RENORBIO

Programa de Pós-graduação em Biotecnologia

PRODUÇÃO, CARACTERIZAÇÃO E APLICAÇÃO DE BIOSSURFACTANTE COMO AGENTE DE REMEDIAÇÃO EM AMBIENTE MARINHO

Darne Germano de Almeida

Recife – PE

2017

DARNE GERMANO DE ALMEIDA

Produção, caracterização e aplicação de biossurfactante como agente de remediação em ambiente marinho

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.

Orientador: Prof[a]. Dr[a]. Leonie Asfora Sarubbo
Co-orientador: Prof[a]. Dr[a]. Juliana Moura de Luna

Recife
2017

Dados Internacionais de Catalogação na Publicação (CIP)
Sistema Integrado de Bibliotecas da UFRPE
Biblioteca Central, Recife-PE, Brasil

A447p Almeida, Darne Germano de
Produção, caracterização e aplicação de biossurfactante como agente de remediação em ambiente marinho / Darne Germano de Almeida. – 2017.
141 f. : il.

Orientador: Leonie Asfora Sarubbo.
Coorientador: Juliana Moura de Luna.
Tese (Doutorado) – Programa de Pós-Graduação em Biotecnologia – RENORBIO, Recife, BR-PE, 2017.
Ponto focal em Pernambuco – Universidade Federal Rural de Pernambuco.
Inclui referências, anexo(s) e apêndice(s).

1. Biossurfactante 2.Candida tropicalis 3. biorremediação I. Sarubbo, Leonie Asfora, orient. II. Luna, Juliana Moura de III. Título

CDD 620.8

DARNE GERMANO DE ALMEIDA

PRODUÇÃO, CARACTERIZAÇÃO E APLICAÇÃO DE BIOSSURFACTANTE COMO AGENTE DE REMEDIAÇÃO EM AMBIENTE MARINHO

Aprovada em 22/02/2017

Banca examinadora:

_____
Profa. Dra. Leonie Asfora Sarubbo (Presidente e Orientadora)
Universidade Católica de Pernambuco – UNICAP

_____
Profa. Dra. Ana Lúcia Figueiredo Porto (Titular Interno)
Universidade Federal Rural de Pernambuco – UFRPE/ RENORBIO

_____
Prof. Dr. Valdemir Alexandre dos Santos (Titular Externo)
Universidade Católica de Pernambuco – UNICAP

_____
Dra. Raquel Diniz Rufino (Titular Externo)
Universidade Católica de Pernambuco – UNICAP

_____
Prof. Dr. Adalberto Pessoa Junior (Titular Externo)
Universidade de São Paulo – USP

Recife-PE
2017

AGRADECIMENTOS

À Deus, por ter me dado inteligência e disposição para realização desta pesquisa.

À minha orientadora, Profa. Dra. Leonie Asfora Sarubbo, pelo seu excelente empenho na minha orientação, sempre me aconselhando e ajudando a desenvolver a minha habilidade de pesquisador.

À Profa. Dra. Juliana Moura de Luna, pois sua participação foi fundamental para a realização do projeto, passando tranquilidade e apoio.

À Rede nordeste de Biotecnologia (RENORBIO), pela oportunidade de participar do excelente Programa de Doutorado e ter possibilitado meu crescimento científico.

À Profa. Dra. Aurea Wischral, Representante Estadual do Ponto Focal Pernambuco da RENORBIO, pelas valiosas contribuições dadas durante o processo de doutorado.

À Universidade Federal Rural de Pernambuco e à Reitora Profa. Dra. Maria José de Sena, por me possibilitar grandes realizações no desenvolvimento desta tese.

Ao Magnífico Reitor da Universidade Católica de Pernambuco, Pe. Pedro Rubens Ferreira Oliveira, S.J., pelo acesso aos laboratórios para realização deste trabalho.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), à Fundação de Amparo à Ciência e Tecnologia de Pernambuco (FACEPE) e à Financiadora de Estudos e Projetos (FINEP) pelo apoio financeiro concedido durante essa pesquisa. À Fundação de Amparo à Ciência e Tecnologia de Pernambuco (FACEPE) pela concessão da bolsa de estudo para a realização desta pesquisa.

À minha querida e estimada amiga e colega Rita de Cássia Freire Soares da Silva, pela ajuda, parceria, cumplicidade e companheirismo devotados durante todas as etapas experimentais e escritas do meu doutorado.

A todos que fazem parte do Grupo de Pesquisa em Biossurfactantes, em especial, a Dra. Raquel Diniz Rufino, Pedro Pinto Brasileiro, Fernanda Cristina Padilha, Nathalia Maria Padilha, Hugo Morais, Elias José da Silva e aos demais igualmente importantes pelo carinho, compreensão e disponibilidade em me ajudar nos experimentos.

Enfim, a todos que de alguma forma tornaram este caminho mais fácil de ser percorrido.

Dedico esta tese aos meus pais Dário Germano de Almeida e Audilene Maria de Lima, que ajudaram na minha formação de caráter e na minha educação; ao meu irmão Demis Germano de Almeida; à minha sobrinha Beatriz e à minha querida Maria da Glória Conceição da Silva.

"Infelicidade é se lamentar por tudo aquilo que não se tem; Felicidade é agradecer por tudo aquilo que se tem".

Darne Almeida.

RESUMO

A contaminação por petróleo e seus derivados causam prejuízos graves, o que tem despertado grande atenção para o desenvolvimento e aplicação de tecnologias inovadoras para a remoção desses contaminantes. Nesse sentido, este trabalho teve por objetivo produzir um biossurfactante de *Candida tropicalis* UCP0996 a partir de resíduos industriais como substratos para aplicação como agente de remediação. A otimização da produção do biossurfactante foi avaliada quanto à influência das variáveis concentrações de melaço, milhocina, óleo de canola residual e tamanho do inóculo sobre as variáveis resposta tensão superficial e rendimento em biossurfactante. As condições ótimas selecionadas para o processo fermentativo foram 2,5% de óleo de canola residual, 2,5% de milhocina, 2,5% de melaço e tamanho do inóculo de 2%, com redução da tensão superficial e rendimento de 29,98 mN/m e 4,19 g/L, respectivamente. O biossurfactante foi produzido em biorreatores, alcançando rendimentos de 5,87 g/L (biorreator de 2 L) e 7,36 g/L (biorreator de 50 L). A capacidade tensoativa e emulsificante do biossurfactante foi investigada sob condições extremas de temperatura, salinidade, pH e tempo de aquecimento, indicando sua estabilidade. A investigação da composição química por espectroscopia no infravermelho por transformada de Fourier (FTIR), ressonância magnética nuclear de prótons (^{1}H RMN) e cromatografia gasosa e acoplada a espectroscopia de massa (GC-MS) revelou que o biossurfactante estudado é um glicolipídeo de natureza aniônica com concentração micelar crítica (CMC) de 600 mg/L e de baixa hidrofobicidade. Após a caracterização, a biomolécula teve sua toxicidade investigada frente ao microcrustáceo *Artemia salina,* demonstrando ser inócua frente a este indicador ambiental. Em seguida, o biossurfactante foi submetido a diferentes metodologias para ser formulado como aditivo comercial. A biomolécula manteve-se estável ao longo de 120 dias à temperatura ambiente após adição de sorbato de potássio como conservante. A aplicação da biomolécula em processos de remoção e degradação de petroderivado demonstrou sua capacidade de dispersar cerca de 71% do óleo de motor em água do mar, de remover 67% do óleo adsorvido em superfície porosa e de aumentar a degradação do óleo pelos micro-organismos marinhos autóctones. Com base nos resultados, foi possível estabelecer o potencial biotecnológico do produto obtido para aplicação na área industrial e ambiental, em substituição aos surfactantes sintéticos.

Palavras-chave: Biossurfactante, *Candida tropicalis,* resíduos industriais, biorremediação, petróleo.

ABSTRACT

Contamination by petroleum and its by-products causes serious damage, which has awakened great attention to the development and application of innovative technologies for the removal of these contaminants. In this sense, this work aimed to produce a biosurfactant of *Candida tropicalis* UCP0996 from industrial residues as substrates for application as a remediation agent. Biosurfactant production optimization was evaluated for the influence of the variables concentrations of molasses, corn steep liquor, residual canola oil and inoculum size on the response variables of surface tension and biosurfactant yield. The optimum conditions selected for the fermentative process were 2.5% of residual canola oil, 2.5% of corn steep liquor, 2.5% of molasses and 2% of inoculum size, with reduction of surface tension and yield of 29.98 mN/m and 4.19 g/L, respectively. The biosurfactant was produced in bioreactors, yielding yields of 5.87 g/L (2 L bioreactor) and 7.36 g/L (50 L bioreactor). The tensioactive and emulsifying capacity of the biosurfactant was investigated under extreme conditions of temperature, salinity, pH and heating time, indicating their stability. Chemical composition investigation of the by Fourier transform infrared spectroscopy (FTIR), proton nuclear magnetic resonance (^{1}H NMR) and gas chromatography coupled to mass spectrometry (GC-MS) revealed that the biosurfactant studied is an anionic glycolipid with critical micelle concentration (CMC) of 600 mg/L and low hydrophobicity. After the characterization, the biomolecule had its toxicity investigated against the microcrustacean *Artemia salina*, proving to be innocuous against this environmental indicator. The biosurfactant was then subjected to different methodologies for the formulation of a commercial additive. The biomolecule remained stable for 120 days at room temperature after addition of potassium sorbate as a preservative. The application of the biomolecule in petroderivative removal and degradation processes demonstrated its ability to disperse about 71% of the motor oil into seawater, to remove 67% of the oil adsorbed on a porous surface and to increase the degradation of the oil by microorganisms. Based on the results, it was possible to establish the biotechnological potential of the product obtained for application in the industrial and environmental area, replacing the synthetic surfactants.

Keywords: Biosurfactant, *Candida tropicalis*, industrial waste, bioremediation, petroleum.

LISTA DE FIGURAS

Figura 1 – Estruturas gerais de biossurfactantes, de acordo com a composição de suas porções hidrófilas, sendo não iônicas, aniônicas, catiônicas ou anfotéricas 21
Figura 2 – Estruturas químicas dos principais tipos de biossurfactantes 23
Figura 3 – Esquema Ilustrativo mostrando as regiões nas quais ocorre a formação de micelas (concentração micelar crítica – CMC) 25
Figura 4 – Metabolismo intermediário relacionado à síntese de precursores de biossurfactantes utilizando carboidratos como substrato. Enzimas-chave para o controle do fluxo de carbono: (A) Fosfofrutoquinase; (B) Piruvato quinase; (C) Isocitrato desidrogenase 31

LISTA DE TABELAS

Tabela 1 – Principais classes de biossurfactantes e respectivos micro-organismos produtores 22
Tabela 2 – Patentes sobre biossurfactantes produzidos por micro-organismos e suas aplicações 32
Tabela 3 – Empresas produtoras, tipos de biossurfactantes comercialmente disponíveis e potenciais aplicações 35
Tabela 4 – Empresas brasileiras envolvidas na comercialização de tensoativos no país 36
Tabela 5 – Aplicações de biossurfactantes para usos industriais e processos ambientais 41
Tabela 6 – Aplicações convencionais de biossurfactantes na indústria do petróleo. 43
Tabela 7 – Biossurfactantes, micro-organismos produtores e suas aplicações na biorremediação de ambientes contaminados com óleo 44

LISTA DE SIGLAS E ABREVIATURAS

^{1} H NMR – *Proton Nuclear Magnetic Resonance*

ABRABI – Associação Brasileira das Empresas de Biotecnologia

ACS – *Allied Carbon Solutions*

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*

GRAS – *Generally Regarded As Safe*

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*

OD - *Optical Density*

OOIP – *Original Oil in Place*

PAC – Programa de Aceleração do Crescimento

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*

TMS – *Tetramethylsilane*

YMA – *Yeast Mold Agar*

YMB – *Yeast Mold Broth*

SUMÁRIO

1. INTRODUÇÃO 14

2. OBJETIVOS 17

2.1. OBJETIVO GERAL 17
2.2. OBJETIVOS ESPECÍFICOS 17
3. REVISÃO DE LITERATURA 18

3.1. Petróleo e meio ambiente 18
3.2. Biossurfactantes 20
3.2.1. Classificação 21
3.2.2. Propriedades e características dos biossurfactantes 24
3.2.3. Micro-organismos produtores de biossurfactantes 26
3.2.4. Função fisiológica dos biossurfactantes 28
3.2.5. Biossíntese de biossurfactantes 30
3.3. Patentes em biossurfactantes 31
3.4. Mercado de biossurfactantes 33
3.5. Resíduos industriais utilizados na produção de biossurfactantes 37
3.5.1. Óleos vegetais para a produção de biossurfactantes 37
3.5.2. Milhocina 38
3.5.3. Melaço 38
3.6. Aplicações industriais e ambientais dos biossurfactantes 39
3.6.1. Aplicações terapêuticas 39
3.6.2. Biossurfactantes na agricultura 39
3.6.3. Biossurfactantes na mineração 40
3.6.4. Biossurfactantes e cosméticos 40
3.6.5. Biossurfactantes e indústria de alimentos 40
3.7. Perspectivas de utilização de biossurfactantes na indústria do petróleo 42
3.8. Aplicação de biossurfactantes em processos de biorremediação 43
3.8.1. *Toxicidade de biossurfactantes sobre organismos no processo de biorremediação* 45
4. REFERÊNCIAS 47

5. ARTIGOS DERIVADOS DA TESE 56

5.1. CAPÍTULO 1 *Biosurfactants: Promising Molecules for Petroleum Biotechnology Advances* 56
5.2. CAPÍTULO 2 *Response Surface Methodology for Optimizing the Production of Biosurfactant by Candida tropicalis on Industrial Waste Substrates* 71
5.3. CAPÍTULO 3 *Production, characterization and commercial formulation of a biosurfactant from Candida tropicalis UCP 0996 and its application in decontamination of pollutants generated by the petroleum industry* 85
5.4. CAPÍTULO 4 *Formulação e processo de obtenção de aditivos estáveis à base de biossurfactantes para dispersantes de petróleo e seus derivados* 124
6. CONCLUSÕES 131

ANEXOS 132

APÊNDICES 137

1. INTRODUÇÃO

Os acidentes ocorridos com derramamentos de petróleo e seus derivados no Brasil, no período de 1975 a 2012, atingiram milhões de litros que contaminaram solos, rios e mares. Nos ecossistemas marinhos, a contaminação por estes compostos forma uma fina camada na superfície da água, impedindo a troca de gases entre o ar e a água e evitando a passagem de luz solar para as comunidades fitoplanctônicas, afetando os processos de respiração e de fotossíntese, causando um colapso fundamental na cadeia alimentar (ASIMIEA et al., 2011; SOUZA et al., 2014).

Várias classes de compostos surfactantes têm sido amplamente utilizadas na remoção de contaminantes hidrofóbicos. No entanto, esses produtos químicos, em sua grande maioria, são de origem sintética e podem apresentar riscos toxicológicos potenciais aos organismos aquáticos devido à sua natureza recalcitrante e persistente, tornando-se mais um problema para o ambiente (OKOLIEGBE; AGARRY, 2012; RICO-MARTÍNEZ et al., 2012). Sendo assim, mais atenção tem sido dada às alternativas biológicas (MALIK; AHMED, 2012).

As tecnologias sustentáveis vêm recentemente impulsionando a busca de compostos naturais e biodegradáveis para remediar locais contaminados por hidrocarbonetos. Biossurfactantes têm se mostrado excelentes dispersantes, em comparação aos surfactantes químicos, além de demonstrarem eficácia mais elevada como agentes de remediação, principalmente devido às suas características de baixa toxicidade, elevada biodegradabilidade e maior resistência às variações do ambiente (SANTOS et al., 2013; SILVA et al., 2014). Estas biomoléculas têm sido amplamente utilizadas na remediação de água do mar por serem capazes de aumentar a biodisponibilidade de hidrocarbonetos insolúveis. A biodegradação por populações microbianas autóctones é o mecanismo primário pelo qual estes contaminantes são removidos do ambiente. A adição de biotensoativos estimula e acelera este processo, devido a um aumento na solubilização de compostos hidrofóbicos (PANIAGUA-MICHEL; ROSALES, 2015).

Acredita-se que os biossurfactantes são secretados no meio de cultivo facilitando a translocação de substâncias hidrofóbicas através da superfície celular de micro-organismos (CAMPOS et al., 2013; SOBRINHO et al., 2013b). Estas moléculas são principalmente produzidas em condições aeróbias utilizando fontes de

carbono, solúveis e insolúveis em água como matéria-prima. O processo é economicamente viável e ambientalmente atrativo quando resíduos industriais são utilizados (DZIEGIELEWSKA; ADAMCZAK, 2013; MAKKAR et al., 2011).

Com os avanços na biotecnologia industrial, vários processos para obtenção de diferentes tipos de surfactantes microbianos foram intensificados (SANTOS et al., 2016; SINGH et al., 2012). 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).

Nos últimos anos, um número crescente de aspectos relacionados com a produção de biossurfactantes a partir de leveduras tem sido o tema de pesquisas, dada sua importância industrial e a vantagem de serem consideradas seguras (não patogênicas e não tóxicas). Os principais gêneros que vêm sendo relatados na literatura como produtores de biotensoativos são *Candida* sp., *Pseudozyma* sp. e *Yarrowia* sp. e seus biossurfactantes caracterizados têm sido identificados como soforolipídeos, lipídeos de manosileritritol, complexos de lipídeos-proteínas-carboidratos, complexos de proteínas-carboidratos, mannano-proteína, mannano-lipídeo-proteína e liposan (AMARAL et al., 2010; SILVA et al., 2014). Numerosos exemplos demonstram o potencial de aplicação de biossurfactantes isolados de espécies do gênero *Candida* em processos de descontaminação ambiental. No que diz respeito à biorremediação em água do mar, foram atingidos percentuais de remoção de óleo em torno de 92% (BATISTA et al., 2010; LUNA et al., 2011; SOBRINHO et al., 2008).

