying oil, 3% corn steep liquor and 0.2% on two cabbages species

Cabbage Seeds	Phytotoxicity parameters (%)	Biosurfactant solutions			
		Cell-free broth	Isolated biosurfactant at ½ x CMC	Isolated biosurfactant at the CMC	Isolated biosurfactant at 2 x CMC
***Brassica oleracea var. botrytis* L.**	**Germination index**	80±0.51	70±0.15	50±0.45	25±0.15
	Root growth	81	81	62	42
	Seeds germinated	99	86	81	59
Brassica oleracea var.capitata	**Germination index**	80±0.61	56±0.39	35±0.31	19±0.25
	Root growth	82	68	48	34
	Seeds germinated	98	83	73	55

Table 5

Removal of motor oil adsorbed in standard sand samples by the biosurfactant produced by *P. cepacia* CCT6659 cultivated in mineral medium supplemented with 2% waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$ and by distilled water (as the control)

Removal agent	Motor oil removal from sand (%)	
	40/50 mesh (0.3-0.42 mm)	20/30 mesh (0.6-0.85 mm)
Biosurfactant (cell-free broth)	84.0±0.6	76.1±0.3
Solution of the isolated biosurfactant at ½ x CMC	82.0±0.3	94.8±0.3
Solution of the isolated biosurfactant at the CMC	92.8±0.6	92.3±0.5
Solution of the isolated biosurfactant at 2 x CMC	95.0±0.7	96.3±0.3
Control (distilled water)	20.0±0.1	15.2±0.2

Table 6

Application of the biosurfactant from *P. cepacia* CCT6659 in hydrophobic contaminant spreading and hydrophobic contaminant recovering from the walls of the beakers

Tests	Biosurfactant solutions			
	Cell-free broth	**Isolated biosurfactant at ½ x CMC**	**Isolated biosurfactant at the CMC**	**Isolated biosurfactant at 2 x CMC**
Hydrophobic contaminant spreading	81±2.5	52±2.7	75±3.0	80±2.2
Hydrophobic contaminant recovery	90±1.9	78±2.8	80±1.8	85±2.3
Control (distilled water)	2.0±0.1	1.5±0.2	1.7.0±0.1	2.3±0.2

Figure captions

Fig. 1. Growth, pH, surface tension and biosurfactant concentration profiles of *P. cepacia* CCT6659 grown in mineral medium supplemented with 2% waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$ during 60 h at 250 rpm and 28ºC

Fig. 2. TLC of the biosurfactant from *P. cepacia* CCT6659 grown in mineral medium supplemented with 2% waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$ during 60 h at 250 rpm and 28ºC. (A) Crude extract forms a viscous sticky oily residue with brown colour. (B) Crude extract after partial purification and (C) off-white powder after final purification.

Fig. 3. ^{1}H NMR spectrum (CD_3OD, 300 MHz) of the isolated biosurfactant from *P. cepacia* CCT6659 cultivated in mineral medium supplemented with 2% waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$

Fig. 4. FTIR spectrum for biosurfactant extract produced by *P. cepacia* CCT6659 cultivated in mineral medium supplemented with 2% waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$

Fig. 5. GC-MS separation of the biosurfactant produced by the *P. cepacia* CCT6659 showing peaks for the octadecanoic acid

Fig. 1.

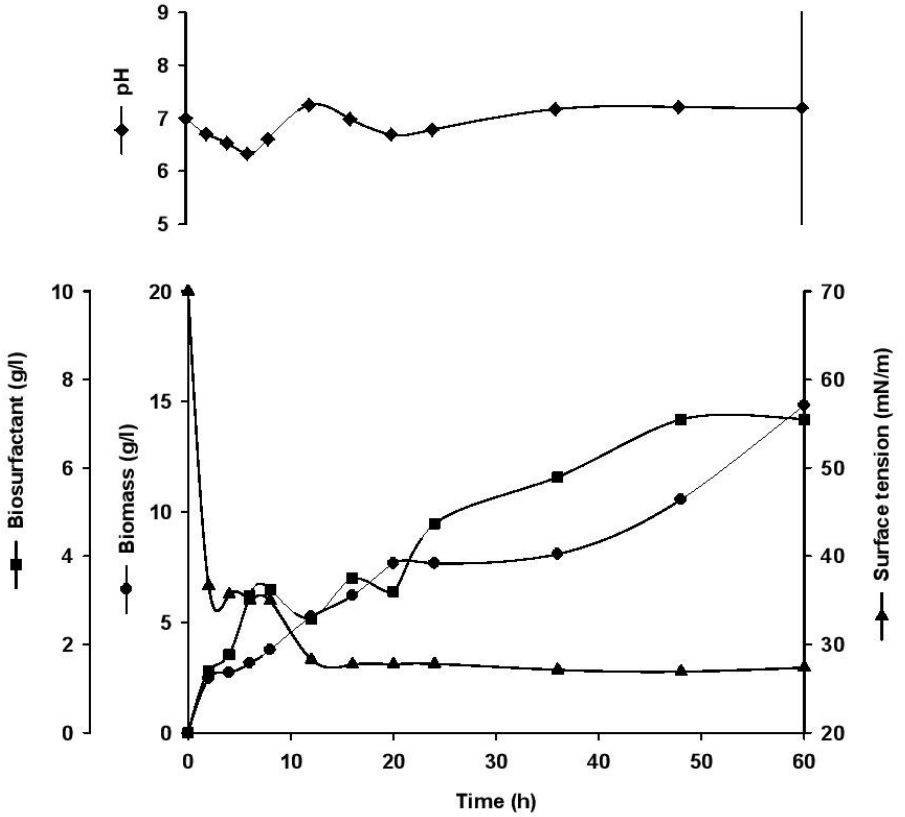

pH
9
8
7
6
5
Biosurfactant (g/l)
Biomass (g/l)
Surface tension (mN/m)
10
8
6
4
2
0
20
15
10
5
0
70
60
50
40
30
20
0
10
20
30
40
50
60
Time (h)

Fig. 2.

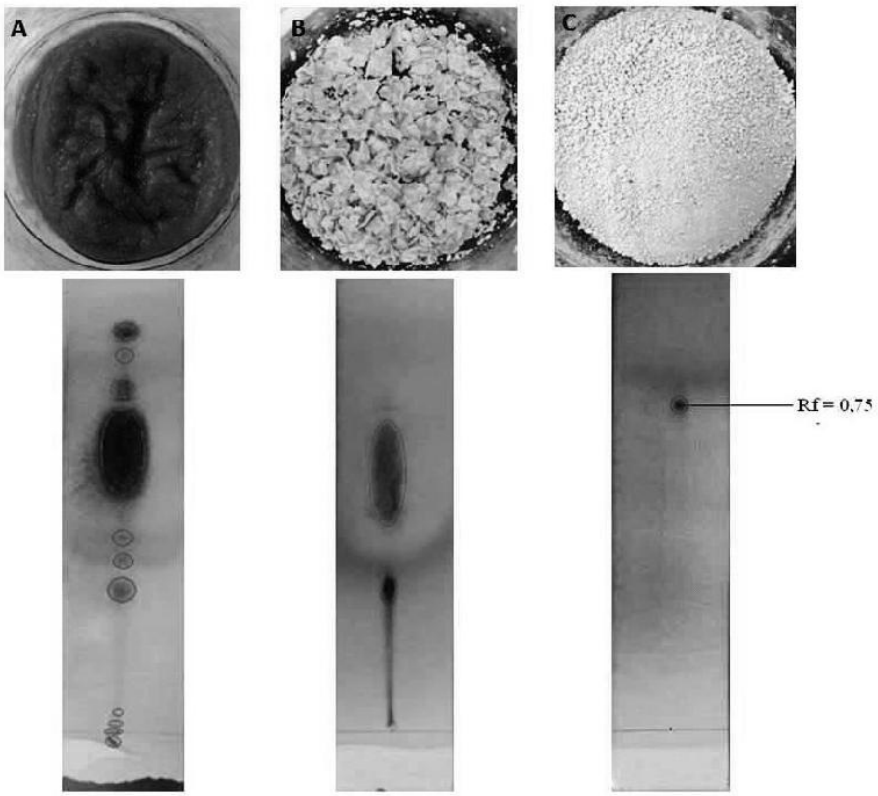


Fig. 3.