Portanto, faz-se necessário o desenvolvimento de estratégias tecnológicas inovadoras para prevenir problemas indesejáveis causados por possíveis acidentes ambientais provocados pelo derramamento de óleos. Neste trabalho, estudou-se a produção de um novo biossurfactante aproveitando resíduos industriais por meio de

um planejamento fatorial para maximização da produção, bem como as propriedades do biossurfactante, seu isolamento, caracterização, toxicidade, além da formulação de um aditivo comercial para aplicação como agente de biorremediação em derramamentos de derivados de petróleo em água do mar.

2. OBJETIVOS

2.1. OBJETIVO GERAL

Produzir um biossurfactante com potencial de aplicação na remoção de derivado de petróleo em água do mar.

2.2. OBJETIVOS ESPECÍFICOS

- Selecionar as melhores condições de cultivo e o meio de produção do biossurfactante utilizando um Delineamento Composto Central Rotacional (DCCR) como ferramenta estatística.
- Descrever as curvas de crescimento do micro-organismo crescendo em resíduos industriais e de produção do biossurfactante.
- Avaliar a estabilidade do biossurfactante quanto à tensão superficial e a atividade de emulsificação frente a diferentes faixas de pH e temperatura, na presença de NaCl e em intervalos de tempo sob aquecimento.
- Determinar a capacidade tensoativa e emulsificante do biossurfactante.
- Caracterizar quimicamente o biossurfactante e determinar sua carga iônica.
- Avaliar a toxicidade do biossurfactante frente ao microcrustáceo *Artemia salina*.
- Realizar o “scale-up” de produção do biossurfactante em biorreatores de 2L e 5L.
- Formular um biossurfactante comercial e avaliar sua estabilidade (efeitos do pH, da adição de NaCl, do tempo sob aquecimento e da temperatura).
- Realizar testes de lavagem de composto hidrofóbico adsorvido em pedras marinhas com o biossurfactante formulado.
- Realizar testes de deslocamento (dispersão/agregação) de composto hidrofóbico em água do mar pelo biossurfactante formulado.
- Avaliar o potencial de aplicação do biossurfactante como agente de biorremediação em água do mar contaminada com derivado de petróleo.

3. REVISÃO DE LITERATURA

3.1. Petróleo e meio ambiente

O petróleo é uma fonte essencial de energia e força motriz do desenvolvimento econômico. Atualmente não existe nenhuma outra fonte de energia disponível que possa competir ou substituir completamente o petróleo, tornando o mundo fortemente dependente deste recurso para suprir as suas necessidades básicas de calor, luz e transporte (ALMEIDA et al., 2016; SILVA et al., 2014). A demanda energética mundial aponta para um crescimento anual de 1,7 % no número de barris de petróleo até 2030, quando alcançará um consumo de 15,3 bilhões de toneladas ao ano. Segundo o Departamento de Energia dos Estados Unidos, os combustíveis fósseis constituem 83 % de todas as fontes primárias de energia deste país, das quais somente o petróleo representa 57 % desses recursos. Diante dessa realidade, a possibilidade de contaminação ambiental torna-se real e iminente (ALMEIDA et al., 2016; SANTOS et al., 2016).

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 uma das grandes pautas de discussão tanto de países industrializados como dos países em desenvolvimento, uma vez que a poluição por petróleo têm efeitos prejudiciais para o meio ambiente e provoca danos significativos aos organismos residentes (ALMEIDA et al., 2017). As principais fontes de hidrocarbonetos nos oceanos são provenientes de operações rotineiras de lavagem de navios, vazamento de óleo natural no fundo do mar ou de acidentes durante a exploração e transporte de petróleo (SILVA et al., 2014). Estima-se, por exemplo, que metade da produção mundial de petróleo (cerca de três bilhões de toneladas/ano) é transportada por navios, o que tem contribuído para um aumento nos níveis de contaminação por hidrocarbonetos devido aos derrames acidentais (SILVA et al., 2014; SOUZA et al., 2014).

Os meios de comunicação têm constantemente noticiado o vazamento de milhares de toneladas de óleo que alcançam o ambiente marinho. Um dos maiores derramamentos de petróleo do mundo ocorreu em 2010 no Golfo do México, ao longo da costa dos estados da Louisiana e Mississippi (EUA), depois da explosão de uma plataforma de petróleo. Após afundar, os dutos abertos da plataforma na área de perfuração (a cerca de 1,5 km de profundidade) continuaram liberando óleo no mar por um período de três meses antes de finalmente serem contidos. Relatórios

oficiais indicaram a fuga de mil barris de petróleo por dia, com um total estimado de três a quatro milhões de barris de petróleo derramados, tornando este o maior desastre ambiental da história dos Estados Unidos (ROCHA e SILVA et al., 2013; SILVA et al., 2014). Em julho de 2010, outro derramamento de óleo, estimado em 1.500 toneladas de petróleo bruto, causou sérios problemas ambientais à costa de Dalian, na China (MAKKAR et al., 2011). No Brasil, um dos maiores derramamentos de óleo ocorreu em novembro de 2011 na plataforma de perfuração Sedco 706, operada pela Chevron-Texaco, na Baía de Campos, Rio de Janeiro. Uma semana após o acidente, a mancha de óleo formada já ocupava uma área de 163 km², com um volume total de 5.943 litros de óleo vazados (SOUZA et al., 2014). Outro acidente impactante ocorreu em janeiro de 2000, quando mais de 1,3 milhão de litros de óleo pesado vazaram de um duto da refinaria da Baía de Guanabara, também no Rio de Janeiro, causando prejuízos às áreas de manguezais preservadas (ALMEIDA et al., 2017; SILVA et al., 2014).

A remediação de locais contaminados por petróleo e seus derivados é geralmente realizada por meio de métodos físicos, químicos ou biológicos. Os métodos físicos e químicos convencionais disponíveis removem rapidamente a maioria do óleo vazado, mas na maior parte dos casos, esta remoção apenas transfere os contaminantes de um ambiente para outro, produzindo, em muitos casos, subprodutos tóxicos (ALMEIDA et al., 2016). Deste modo, o petróleo não pode ser completamente removido do ambiente pelos métodos físicos e químicos. Além disso, as novas diretrizes de recuperação de águas e solos têm restringido o uso de diversos produtos químicos devido às consequências negativas desta abordagem. Sendo assim, mais atenção tem sido dada à exploração de alternativas biológicas (LIN et al., 2014; MALIK; AHMED, 2012).

Dentre as técnicas de remediação disponíveis, a biorremediação tem se destacado como um conjunto de tecnologias ambientalmente sustentáveis, que tem sido utilizada para a remoção de contaminantes por meio da capacidade natural de degradação de micro-organismos, seja pela conversão completa destas substâncias em dióxido de carbono e água ou pela conversão parcial destes contaminantes em compostos menos tóxicos (MHATRE; KUNDE, 2014; SILVA et al., 2015). Esta técnica pode ser conduzida por meio de duas diferentes abordagens:

1. Bioestimulação, que consiste em estimular o crescimento de micro-organismos presentes no local contaminado. Este processo envolve basicamente a introdução

de oxigênio e de nutrientes para a degradação do contaminante, bem como de substâncias para corrigir o pH (SILVA et al., 2015);

2. Bioaumentação, na qual os micro-organismos autóctones são adicionados ao ambiente contaminado para acelerar e completar a degradação do poluente (SOUZA et al., 2014).

A biorremediação desempenhou um importante papel na limpeza do derramamento de milhões de litros de petróleo causado pelo acidente com o navio Exxon Valdez, no Golfo do Alasca, em 1989, dando origem ao desenvolvimento dessa tecnologia, a qual demonstrou ser bastante viável e de aplicação efetiva no tratamento de futuros derramamentos de óleo em circunstâncias apropriadas (SANTOS et al., 2016). Neste acidente, a primeira medida tomada foi lavagem física com jatos de água a alta pressão. Em seguida, surfactantes químicos foram aplicados nas áreas poluídas para acelerar a atividade dos micro-organismos degradadores de petróleo. Após três semanas, as áreas tratadas com os surfactantes estavam significativamente mais limpas do que as áreas controle. No entanto, foi difícil avaliar os exatos efeitos do tratamento devido à heterogeneidade da contaminação. De qualquer forma, outros estudos demonstraram a importância do uso de surfactantes para aumentar a biodegradação do petróleo (SANTOS et al., 2016; SATPUTE et al., 2010).

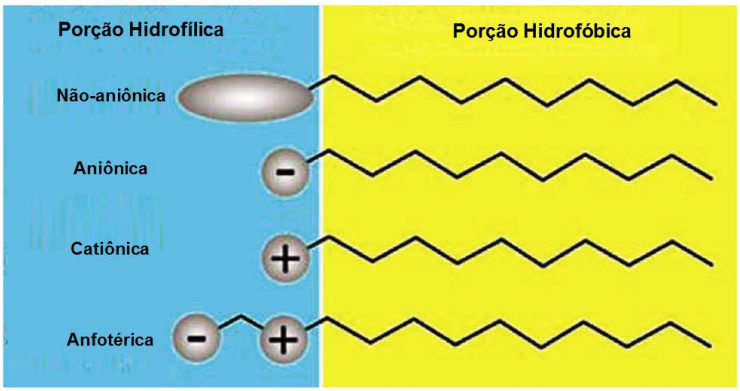
Embora a utilização de surfactantes químicos melhore a solubilidade de contaminantes hidrofóbicos gerados pela indústria do petróleo, eles representam um problema adicional para o ambiente, pois, em sua grande maioria, são compostos persistentes e recalcitrantes. Nesse sentido, a utilização de biossurfactantes surge como uma alternativa mais atrativa e segura para a remoção de hidrocarbonetos, tornando a técnica de biorremediação ainda mais atrativa (APARNA et al., 2011; SILVA et al., 2014; SOUZA et al., 2014).

3.2. Biossurfactantes

Os biossurfactantes são compostos naturais anfipáticos, cujas estruturas moleculares possuem simultaneamente grupos hidrofílicos e hidrofóbicos. A porção apolar é frequentemente uma cadeia de hidrocarboneto, enquanto que a porção polar pode ser iônica (catiônica ou aniônica), não iônica ou anfotérica, como ilustrado na Figura 1. Praticamente todos os biossurfactantes exibem estruturas aniônicas ou não iônicas, muito embora alguns casos relatem a presença de grupos

hidrofílicos contendo nitrogênio, o qual confere características catiônicas à molécula (CAMPOS et al., 2013; SANTOS et al., 2016).

Figura 1 - Estruturas gerais de biossurfactantes, de acordo com a composição de suas porções hidrófilas, sendo não iônicas, aniônicas, catiônicas ou anfotéricas



Fonte: CAMPOS et al. (2013)

Os biossurfactantes se particionam preferencialmente nas interfaces entre fases fluidas com diferentes graus de polaridade e exibem propriedades como adsorção, formação de micelas, formação de macro ou micro emulsões, ação espumante, solubilidade e detergência, todas ligadas à capacidade de redução da tensão superficial por essas moléculas (KAPADIA; YAGNIK, 2013).

Muitos micro-organismos são conhecidos por secretarem biossurfactantes no meio de cultivo quando se alimentam de substâncias que são imiscíveis em água, facilitando a translocação de substratos insolúveis através das membranas celulares. Estas moléculas são principalmente produzidas por micro-organismos aeróbicos utilizando fontes de carbono, tais como carboidratos, hidrocarbonetos, gorduras, óleos, dentre outras como matéria-prima (CAMPOS et al., 2013; SOBRINHO et al., 2013b).

3.2.1. Classificação

Os biossurfactantes são geralmente classificados de acordo com sua origem microbiana e com sua composição química (Tabela 1) (SOBRINHO et al., 2013b). As principais classes incluem glicolipídeos, lipopeptídeos, lipoproteínas, ácidos graxos, fosfolipídeos, surfactantes poliméricos e surfactantes particulados (Figura 2). De um modo geral, os biossurfactantes contêm uma ou várias porções lipofílicas e hidrofílicas semelhantes a todas as moléculas tensoativas (KAPADIA; YAGNIK,

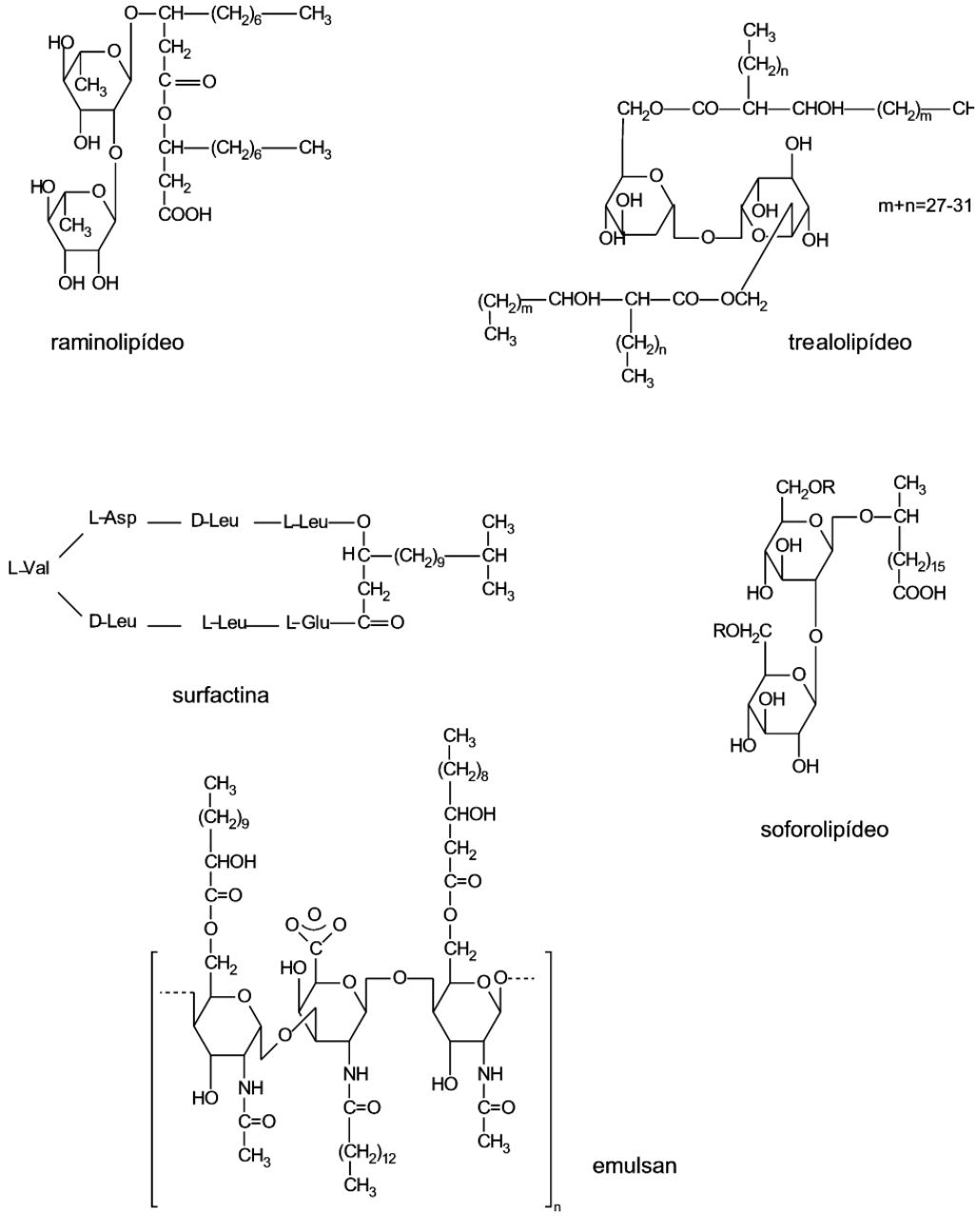
2013; SOBRINHO et al., 2013b). A porção lipofílica pode ser uma cadeia de um ácido graxo com 10-18 átomos de carbono ou mais; ou pode ser uma proteína ou peptídeo com uma elevada proporção de aminoácidos contendo cadeias laterais hidrofóbicas. A porção hidrofílica pode ser um éster, um grupo hidroxila, um grupo fosfato, um grupo carboxílico, um carboidrato ou um peptídeo/proteína com elevada proporção de aminoácidos de cadeias laterais hidrofílicas (CAMPOS et al., 2013).

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

Classe/Tipo de Biossurfactante	**Micro-organismos**
Glicolipídeos	
Raminolipídeos	*Pseudomonas aeruginosa*
Soforolipídeos	*Candida bombicola, Candida apicola*
Trealolipídeos	*Rhodococcus erythropolis, Mycobacterium sp.*
Lipopeptídeos e lipoproteínas	
Peptídeo-lipídeo	*Bacillus licheniformis*
Viscosina	*Pseudomonas fluorescens*
Serrawettin	*Serratia marcenscens*
Surfactina	*Bacillus subtilis*
Subtilisina	*Bacillus subtilis*
Gramicidina	*Bacillus brevis*
Polimixina	*Bacillus polymyxia*
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*
Surfactante particulado	
Vesícula	*Acinetobacter calcoaceticus*
Células	Diversas bactérias

Fonte: Adaptado de SILVA et al., 2014.

Figura 2 – Estruturas químicas dos principais tipos de biossurfactantes



Fonte: Adaptado de KAPADIA e YAGNIK, 2013.

Os biossurfactantes também podem ser classificados de acordo com seus pesos moleculares, sendo divididos em compostos de baixo peso molecular, os quais incluem fosfolipídeos, glicolipídeos e lipopeptídeos; e em compostos de alto peso molecular, os quais compreendem os polissacarídeos anfipáticos, proteínas, lipoproteínas, lipopolissacarídeos ou misturas complexas destes biopolímeros. Os biossurfactantes de alto peso molecular são mais eficazes na estabilização de emulsões de óleo em água, enquanto que os biossurfactantes de baixo peso

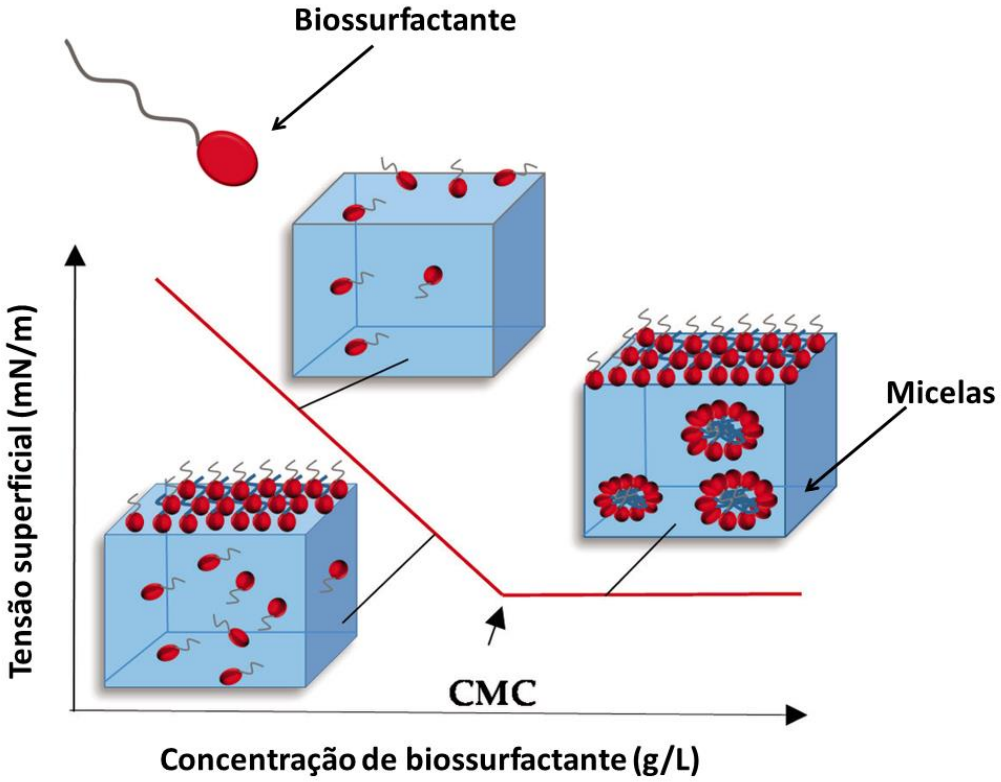
molecular são mais eficientes na redução das tensões superficiais e interfaciais (CAMPOS et al., 2013; SOBRINHO et al., 2013b).

3.2.2. Propriedades e características dos biossurfactantes

Os biossurfactantes atuam como agentes que facilitam a 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 representam vantagens sobre os surfactantes convencionais, fornecendo novas possibilidades para aplicações industriais e ambientais (SANTOS et al., 2016) sendo elas:

1. 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 (SANTOS et al., 2016). Tensão superficial é a força de atração existente entre as moléculas dos líquidos. Esta tensão 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, vão formando 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) (Figura 3).

Figura 3 – Esquema Ilustrativo mostrando as regiões nas quais ocorre a formação de micelas na concentração micelar crítica – CMC



Fonte: SANTOS et al. (2016)

2. Tolerância a condições extremas de temperatura, pH e salinidade: vários biossurfactantes apresentam elevada estabilidade a diferentes valores de temperatura e de pH, podendo ser utilizados em ambientes com condições adversas. O biossurfcatante da levedura *Candida lipolytica* UCP 0988 mostrou-se estável após o tratamento com temperaturas chegando a 120 °C, e apresentou propriedades praticamente 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 % já é suficiente para inativar a maioria dos surfactantes convencionais (CAMPOS et al., 2013).
3. Biodegradabilidade: diferentemente 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., 2016).

4. Baixa toxicidade: os biossurfactantes também têm recebido maior atenção devido à crescente preocupação da população com os efeitos alérgicos dos produtos sintéticos; além disto, sua baixa toxicidade permite o seu uso em alimentos, cosméticos e produtos farmacêuticos (CAMPOS et al., 2013).
5. 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 a outra, 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).
6. Não serem derivados do petróleo: este fator é bastante importante à medida que os preços do petróleo aumentam por 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 (SANTOS et al., 2016; SILVA et al., 2014).

Todas estas vantagens acima contribuem para a aplicação de biossurfactantes em diferentes indústrias.

3.2.3. Micro-organismos produtores de biossurfactantes

Uma variedade de micro-organismos, tais como bactérias, leveduras e fungos filamentosos, são capazes de produzir biossurfactantes com diferentes estruturas moleculares. Além da habilidade genética, outros fatores, como as condições ambientais e o tipo de substrato utilizado, determinam o grau de produção de biossurfactantes por micro-organismos (DAS; CHANDRAN, 2011). Dentre as principais espécies e gêneros investigados, destacam-se: *Bacillus subtilis, Pseudomonas aeruginosa, Arthrobacter*, *Acinetobacter calcoaceticus*, *Candida lipolytica*, *Candida bombicola*, dentre outras (CAMPOS et al., 2015).

Dentre as bactérias, a literatura descreve as dos gêneros *Pseudomonas* e *Bacillus* como grandes produtores de biossurfactantes. O gênero *Pseudomonas* é

conhecido por sua capacidade de produzir grandes quantidades de glicolipídeos, dos quais, os mais bem estudados e descritos na literatura são os raminolipídeos, produzidos pela bactéria Gram-positiva *Pseudomonas aeruginosa* (SANTOS et al., 2016). Dentre as bactérias Gram-positivas, *Bacillus subtilis* é a mais amplamente estudada e é conhecida por sua eficiência na produção de um tipo de lipopeptídeo com excelente atividade superficial denominado surfactina. Este biossurfactante contém sete aminoácidos ligados aos grupos carboxila e hidroxila do ácido C_{14} e é reportado pela literatura como um dos mais poderosos tensoativos naturais conhecidos (LIU et al., 2015).

Embora a maioria dos biossurfactantes investigados seja obtida a partir de bactérias, a provável natureza patogênica de algumas linhagens produtoras restringe a ampla aplicação desses compostos (SHARMA et al., 2016; TORIBIO et al., 2010), tornando-os inadequados para utilização em indústrias como a de alimentos, farmacêutica ou de cosméticos (SANTOS et al., 2016). Sendo assim, um número crescente de aspectos relacionados à produção de biossurfactantes por leveduras tem sido tema de pesquisas nas últimas décadas, dada a importância industrial das leveduras e seu potencial para a produção de produtos biotecnológicos (AMARAL et al., 2010). Uma vantagem chave da utilização de leveduras reside no seu *status Generally Regarded As Safe* (GRAS) ou "geralmente considerado como seguro" (GRAS). Os organismos com o *status* GRAS, tais como *Yarrowia lipolytica*, *Saccharomyces cerevisiae* e *Kluyveromyces lactis*, não oferecem riscos de toxicidade ou patogenicidade, o que permite a sua utilização nas indústrias alimentar e farmacêutica (CAMPOS et al., 2013).

Várias espécies do gênero *Candida*, incluindo *Candida bombicola* (LUNA et al., 2016; ROELANTS et al., 2013), *Candida glabrata* (GUSMÃO et al., 2010; LUNA et al., 2009), *Candida lipolytica* (RUFINO et al., 2014; SANTOS et al., 2013), *Candida sphaerica* (LUNA et al., 2015; SOBRINHO et al., 2013a), *Candida utilis* (CAMPOS et al., 2013), *Candida guilliermondii* (SITOHY et al., 2010), *Candida antarctica* (KIM et al., 2002; HUA et al., 2003) e *Candida tropicalis* (BATISTA et al., 2010; PRIJI et al., 2013) são conhecidos por produzir biossurfactantes. Dentre estas, *Candida bombicola* e *Candida lipolytica* estão entre as mais comumente estudadas para a produção de biossurfactantes (CAMPOS et al., 2013; SILVA et al., 2014). Os glicolipídeos mais comuns produzidos por este gênero são os soforolipídeos. Este biossurfcatante é composto por um açúcar dissacarídeo (2´-O-*β*-D-glicopiranosil-1-*β*-

D-glicopiranose) unido por ligação *β*-glicosídica a um ácido graxo de cadeia longa. *Candida bombicola* se destaca dentre as leveduras utilizadas na produção deste biossurfactante, para o qual já foram registrados valores de tensão superficial de aproximadamente 33 mN/m e altos rendimentos (SANTOS et al., 2016). Outro biossurfactante bastante promissor produzido por leveduras são os lipídeos de manosileritritol, os quais são abundantemente produzidos pela levedura *Candida antarctica* a partir de óleos vegetais (AL-BAHRY et al., 2013).

A levedura *Candida tropicalis* tem sido amplamente estudada por vários pesquisadores como uma potente linhagem com capacidade para biodegradar hidrocarbonetos (ALMEIDA et al., 2017; CHANDRAN; DAS, 2011; FARAG; SOLIMAN, 2011). Estudos mais recentes, no entanto, também têm revelado que esta espécie tem a capacidade metabólica para produzir biossurfactante quando cultivada em substratos imiscíveis em água (CHANDRAN; DAS, 2012; VERMA et al., 2015). Haba et al. (2000), em um trabalho preliminar, conseguiram uma redução para 35 mN/m na tensão superficial do meio, contendo óleo de fritura utilizado como substrato para a produção de biossurfactante de *C. tropicalis* CECT 1440. Em outro estudo, Batista et al. (2010), obtiveram um rendimento de 3,61 g/L de biossurfactante de *C. tropicalis* cultivada em meio também contendo óleo de fritura como substrato, reduzindo a tensão superficial para 33,66 mN/m.

3.2.4. Função fisiológica dos biossurfactantes

Embora o papel fisiológico exato dos biossurfactantes ainda não tenha sido completamente elucidado, algumas funções têm sido atribuídas. A vertente mais forte sugere que o papel fisiológico dos biossurfactantes em micro-organismos é facilitar o contato das células com os substratos hidrofóbicos (MATVYEYEVA et al., 2014). As superfícies da maioria dos micro-organismos são hidrofílicas, o que lhes permite interagir de modo eficaz com compostos solúveis em água e garantir o funcionamento normal dos sistemas enzimáticos ligados às suas membranas. No entanto, estes mesmos sistemas encontram dificuldades para interagir com substratos hidrofóbicos, tais como os produtos petrolíferos. Por outro lado, há diversos grupos de micro-organismos capazes de assimilar hidrocarbonetos de petróleo como fonte de carbono e energia (MATVYEYEVA et al., 2014; WEI et al., 2004). Como os sistemas enzimáticos para o catabolismo de hidrocarbonetos estão

localizados no citoplasma das células, a sua capacidade para assimilar estes compostos contam com três principais vias de absorção:

1. Canais de parede celular de natureza polissacarídica lipofílicas, ou seja, com teor maior de substâncias hidrofóbicas com elevada afinidade por hidrocarbonetos: por esta via, verifica-se um aumento significativo da porção lipídica do polissacarídeo de membrana do micro-organismo crescendo em petróleo ou derivados, indicando que o complexo polissacarídeo-ácido graxo presente na superfície celular está envolvido no transporte de hidrocarbonetos. Este mecanismo é muito característico de leveduras e de algumas bactérias do gênero *Arthrobacter* (MATVYEYEVA et al., 2014);

2. Síntese de compostos emulsionantes e solubilizantes de hidrocarbonetos: a função chave dos biossurfactantes extracelulares é provocar a dispersão de hidrocarbonetos no meio aquoso. Este tipo de mecanismos é o mais bem estudado e é característico de micro-organismos que possuem processos de oxidação mais ativos, tais como os dos gêneros *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Arthrobacter*, *Serratia*, algumas bactérias dos gêneros *Rhodococcus*, *Gordonia* e *Candida* (BENINCASA et al., 2004; WEI et al., 2004).

3. Parede celular altamente hidrofóbica: compostos lipofílicos proporcionam um contato mais direto com as moléculas de hidrocarbonetos. Espécies do gênero *Rhodococcus* têm alta afinidade por hidrocarbonetos pela alta hidrofobicidade de suas paredes celulares, cuja espessura é relativamente alta, permitindo uma difusão passiva efetiva e acumulação de hidrocarbonetos na célula (MATVYEYEVA et al., 2014).

Outras funções fisiológicas são atribuídas aos biossurfactantes, a saber:

1. Aderência-liberação da célula a superfícies: uma das mais importantes estratégias de sobrevivência dos micro-organismos é sua habilidade em colonizar um nicho ecológico onde possa se multiplicar. O elemento chave nesta estratégia são estruturas da superfície celular responsáveis pela aderência das células às superfícies. Os micro-organismos podem utilizar biossurfactantes ligados à parede para regular as propriedades da superfície celular, visando aderir ou se desligar de um determinado local, de acordo com sua necessidade para encontrar novos habitats com maior disponibilidade de nutrientes ou se livrar de ambientes desfavoráveis (LUNA et al., 2010);

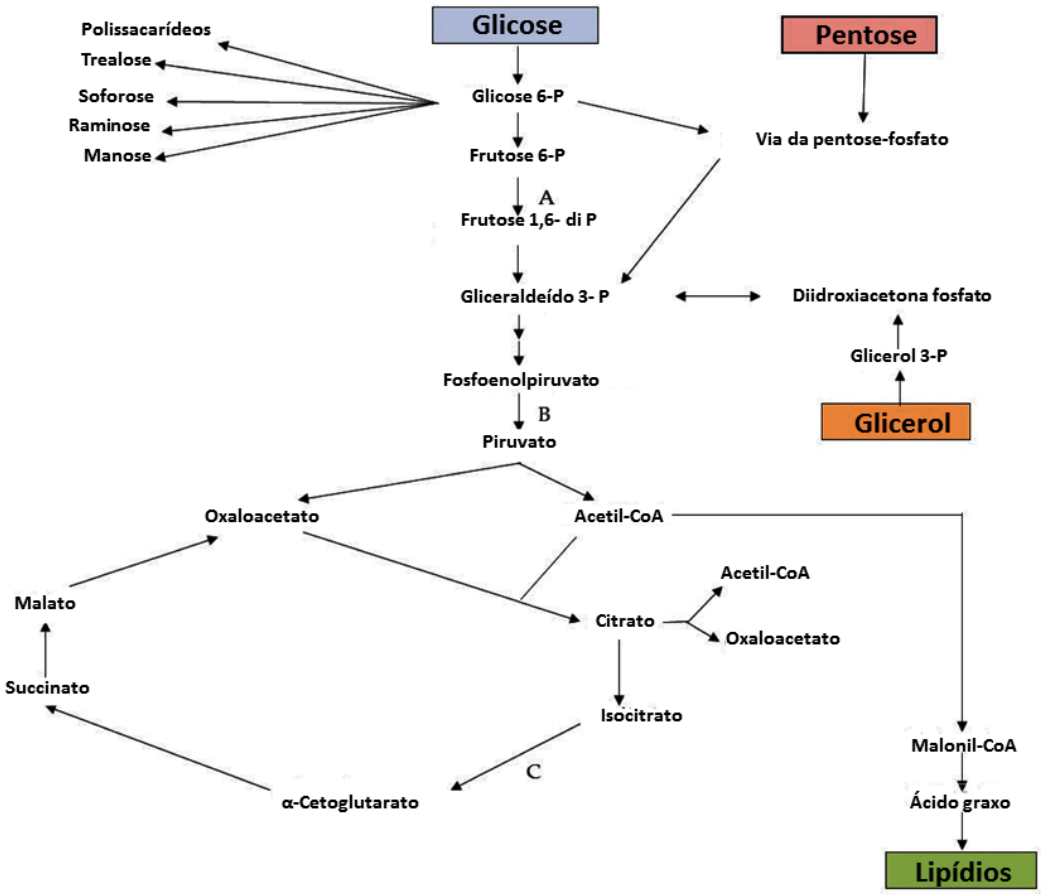
2. Atividade antibiótica: demonstrada por vários biossurfactantes, principalmente da classe dos lipopeptídios e glicopeptídios. Os raminolipídeos de *P. aeruginosa* e a surfactina de *B.subtilis* funcionam como antibióticos, solubilizando os principais componentes das membranas celulares microbianas. Através da excreção destes biossurfactantes no meio, os micro-organismos adquirem maior chance de sobrevivência e maior competitividade na busca por nutrientes (QUADROS et al., 2011).