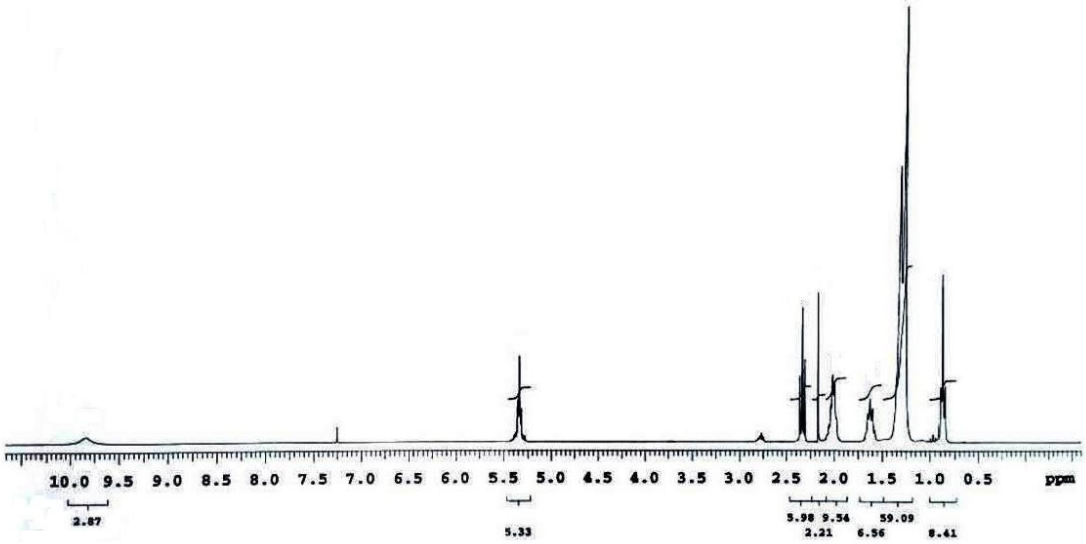

10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm
2.87
5.33
5.98 9.54
2.21
59.09
6.56
8.41

Fig. 4.

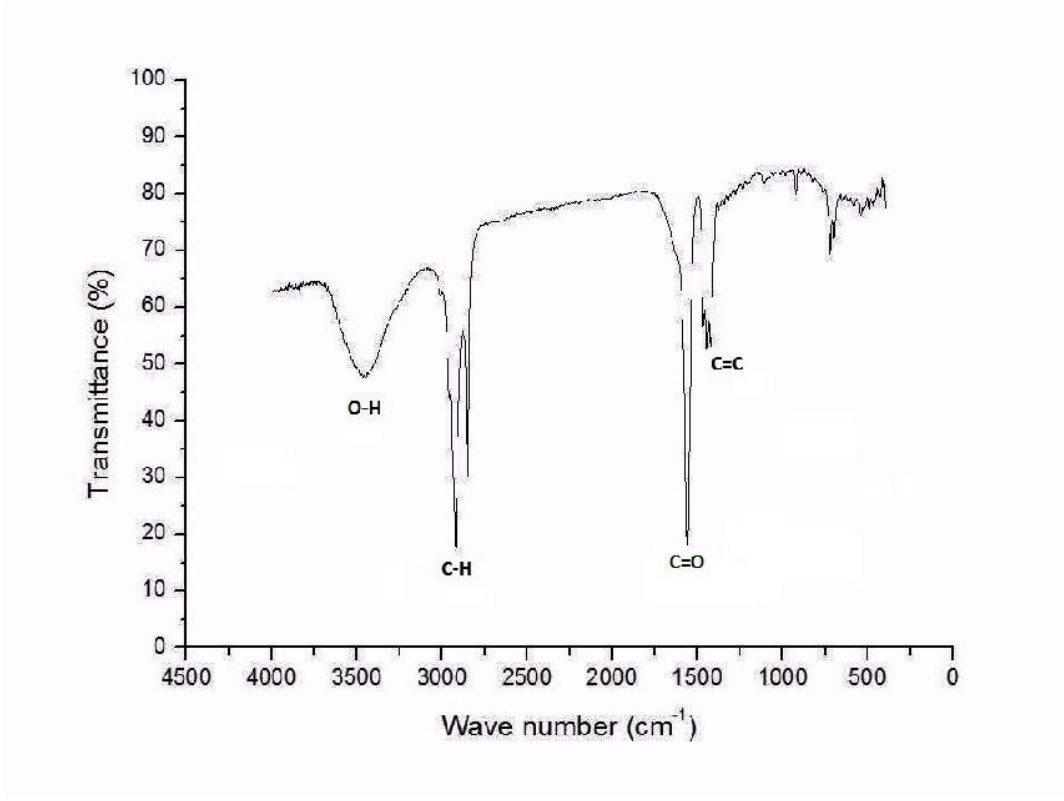
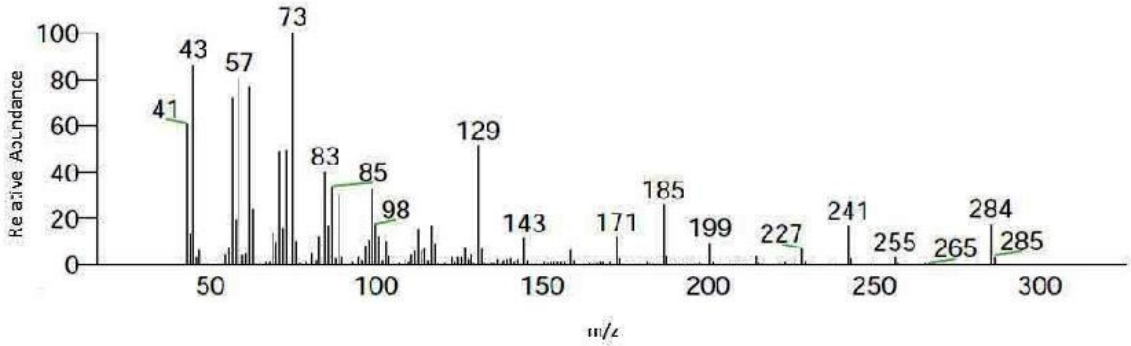

Transmittance (%)
100
90
80
70
60
50
40
30
20
10
0
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Wave number (cm-1)
O-H
C-H
C=O
C=C

Fig. 5.


Relative Abundance
100
80
60
40
20
0
50
100
150
200
250
300
m/z
41
43
57
73
83
85
98
129
143
171
185
199
227
241
255
265
284
285

HIGHLIGHTS

1. *Pseudomonas cepacia* CCT6659 produced a biosurfactant in a low-cost medium.
2. The biosurfactant is stable under extreme conditions of pH, temperature and NaCl.
3. The biosurfactant did not show toxicity in soil and water.
4. The biosurfactant recovered 75% of oil from sand and displaced 81% of oil in water.
5. The new biosurfactant shows a glycolipidc nature.

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5.4 CAPÍTULO 4

Artigo intitulado "*Formulated biosurfactant for application in the descontamination of petroderivatives*", a ser submetido à revista Frontiers in Microbiology.

Formulated biosurfactant for application in the descontamination of petroderivatives

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Abstract

Biosurfactants are multifunctional molecules that due to their amphipathic nature have great potential of application in several sectors of the industry, especially in the petroleum sector. In this study, it was investigated biosurfactant fermentative production by *Pseudomonas cepacia* CCT6659 strain using industrial residues in semi-industrial 50L bioreactor under controlled parameters. Yield values of 40.5 g/L were achieved in the scale up, with a reduction on surface tension to 29 mN/m. Biosurfactant was formulated using the methods of Addition of preservative, Tindalization and Fluent steam. After this, formulated biosurfactant samples were stored for 120 days and the product tenso-active-properties and stability were evaluated. Biosurfactant presented stability in all formulation methods, demonstrating be economically viable for large-scale application, due to the low cost of obtaining the formulated product, estimated at around US $ 0.14-0.15/L. The formulated product was evaluated in oil removal in soil, reaching a removal of 76.55%; marine stones, with a removal of 84.5%; and on bioremediation processes, promoting an oil biodegradation of 70% in seawater. Therefore, the formulated product has suitable conditions for application in extreme conditions, such as in the petroleum industry and in the decontamination of petroderivatives in the environment and can be produced at a reduced price compared to those commercially available on the world market.

Keywords: Biosurfactants price; *Pseudomonas cepacia* CCT6659; Formulation; Scale up; Petroderivatives.

1. Introduction

Each year, about 5 million tons of oil are transported by ships to supply the major industries with theoretical commitment of safety standards to avoid possible spills. Moreover, the huge drilling platforms in the oceans should have the responsibility to maintain a structure with assurance mechanisms to prevent leaks on a large scale (Spier et al., 2013). Despite efforts in the creation of laws over the decades, when one speaks of petroleum and sustainable development, maritime transport with oil cargo will continue to exist, along with the potential risk of accidents involving oil and by-product spills (Freitas et al., 2016). In 2010, the environmental disaster in the Gulf of Mexico, for example, released 780 million gallons of the mixture of oils and hydrocarbons from Deepwater Horizon platform, triggering an imbalance in marine fauna and flora for 1.5 km deep (Spier et al., 2013; Silva et al., 2014). Thus, the possibility of environmental contamination is real and imminent, with an urgent need for the development of novel technologies that can contain possible contaminations. (Silva et al., 2014; Freitas et al. 2016).