3.2.5. Biossíntese de biossurfactantes

Diversas vias metabólicas estão envolvidas na biossíntese de precursores para a produção de biossurfactantes e são dependentes da natureza das fontes de carbono empregadas no cultivo microbiano. Substratos hidrofílicos são utilizados principalmente por micro-organismos para o metabolismo celular e para a síntese da porção polar do biossurfactante, enquanto substratos hidrofóbicos são utilizados exclusivamente para a produção da porção apolar do biossurfactante (SANTOS et al., 2016). Quando os carboidratos são utilizados como única fonte de carbono para a produção de um glicolipídeo, o fluxo de carbono é regulado de tal forma que ambas as vias lipogênicas (formação de lipídeos) e a formação da porção hidrofílica, através da via glicolítica, são supridas pelo metabolismo microbiano (HARITASH; KAUSHIK, 2009), como ilustrado na Figura 4. Um substrato hidrofílico, tal como glicose ou glicerol, é degradado até formar intermediários da via glicolítica, tal como glicose 6-fosfato, que é um dos principais precursores de carboidratos encontrados na parte hidrofílica de um biossurfactante. Para a produção de lipídeos, a glicose é oxidada à piruvato, através da glicólise, e o piruvato é então convertido em acetil-CoA, que produz malonil-CoA que, quando unido com oxaloacetato, é convertido em ácido graxo, que é um dos precursores para a síntese de lipídeos (SANTOS et al. 2016). No entanto, quando se utiliza um hidrocarboneto como fonte de carbono, o mecanismo microbiano dirige-se principalmente para à via lipolítica e à gliconeogênese (formação de glicose através de diferentes precursores de hexose), permitindo, assim, a sua utilização para a produção de ácidos graxos ou açúcares. A via da gliconeogênese é ativada para a produção de açúcares. Esta via consiste na oxidação de ácidos graxos, liberando como produto acetil-CoA. Começando com a formação de acetil-CoA, as reações envolvidas na síntese de precursores polissacáridos, tais como glicose-6-fosfato, são essencialmente o inverso das

envolvidas na glicólise. Contudo, as reações catalisadas pela piruvato quinase e pela fosfofrutoquinase são irreversíveis. Assim, são necessárias outras enzimas exclusivas para o processo de gliconeogênese para evitar tais reações (TOKUMOTO et al., 2009).

Figura 4 – Metabolismo intermediário relacionado à síntese de precursores de biossurfactantes utilizando carboidratos como substrato. Enzimas-chave para o controle do fluxo de carbono: (A) Fosfofrutoquinase; (B) Piruvato quinase; (C) Isocitrato desidrogenase



Fonte: SANTOS et al. (2016)

3.3. Patentes em biossurfactantes

A vasta diversidade estrutural que caracteriza os biossurfactantes, bem como suas diversas propriedades, tem levado a uma infinidade de pedidos de patentes por parte de empresas interessadas e de pesquisadores (MARCHANT; BANAT, 2012; LUNA et al., 2013). Várias patentes sobre a produção de biossurfactantes têm sido emitidas a partir de uma vasta gama de micro-organismos, incluindo *Pseudomonas*

spp., *Bacillus* spp., *Acinetobacter* spp. e *Candida* spp., relacionadas a muitas aplicações industriais (SACHDEV; CAMEOTRA, 2013). De acordo com Müller et al. (2012), a pesquisa de patentes no *Instituto Europeu de Patentes* utilizando os termos "biossurfactantes", "raminolipídeos", "soforolipídeos" e "lipídeos de manosileritritol" mostrou um grande aumento no número de patentes a partir do ano 2000. Dados revelaram que mais de 250 patentes foram emitidas no mundo sobre biossurfactantes e bioemulsificantes, das quais 33% estão relacionadas à utilização destes compostos na indústria de petróleo, 15 % para uso em cosméticos, 12 % para utilização como agente antimicrobiano e aplicações biomédicas e 11 % em usos relacionados à biorremediação. Patentes relacionadas aos biossurfactantes soforolipídeos, surfactinas e raminolipídeos representaram 24 %, 13 % e 12 % do número total de patentes, respectivamente. Estes números, contudo, podem estar subestimados, uma vez que muitas patentes não descrevem e nem especificam o micro-organismo produtor, referindo-se à descrição geral de um "biossurfactante" (ALMEIDA et al., 2016; RANDHAWA; RAHMAN, 2014; REIS et al., 2013; SHETE et al., 2006). A Tabela 2 lista algumas das importantes patentes emitidas nos últimos anos.

Tabela 2 – Patentes sobre biossurfactantes produzidos por micro-organismos e suas aplicações

Micro-organismo/ Tipo de biossurfactante	Titular da patente	Título da patente	N° da publicação	Data da Publicação
Produtor de soforolipídeo	Borzeix F.	Soforolipídeo como agente de estimulação do metabolismo de fibroblastos dérmicos	US 6057302 A	02 de maio de 2000
Produtor de soforolipídeo	Borzeix F., Concaix	Utilização de soforolipídeos, compreendendo diacetil lactonas como agente para estimular o metabolismo dos fibroblastos da pele	US 6596265 B1	22 julho de 2003
Novas linhagens de bactérias degradadoras de hidrocarbonetos capazes de produzir biossurfactantes	Robin L. Brigmon, Sandra Story, Denis Altman, Christopher J. Berry	Surfactante biocatalisador para remediação de materiais orgânicos recalcitrantes e metais pesados	PI 0519962-0 A2	28 junho de 2005
Produtor de soforolipídeo	Gross R.A., Shah V., Doncel G.F.	Propriedades espermicidas e virucidas de várias formas de	WO 2005089522 A2	29 de setembro de 2005

soforolipídeos

C. albicans, C. rugosa, C. tropicalis, C. lipolytica, C. torulopsis	Awada S., Spendlove R., Awada M.	Biossurfactantes microbianos como agentes para controlar pragas	US 20050266036 A1	01 de dezembro de 2005
Pseudomonas aeruginosa	Silvanito Alves Barbosa, Roberto Rodrigues de Souza	Produção de biossurfactantes para o desenvolvimento de detergentes biodegradáveis	PI 1102592-1 A2	16 de maio de 2011
Produtor de soforolipídeo	Cox T.F., Crawford R.J., Gregory L.G., Hosking SL, Kotsakis	Composição de detergente suave para a pele, espumante	WO2011120776 A1	06 outubro de 2011
Streptomyces sp.	Ana LF Porto, Eduardo F Santos, Leonie A Sarubbo	Biossurfactante e processo de produção	PI 1105951-6 A2	28 de novembro de 2011
Candida guilliermondii	Leonie A. Sarubbo, Valdemir A. Santos, Raquel D. Rufino, Juliana M. Luna	Processo de produção de biossurfactante produzido por *C. guilliermondii* utilizando resíduos agro-industriais	BR102012023115	13 setembro de 2012
Candida bombicola ATCC 22214	Soetaert W., De M.S., Saerens K., Roelants S., Van B.I.	Produção modificada de soporolipídeos por cepas de levedura e seu uso	EP 2580321 A1	17 abril de 2013
Produtor de lipopeptídeo	X. Vecino, R. Dvesa-Rey, J.M. Cruz, A.B. Moldes	Método para separar os tensoativos presentes nos licores de lavagem de milho e utilizações	WO2014044876 A1	27 março de 2014

Fonte: Adaptado de SILVA et al. (2014).

3.4. Mercado de biossurfactantes

O aumento da consciência ambiental tem sido o principal motivador na procura de um substituto para os surfactantes químicos (MARCHANT; BANAT, 2012). De acordo com estudos recentes, o mercado global para os substitutos de base biológica 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. Para 2018, a estimativa é que se atinja um montante de até US $ 2.210,5 milhões, e em 2020, 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% entre os anos de 2014 e 2020 (ALMEIDA et al., 2016; GUDIÑA et al., 2015; SEKHON et al., 2012).

Ainda de acordo com mesmo estudo, 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 naquele ano. A América do Norte foi o segundo maior consumidor de biossurfactantes neste mesmo ano, com uma participação de mais de um quarto. Já o bloco Ásia-Pacífico teve um mercado relativamente pequeno em 2013, mas a previsão é que ganhe participação significativa até 2019, devido à presença de grandes indústrias na região (ALMEIDA et al., 2016).

A preocupação global com a sustentabilidade tornou-se um diferencial competitivo para empresas que aplicam seus conceitos no processo produtivo, uma vez que a preocupação com o futuro ambiental do planeta deixou de ser opção, passando a constituir uma tendência emergencial de empresas e consumidores. Nesse sentido, uma das grandes vantagens de empresas do setor de biotecnologia frente à concorrência é a natureza biodegradável e atóxica de seus produtos e a utilização de resíduos industriais como parte de seu processo produtivo. Diante deste cenário, espera-se que o mercado de biossurfactantes ultrapasse e supere o mercado de surfactantes sintéticos (ALMEIDA et al., 2016; SILVA et al., 2014). A Tabela 3 sumariza os principais fabricantes comerciais de biossurfactantes no mundo com uso potencial em diversas indústrias.

No Brasil, a oportunidade de explorar a produção de bioprodutos se mostra muito atrativa e promissora. De acordo com dados levantados pela Associação Brasileira das Empresas de Biotecnologia (ABRABI), o faturamento anual do setor de Biotecnologia no país está estimado entre R$ 5,4 bilhões e R$ 9 bilhões, com um PIB de aproximadamente 2,8% ligado ao negócio de biotecnologia (FILHO, 2015). Um dos grandes fatores que influenciaram estes números foi o incentivo de ações de fomento voltadas para o desenvolvimento do setor. Conforme informações do Ministério da Ciência e Tecnologia, em 2007 o Brasil instituiu o Plano de Ação 2007-2010, integrado ao conjunto de ações do Programa de Aceleração do Crescimento

(PAC), promovendo, dentre outros, o estímulo ao desenvolvimento da biotecnologia por meio da identificação da demanda biotecnológica nacional e criando ferramentas para transformar o conhecimento acumulado nas universidades em produção industrial, além de estimular as empresas a incorporarem as atividades de pesquisa, desenvolvimento e inovação (PD&I) no seu processo produtivo. De acordo com a Sociedade Brasileira de Biotecnologia, existem atualmente no Brasil pouco mais de 300 empresas especializadas em biotecnologia, com incubadoras localizadas próximas a Centros de Pesquisa (FILHO, 2015; FONSECA, 2010).

Tabela 3 – Empresas produtoras, tipos de biossurfactantes comercialmente disponíveis e potenciais aplicações

Empresa	Biossurfactantes	Aplicações
Fraunhofer IGB – Alemanha	Glicolipídeos e lipídeos de celobiose	Produtos de limpeza, líquidos para lavagem de louças, produtos farmacêuticos (propriedades bioativas)
AGAE Technologies – EUA	Raminolipídeos	Farmacêutica, cosmética, cuidados pessoais, biorremediação (*in situ* e *ex situ*), Recuperação melhorada de óleo (EOR, do inglês)
TeeGene Biotech – UK	Raminolipídeos e Lipopeptídeos	Produtos farmacêuticos, cosméticos, antimicrobianos e ingredientes anticancerígenos,
Jeneil Biosurfactant – EUA	Raminolipídeos	Limpeza e recuperação de óleo de tanques de armazenamento, EOR
Allied Carbon Solutions (ACS) Ltd – Japão	Sophorolipideos	Produtos agrícolas, pesquisa ecológica
Rhamnolipid Companies – USA	Raminolipídeos	Agricultura, cosméticos, EOR, biorremediação, produtos alimentares, produtos farmacêuticos
Saraya Co. Ltd. – Japão	Sophorolipideos	Produtos de limpeza, produtos de higiene
BioFuture – Irlanda	Raminolipídeos	Lavagem de tanques de óleo combustível
Logos Technologies – EUA	Raminolipídeos	EOR
TensioGreen – EUA	Raminolipídeos	Indústria do petróleo, EOR
Synthezyme – EUA	Soforolipídeos	Emulsificação de óleo bruto, petróleo e gás
EcoChem Organics Company – Canadá	Raminolipídeos	Agente dispersivo de hidrocarbonetos insolúveis em água
EnzymeTechnologies – EUA	Biossurfactante bacteriano, (desconhecido)	Remoção de óleo; Recuperação e processamento de óleo, EOR

Fonte: Adaptado de RANDHAWA e RAHMAN (2014).

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.

Os únicos registros identificados para o setor de tensoativos no país englobam um total de nove empresas relacionadas com a produção e comercialização de surfactantes sintéticos (Tabela 4). A maior parte destas empresas está concentrada no estado de São Paulo, seguido pelo estado do Rio de Janeiro. Deste modo, esforços para o desenvolvimento de tecnologias de produção de biossurfactantes e biodetergentes permitirá o acesso a um produto inovador em uma área ainda pouco explorada no país.

Tabela 4 – Empresas brasileiras envolvidas na comercialização de tensoativos no país

Nome	Tipo de empresa	Localização
Rhodia do Brasil Ltda	Fabricante, Importador	SP
Herga Indústrias Químicas Ltda	Importador, Fabricante	RJ
Ata Assessoria Indústria e Comércio de Tensoativos Ltda	Fabricante	SP
Dinaco Importação Comércio S/A	Distribuidor, Importador	SP
Champion Technologies do Brasil Serviços e Produtos Químicos Ltda	Fabricante	RJ
Fortinbrás Comercial e Industrial Ltda	Distribuidor	SP
Oxiteno S/A Indústria e Comércio	Fabricante	SP
Unikrafht Indústria e Comércio de Produtos Químicos Ltda	Fabricante, Prestador de Serviços	SP
Braschemical Representação Ltda	Distribuidor, Importador	SP
ITW Chemical	Fabricante	SP

FONTE: Adaptado de < www.nei.com.br > Acesso em: 10 de janeiro 2017.

As indústrias estão atualmente tentando substituir alguns ou todos os surfactantes químicos por biossurfactantes (ALMEIDA et al, 2016). No entanto, o alto custo de produção é ainda uma grande desvantagem. O fator chave que regula o sucesso da produção de biossurfactantes é o desenvolvimento de um processo econômico que utilize materiais de baixo custo e que ofereça um rendimento elevado. De fato, a escolha de um substrato de baixo custo é importante para a

economia do processo, uma vez que o substrato é responsável por até 50 % do custo final de produção. Felizmente, os biossurfactantes podem ser produzidos a partir de recursos renováveis. Países essencialmente agrícolas, como o Brasil, têm fácil acesso a subprodutos agroindustriais (RUFINO et al., 2014).

3.5. Resíduos industriais utilizados na produção de biossurfactantes

As indústrias produzem diariamente grandes quantidades de resíduos sólidos e líquidos, resultantes da produção e processamento de alimentos e produtos agroindustriais, que quando descartados, geram grande poluição e representam um grande desperdício de nutrientes. No entanto, a necessidade de preservação ambiental tem estimulado cada vez mais a recuperação, reciclagem e reutilização de diversos resíduos (EZEJIOFOR et al., 2014).

Os resíduos industriais têm particularmente despertado grande interesse dos pesquisadores como alternativa de substratos de baixo custo para a produção de biossurfactantes, utilizando processos fermentativos. A escolha do resíduo para este fim deve garantir um balanço de nutrientes que suporte o crescimento microbiano e a produção do biotensoativo. Os resíduos industriais com altos teores de carboidratos ou lipídeos vêm se destacando como uma alternativa mais promissora para a produção de biossurfactantes (MAKKAR et al., 2011).

A literatura descreve uma variedade de resíduos utilizados na produção de biossurfactantes, tais como óleos vegetais, efluentes oleosos, efluentes amiláceos, gordura animal, gordura vegetal, resíduos de óleo de fritura vegetal, melaço, resíduos da indústria de laticínios, milhocina, manipueira, glicerol, dentre outros (SANTOS et al., 2016; SILVA et al., 2014). Alguns dos resíduos industriais mais utilizados por pesquisadores brasileiros para a produção de biossurfactantes são detalhados a seguir.

3.5.1. Óleos vegetais para a produção de biossurfactantes

Óleos vegetais constituem uma fonte de carbono lipídico e são principalmente constituídos por ácidos graxos saturados ou insaturados com cadeias contendo entre 16 e 18 átomos de carbono (MAKKAR et al., 2011). Diferentes tipos de óleos tais como óleo de girassol e de azeite, dentre outros, demonstraram serem substratos adequados, servindo como fontes de energia e de carbono para a

produção de biossurfactantes. As linhagens de *P. aeruginosa* produzem um excelente biossurfactante crescendo tanto em óleo residual de milho, como de soja e de canola (SANTOS et al. 2016). A combinação de glicose e óleo de canola foi utilizada para a produção bem sucedida de um biossurfactante pela levedura *C. lipolytica* (SARUBBO et al., 2007). Óleo de canola residual também tem foi relatado como adequado para o crescimento microbiano e a produção de até 8,0 g/L de biossurfactante (SILVA et al. 2013).

3.5.2. Milhocina

A agroindústria de produtos à base de milho, através das etapas de processamento húmido, que resulta em resíduos sólidos e líquidos, os quais, quando dispostos de forma inadequada, se tornam uma fonte de contaminação. A milhocina é um destes resíduos, resultante da água de lavagem, a qual contém 40 % de material sólido. Este subproduto consiste de 21 % a 45 % de proteínas, de 20 % a 26 % de ácido lático, 8 % de cinzas (contendo Ca^{2+}, Mg^{2+}, K^{+}, etc.), 3 % de carboidratos e um baixo teor de gordura (de 0,9 % a 1,2 %) (HELMY et al., 2011). Milhocina tem se mostrado uma excelente fonte de nutrientes de baixo custo para a produção de glicolípideos por *C. sphaerica* UCP 0995. O biossurfactante desta linhagem mostrou potencial para mobilização e remoção de até 95% de óleo de motor em areia, demonstrando potencial para processos de biorremediação (LUNA et al., 2015).

3.5.3. Melaço

O melaço é um subproduto do processamento da cana-de-açúcar e da beterraba. Este resíduo tem como composição média 75 % de matéria seca, 9 % - 12 % de matéria orgânica não açucarada, 2,5 % de proteína e 1,5 % - 5,0 % potássio, bem como magnésio, fósforo e cálcio (1%). Os teores de inositol, biotina, tiamina e ácido pantotênico (1 % - 3 %) conferem ao melaço sua consistência e cor acastanhada. O elevado teor de açúcar (48 % - 56 %) torna o melaço adequado para a produção de biossurfactantes por diferentes micro-organismos. Freitas et al. (2016) utilizaram melaço, bem como outras fontes de carbono para produzir um biossurfactante pela linhagem de *Candida bombicola* URM 3718. O biossurfactante obtido apresentou excelente estabilidade sob condições extremas de temperatura,

pH e de salinidade e potencial para aplicação na remediação ambiental (SANTOS et al., 2016).

3.6. Aplicações industriais e ambientais dos biossurfactantes

Os biossurfactantes apresentam potencial para uma variedade de aplicações em diversos setores da indústria, tais como a de alimentos, bebidas, cosméticos, detergentes, têxteis, tintas, mineração, celulose, farmacêutica e etc (SILVA et al., 2014). No entanto, o principal mercado que tem se mostrado mais viável para a aplicação destas biomoléculas é a indústria do petróleo, na qual os biossurfactantes podem ser utilizados na extração de óleo bruto de reservatórios de petróleo, na limpeza de tanques de armazenamento, no tratamento de resíduos oleosos e em processos de biorremediação de solo e da água contaminados (ALMEIDA et al., 2016). A seguir são relacionadas algumas das principais aplicações de biossurfactantes em diferentes indústrias.

3.6.1. Aplicações terapêuticas

A surfactina, um dos mais conhecidos biossurfactantes, apresenta várias aplicações farmacêuticas, como a inibição da formação de coágulos, formação de canais iônicos em membranas, atividade antibacteriana e antifúngica, e atividade antiviral e antitumoral. Já o biossurfactante produzido por *Rhodococcus erythropolis* demonstrou potencial de inibição do vírus do herpes simples e vírus parainfluenza. A iturina, lipopeptídeo produzido por *Bacillus subtilis*, demonstrou atividade antifúngica, afetando a morfologia e a estrutura da membrana celular de leveduras. A inibição da adesão de bactérias entéricas patogênicas por biossurfactante produzido por *Lactobacillus* foi também demonstrada (BANAT et al., 2014; ŁAWNICZAK et al., 2013; MNIF; GHRIBI, 2015).

3.6.2. Biossurfactantes na agricultura

Os biossurfactantes são utilizados na agricultura especialmente em formulações de herbicidas e pesticidas. Os compostos ativos destas formulações são geralmente hidrofóbicos, sendo necessário o uso de agentes emulsificantes para dispersá-los em soluções aquosas. Os biossurfactantes obtidos de *Bacillus* têm sido utilizados para emulsificar as formulações de pesticidas organofosforados

imiscíveis. Já os raminolipídeos possuem potencial para o controle biológico de fitopatógenos que produzem zoósporos (SACHDEV; CAMEOTRA, 2013).

3.6.3. Biossurfactantes na mineração

Biossurfactantes produzidos por linhagens de *Pseudomonas* sp. e *Alcaligenes* sp. foram utilizados para flotação e na separação de formações de minerais de calcita e scheelita, alcançando percentuais de recuperação de 95% para $CaWO_4$ e de 30% para $CaCO_3$, respectivamente, ressaltando que reagentes químicos convencionais são incapazes de separar estes dois minerais. O biodispersan, um polissacarídeo aniônico produzido por *A. calcoaceticus*, foi avaliado na prevenção da floculação e dispersão de misturas de pedra calcária e água. Já os biossurfactantes de *C. bombicola* demonstraram eficiência na solubilização de carvão (SARUBBO et al., 2015).

3.6.4. Biossurfactantes e cosméticos

Devido a sua compatibilidade com a pele, os biossurfactantes podem ser utilizados em produtos de higiene e cosméticos. Alguns soforolipídeos apresentam excelente compatibilidade dérmica, podendo ser utilizados como hidratante em cremes faciais e outros podem ser utilizados como umectantes para incorporação em produtos de maquiagem. A KAO Co. Ltda desenvolveu um processo fermentativo para produção de soforolipídeos, que posteriormente sofrem esterificação, resultando em um produto com aplicação em batons e como hidratante para pele e cabelos (VIJAYAKUMAR; SARAVANAN, 2015).

3.6.5. Biossurfactantes e indústria de alimentos

Na área de alimentos, a emulsificação tem um papel importante na formação da consistência e textura, bem como na dispersão de fases e na solubilização de aromas. Os biossurfactantes são utilizados como emulsionantes no processamento de matérias-primas para aplicação na panificação e em produtos derivados de carne, onde influenciam nas características reológicas da farinha e na emulsificação de gorduras. O bioemulsificante produzido por *Candida utilis* tem sido utilizado em molhos prontos para saladas (CAMPOS et al., 2013).

A Tabela 5 sumariza as principais aplicações de biossurfactantes em diferentes indústrias e processos ambientais.

Tabela 5 – Aplicações de biossurfactantes para usos industriais e processos ambientais

Indústria/processo	Aplicação	Papel dos biossurfactantes
Petróleo	Recuperação microbiana melhorada de petróleo (MEOR); Desemulsificação.	Melhora da drenagem de óleo em poços de perfuração; estimulando a liberação de óleo aprisionado de dentro dos capilares; redução de viscosidade de óleo; diminuição da tensão interfacial e dissolução de óleo; desemulsificação de emulsões de óleo; solubilização de óleo.
Descontaminação ambiental	Biorremediação; Processos de limpeza de derramamento de óleo.	Emulsificação de óleo; redução da tensão interfacial; dispersão de óleos; solubilização de óleos; dispersão, detergência; agente espumante; detergente.
Processos biológicos	Microbiológica	Estudo de comportamento fisiológico, tais como mobilidade celular, comunicação celular, adesão de nutrientes, competição celular-celular; patogênese vegetal e animal.
Bioprocessamento	Processos de *down-stream*	Biocatálise em sistemas aquosos de duas fases e microemulsões; biotransformações; recuperação de produtos intracelulares; aumento da produção de enzimas extracelulares e produtos de fermentação.
Agricultura	Biocontrole; Fertilizantes	Facilitação de mecanismos de biocontrole de micro-organismos, tais como parasitismo, antibiose, competição, resistência sistêmica induzida e hipovirulência; suspensão de pesticidas e fertilizantes em pó; emulsificação de soluções de pesticidas; facilitação de mecanismos de eliminação de patógenos de plantas; aumento da biodisponibilidade de nutrientes para micro-organismos benéficos associados a plantas.
Cosmético	Produtos de saúde e beleza	Emulsionante, agente espumante, solubilizante, agentes molhante, limpadores, agente antimicrobiano.
Mineração	Remoção de metais pesados; Recuperação do solo; Flotação.	Coletor na flotação; remoção de íons metálicos de soluções aquosas, solo e de sedimentos; sequestrante de metais pesados; inibidor de corrosão.
Alimentos	Emulsificação e desemulsificação; Ingrediente funcional.	Solubilização de óleos aromatizados; controle da consistência; agente emulsificante; agente molhante; dispersante;

		detergência; formação de espuma; agente espessante.
Medicina	Produtos farmacêuticos; Produtos terapêuticos.	Agentes anti-aderentes; agentes antifúngicos; agentes antibacterianos; agentes antivirais; vacinas; terapia génica; moléculas imunomoduladoras.
Limpeza	Detergentes para lavagem	Detergentes e desinfectantes para lavagem, molhagem, espalhamento.
Têxteis	Preparação de fibras; Tingimento e impressão; Acabamento têxtil.	Molhagem; penetração; solubilização; emulsificação; detergência e dispersão; umidificação e emulsificação em formulações de acabamento; amaciamento.

FONTE: Adaptado de SANTOS et al. (2016).

3.7. Perspectivas de utilização de biossurfactantes na indústria do petróleo

Os biossurfactantes têm uma vasta gama de aplicações biotecnológicas na indústria do petróleo. Todas as operações, incluindo a exploração e produção de petróleo, refino, transporte, síntese e manipulação de produtos, gestão de resíduos de petróleo e os processos de remediação, podem ser melhoradas e otimizadas pela utilização de algum tipo de biossurfactante (SILVA e al., 2014).

A recuperação microbiana melhorada de petróleo (MEOR) consiste em uma tecnologia de recuperação terciária de óleo que utiliza micro-organismos ou produtos de seu metabolismo para a recuperação de óleo residual. Estes micro-organismos produzem compostos tensoativos que reduzem a tensão superficial da interface óleo-rocha, reduzindo as forças capilares que impedem a movimentação do óleo através dos poros da rocha. Os biossurfactantes também auxiliam na emulsificação e na quebra dos filmes de óleo das rochas. A utilização de biossurfactantes em MEOR envolve várias estratégias, como a injeção de micro-organismos produtores de biossurfactantes no reservatório; injeção de nutrientes no reservatório, para estimular o crescimento de micro-organismos autóctones produtores de biossurfactantes; ou, ainda, a produção de biossurfactantes em biorreatores e posterior injeção no reservatório (ALMEIDA et al., 2016).

Resíduos de óleos pesados que sedimentam no fundo de tanques de estocagem são altamente viscosos e podem se tornar depósitos sólidos que não são removidos através do bombeamento convencional. A remoção requer lavagem com solventes ou limpeza manual, ambas perigosas, demoradas e caras. Um processo

alternativo de limpeza é a utilização de biossurfactantes, os quais promovem a diminuição na viscosidade e a formação de emulsões água-óleo, facilitando o bombeamento dos resíduos e a recuperação do óleo bruto após a quebra da emulsão. Os sólidos resultantes carregam uma quantidade limitada de óleo residual pela ação detergente do biossurfactante, tornando o descarte destes resíduos menos problemático (SILVA et al, 2014). A Tabela 6 apresenta uma lista de aplicações de biossurfactantes nas quatro principais atividades relacionadas à indústria de petróleo.

Tabela 6 – Aplicações convencionais de biossurfactantes na indústria do petróleo

Etapas da cadeia de produção de petróleo	Aplicações
Extração	Modificação da molhabilidade do reservatório; Redução da viscosidade de óleo; Lama de perfuração; Controle de deposição de parafina/asfalteno; Mobilização de óleo.
Transporte	Redução da viscosidade; Estabilização de emulsões; Depósito de parafina/asfalto.
Limpeza de tanques de estocagem de óleo	Redução da viscosidade de óleo; Emulsificação de óleo; Dispersão de hidrocarbonetos.
Tratamento de resíduos de óleo	Solubilização e mobilização de óleo.

FONTE: Adaptado de ALMEIDA et al. (2016).

3.8. Aplicação de biossurfactantes em processos de biorremediação

O papel mais comum dos biossurfactantes na biorremediação é aumentar a dispersão dos contaminantes hidrofóbicos na fase aquosa e a biodisponibilidade destes aos micro-organismos, com sua subsequente remoção através da biodegradação (APARNA et al., 2011; OLKOWSKA et al., 2012). Um dos mecanismos de ação dos biossurfactantes para o aumento da biodisponibilidade de substratos hidrofóbicos está relacionado com a redução da tensão superficial do meio em torno do micro-organismo, bem como uma redução na tensão interfacial entre a parede celular e as moléculas de hidrocarbonetos. A interação entre o biossurfactante e a superfície celular provoca alterações na membrana celular, facilitando a aderência dos hidrocarbonetos devido ao aumento da hidrofobicidade. Os biossurfcatantes facilitam a formação de gotículas de óleo menores,

aumentando, por sua vez, a área superficial do óleo, facilitando o acesso a um maior número de bactérias e a consequente biodegradação (SANTOS et al., 2016).

Numerosos exemplos já demonstraram o potencial de aplicação de biossurfactantes na descontaminação ambiental. Sobrinho et al. (2008) testaram um biossurfactante produzido por *Candida sphaerica* para a remoção de óleo de motor de solo e de água do mar e encontraram taxas de remoção de 75% e de 92% de óleo em solo argiloso e siltoso, respectivamente; Em testes realizados com água do mar, o biossurfactante apresentou uma eficiência na dispersão de óleo de 75%, demonstrando seu potencial de aplicação como coadjuvante em processos biotecnológicos de descontaminação ambiental. Batista et al. (2010) investigaram a aplicação de um biossurfactante produzido por *Candida tropicalis* para remoção de óleo de motor em areia e encontraram taxas de remoção de 78% a 97%, demonstrando potencial considerável em relação à biorremediação de solo. Gusmão et al. (2010) investigaram a aplicação de um biossurfactante bruto produzido por *Candida glabrata* UCP1002 em um sistema solo-água-contaminante hidrofóbico e encontraram uma taxa de remoção de até 92,6%. Luna et al. (2011) avaliaram um novo biossurfactante, denominado Lunasan, produzido por *Candida sphaerica* UCP 0995. Este biossurfactante removeu 95% do óleo de motor adsorvido em areia, demonstrando potencial considerável para uso em processos de biorremediação. A Tabela 7 oferece uma lista de diferentes biossurfactantes e seus micro-organismos produtores com potencial de aplicação na biorremediação de ambientes poluídos por petróleo.

Tabela 7 – Biossurfactantes, micro-organismos produtores e suas aplicações na biorremediação de ambientes contaminados com óleo

Micro-organismos	Tipo de biossurfactante	Aplicações
Rhodococcus erythropolis 3C-9	Glicolipídio e trealose	Operações de limpeza de derramamento de óleo
Pseudomonas aeruginosa S2	Raminolipídeo	Biorremediação de locais contaminados com óleo
Rhodococcus sp. TW53	Lipopeptídeo	Biorremediação da poluição marinha por óleo.
R. wratislaviensis BN38	Glicolipídio	Aplicações de biorremediação
Bacillus subtilis BS5	Lipopeptídeo	Biorremediação de locais contaminados por hidrocarbonetos
Azotobacter chroococcum	Lipopeptídeo	Aplicações ambientais
Pseudomonas aeruginosa BS20	Raminolipídeo	Biorremediação de locais contaminados por hidrocarbonetos
Micrococcus luteus BN56	Tetraéster de trealose	Biorremediação de ambientes contaminados com óleo

Nocardiopsis alba MSA10	Lipopeptídeo	Biorremediação
Pseudoxanthomonas sp. PNK-04	Raminolipídeo	Aplicações ambientais
Pseudomonas alcaligenes	Raminolipídeo	Aplicações ambientais
Nocardiopsis lucentensis MSA04	Glicolipídio	Biorremediação de ambientes marinhos
Calyptogena soyoae	Lipídio de manosileritritol	Biorremediação de ambientes marinhos
Pseudozyma hubeiensis	Glicolipídio	Biorremediação da poluição marinha por óleo
Pseudomonas cepacia CCT6659	Raminolipídeo	Biorremediação de ambientes marinhos e do solo
Candida bombicola	Soforolipídeos	Aplicações ambientais
C. glabrata UCP1002	Complexo proteico-carboidrato-lipídio	Recuperação de óleo em areia
C. lipolytica UCP0988	Soforolipídeos	Recuperação de óleo
C. lipolytica UCP0988	Soforolipideos	Remoção de óleo
C. sphaerica UCP0995	Complexo proteico-carboidrato-lipídio	Remoção de óleo em areia
C. lipolytica UCP0988	Soforolipídeos	Controle da poluição ambiental por óleo
C. sphaerica UCP0995	Complexo proteico-carboidrato-lipídio	Processos de biorremediação
C. glabrata UCP1002	Complexo proteico-carboidrato-lipídio	Remoção de óleo
C. guilliermondii UCP0992	Complexo de glicolipídio	Remoção de óleo de motor em areia
C. tropicalis UCP0996	Complexo proteico-carboidrato-lipídio	Remoção de petróleo e óleo de motor adsorvido em areia
C. lipolytica UCP0988	Soforolipídeos	Remoção de petróleo e óleo de motor adsorvido em areia
C. sphaerica UCP0995	Complexo proteico-carboidrato-lipídio	Remoção de óleo

FONTE: Adaptado de SILVA et al. (2014).

3.8.1. *Toxicidade de biossurfactantes sobre organismos no processo de biorremediação*

A toxicidade de biossurfactantes no ambiente não é bem conhecida. Edwards et al. (2003), em uma comparação da toxicidade de três surfactantes sintéticos e de três biossurfactantes, concluíram que os biossurfactantes foram menos tóxicos do que os surfactantes sintéticos para algumas espécies de invertebrados. No entanto, os riscos ambientais decorrentes da utilização de biossurfactantes, avaliados através da análise de composição da comunidade microbiana, não foram ainda suficientemente estudados (FRANZETTI et al., 2006). A menor toxicidade e maior biodegradabilidade dos biossurfactantes em comparação com os seus homólogos químicos (os quais podem apresentar riscos para os organismos aquáticos,

individualmente e, quando misturado com óleo (BERNINGER et al., 2011; RICO-MARTÍNEZ et al., 2012) é a principal razão para a sua elevada aceitabilidade. No entanto, essas características são assumidas muitas vezes como a única consequência direta da sua origem natural (FRANZETTI et al., 2012).

Vários biossurfactantes não são tóxicos para os micro-organismos do ambiente em concentrações perto dos seus valores de CMC, muito embora, outros possam apresentar toxicidade à microbiota nativa, provocando uma possível inibição da biorremediação (VAN HAMME; WARD, 1999). Além disso, uma taxa reduzida de biorremediação na presença de biossurfactantes pode ser obtida pelo aumento da toxicidade decorrente do contaminante hidrofóbico, devido à sua maior pseudo solubilidade. Alguns biossurfactantes ou contaminantes pseudo solubilizados podem apresentar uma toxicidade seletiva para algumas linhagens puras de micro-organismos, mas pode ter um impacto inibidor limitado em um sistema envolvendo uma população microbiana autóctone diversificada (SINGH et al., 2007). Por estas razões, as características ambientais de novos biossurfactantes devem ser cuidadosamente consideradas e investigadas antes de sua aplicação no meio ambiente (FRANZETTI et al., 2012; SILVA et al. 2014).