Contamination by petroleum and its by-products can be treated through physical, chemical, or biological methods. Conventional physical and chemical methods could rapidly remove the majority of leaked oil, but in most cases the removal just transfer contaminants from one environment to another, even produce toxic by-products. More importantly, oil could not be completely cleaned up by physical and chemical methods. Due to negative consequences of the physicochemical approach, more attention is now given to the exploitation of biological alternatives. Thus, bioremediation has gained ground in recent years (Freitas et al., 2016; Almeida et al., 2017). In this sense, the innovative biosurfactants have replacing the chemical dispersants because of its environmental benefits such as

biodegradability, low toxicity and stability under extreme environmental conditions, allowing also their use in industrial applications (Marchant; Banat, 2012).

The global concern with sustainability has become a competitive advantage for companies that apply their concepts in the production process, given that concern about the environmental future of the planet ceased to be an option, constituting an emergency trend for companies and consumers. In this scenario, it is expected that the biosurfactants market overcome the synthetic surfactants market. In this sense, one of the great advantages of biotech-sector companies compared to its competitors is biodegradable and non-toxic nature of its products and the using of industrial waste in the production process (Silva et al. 2014; Almeida et al., 2016).

Large-scale biosurfactant production seem to be sound effective strategy to overcome the competitiveness with their synthetic counterparts. In order to develop suitable technology for possible commercialization, it is essential to carry out tests in bioreactors that are systems which allow a larger control of parameters affecting rates of microbial growth. Then scale up in bioreactor leading to an operational facilitation of technical and economic view-point in the implementation of the industrial-scale production (Chikere et al., 2012; Luna et al., 2015).

But stability is an essential factor to enable large-scale production, mainly of a biotech product that need a certain time to be produced before the urgent application in an oil disaster. All the oil must be removed from the ocean at about 24 hours after pouring. Therefore, biosurfactant durability must be high to maintain the product in stock, so that it is readily available for the purpose of immediate use (Marchant; Banat, 2012).

The stability studies of biosurfactants are based on the following approaches: 1. Long-term stability study - study designed to verify physical, chemical, biological and microbiological characteristics of biosurfactants formulated, after period for expected validity. The results are used to establish or confirm expiry date and recommend storage conditions. 2.

Accelerated stability study - study designed to accelerate possible chemical degradation or physical changes in biosurfactant in conditions forced storage. The data thus obtained can be used to assess the impact of short exposure to adverse conditions outside those idealized for activity of the bio-product (Freitas et al., 2016).

Given the above the aim of the study was to formulate a commercial biosurfactant produced by the *Pseudomonas cepacia* CCT6659 and evaluate the scale up of production in order to apply this product as a dispersing agent in the removal of oil spilled by industries and bioremediation processes.

2. Materials and methods

2.1. Microorganism

Pseudomonas cepacia CCT6659 was obtained from the culture collection of Fundação André Tosello de Pesquisa e Tecnologia, Campinas city, São Paulo, Brazil. The cultures were maintained in nutrient agar slants at 4°C. For pre-culture, the strain from a 24-h culture on nutrient agar was transferred into 50 ml nutrient broth to prepare the seed culture. The cultivation condition for the seed culture was 28°C, 150 rpm, and 10-14h of incubation time.

2.2. Materials

Canola waste frying oil was obtained from a local restaurant in the city of Recife, state of Pernambuco, Brazil, stored according to the supplier's recommendations and used without any further processing. Corn steep liquor was obtained from Corn Products do Brasil in the municipality of Cabo de Santo Agostinho, state of Pernambuco, Brazil. Seawater was collected near the Suape Port, located in the municipality of Cabo de Santo Agostinho, in Pernambuco state, Brazil. Water samples were stored in plastic bottles of 5 L.

2.3. Scale up of biosurfactant production

The production of biosurfactant was initially performed in a basal medium previously described by Silva et al. (2013). Initial pH was adjusted to 7.0. 500-mL shake flasks were kept under 250 rpm orbital agitation for 60 h at 28°C. Biosurfactant production was carried out on semi-industrial scale in a 50 L bioreactor MA 502/50 L (Marconi LTDA, Brazil), containing 30 L production medium. The culture medium was aseptically inoculated with a 24 h-inoculum. Bioreactor was kept under controlled parameters using the same conditions above and aeration rate 1.0 vvm. At the end of fermentation, samples were taken from the liquid culture to determine the surface tension, biosurfactant yield, emulsification and dispersion index.

2.4. Formulation

After fermentation, the cell-free broth was submitted to different conservation methods: (a) addition of 0.2% potassium sorbate; (b) heating to 80°C for 30 min (fluent vapor), followed by the addition of 0.2% potassium sorbate; and (c) sterilization at 121°C for 30 min over three consecutive days (fractionated tyndallization). After the treatment of the crude biosurfactant in each conservation methods, the broth was stored at room temperature (28–30°C) for 120 days, with samples withdrawn at 15, 30, 45, 90, and 120 days (long term stability study). After each storage time, biosurfactant was subject to changes on pH (5.0, 7.0 and 9.0), addition of NaCl (1, 3 and 5% w/v) and heating at 40°C and 50°C. Biosurfactant properties were checked by surface tension determination, emulsification activity and the dispersant capacity of motor oil in seawater to select the best conservation method (accelerated stability study).

2.5. Determination of surface tension

Surface tension of the biosurfactant was determined with a Tensiometer (Sigma 700, KSV Instruments Ltd., Finland), using the Du Nouy ring method at room temperature (Silva et al., 2014a).

2.6. Isolation of biosurfactant

Biosurfactant was extracted from the cell-free broth after cell removal by centrifugation at 5000×g for 20 min. The supernatant pH was adjusted to 2.0 with 6.0 M HCl, and an equal volume of $CHCl_3$/CH_3OH (2:1, v/v) was added. The mixture was vigorously shaken for 15 min and allowed to set until phase separation. The organic phase was removed and the operation was repeated twice. The pooled product from organic phase was dried in an oven until complete evaporation of the solvent at 80°C to a constant weight (Silva et al. 2013).

2.7. Emulsification Activity with Motor Oil

The emulsification index was determined using the method described by Cooper and Goldenberg (1987). Motor oil used as contaminant was obtained from a local automotive manufacturer in the city of Recife, Brazil. This oil is commercially available for use in flex engines (gasoline,VNG and alcohol), type SAE20W-50, with synthetic guard (PETROBRAS, Brazil). It consists of a paraffinic base lubricating oil (a complex mixture of hydrocarbons) and performance enhancing additives. The viscosity of the oil is 98.0 cSt (at 40°C) and its density is 0.9420 g/mL (at 20°C). The assays were carried out in triplicate and did not vary more than 5%.

2.8. Oil Displacement Test

The oil displacement test was carried out by slowly dropping 80 µL of motor oil onto the surface of 40 mL of seawater in a Petri dish (15 cm in diameter) until covering the entire surface area of the water. This was followed by the addition of 10 µL of the biosurfactant onto the surface of the oil layer. The mean diameter of the clear zones of triplicate experiments was measured and calculated as the rate of the Petri dish diameter (Ohno et al., 1993).

2.9. Removal of motor oil from packed columns through static assay

The oil removability of the biosurfactant was evaluated using three different types of soil textures (sandy, clayey and silty) as Rufino et al., (2013). Glass columns measuring 55 cm in height X 6 cm in diameter were initially filled with a mixture of approximately 200 g of the soil containing 20 g of motor oil (15 cSt). The columns used in the experiments are illustrated in Fig. 1. The surface was then inundated with 200 mL of the formulated biosurfactant and isolated biosurfactant solutions at ½xCMC, 1xCMC and 2xCMC under the action of gravity. Percolation of the biosurfactant solution was monitored in 5-min intervals for 24 h, when no further percolation of the solution was observed. Following the washing of the columns, the soil samples were washed with 20 mL of hexane for the removal of residual oil. The solvent was evaporated at 50°C and the amount of oil removed was determined by gravimetry.

Insert Figure 1

2.10. Wash hydrophobic compound adsorbed on marine stones

The removal of motor oil adsorbed to the marine stones was evaluated by soaking the material in the contaminant until complete coverage and recording the volume spent. The

material was then carefully placed in a 100 mL beaker with the aid of a pincers and submitted to washing with the formulated biosurfactant and with the isolated biosurfactant at ½xCMC, 1xCMC, 2xCMC and 5xCMC, as illustrated in Fig. 2. After the washing process, the percentage of removal was calculated. Following the washing of the porous surface, the samples were treated with 50 mL of hexane twice for the removal of residual oil. The solvent was evaporated at 50 ºC and the amount of oil removed was determined by gravimetry (Sarubbo et al. 2015).