4. REFERÊNCIAS

AL-BAHRY, S.N. et al. Biosurfactant production by *Bacillus subtilis* B20 using date molasses and its possible application in enhanced oil recovery. **International Biodeterioration & Biodegradation**, v. 81, p. 141–146, 2013.

ALMEIDA, D.G. et al. Biodegradation of marine fuel MF-380 by microbial consortium isolated from seawater near the petrochemical Suape Port, Brazil. **International Biodeterioration & Biodegradation**, v.116, p. 73–82, 2017.

ALMEIDA, D.G. et al. Biosurfactants: Promising Molecules for Petroleum Biotechnology Advances. **Frontiers in Microbiology**, v. 7, p. 1718, 2016.

AMARAL, P.F.; et al. Biosurfactants from yeasts: characteristics, production and application. **Advances in Experimental Medicine and Biology**, v. 672, p. 236–249, 2010.

APARNA, A.; SRINIKETHAN, G.; HEDGE, S. Effect of addition of biosurfactant produced by *Pseudomonas* ssp. on biodegradation of crude oil. In: INTERNATIONAL PROCEEDINGS OF CHEMICAL, BIOLOGICAL & ENVIRONMENTAL ENGINEERING. 2011, *Proceedings of the 2nd International Proceedings of Chemical,* Singapore: p. 71–75.

ASIMIEA, O.A. et al. The impact of hydrocarbon waste from brass oil terminal on the phytoplancton and periphyton communities of lower Brass river, Niger delta, Nigeria. **Journal of Emerging Trends in Engineering and Applied Sciences,** v. 2, p. 729–733, 2011.

BANAT, I.M.; DE RIENZO, M.A.D.; QUINN, G.A. Microbial biofilms: Biosurfactants as antibiofilm agents. **Applied Microbiology and Biotechnology**, v. 98, p. 9915–9929, 2014.

BATISTA, R.M. et al. Effect of medium components on the production of a biosurfactant from *Candida tropicalis* applied to the removal of hydrophobic contaminants in soil. **Water Environment Research,** v. 82, p. 418–425, 2010.

BENINCASA M. et al. Chemical structure, surface properties and biological activities of the biosurfactant produced by *Pseudomonas aeruginosa* LBI from soapstock.

Antonie van Leeuwenhoek, v. 85, p. 1–8, 2004.

BERNINGER, J.P.; WILLIAMS, E.S.; BROOKS, B.W. An initial probabilistic hazard assessment of oil dispersants approved by the United States National Contingency Plan. **Environmental Toxicology and Chemistry**, v. 30, p. 1704–1708, 2011.

BEZZA, F.A. et al. Production and applications of lipopeptide biosurfactant for bioremediation and oil recovery by *Bacillus subtilis* CN2. **Biochemical Engineering Journal**, v. 101, p. 168–178, 2015.

CAMPOS, J.M. et al. Microbial biosurfactants as additives for food industries. **Biotechnology Progress**, v. 29, p. 1097–1108, 2013.

CAMPOS, J.M. et al. Formulation of mayonnaise with the addition of a bioemulsifier isolated from *Candida utilis*. **Toxicology Reports,** 2, 1164–1170, 2015.

CHANDRAN, P.; DAS, N. Role of sophorolipid biosurfactant in degradation of diesel oil by *Candida tropicalis*. **Bioremediation Journal**, v. 16, p. 19–30, 2012.

CHANDRAN, P.; DAS, N. Degradation of diesel oil by immobilized *Candida tropicalis* and biofilm formed on gravels. **Biodegradation,** v. 22, p. 1181–1189, 2011.

DAS, N.; CHANDRAN, P. Microbial degradation of petroleum hydrocarbon contaminants: an overview. **Biotechnology Research International**, v. 2011, p. 1–13, 2011.

DZIEGIELEWSKA, E.; ADAMCZAK, M. Evaluation of wast products in the synthesis of surfactants by yeasts. **Chemical Papers,** v. 67, p. 1113–1122, 2013.

EDWARDS, K.R.; LEPO, J.E.; LEWIS, M.A. Toxicity comparison of biosurfactants and synthetic surfactants used in oil spill remediation to two estuarine species. **Marine Pollution Bulletin**, v. 46, p. 1309–1316, 2003.

EZEJIOFOR T.I.N., ENEBAKU, U.E.; OGUEKE, C. Waste to wealth- value recovery from agrofood processing wastes using biotechnology: a review. **British Biotechnology Journal**, v. 4, n.4, p. 418-481, 2014.

FARAG, S.; SOLIMAN, N.A. Biodegradation of crude petroleum oil and

environmental pollutants by *Candida tropicalis* strain. **Brazilian Archives of Biology and Technology**, v. 54, p. 821–830, 2011.

FILHO, G.A. et al. **Biotecnologia e (Bio) Negócio no Amazonas**. Manaus: EDUA. p. 224, 2015.

FONSECA, M.G.D. **Biotecnologia no Brasil: uma avaliação do seu potencial empresarial e industrial**. Brasília: SENAI.DN, p. 77, 2010.

FRANZETTI, A. et al. Environmental features of two commercial surfactants widely used in soil remediation. **Chemosphere**, v. 62, p. 1474–1480, 2006.

FRANZETTI, A. et al. Environmental fate, toxicity, characteristics and potential applications of novel bioemulsifiers produced by *Variovorax paradoxus* 7bCT5. **Bioresource Technology**, v.108, p. 245–251, 2012.

FREITAS, B.G. et al. Formulation of a commercial biosurfactant for application as a dispersant of petroleum and by-products spilled in oceans. **Frontiers in Microbiology**, v.7, p.1646, 2016.

GAO, R. et al. Production of sophorolipids with enhanced volumetric productivity by means of high cell density fermentation. **Applied Microbiology and Biotechnology,** v. 97, p. 1103–1111, 2013.

GUDIÑA, E.J. et al. Bioconversion of agro-industrial by-products in rhamnolipids toward applications in enhanced oil recovery and bioremediation. **Bioresource Technology**, v. 177, p. 87–93, 2015.

GUSMÃO, C.A.B.; RUFINO, R.D.; SARUBBO, L.A. Laboratory production and characterization of a new biosurfactant from *Candida glabrata* UCP 1002 cultivated in vegetable fat waste applied to the removal of hydrophobic contaminant. **World Journal of Microbiology and Biotechnology**. v. 26, p. 1683–1692, 2010.

HABA, E. et al. Screening and production of rhamnolipids *Pseudomonas aeruginosa* 47T2 NCIB 40044 from waste frying oils. **Journal of Applied Microbiology,** v. 88, p. 379–387, 2000.

HARITASH, A.K.; KAUSHIK, C.P. Biodegradation aspects of polycyclic aromatic

hydrocarbons (PAHs): A review. **Journal of Hazardous Materials**, v. 169, p. 1–15, 2009.

HELMY, Q. et al. Strategies toward commercial scale of biosurfactant production as potential substitute for it's chemically counterparts. **International Journal of Biotechnology**, v. 12, p. 66–86, 2011.

HUA, Z. et al. Influence of biosurfactants produced by *Candida antarctica* on surface properties of microorganism and biodegradation of n-alkanes. **Water Research**, v. 37, p. 4143–4150, 2003.

KAPADIA, S.G.; YAGNIK, B.N. Current trend and potential for microbial biosurfactants. **Asian Journal of Experimental Biological Sciences**, v. 4, 1–8, 2013.

KIM, H. et al. Extracellular production of a glycolipid biosurfactant, mannosylerythritol lipid, from *Candida antarctica*. **Biotechnology Letters**, v. 24, p. 225–229.

ŁAWNICZAK, L.; MARECIK, R.; CHRZANOWSKI, L. Contributions of biosurfactants to natural or induced bioremediation. **Applied Microbiology and Biotechnology**, v. 97, p. 2327–2339, 2013.

LIN, C.W. et al. Microbial communities and biodegradation in lab-scale BTEX-contaminated groundwater remediation using an oxygen-releasing reactive barrier. **Bioprocess and Biosystems Engineering,** v. 33, p. 383–391, 2010.

LIU, J. et al. Chemical structure, property and potential applications of biosurfactants produced by *Bacillus subtilis* in petroleum recovery and spill mitigation. **International Journal of Molecular Sciences**, v. 16, n. 3, p. 4814–4837, 2015.

LUNA, J.M. et al. Environmental applications of the biosurfactant produced by *Candida sphaerica* cultivated in low-cost substrates. **Colloids and Surfaces A: Physicochemical and Engineering**, v. 480, p. 413–418, 2015.

LUNA, J.M., et al. Production of a biosurfactant from Candida bombicola URM 3718 for environmental applications. **Chemical Engineering Transactions**, v. 49, p. 583–588, 2016.

LUNA, J.M.; SARUBBO, L.A.; CAMPOS-TAKAKI, G.M. A new biosurfactant produced by *Candida glabrata* UCP 1002: characteristics of stability and application in oil recovery. **Brazilian Archives of Biology and Technology**, v. 49, p. 785–793, 2009.

LUNA, J.M. et al. Characterisation, surface properties and biological activity of a biosurfactant produced from industrial waste by *Candida sphaerica* UCP0995 for application in the petroleum industry. **Colloids and Surfaces B: Biointerfaces,** v. 102, p. 202–209, 2013.

LUNA, J.M. et al. Evaluation antimicrobial and antiadhesive properties of the biosurfactant lunasan produced by *Candida sphaerica* UCP 0995. **Current Microbiology,** v. 62, p. 1527–1534, 2011.

MAKKAR, R.S. et al. Advances in utilization of renewable substrates for biosurfactant production. **Applied Microbiology and Biotechnology,** v. 1, p. 1–5, 2011.

MALIK, Z.A.; AHMED, S. Degradation of petroleum hydrocarbons by oil field isolated bacterial consortium. **African Journal of Biotechnology,** v. 11, p. 650–658, 2012.

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

MATVYEYEVA, O.L.; VASYLCHENKO, O.A.; ALIIEVA, O.R. Microbial biosurfactants role in oil products biodegradation. **International Journal of Environmental Bioremediation & Biodegradation**, v. 2, p. 69–74, 2014.

MHATRE, B.A.; KUNDE, R. Biodegradation of diesel using microbes from a clam (Meretrixmeretrix) shell. **Indian Journal of Geo-Marine Sciences,** v. 43, p. 877–881, 2014.

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

MÜLLER, M.M. et al. Rhamnolipids—nextgenerationsurfactants? **Journal of Biotechnology**, v. 162, p. 366–380, 2012.

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

OKOLIEGBE, I.N.; AGARRY, O.O. Application of microbial surfactant (a review). **Scholarly Journals of Biotechnology,** v.1, p. 15–23, 2012.

OLKOWSKA, E.; POLKOWSKA, Z.; NAMIÉSNIK, J. Analytical procedures for the determination of surfactants in environmental samples. **Talanta**, v. 88, p. 1–13, 2012.

PACWA–PLOCINICZAK, M.; et al. Environmental applications of biosurfactants: Recent advances. **International Journal of Molecular Sciences,** v. 13, p. 633–654, 2011.

PANIAGUA-MICHEL, J.; ROSALES, A. Marine bioremediation - a sustainable biotechnology of petroleum hydrocarbons biodegradation in coastal and marine environments. **Journal of Bioremediation & Biodegradation,** v. 6, p. 2, 2015.

PI, G. et al. Preparation of oil-in-seawater emulsions based on environmentally benign nanoparticles and biosurfactant for oil spill remediation. **ACS Sustainable Chemistry & Engineering,** v. 3 p. 2686–2693, 2015.

PRIJI, P. et al. *Candida tropicalis* BPU1, a novel isolate from the rumen of the Malabari goat, is a dual producer of biosurfactant and polyhydroxybutyrate. **Yeast,** v. 30, p. 103–110, 2013.

QUADROS, C.P.; DUARTE, M.C.T.; PASTORE, G.M. Biological activities of a mixture of biosurfactant from *Bacillus subtilis* and alkaline lipase from *Fusarium oxysporum*. **Brazilian Journal of Microbiology**, v. 42, n. 1, p. 354–361, 2011.

RANDHAWA, K.K.S.; RAHMAN, P.K.S.M. Rhamnolipid biosurfactants — past, present, and future scenario of global market. **Frontiers in Microbiology**, v. 5, p. 454, 2014.

REIS, R.S. et al. Biosurfactants: production and applications. In: CHAMY, R.; ROSENKRANZ, F. (Eds.). **Biodegradation—Life of Science**. Rijeka: InTech, p. 31–61, 2013.

RICO-MARTÍNEZ, R. et al. Synergistic toxicity of Macondo crude oil and dispersant Corexit 9500A ® to the *Brachionus plicatilis* species complex (Rotifera). **Environmental Pollution,** v. 173, p. 5-10, 2012.

ROCHA e SILVA, N.M.P. et al. Screening of *Pseudomonas* species for biosurfactant production using low-cost substrates. **Biocatalysis and Agricultural Biotechnology,** v. 3, p. 132–139, 2013.

ROELANTS, S.L. et al. *Candida bombicola* as a platform organism for the production of tailor-made biomolecules. **Biotechnology and Bioengineering**, v. 110, p. 2494–503, 2013.

RUFINO, R.D. et al. Characterization and properties of the biosurfactant produced by *Candida lipolytica* UCP 0988. **Electronic Journal of Biotechnology**, v.17, p. 34–38, 2014.

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

SANTOS, D.K.F. et al. Synthesis and evaluation of biosurfactant produced by *Candida lipolytica* using animal fat and corn steep liquor. **Journal of Petroleum Science and Engineering**, v. 105, p. 43–50, 2013.

SANTOS, D.K.F. et al. Biosurfactants: multifunctional biomolecules of the 21st century. **International Journal of Molecular Sciences,** v.17, p. 401, 2016.

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

SARUBBO, L.A. et al. Some aspects of heavy metals contamination remediation and role of biosurfactants. **Journal of Chemical Ecology**, v. 31, 2015.

SATPUTE, S.K. et al. Biosurfactants, bioemulsifiers and exopolysaccharides from marine microorganisms. **Biotechnology Advances,** v. 28, p. 436–450, 2010.

SEKHON, K.K.; KHANNA, S.; CAMEOTRA, S.S. Biosurfactant production and potential correlation with Esterase activity. **Journal Petroleum & Environmental**

Biotechnology, v.3, p. 7, 2012.

SHARMA, D.; SAHARAN, B.S.; KAPIL, S. Biosurfactants of lactic acid bacteria. In: SHARMA, D.; SAHARAN, B.S.; KAPIL, S. (Eds.). **SpringerBriefs in Microbiology**. New York, NY: Springer, 2016.

SHETE, A.M.; et al. Mapping of patents on bioemulsifier and biosurfactant: a review. **Journal of Scientific & Industrial Research**, v. 65, p. 91–115, 2006.

SILVA, D.S.P., et al. Bio-removal of diesel oil through a microbial consortium isolated from a polluted environment. **International Biodeterioration & Biodegradation**, v. 97, p. 85 – 89, 2015.

SILVA, R.C.F.S. et al. Applications of biosurfactants in the petroleum industry and the remediation of oil spills. **International Journal of Molecular Sciences,** v. 15, p. 12523–12542, 2014.

SINGH, A. et al. Potential applications of bioprocess technology in petroleum industry. **Biodegradation,** v. 23, p. 865–880, 2012.

SITOHY, M.Z. et al. Bioconversion of soy processing waste for production of surfactants. **African Journal of Microbiology Research**, v. 4, p. 2811–2821, 2010.

SOBRINHO, H.B.S. et al. Application of biosurfactant from *Candida sphaerica* UCP 0995 in removal of petroleum derivative from soil and sea water. **Life Sciences**, v.7, 559–569, 2013a.

SOBRINHO, H.B. et al. Biosurfactants: Classification, Properties and Environmental Applications. In: **Recent Developments in Biotechnology.** Texas, USA: Studium Press LLC, v. 11, p. 1–29, 2013b.

SOBRINHO, H.B.S. et al. Utilization of two agroindustrial by-products for the production of a surfactant by *Candida sphaerica* UCP0995. **Process Biochemistry,** v. 43, p. 912–917, 2008.

SOUZA, E.C. et al. Biosurfactant-enhanced hydrocarbon bioremediation: An overview. **International Biodeterioration & Biodegradation,** v. 89, p. 88– 94, 2014.

TOKUMOTO, Y. et al. Structural characterization and surface-active properties of a succinoyl trehalose lipid produce by *Rhodococcus* sp. SD-74. **Journal of Oleo Science,** v. 58, p. 97–102, 2009.

TORIBIO, J.; ESCALANTE, A.E.; SOBERÓN-CHÁVEZ, G. Rhamnolipids: Production in bacteria other than *Pseudomonas aeruginosa*. **European Journal of Lipid Science and Technology**, v. 112, p. 1082–1087, 2010.

VAN HAMME, J.D.; Ward, O.P. Influence of chemical surfactants on the biodegradation of crude oil by a mixed-bacterial culture. **Canadian Journal of Microbiology,** v. 45, p. 130–137, 1999.

VERMA, A. et al. Multifactorial approach to biosurfactant production by adaptive strain *Candida tropicalis* MTCC 230 in the presence of hydrocarbons. **Journal of Surfactants and Detergents**, v. 18, p. 145–153, 2015.

VIJAYAKUMAR, S.; SARAVANAN, V. Biosurfactants-types, sources and applications. **Research Journal of Microbiology**, v. 10, p. 181–192, 2015.