Insert Figure 2

2.11. Bioremediation test

Bioremediation tests were performed according to the method mentioned in the Standard Methods for the Examination of Water and Wasterwater (APHA, 2005). In brief, 250 ml Erlenmeyer flasks were filled with 100 ml fresh seawater obtained from the Suape Petrochemical Complex, Pernambuco State, Brazil, 1.0% of motor oil, and formulated biosurfactant solutions and isolated-biosurfactant concentrations at ½x CMC, 1xCMC, 3xCMC and 5xCMC . The flasks were incubated at 28 °C on an orbital shaker rotating at 150 rpm. Shake flasks were withdrawn after 1, 7, 14, 21 and 28 days of incubation and then analyzed for the number of microorganisms using the most probable number (MPN).

3. Results and Discussion

3.1. Scale up of biosurfactant production

Application efficiency of bioreactors and its combination with industrial and agricultural wastes use has becoming important tools towards a high yield and low cost for

biosurfactant production, especially if the process is going to be implemented on an industrial scale (Luna et al, 2015; Marti et al., 2014). Table 1 compare the results of the biosurfactant produced by *P. cepacia* CCT6659 in shake flask and semi-industrial 50-L bioreactor, where the yield showed an increase of around five times. This great result are probably due to the better agitation, aeration and temperature controls, once bioreactors are systems completely closed, favoring a greater cell growth. Furthermore, the cultivation conducted in shaker uses orbital shaking while the bioreactor uses mechanical agitation, where the oxygen supply is continuous, allowing better biosurfactant yields (Luna et al., 2015).

Insert Table 1

In a similar study, Zhu et al. (2007) achieved a yield of 12.47 g/L rhamnolipid after cultivation of *P. aeruginosa* zju.u1M using waste frying oil in shaking flasks, while in 50-L bioreactor, the yield reached 20 g/L. In the present study, five times the yield was achieved. Furthermore, the biosurfactant obtained from *P. cepacia* kept its surface-active properties (surface tension reduction, emulsification and dispersion) (Table 1). These results demonstrated the feasibility to using industrial waste for production of efficient biosurfactants on industrial scale.

3.2. Formulation

According Souza et al. (2014), one of the main requirements for biosurfactant formulation for industrial and biotechnological applications is that it should be stable over time and their properties should not significantly change with environmental variations. As can be seen in Figure 3, surface tension reduction capacity of biosurfactant through the three conservation methods investigated remained stable, especially in the potassium sorbate

addition method (Figure 3A), maintaining the surface tension practically around 29 mN/m over the 120 day test. With respect to the property of emulsification it was observed that, generally, the formulated biosurfactant remained stable in all conditions tested (pH, salinities and temperatures) reaching high levels (100%) of motor oil emulsification in all conservation methods, during 120 days of storage (Figures 4A – 4C). The dispersant capacity of the formulated biosurfactant had behavior different in the methods tested (Figure 5A – 5C). Fractionated tyndallization method showed greater stability, reaching a maximum dispersion of motor oil about 80% over 120 days. Freitas et al. (2016) used the same conservation procedures in a biosurfactant from *Candida bombicola*, checking that the most appropriate methods were the addition of potassium sorbate and fluent vaporization. In this study, biosurfactant of *Pseudomonas cepacia* UCP 6659 was stable in all investigated methods, however, considering the importance of the maintenance of the properties through out the storage time, the most appropriate conservation methods were tyndalization (based on the best dispersant capacity) and sorbate, respectively. The dispersant capacity of a biosurfactant is of extreme importance when the intention is to treat marine environments contaminated with hydrocarbons, as this property helps accelerate the natural dispersion and degradation of the oil spill by breaking down the droplets, consequentially promoting a larger surface area for all degradation processes or photooxidation (Hazra et al., 2012; Freitas et al., 2016).

Insert Figure 3, Figure 4 and Figure 5

3.3. Cost estimate of the commercial production of the formulated biosurfactant

Biosurfactants are emerging as a promising alternative to chemical surfactants. However, economical large scale production of biosurfactants is still a challenge. According to the literature, the typical cost of a commercialized biosurfactant ranges from approximately

US$ 13.94/mg for surfactin (98% purity) available from Sigma Chemical Company; US$ 50/Kg for RAG-1 emulsan marketed by Petroferm Research Inc; US$ 2.5–6.3/kg for sophorolipids offered by Sophoron TM at “Saraya” (Japan) and“ Soliance” (France); US$ 20–22.7/mg for rhamnolipid manufactured by Sigma-aldrich and AGAE technologies (USA) (Randhawa and Rahman, 2014; Dhanarajan and Sen, 2015; Freitas et al., 2016). Rhamnolipids are also available from Ecover (Boulogne-sur-Mer, France), Jeneil Biosurfactant Inc. (Saukville,Wisconsin, USA) and Rhamnolipid Holdings Inc. (New York, USA) with a production cost 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). Biosurfactants with a high degree of purity above are economically viable only for medical and food products. However, for environmental uses, biosurfactants do not need to be pure and substrates with high costs can be replaced by industrial waste for their synthesis, making the use of crude fermentation broths a viable solution (Santos et al., 2016b). In this sense, Freitas et al. (2016) evaluated the commercial application of fermented broth of *Candida bombicola* formulated with potassium sorbate at an industrial level, which estimated a cost of only US$ 0.1 – 0.22/L. In the present study, the biosurfactant from *P. cepacia* was shown to be even more profitable, which was obtained with a shorter fermentation time, further reducing of the final cost of the crude product, estimated at around US$ 0.11/L. In order to calculate the final market price of the formulated product, the costs of the conservation methods used are considered (Table 2).

Insert Table 2

As can be seen, all methods of preservation are economically feasible however the method of tindalization has some additional advantages:

1. Sterilization of the product, avoiding problems of contamination during storage;

2. Stability at high temperatures, allowing the application of the product in environments of extreme conditions.

3.4. Removal of motor oil from packed columns through static assay

The crude and the isolated biosurfactant produced by *P. cepacia* were able to remove the motor oil from soils-packed columns (Table 3). The removal of the oil by the isolated biosurfactant varied depending on the concentration employed and of the soil type. However, the formulated biosurfactant was more effective, reaching a removal of 76.55%, considered the best result. Bench scale laboratory studies utilising soils-packed columns are suitable to evaluate microbial recuperation of oil (MEOR) for several reasons: it is an economic model; a battery of columns can be set up simultaneously; and they can simulate the oil recovery operations usually conducted in reservoirs (Sarubbo et al. 2015). Studies carried out by Rufino et al. (2013) also using biosurfactants, isolade from *Candida lipolytica*, in packed columns demonstrated a maximum oil removal of 30% from clay, using cell free broth; and 33,1% and 37,3% from sand, using isolated biosurfactant concentrations at 1xCMC and 3xCMC, respectively.

Insert Table 3

3.5. Wash hydrophobic compound adsorbed on marine stones

The literature on the removal of oil from porous surface is scarce. However, Sarubbo et al. (2015) assessing the oil removal capacity of biosurfactant from *Pseudomonas* sp, found as a result, 46.1%, 47.8% and 70% motor oil removal impregnated in marine stones using ½xCMC, 1xCMC and free broth of cells, respectively. Moreover, a 81,3% and 68,62%

removal rate of motor oil adsorbed to a porous surface has been reported for the cell-free broth and the isolated biosurfactant at a concentration of 0.5% produced by *P.cepacia*, respectively (Rocha e Silva et al., 2013). In the present study, a removal of 84.5% was found as the best result (Table 4), demonstrating the application feasibility of *P.cepacia* biosurfactant (formulated and isolated) as dispersing agent adequate to cleanup of extremely sensitive ecosystem contaminated by hydrophobic pollutants, such as coral reefs that are delicate environments and of difficult access.

Insert Table 4

3.6. *Bioremediation test*

The effect of the biosurfactant from *P.cepacia* on the biodegradation of motor oil through the use of indigenous marine bacteria and fungi was evaluated over a 28-day period (Figs. 6A – 6F). As can be seen, formulated biosurfactant use as well as concentration increase of the isolated biosurfactant stimulated the autochthonous microorganism growth and, as a consequence, provoked an increase in the biodegradation of motor oil. Bacteria showed maximum growth up to the 14^{th} day, declining afterwards. On the other hand, the fungi presented a marked increase, starting from the 14^{th} day, which may be related to lower competition as a result of the decline in the number of bacteria from this period.

Insert Figure 6

At the concentration of only ½ x CMC, the biosurfactant already stimulated a 63% biodegradation, while at high concentrations (3xCMC and 5xCMC), the biodegradation of the oil was equivalent (slightly above 70%), demonstrating its effectiveness in low

concentrations, being one advantage in terms of industrial production in the development of a bioremediation agent using this biomolecule. Formulated biosurfactant stimulated an optimum biodegradation rate of about 70% removal. Santos et al. (2016a) investigated the biosurfactant of *Candida lipolytica* UCP 0988 produced in medium containing animal fat and corn steep liquor as a bioremediation agent and proved that the biotensoativo stimulated the degradation of motor oil by the native microorganisms (bacteria and fungi) in the sea water. Rocha e Silva et al. (2013) had already demonstrated the potential of *P.cepacia* biosurfactant in the solubilization of motor oil. They also obtained an acceleration on the growth of indigenous microorganisms throughout 30 days of cultivation in the presence of the biosurfactant at both the concentration of 1/2 the CMD and the full CMD.

4. Conclusions

The greatest impact of this work resided in the commercial formulation of a low cost biosurfactant using successfully industrial waste products. The present findings demonstrate that scale-up promoted an excellent of biosurfactant yield increase, increasing the chances of an actual industrial application on a large scale. Biosurfactant formulated maintained its tensioactive properties over a long storage period and oil-removal results from soil and water contaminated clearly demonstrate the viability of application of this biomolecule as an agent for remediation processes. Commercialization possibility of the formulated product can be considered promising, once its obtaining process proved viable at a compatible price in the current biosurfactants and biodetergents market.

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

SPIER, C.; STRINGFELLOW, W.T.; HAZEN, T.C.; CONRAD, M. Distribution of hydrocarbons released during the MC252 oil spill in deep offshore waters. **Environmental Pollution**, v. 173, p. 224–302, 2010.

ZHU, Y.; GAN, J-J; ZHANG, G-L; YAO, B.; ZHU, W-J; MENG, Q. Reuse of waste frying oil for production of rhamnolipids using *Pseudomonas aeruginosa* zju.u1M. **Journal of Zhejiang University - Science A**, v.8, p.1514–1520, 2007.

Table 1. Evaluation of surface tension, biosurfactant yield, dispersion and emulsification of the biosurfactant from *Pseudomonas cepacia* CCT6659 grown in mineral medium supplemented with 2% canola waste frying oil and 3% corn steep liquor in shake and semi-industrial 50-L bioreactor.

Vessel type	**Biosurfactant concentration (gL^{-1})**	**Emulsification index (%)**	**Dispersion index (%)**	**Surface tension (mNm^{-1})**
0.5-L shake flask	8.01 ± 0.19[a]	90 ± 2.3	65 ± 1.4	27.01 ± 0.04[a]
50-L bioreactor	40.5 ± 0.34	100 ± 1.34	75 ± 1.79	29.03 ± 0.05

[a] Data obtained in from Silva et al. (2013)

Table 2. Average costs of the inputs/treatments used in biosurfactant conservation techniques

Inputs/treatments	Conservation techniques		
	Sorbate	**Tindalization**	**Fluent steam**
Crude biosurfactant	0.11	0.11	0.11
Preservative	0.03	-	0.03
Heat treatment without pressure	-	-	0.01
Sterilization	-	0.04	-
Biodetergent total cost (US$)/Liter	**0.14**	**0.15**	**0.15**

Table 3. Removal of hydrophobic contaminant adsorbed on sand, clay and silty in packed columns by the cell free broth and the isolated biosurfactant produced by *Pseudomonas cepacia* CCT 6659

Removal agent	Removal in sand (%)	Removal in Clay (%)	Removal in Silt (%)
Formulated biosurfactant	68.87±0.2	70.32±0.13	76.55±0.14
½ x CMC (300 mg/l)	63.75±0.17	59.88±0.2	66.30±0.11
CMC (600 mg/l)	64.38±0.19	63.92±0.12	70.02±0.2
2 x CMC (1200 mg/l)	62.01±0.12	59.94±0.21	66.87±0.17
Control (distilled water)	8.73±0.14	5.53±0.15	5.18±0.19

Table 4. Removal of hydrophobic contaminant adsorbed on marine stones by the cell free broth and the isolated biosurfactant produced by *Pseudomonas cepacia* CCT 6659

Removal agent	Removal (%)
Formulated biosurfactant	84.5±0.2
½ x CMC (300 mg/l)	68.4±0.17
CMC (600 mg/l)	71.3±0.19
2 x CMC (1200 mg/l)	70.3±0.12
5 x CMC (3000 mg/l)	69.4±0.11
Control (distilled water)	0.5±0.14

Figure captions

Fig. 1. Glass columns measuring 55 cm in height x 6cm in diameter used in the removal of motor oil through static assay

Fig. 2. Illustration of marine stone surface before (A), during (B) an after (C) washing process with the formulated biosurfactant from *Pseudomonas cepacia*. (D) Oil removed.

Fig. 3. Surface tensions of biosurfactant over 120 days of conservation with (A) the addition of preservative, (B) fluent vaporization and (C) fractionated tyndallization.

Fig. 4. Motor oil emulsification capacity of biosurfactant over 120 days of conservation with (A) the addition of preservative, (B) fluent vaporization and (C) fractionated tyndallisation.

Fig. 5. Motor oil dispersant capacity of biosurfactant over 120 days of conservation with (A) the addition of preservative, (B) fluent vaporization and (C) fractionated tyndallisation.

Fig. 6. Influence of biosurfactant from *P.cepacia* CCT6659 on growth of bacteria and fungi indigenous in seawater. Microbial growth in the (A) biosurfactant absence (B) formulated biosurfactant presence (C) at ½xCMC (D) at 1xCMC (E) at 3xCMC (F) at 5xCMC.

Fig. 1.

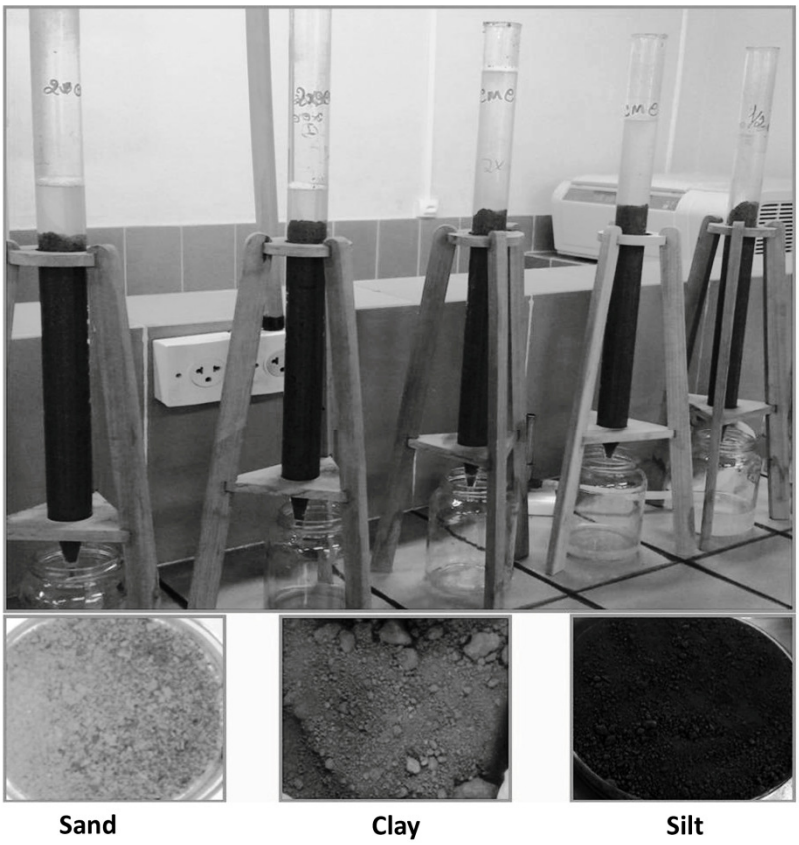


Fig. 2.

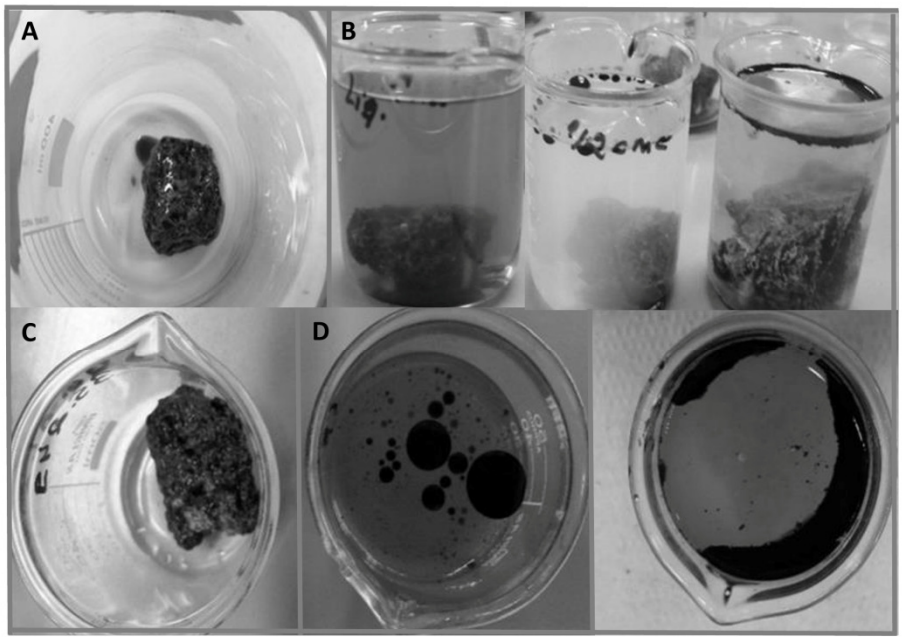


Fig. 3.