WEI, Y.H. et al. Biosurfactant production by *Serratia marcescens* SS-1 and its isogenic strain SMΔR defective in SpnR, a quorum-sensing LuxR family protein. **Biotechnology Letters,** v. 26, p. 799–802, 2004.

5. ARTIGOS DERIVADOS DA TESE

5.1. CAPÍTULO 1

Biosurfactants: Promising Molecules for Petroleum Biotechnology Advances

Artigo de revisão publicado na revista *Frontiers in Microbiology*.

frontiers in Microbiology

REVIEW
published: 31 October 2016
doi: 10.3389/fmicb.2016.01718

Biosurfactants: Promising Molecules for Petroleum Biotechnology Advances

Darne G. De Almeida[1,2], *Rita de Cássia F. Soares Da Silva*[1,2], *Juliana M. Luna*[2,3], *Raquel D. Rufino*[2,3], *Valdemir A. Santos*[2,3], *Ibrahim M. Banat*[2,4] and *Leonie A. Sarubbo*[1,2,3]*

[1] Northeast Biotechnology Network (RENORBIO), Federal Rural University of Pernambuco, Recife, Brazil, [2] Advanced Institute of Technology and Innovation, Recife, Brazil, [3] Center of Sciences and Technology, Catholic University of Pernambuco (UNICAP), Recife, Brazil, [4] Faculty of Life and Health Sciences, School of Biomedical Sciences, University of Ulster, Ulster, UK

OPEN ACCESS

Edited by:
Randhir Makkar, Guild Biosceinces, USA

Reviewed by:
Jun-Jie Zhang, Indiana University School of Medicine, USA
Kamaljeet K. Sekhon Randhawa, Aalborg University Copenhagen, Denmark
Surekha K. Satpute, Savitribai Phule Pune University, India

***Correspondence:**
Leonie A. Sarubbo
leonie@unicap.br

Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: *11 September 2016*
Accepted: *13 October 2016*
Published: *31 October 2016*

Citation:
De Almeida DG, Soares Da Silva RCF, Luna JM, Rufino RD, Santos VA, Banat IM and Sarubbo LA (2016) Biosurfactants: Promising Molecules for Petroleum Biotechnology Advances. Front. Microbiol. 7:1718. doi: 10.3389/fmicb.2016.01718

The growing global demand for sustainable technologies that improves the efficiency of petrochemical processes in the oil industry has driven advances in petroleum biotechnology in recent years. Petroleum industry uses substantial amounts of petrochemical-based synthetic surfactants in its activities as mobilizing agents to increase the availability or recovery of hydrocarbons as well as many other applications related to extraction, treatment, cleaning, and transportation. However, biosurfactants have several potential applications for use across the oil processing chain and in the formulations of petrochemical products such as emulsifying/demulsifying agents, anticorrosive, biocides for sulfate-reducing bacteria, fuel formulation, extraction of bitumen from tar sands, and many other innovative applications. Due to their versatility and proven efficiency, biosurfactants are often presented as valuable versatile tools that can transform and modernize petroleum biotechnology in an attempt to provide a true picture of state of the art and directions or use in the oil industry. We believe that biosurfactants are going to have a significant role in many future applications in the oil industries and in this review therefore, we highlight recent important relevant applications, patents disclosures and potential future applications for biosurfactants in petroleum and related industries.

Keywords: biosurfactants, petroleum biotechnology, emulsified fuels, enhanced oil recovery, bitumen, sulfate reducing bacteria

INTRODUCTION

Petroleum is the most important energy resource and raw material for the chemical industry and has driven the development of the modern world and global intensive economic development for the past century (Okoliegbe and Agarry, 2012; Silva et al., 2014). We depend on it for our basic needs for heat, light and transportation. Prediction of the world energy demand indicates a 1.7% annual increase in the number of oil barrels produced annually between the years 2000 to 2030, while oil consumption is expected to reach 15.3 billion tons annually. If current levels of world consumption are maintained the oil reserves available can allow meeting these demand for approximately 40 years (Elraies and Tan, 2012; Silva et al., 2014).

There is no an energy source available at present that could meet or compete with oil, making the largest energy consumers dependent on countries with large oil reserves (Elraies and Tan, 2012). The US Department of Energy for example, reported that the majority (≈83.0%) of primary energy sources within the US are fossil fuels derived, of which 57.0% are from petroleum products. In 2010 19.2 million cubic meters of petroleum were consumed on daily basis (Santos et al., 2016).

The USA produces 870,000 m^3 of crude oil on daily basis from 530,000 production-wells, the majority of which produce ≤1.59 m^3, therefore high quality easily extractable light crude oils are limited and poses two major issues: first, efficiency and maximization of the overall stages of processing and secondly, the ability to utilize the heavy crude oils, bitumen and tar-sand components (Santos et al., 2016). On the whole petroleum production has been steadily moving toward the extraction of heavy/extra-heavy oils rather than medium to light oils, according to the International Energy Agency. In countries such as China, Canada, Venezuela, Mexico, and the USA; the heavy crude oils represent approximately half of recoverable oil resources. The development of efficient uses for this resource therefore is fast becoming an important technology (Cerón-Camacho et al., 2013).

Petroleum biotechnology has become an emerging technology that aims to implement biological processes to explore, produce, transform, and refine petroleum to generate valuable by-products and to reduce, manage and clean any pollution output and to treat petroleum industrial effluents (Silva et al., 2014). The versatility of microbes and microbial metabolism and their intrinsic ability to mediate transformation of complex raw materials at a wide range under extreme conditions such as high salinity, temperature, pH values, pressure, and hydrophobicity, facilitates the development of these technologies (Montiel et al., 2009). Among the emerging biotechnologies with application prospects in the oil industry, those using biosurfactants have stood out promisingly (Silva et al., 2014).

Biosurfactants are expected to become known as multifunctional materials of the twenty first century as they have applications in different industrial processes as well as potential novel future uses (Marchant and Banat, 2012) mostly due to their diverse structures. Microorganisms produce surface active compounds to enhance both the bioavailability of hydrophobic immiscible and mostly inaccessible substrates allowing better survival under low moisture conditions. Biosurfactant production generally requires the presence of miscible hydrophilic and oily/hydrocarbon type carbon source in the culture medium. The process economics and environmental credentials can make it attractive when using waste products as substrates (Makkar et al., 2011; Dziegielewska and Adamczak, 2013). Currently, the major emerging market for biosurfactants has been the petroleum related industries to allow effective exploration of heavy oil, offering advantages over chemical surfactants in processes involving extraction, transportation, storage and refining. Biosurfactants have also been successfully used in cleaning of oil sludge in storage tanks, microbial-enhanced oil recovery and to facilitate better transportation of heavy crude oil though pipeline (Assadi and Tabatabaee, 2010; Luna et al., 2012; Sobrinho et al., 2013).

This review discuss biosurfactants potential roles and applications within the petroleum industry. Roles in processes of petroleum exploration, treatment, transport, and remediation as well as patents disclosures related to biosurfactants application by petroleum industry and related market trends and future potentials are all described in details.

PETROLEUM BIOTECHNOLOGY

Petroleum is believed to have originated from the organic matter of microorganisms and algae that form the plankton deposited over millennia, which did not undergo oxidation process and accumulated in the bottom of the primitive oceans and was covered by sediment. The interaction between the organic matter, sediments and appropriate thermochemical conditions was fundamental to the beginning of the chain of processes which led to the formation of petroleum (Thomas, 2004). Crude oil usually consists of two or three different components/phases (namely gas, liquid and solid). The petroleum industry uses several separation mechanisms to separate these from one another (Holmager, 2010).

Exploration includes prospecting, seismic and drilling activities (Devold, 2013). Primary recovery mainly uses the reservoir's natural innate energy to displace oil from the porous rocks (Elraies and Tan, 2012) while conventional secondary recovery method involves water and/or gas injections to increase oil displacement, mobility and productivity of the oil well. A significant proportion of crude oil (>50%) however are often unrecoverable by conventional oil recovery methods and remains trapped in reservoirs (Bachmann et al., 2014). Ways to further increasing oil production are often carried out through tertiary enhanced oil recovery (EOR) methods which may result in recovering significant additional portions of the oil remaining after conventional methods (Elraies and Tan, 2012). Petroleum refining, on the other hand, are traditionally based on the use of physicochemical processes including chemical catalysis and distillation that operates under high pressures and temperatures where the crude oil and condensate are processed into a multitude of marketable products with defined specifications, such as gasoline, diesel fuel or raw material for the petrochemical industry (Singh et al., 2012; Devold, 2013).

Biotechnology has played a significant role in enhancing crude oil recovery from depleted oil reservoirs and as a tool to increase stagnant petroleum production as well as in the refining and processing and further managing environmentally safe pollutant remediation and disposal practices (Sen, 2008; Singh et al., 2012). The use of bioprocess in this industry has expanded to the application of technologies related to biodesulfurization, biodemetallation, biodenitrogenation, and biotransformation and into crude oil refining associated with upgrading of fuels, production of fine chemicals, reduction of souring during production, complementing techniques such as microbial enhanced oil recovery (MEOR) and bioremediation (**Figure 1**; Singh et al., 2012; Bachmann et al., 2014). Among the

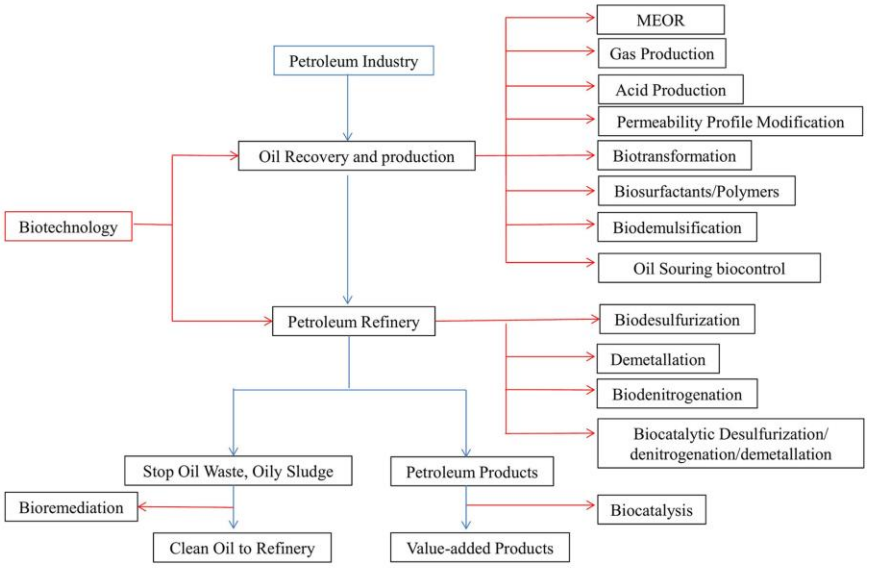


FIGURE 1 | Potential applications of biotechnology in petroleum industry. Blue lines represent the major petroleum processing steps, and the red lines represent the biotechnological applications in the respective steps.

biotechnologies proposed above, those that apply biosurfactants have been the most promising and have received the greatest attention, since biosurfactants' applications can find space in almost all stages of the oil production chain (Silva et al., 2014).

BIOSURFACTANT CHARACTERISTICS CONDUCIVE TO USE IN PETROLEUM INDUSTRY

Surfactants are amphipathic compounds with both hydrophilic and hydrophobic moieties that preferentially partition at the interface between different phases; gas, liquid and solid, and with liquids of different polarities (oil/water and water/oil) and hydrogen bonding. These molecules reduces the surface and interfacial tension, conferring many properties such as detergency, emulsifying, foaming, and dispersing, making them versatile process chemicals (Joshi and Desai, 2010; Silva et al., 2014). Petroleum industry mostly employs petrochemical-based synthetic surfactants as mobilizing agents in their activities (Hazra et al., 2012; Silva et al., 2014). However, demands for sustainable technologies have driven the search for natural, environmental friendly and biodegradable compounds.

Biosurfactants are mainly produced by microbial cultures grown on water immiscible substrates, therefore allowing access to these hydrophobic substrates (such as hydrocarbons) and are generally classified into low molecular-mass molecules (lipopeptides, glycolipids) and high molecular-mass polymers (polymeric and particulate surfactants) (Kapadia and Yagnik, 2013). These molecules offer several advantages over chemical surfactants, such as environmental compatibility, low toxicity, biodegradability, and maintained activity under extreme conditions of temperatures, salinity and pH values (Kapadia and Yagnik, 2013; Santos et al., 2013; Silva et al., 2014). These traits contribute to the relevance of biosurfactants to different industries, especially in the oil industry which has many adverse processes conditions (Silva et al., 2014). Most successful biosurfactants applications that managed to reach the market has been mainly driven by economical production process and cost effectiveness (Banat et al., 2010). This has been facilitated by the lower purity specifications required for such applications, eliminating the purification downstream processing steps which often represent almost 60.0% of the total production costs (Sarubbo et al., 2015). High production cost of biosurfactants has been a major constraining factor that hampers its market growth. Substrate composition accounts for up to 50.0% of the total production costs, the choice of low-cost alternatives therefore is important to the overall economics. Fortunately, biosurfactants can be produced from economical renewable agricultural resources and waste products that can significantly decrease the cost (Helmy et al., 2011; Rufino et al., 2014).

Among the main companies in the global biosurfactants market are Jeneil Biotech, Ecover, Soliance, Saraya, MG Intobio and AGAE Technologies with potential targeted markets

TABLE 1 | Producing companies, types of biosurfactant and potential applications marketed for petroleum industry.

Company	Biosurfactant	Applications
AGAE Technologies—USA	Rhamnolipids (R95, anHPLC/MS grade rhamnolipid)	Enhanced oil recovery (EOR)
Jeneil Biosurfactant—USA	Rhamnolipids	EOR
Rhamnolipid Companies—USA	Rhamnolipids	EOR
Synthezyme—USA	Sophorolipids	Crude oil emulsification
BioFuture—Ireland	Rhamnolipid	Washing fuel oil tanks
Logos Technologies—USA	Rhamnolipids	EOR
TensioGreen—USA	Rhamnolipids	Petroleum Industry, EOR
Synthezyme—USA	Sophorolipids	Oil and gas
EcoChem Organics Company—Canada	Rhamnolipids-based	Water-insoluble hydrocarbons dispersive agent
EnzymeTechnologies—USA	Bacteria biosurfactant, (unknown)	Oil removal; oil recovery and processing, EOR

covering North America, Europe and Asia-Pacifc (Sajna et al., 2015). The most successful efforts to bring biosurfactant into industrial scale were carried out by Jeneil Biosurfactant Co. (Saukville, Wisconsin) who has successfully developed a production process for rhamnolipids based biosurfactant with a capacity to carry out fermentation processes in batches up to 20,000 gallons (Rufino et al., 2014). **Table 1** summarizes commercial manufacturers of different types of biosurfactants and their potential uses in the petroleum industry.

Increased environmental awareness has been the main driver for the search for a replacement to chemical surfactants (Marchant and Banat, 2012). According to recent studies, the global market for these "green" alternatives to synthetic surfactants reached US $ 1735.5 million in 2011. In 2013 the total production was approximately 344 kilo tons. Projections for this market share are even more encouraging as it was estimated that by 2018, to reach a value up to US $ 2210.5 million, and in 2020, US $ 2308.8 million when the worldwide market will reach biosurfactants production about 462 kilo tons. The annual average growth rate is expected to reach 4.3% during 2014–2020 (Sekhon et al., 2012; Gudiña et al., 2015; Grand View Research, 2016).

Also according to the same study, Europe was the largest market of biosurfactants consumers with a consumption of 178.9 kilo tons in 2013, representing over 50% of global consumption. North America was the second largest consumer of biosurfactants in the same year, with a participation of more than a quarter. But the Asia-Pacific block had a relatively small market in 2013, but is forecast to gain significant participation over the next 6 years due to the presence of large industries in the region (Grand View Research, 2016).

Patents on Biosurfactants for Petroleum Industry

The vast structural diversity that characterize biosurfactants leading to a broad range of properties may explain why this group of molecules continues to intrigue scientific interest (Marchant and Banat, 2012; Ławniczak et al., 2013; Luna et al., 2013). This has led to a plethora of patent applications by interested companies and researchers. Several patents have been issued for biosurfactant production from a wide range of microorganisms including *Pseudomonas* spp., *Bacillus* spp., *Acinetobacter* spp. and *Candida* spp. covering many industrial applications (Sachdev and Cameotra, 2013). According to Müller et al. (2012), patents search using the European Patent Office for the terms "biosurfactant", "rhamnolipid", "sophorolipid" and "mannosylerythritol lipid" showed a strong increase in number starting from the year 2000. Data showed >250 patents were issued worldwide on biosurfactants and bioemulsifiers with 33% related to the use of petroleum, followed by 15% for cosmetics, 12% for use as antimicrobial agent and biomedical applications and 11% in uses related to bioremediation. Sophorolipids, surfactin, and rhamnolipids related patent represented 24, 13, and 12% of the total number of patents respectively this may however be an underestimate since many patents do not describe or specify the producing microorganism, referring to the general description of a selected biosurfactant (Shete et al., 2006; Reis et al., 2013; Randhawa and Rahman, 2014).

Patents filed in relation to the petroleum industry have been mainly related to uses linked to their properties including wetting, emulsification, phase separation, solubilization, foaming, de-emulsification, corrosion inhibition, and viscosity reduction of heavy crude oils. These patents outline methods and compositions to facilitate the combustion and transportation of highly viscous hydrocarbon-in-water emulsions and in particular, bioemulsifier-stabilized emulsions of hydrocarbon-in-water (Shete et al., 2006). Other patented applications includes using in separating hydrocarbon values from tar sands (Zajic and Gerson, 1981), crude oil recovery from reservoir by MEOR method (Sheehy, 1992), use as bioemulsifier to stabilize hydrocarbons (Hayes et al., 1988), cleaning of oil-contaminated tankers, transportation of heavy crude, recovery of oil from sludge of oil storage tanks (Bachmann et al., 2014) among many other applications. **Table 2** lists some of the important patents of bioemulsifiers and biosurfactants in the petroleum industry.