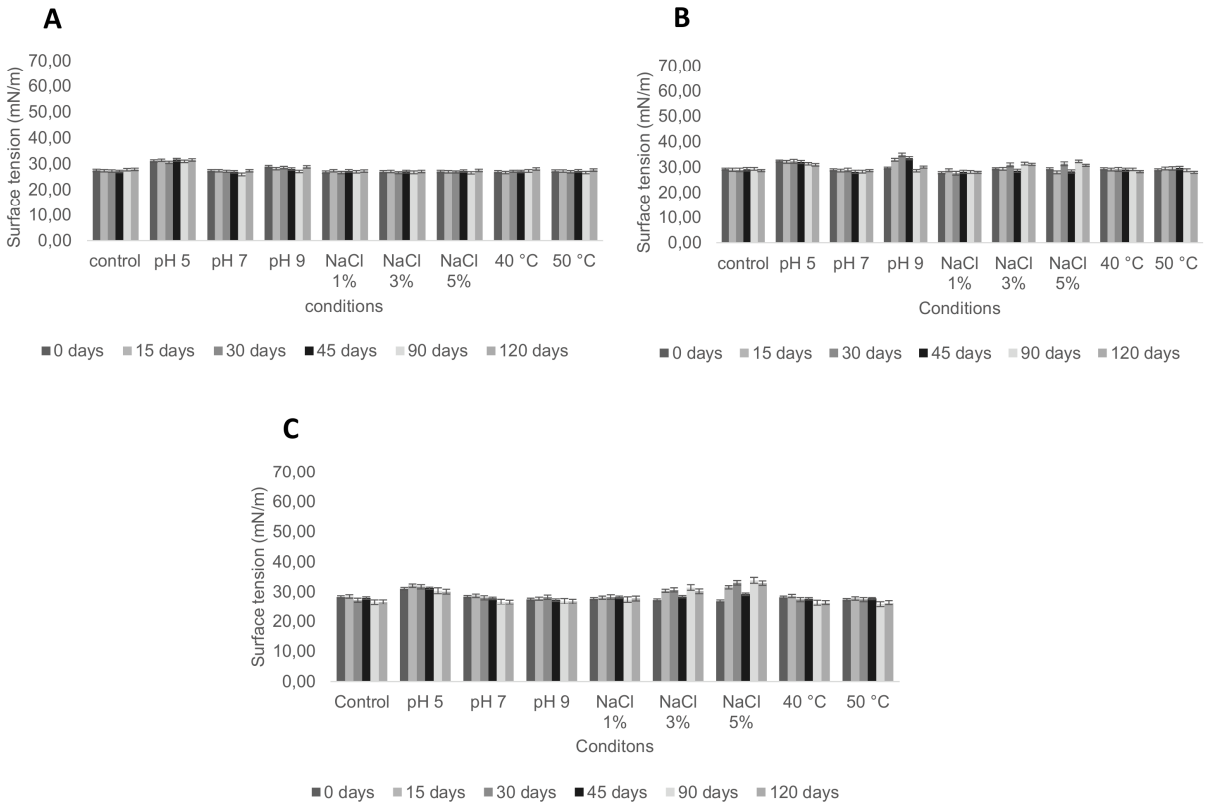

A
Surface tension (mN/m)
70,00
60,00
50,00
40,00
30,00
20,00
10,00
0,00
control
pH 5
pH 7
pH 9
NaCl 1%
NaCl 3%
NaCl 5%
40 °C
50 °C
conditions
0 days
15 days
30 days
45 days
90 days
120 days
B
Surface tension (mN/m)
Conditions
C
Surface tension (mN/m)
Control
Conditons

Fig. 4.

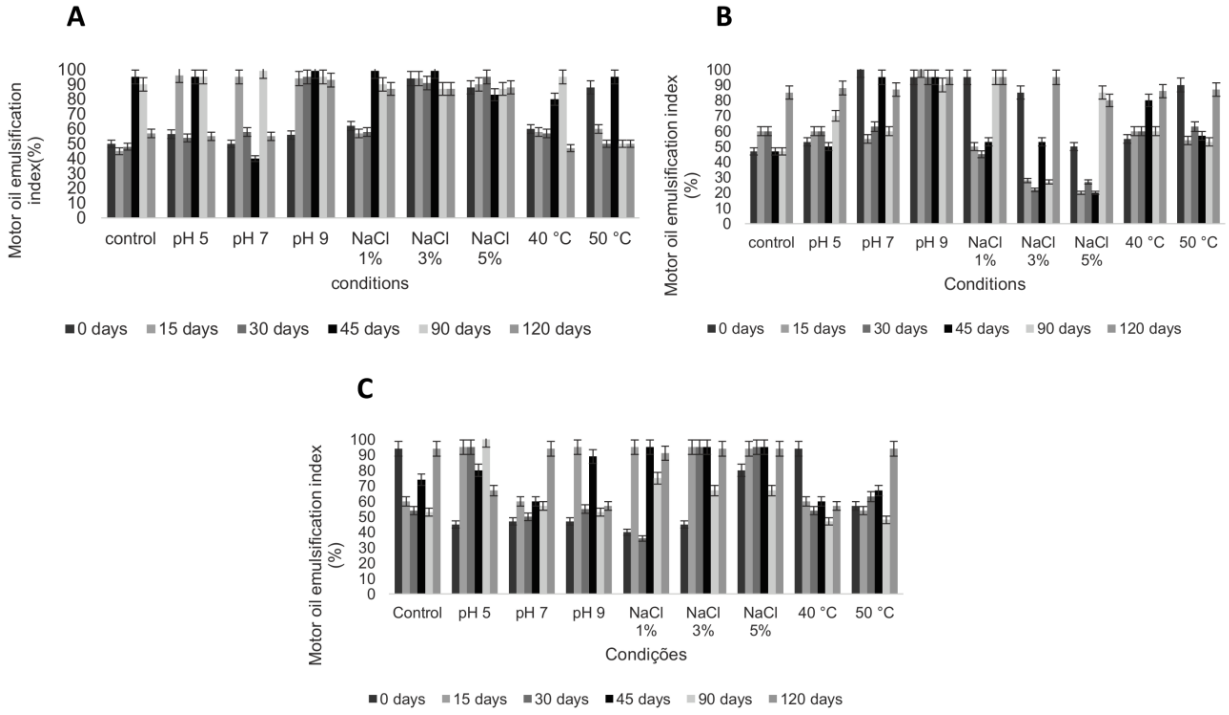

A
Motor oil emulsification index(%)
100
90
80
70
60
50
40
30
20
10
0
control
pH 5
pH 7
pH 9
NaCl 1%
NaCl 3%
NaCl 5%
40 °C
50 °C
conditions
0 days
15 days
30 days
45 days
90 days
120 days
B
Motor oil emulsification index (%)
Conditions
C
Motor oil emulsification index (%)
Control
Condições

Fig. 5.

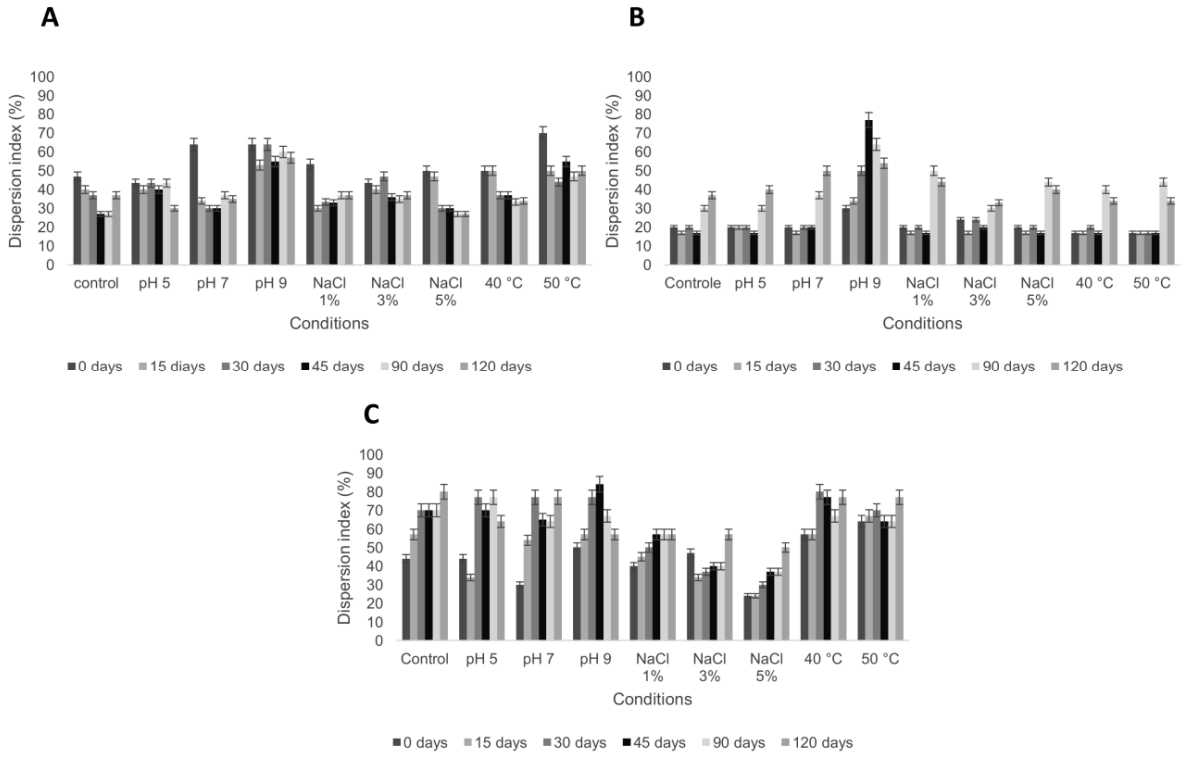
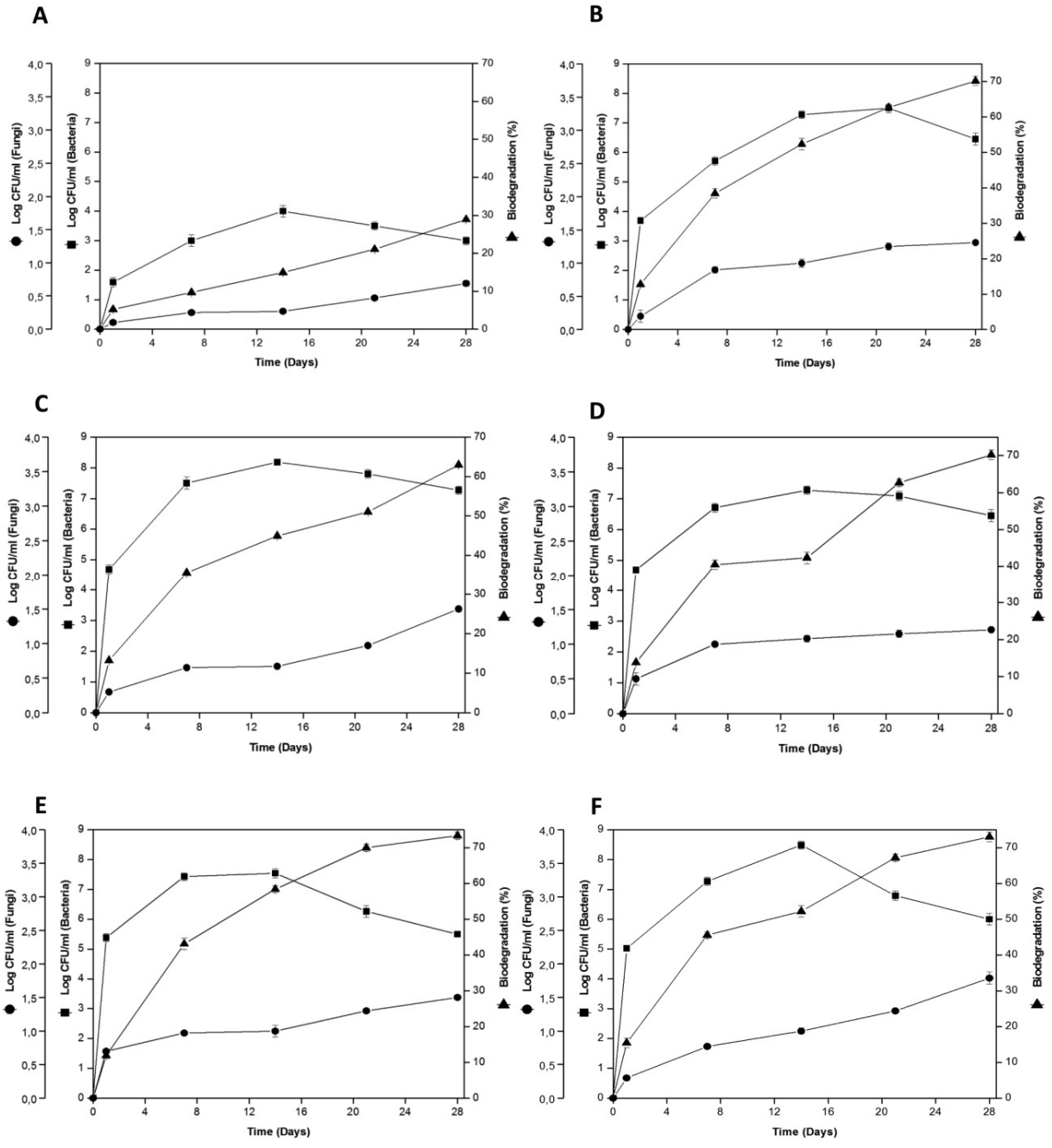

A
Dispersion index (%)
100
90
80
70
60
50
40
30
20
10
0
control
pH 5
pH 7
pH 9
NaCl 1%
NaCl 3%
NaCl 5%
40 °C
50 °C
Conditions
0 days
15 diays
30 days
45 days
90 days
120 days
B
Dispersion index (%)
Controle
pH 5
pH 7
pH 9
NaCl 1%
NaCl 3%
NaCl 5%
40 °C
50 °C
Conditions
0 days
15 days
30 days
45 days
90 days
120 days
C
Dispersion index (%)
Control
pH 5
pH 7
pH 9
NaCl 1%
NaCl 3%
NaCl 5%
40 °C
50 °C
Conditions
0 days
15 days
30 days
45 days
90 days
120 days

Fig. 6.


A
B
C
D
E
F
Log CFU/ml (Fungi)
Log CFU/ml (Bacteria)
Biodegradation (%)
Time (Days)

5.5 CAPÍTULO 5

Patente de Invenção: “*Formulação e processo de obtenção de aditivos estáveis à base de biossurfactantes para dispersantes de petróleo e seus derivados*”, depositada no “Instituto Nacional da Propriedade Industrial”.

< Uso exclusivo do INPI >

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22/07/2014 018140013678 12:32 DESP
BR 10 2014 017963 1

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INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
Sistema de Gestão da Qualidade
Diretoria de Patentes

DIRPA	Tipo de Documento: Formulário	DIRPA	Página: 1/3
Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

Ao Instituto Nacional da Propriedade Industrial:
O requerente solicita a concessão de um privilégio na natureza e nas condições abaixo indicadas:

1. Depositante (71):

1.1 Nome: Centro de Gestão de Tecnologia e Inovação - CGTI

1.2 Qualificação: Pesquisa e desenvolvimento experimental

1.3 CNPJ/CPF: 06062204/0002-14

1.4 Endereço Completo: Rua Padre Roma, 120 Sala 1501 - 1502 Tamarineira Recife PE

1.5 CEP: 52050-150

1.6 Telefone: (81) 3031-8283 1.7 Fax: (81)3031-8283

1.8 E-mail: quilherme@buenomak.com.br

☐ continua em folha anexa

2. Natureza: ☒ Invenção ☐ Modelo de Utilidade ☐ Certificado de Adição

3. Título da Invenção ou Modelo de Utilidade (54):

Formulação e processo de obtenção de aditivos estáveis à base de biossurfactantes para dispersantes de petróleo e seus derivados.

☐ continua em folha anexa

4. Pedido de Divisão: do pedido Nº **Data de Depósito:**

5. Prioridade: ☐ Interna (66) ☐ Unionista (30)

O depositante reivindica a(s) seguinte(s):

País ou Organização do depósito	Número do depósito (se disponível)	Data de depósito

☐ continua em folha anexa

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
Sistema de Gestão da Qualidade
Diretoria de Patentes

DIRPA	Tipo de Documento: Formulário	DIRPA	Página: 2/3
Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

6. Inventor (72):

☐ Assinale aqui se o(s) mesmo(s) requer(em) a não divulgação de seus nome(s), neste caso não preencher os campos abaixo.

6.1 Nome: Leonie Asfora Sarubbo

6.2 Qualificação: quimica industrial

6.3 CPF: 735.801.634-34

6.4 Endereço Completo: rua Arthur Muniz, n. 147, apto. 2001, bairro Boa Viagem,...

6.5 CEP: 51.111-190

6.6 Telefone: (81)3342-0581 6.7 FAX: (81)3342-0581

6.8 E-mail: leonie@unicap.br

☒ continua em folha anexa

7. Declaração de divulgação anterior não prejudicial. ☐
Artigo 12 da LPI – período de graça.
Informe no item 11.13 os documentos anexados, se houver.

8. Declaração na forma do item 3.2 da Instrução Normativa PR nº 17/2013:

☐ Declaro que os dados fornecidos no presente formulário são idênticos ao da certidão de depósito ou documento equivalente do pedido cuja prioridade está sendo reivindicada.

9. Procurador (74):

9.1 Nome:

9.2 CNPJ/CPF: 9.3 API/OAB:

9.4 Endereço Completo:

9.5 CEP:

9.6 Telefone: 9.7 FAX:

9.8 E-mail:

☐ continua em folha anexa

10. Listagem de sequências biológicas.
Informe nos itens 11.9 ao 11.12 os documentos anexados, se houver. ☐

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
Sistema de Gestão da Qualidade
Diretoria de Patentes

DIRPA	Tipo de Documento: Formulário	DIRPA	Página: 3/3
Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

11. Documentos Anexados:

(Assinale e indique também o número de folhas):
(Deverá ser indicado o número total de somente uma das vias de cada documento).

		Documentos Anexados	folhas
☒	11.1	Guia de Recolhimento da União (GRU).	01
☐	11.2	Procuração.	
☐	11.3	Documentos de Prioridade.	
☐	11.4	Documento de contrato de trabalho.	
☒	11.5	Relatório descritivo.	17
☒	11.6	Reivindicações.	06
☐	11.7	Desenho(s) (se houver). Sugestão de figura a ser publicada com o resumo: nº, _____ por melhor representar a invenção (sujeito à avaliação do INPI).	
☒	11.8	Resumo.	01
☐	11.9	Listagem de sequências em arquivo eletrônico: _____nº de CDs ou DVDs (original e cópia).	
☐	11.10	Código de controle alfanumérico no formato de código de barras referente às listagem de sequências.	
☐	11.11	Listagem de sequências em formato impresso.	
☐	11.12	Declaração relativa à Listagem de sequências.	
☑	11.13	Outros (especificar) : contrato social + alteração (62 fls) + cenões dos inventores (6 fls.).	68

12. Total de folhas anexadas: 93 **fls.**

13. Declaro, sob as penas da Lei que todas as informações acima prestadas são completas e verdadeiras.

Recife, 17 de julho de 2014
Local e Data

Assinatura e Carimbo
José Mak
Diretor Superintendente
Centro de Gestão de Tecnologia e Inovação - CGTI

Folha Anexa do Formulário FQ001

6. Inventor (72):

6.1 Nome: Leonie Asfora Sarubbo
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6.1 Nome: Valdemir Alexandre dos Santos
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6.3 CPF: 079.743.104-72
6.4 Endereço completo: Rua Leonardo da Vince, n. 112, Apto. 2121, bairro Imbiribeira, cidade Recife, estado Pernambuco.
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6.8 E-mail: valdemir.alexandre@hotmail.com

6.1 Nome: Darne Germano de Almeida
6.2 Qualificação: biólogo
6.3 CPF: 054.947.544-30
6.4 Endereço completo: Rua Cuba, n.16, bairro Sucupira, cidade Jaboatão dos Guararapes, estado Pernambuco.
6.5 CEP: 54.280-245
6.6 Telefone: (81) 3455-0600
6.7 Fax: (81) 3455-0600
6.8 E-mail: darnebio@yahoo.com.br

6.1 Nome: Rita de Cássia Freire Soares da Silva
6.2 Qualificação: bióloga
6.3 CPF: 027.931.224-59
6.4 Endereço completo: Rua Jaqueline, n. 94, bairro Sucupira, cidade Jaboatão dos Guararapes, estado Pernambuco.
6.5 CEP: 54280-660
6.6 Telefone: (81) 3254-5759
6.7 Fax: (81) 3254-5759
6.8 E-mail: ritaf78@gmail.com

RESUMO

Patente de Invenção: "FORMULAÇÃO E PROCESSO DE OBTENÇÃO DE ADITIVOS ESTÁVEIS À BASE DE BIOSSURFACTANTES PARA DISPERSANTES DE PETRÓLEO E SEUS DERIVADOS".

A presente invenção, que pertence as áreas de microbiologia aplicada e biotecnologia refere-se a formulação e ao processo para a obtenção de dez diferentes biossurfactantes, três produzidos por espécies da bactéria *Pseudomonas*, seis por espécies da levedura *Candida* e um produzido por uma espécie da bactéria *Bacillus,* formulados como aditivos estáveis e de longo tempo de vida útil para aplicação na remediação de áreas contaminadas por petróleo e derivados. Nos processos de obtenção destes biossurfactantes foram utilizados resíduos industriais para minimização dos respectivos custos de produção, foram testadas e maximizadas as capacidades de redução da tensão superficial, de emulsificação e de dispersão de óleos em água, verificadas as estabilidades ao longo do tempo de estocagem e adotados procedimentos inéditos para o aumento na conservação de cada um dos biossurfactantes desenvolvidos de forma a obter-se as menores perdas possíveis de suas propriedades surfactantes.

6. CONCLUSÕES

- O utilização do delineamento composto central rotacional (DCCR) contribuiu positivamente em desenvolver as condições ideais de cultivo para maximizar a produção de biossurfactantes.
- O biossurfactante produzido *Pseudomonas cepacia* CCT6659 demonstrou atividade de superfície satisfatória e elevada atividade emulsionante e dispersante para derivados de petróleo.
- O biossurfactante de *P. cepacia* demonstrou estabilidade frente a condições extremas de pH, temperatura e concentrações de NaCl.
- O biossurfactante demonstrou baixa toxicidade frente ao microcrustáceo *Artemia salina* e as sementes de *Brassica oleracea*.
- A caracterização química revelou a natureza glicolipídica e aniônica do biossurfactante.
- O biossurfactante formulado manteve as suas propriedades tensoativas estáveis durante um longo período de armazenamento, permitindo sua produção industrial associada a uma logística de estoque para aplicação imediata.
- O aumento da escala de produção em biorreator semi industrial promoveu um excelente rendimento de biossurfactante, aumentando as chances de uma aplicação real em larga escala.
- O biossurfactante bruto ou isolado demonstrou potencial de aplicação como agente surfactante e emulsificante em ambientes de condição extrema, como a indústria de petróleo, especialmente na mobilização e recuperação de óleo, na limpeza de tanques de estocagem e na remediação de derramamentos de petroderivados em solos e água.
- A possibilidade de comercialização do produto formulado pode ser considerada promissora, uma vez que seu processo de obtenção se mostrou viável a um preço compatível no atual mercado de biossurfactantes.

ANEXOS

Programa de Pós-Graduação em Biotecnologia
Universidade Federal Rural de Pernambuco - UFRPE
Pró-Reitoria de Pesquisa e Pós-Graduação
Rua Dom Manoel de Medeiros, s/n – Dois Irmãos
52171-900, Recife-PE - Telefone: (81) 3320.6050 - E-mail: renorbio@prppg.ufrpe.br

DECLARAÇÃO

Declaramos, para os devidos fins, que **Rita de Cássia Freire Soares da Silva**, ingressa no doutorado do Programa de Pós-Graduação em Biotecnologia da Rede Nordeste de Biotecnologia (RENORBIO) em março de 2013, sob o número de matrícula 13023145, defendeu a tese intitulada **"Produção e formação de biossurfactante de *Pseudomonas cepacia* para aplicação na remoção de poluentes ambientais gerados na indústria de petróleo"**, desenvolvida na Instituição Associada Universidade Católica de Pernambuco, sob a orientação da Profa. Dra. Leonie Asfora Sarubbo, no dia 21 de fevereiro de 2017, a qual foi aprovada pela banca composta pelos professores doutores Leonie Asfora Sarubbo da Universidade Católica de Pernambuco, presidente, Ana Lúcia Figueiredo Porto do RENORBIO da Universidade Federal Rural de Pernambuco, como titular, Juliana Moura de Luna da Universidade Católica de Pernambuco, como titular, Thayza Cristina de Montenegro Stamford da Universidade Federal de Pernambuco, como titular, e Adalberto Pessoa Júnior – USP, como titular, estando apta a receber o Diploma de Doutora em Biotecnologia, Área de Concentração em Biotecnologia Industrial, após a entrega da versão final da tese.

Recife 21 de fevereiro de 2017.

Profa. Dra. Aurea Wischral
Coordenadora do Programa de Pós-Graduação
em Biotecnologia da RENORBIO-PERNAMBUCO

Declaração do CGTI

Certificados

Apêndices: Primeira página dos artigos de Darne e Khadydja