MAIN APPLICATIONS OF BIOSURFACTANTS IN THE PETROLEUM INDUSTRY

Biosurfactants have a wide range of biotechnological applications in the petroleum industry. All the operations including

TABLE 2 | Patents issued on the application of biosurfactants relevant to the petroleum industry.

Biosurfactants /Organisms	Title of Patent	Patent No.	Author and Year	Applications
Glycolipids	Method and installation for flooding petroleum wells and oil-sands	CA 1119794	Wagner et al., 1982	Recovery of oil from an oil well or oil sands
Biosurfactant-producing microorganisms mixtures	Enhanced oil recovery process using microorganisms	US 4450908	Hitzman, 1984	Enhanced oil recovery
Biosurfactant-producing endogenous microorganisms	Recovery of oil from oil reservoirs	US 5083610	Sheehy, 1992	Oil recovery
Injecting microbial nutrients to stimulate biosurfactant production	Nutrient injection method for subterranean microbial processes	US 5083611	Clark and Jenneman, 1992	Enhanced oil recovery (MEOR).
Lipopeptide	Biosurfactant and enhanced oil recovery	US 4522261	McInerney et al., 1985	Oil recovery
Mixture of microbes, enzymes, surfactants and chemicals.	System and process for in tank treatment of crude oil sludges to recover hydrocarbons and aid in materials separation	US 6033901	Powell, 2000a	Removing of crude oil sludge from oil tank
Treatment fluid containing biosurfactant	System and process for in tank treatment of crude oil sludges to recover hydrocarbons and aid in materials separation	US 6069002	Powell, 2000b	Recover of hydrocarbon
Any biosurfactant producer	Extraction of bitumen from bitumen froth and biotreatment of bitumen froth tailings generated from tar sands	CA 2350907	Duyvesteyn et al., 2000	Extraction and recovery of bitumen
Surface-active agents by exogenous microorganisms	Methods for improved hydrocarbon and water compatibility	US 7992639	Fallon, 2011	MEOR
Stimulation of bacteria with nutrients for production of surfactants	System and method for preparing near-surface heavy oil for extraction using microbial degradation	US 7922893	Busche et al., 2011	MEOR
Consortium including surfactant producer bacteria	Biological enhancement of hydrocarbon extraction	US 7472747	Brigmon and Berry, 2009	MEOR
Viscoelastic surfactants	Bacteria-based and enzyme-based mechanisms and products for viscosity reduction breaking of viscoelastic fluids	US7052901	Crews, 2006	MEOR
Microbial consortia	Process for stimulating microbial activity in a hydrocarbon-bearing, subterranean formation	US 6543535	Converse et al., 2003	MEOR

exploration and production of oil, refining, transportation, product handling, oil waste management, and responses dealing with accidental pollution or release incidents can be improved, optimized or augmented by the use of some kind of biosurfactant. **Table 3**, adapted from Silva et al. (2014), presents a list of biosurfactant applications in the four main activities carried out by oil industry.

The mechanism behind biosurfactant-enhanced removal and recovery of oil has been proposed to take place through solubilization, mobilization, or emulsification, increasing the area of contact of hydrocarbons (Joseph and Joseph, 2009; Santos et al., 2016). Solubilisation capacity measures a surfactant's ability to increase the solubility of hydrophobic components in an aqueous phase. A significant increase in this capacity occurs when micelles are formed as a result of the partitioning of the hydrocarbon in the hydrophobic part of the micelles. In such a process, higher concentrations of biosurfactants are usually required as hydrocarbon solubility wholly depends on the biosurfactant concentration. Mobilization on the other hand involves both displacement and dispersion. Displacement occurs when hydrocarbon droplets are released from the porous medium as a result of the reduction in interfacial tension. It can also occurs when entrapped hydrocarbon undergoes displacement when sufficient reduction of the interfacial tension between the aqueous and oil phases takes overcoming the capillary forces that cause the formation of residual saturation. Displacements therefore are only related to the interfacial tension between aqueous and hydrophobic phases and not emulsion formation. Dispersion in comparison is a process by which hydrocarbons are dispersed into aqueous phases due to emulsions formation and therefore is linked to both the surfactant concentration and interfacial tension (Sarubbo et al., 2015; Santos et al., 2016).

Biosurfactants for Extraction of Crude Oil

Oil production strategies traditionally consist of primary depletion followed by secondary recovery and in some cases tertiary recovery processes. In the primary recovery, the initial

TABLE 3 | Biosurfactants' applications within the main four petroleum production processes.

Step in petroleum production chain	Applications
Extraction	Reservoir wettability modification
	Oil viscosity reduction
	Drilling mud
	Paraffin/asphalt deposition control
	Enhanced oil displacement
Transportation	Oil viscosity reduction
	Oil emulsion stabilization
	Paraffin/asphalt deposition
Oil tank/container cleaning	Oil viscosity reduction
	Oily sludge emulsification
	Hydrocarbon dispersion
Oil waste treatment	Solubilization and mobilization oil

oil is extracted under natural pressure often only recovering 10–20% of the original oil in place (OOIP; Elraies and Tan, 2012; Bachmann et al., 2014). When oil yields fall due to natural pressure reductions in a reservoir's, secondary recovery technologies are used through either water and/or gas injection. Secondary recovery can lead to an increase of total recovery up to 40–50% of OOIP (Bachmann et al., 2014). Approximately half of the oil in the reservoir remains trapped in small pores of the rock formation. Poor displacement efficiency is attributed to the high forces of capillarity due to surface and interfacial forces, viscosity forces and reservoir heterogeneities (Elraies and Tan, 2012; Santos et al., 2016). Tertiary or enhanced oil recovery methods include chemical and or thermal treatment technologies. Thermal processes are the most common through steam, hot water or combustible gas injection to elevate the temperature of oil and gas in the reservoir facilitating their flow to the production wells. Chemical processes consists of injecting hydrocarbon solvents, surfactants, gas, or combinations thereof to mobilize the residual oil through lowering interfacial tension between oil and water (Elraies and Tan, 2012; Bachmann et al., 2014). This technology is however quite expensive as well as environmentally hazardous which led to the search for eco-friendly and cost-effective alternatives to both thermal and chemical EOR methods (Perfumo et al., 2010).

MEOR is the tertiary recovery of oil in which microbes or their metabolic products are used to enhance recovered residual oil. It usually is less-expensive when compared to chemically-enhanced oil recovery particularly when microorganisms are used to produce sufficient products such as polymers and biosurfactants starting with low-cost substrates raw materials (Sarafzadeh et al., 2014; Silva et al., 2014). Biosurfactants mainly improve hydrocarbon mobilization thereby enhancing crude oil recovery from reservoirs (Perfumo et al., 2010). There are three main strategies for biosurfactants use in MEOR as shown in **Figure 2** they include:

(1) Production *ex situ* in industrial setting using bioreactors (batch or continuous culture) followed by subsequent injection into the reservoir along with the water flood (otherwise known as *ex situ* MEOR) (Al-Bahry et al., 2013; Bachmann et al., 2014). Of course biosurfactant production is dependent on the medium composition under controlled setting which is also important for surface-active agent production by the exogenous mixed populations of microorganisms growing *in situ* or added in injection flood waters containing hydrophobic substrate. Excess of carbon/energy source promotes the production of surface-active agents (Fallon, 2011).

(2) Microbial augmentation through injecting biosurfactants producing microorganisms at the cell/oil interface within the reservoir formation. This introduces metabolically active cells into the reservoir to allow *in situ* spreading (Al-Bahry et al., 2013; Bachmann et al., 2014). These microbial cells would play a significant role in the surface interactions at interphases between oil and water where they usually prefer to be. It has been reported that at the oil/water interphase, the formed emulsions are proportional to the total biomass produced with increased quality of emulsion at higher quantity of biomass (Bachmann et al., 2014).

(3) Nutrients augmentation; injecting essential elements (with or without growth inhibitors for unwanted type of microbial strains) into the reservoir to stimulate the growth of desired indigenous microorganisms producing biosurfactant. Microbial population grows exponentially under favorable conditions producing metabolic products and gases to increase residual oil mobilization within the oil well (Al-Bahry et al., 2013; Bachmann et al., 2014).

All the above strategies increases petroleum yields from a depleted reservoir by decreasing oil-rock surface and interfacial tension and reducing the capillary forces which may impede oil movement through the rock pores. Biosurfactants also enhances the formation of stable water-oil emulsions and the breakdown of the oil film in the rocks which is important for a maximizing oil extraction ultimately extending the reservoir life time (Korenblum et al., 2012; Al-Bahry et al., 2013; Bachmann et al., 2014). The application of MEOR technology however has some disadvantages which includes increased corrosive action against nor resistant equipment due to the introduction of air deployed in aerobic MEOR or logistical problems encountered when high nutrients additives through down-hole piping. Limitations can also be encountered in providing positive pressure to maintain allochthonous microorganisms introduced in the field to produce biosurfactants to enhance oil recovery. Finally most published literature does not include reservoirs physiological and biochemical characteristics of the microflora controlling biological mechanisms nor does it include any details on process economics.

Biosurfactants Uses to Enhance Crude Oil Transportation through Pipelines

Crude oil is often transported in pipelines from the extraction fields to shipping ports or refineries over long distances. Such transportation particularly for heavy or extra-heavy crudes often represents operational challenges limiting its economic viability. High degree of viscosity due to high paraffins and

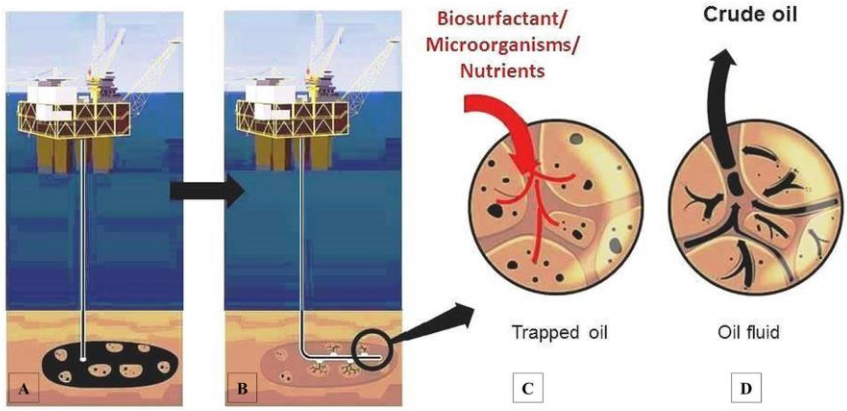


FIGURE 2 | Process of microbial recovery of crude oil using biosurfactant. (A) Oil extraction using natural pressure of the reservoir. **(B)** Oil well pressure decreased. **(C)** Main strategies of biosurfactant used to the oil release. **(D)** Oil well pressure restored facilitating oil extraction.

asphaltenes content in heavy crude oil can decrease its flow which often leads to sludge deposition on the inner walls leading to pressure reductions that ultimately can lead to pipeline plugging problems (Perfumo et al., 2010; Cerón-Camacho et al., 2013). Asphaltenes in particular precipitate in metal pipelines under acidic conditions and in the presence of ferric ions forming asphaltene mud which would deposit in the pipeline obstructing the flow of crude oil. The presence of paraffin in crude oil would decrease the fluidity of oil due to the high content in cyclic hydrocarbons that can solidify and deposit at room temperature, resulting in pipeline blockages in transportation (Assadi and Tabatabaee, 2010). Heating or diluting with solvents, such as xylene and toluene, are traditionally employed to reduce oil viscosity and dissolve any semisolid obstructions, this however of course increases the production cost and generates solvent containing toxic waste residue (Assadi and Tabatabaee, 2010; Mulligan et al., 2014).

A promising technology has been recently developed through the production of a stable oil-in-water emulsion using bioemulsifier biosurfactants to facilitate oil mobility. Such bioemulsifiers are high-molecular weight biosurfactants with different properties compared to low molecular weight glycolipids and lipopeptides. They have a great capacity to stabilize oil-in-water emulsions, but are not effective in reducing interfacial tensions. They also bind tightly to oil droplets and form an effective barrier that prevents drop coalescence due to the high number of reactive groups in their molecules (Perfumo et al., 2010). Emulsan and its analogs, such as alasan and biodispersan, are certainly the most powerful among the bioemulsifiers synthesized by different *Acinetobacer* strains (Mulligan et al., 2014). Bioemulsifier have been extensively studied and have shown potential applications in the formation of heavy oil-water emulsions useful for viscosity reduction during crude transport in pipelines (Assadi and Tabatabaee, 2010; Perfumo et al., 2010; Mulligan et al., 2014). It was reported that such emulsion can under optimal conditions be transported for 26,000 miles. Once reaching destination, the emulsion can either be utilized directly without dewatering or treated with specific enzymes to break the emulsion before use (Mulligan et al., 2014). Amani and Kariminezhad (2016) investigated removing crude oil from a stainless steel tubing using an emulsan type biosurfactant produced by *Acinetobacter calcoaceticus* PTCC1318 and reported successful tube cleaning at the room temperature and suggested suitability for use in pipeline transportation.

The difficulties encountered with such applications however can include the need for high volume or concentration of active materials to be added, or ensuring mixing and continued high pressure into such pipelines. Other concerns may be historical deposition of blockages of transporting pipelines that may need physical clearing methods or use of this technology as a preventative measure to combat such deposition or blockage of new commissioned pipelines.

Biosurfactants Use in Oil Storage Tank Cleaning

Large amounts of oil are stored in oil tanks in refineries or transported by oil tankers, barges, and trucks over extended periods. Most such storage tanks and containers are subject to regular cleaning and or maintenance schedule which has often becomes an increasing problem involving hazardous practices and or generating large amounts of hazardous waste (Perfumo et al., 2010; Matsui et al., 2012; Mulligan et al., 2014). The oil sludge fractions that build up at the walls and bottom of the storage tanks are also highly viscous or semisolid and cannot be removed by conventional pumping. The removal of this sludge materials are often carried out manually and may involve

the use of steam or hot water or solvents and are hazardous, both time and labor intensive, expensive and usually results in the production of large amounts of waste material for disposal (Perfumo et al., 2010; Matsui et al., 2012).

The use of biosurfactants for cleaning oil storage sludge tanks was proposed for the first time in 1981 as an alternative to traditional methods (Gutnick and Rosenberg, 1981). Ten years later Banat et al. (1991) described microbial biosurfactants applications in oil storage tank cleaning up technology. A field trial was carried out at the Kuwait Oil Company demonstrated that the biosurfactants can effectively drive the cleaning activity of the storage tank. This was carried out through the addition of two tons of rhamnolipid biosurfactants containing culture broth and through energy input to create a liquid vortex within the tank continuously for 5 days at ambient temperatures of 40–50°C. This effectively lifted and mobilized oil sludge from the bottom of the tank and solubilized it within the formed emulsion. The treatment technology recovered 91% of hydrocarbons in the sludge and the value of the recovered crude was estimated to cover the cost of the cleaning operation (Galabova et al., 2014; Mulligan et al., 2014). The recovered hydrocarbon had excellent properties and could be sold after being blended with fresh crude (Banat et al., 1991). An improved process encompassing this technology was patented in 2004 by Idrabel Italia (Italy) and Jeneil Biosurfactant Company (United States). As a result of the implementation of the proposed process, the recovery of oil has generally been > 90% of the total sludge volume with a reduction of material to be disposed of to values <5% of the original sludge volume (Galabova et al., 2014). It is however important to note that the application of these technologies requires significant engineering expertise to ensure the delivery of the active ingredient and energy input that is required for mixing tanks content all of which within a highly controlled and regulated environment of in terms of safety provision and consequences or accidental hazardous practices in oil refineries and installations.

Diab and El Din (2013) also evaluated the effect of *P. aeruginosa* SH 29 biosurfactant in cleaning oil-contaminated vessels. They reported successful oil removal from the vessels bottom and walls within 15 min of application under laboratory conditions, floating as a supernatant distinct phase. They concluded suitability of the product and process for use in vessels used for the transportation and storage of crude oil. Similar observations were reported by Rocha e Silva et al. (2013) using biosurfactant from *Pseudomonas cepacia* CCT6659 for cleaning oil covered beaker walls. Matsui et al. (2012) also carried out a successful oil tank bottom sludge cleaning process using a biosurfactant produced by an actinomycete *Gordonia* sp. and reporting dispersion activity greater than that achievable with a chemical or plant-derived surfactant. Most industrial operators currently working in the field of dispersion and oil sills control have highly effective chemical dispersants for deployment when needed, all of which have official approval. For biosurfactants to replace these chemical dispersants they have to present significant clear advantages in addition to biodegradability, and at present these are probably limited, since biosurfactants are less efficient dispersants than current chemical products and are certainly more expensive to produce on a large-scale.

Biosurfactants for Oil Waste Treatment

During oil exploration, storage, transport and refining processes a considerable amount of oily sludge is generated by the petroleum industry (Hu et al., 2013). The disposal of such residues has always been a major issue faced by petroleum industries (Joseph and Joseph, 2009). For example, the annual output of oil sludge in China's refineries was estimated to approximately be one million tons, mainly derived from the cleaning process of oil storage tanks (Liu et al., 2011). In India, about 28,000 tons of oily sludge are generated by the refineries industries per annum (Joseph and Joseph, 2009). Oily sludge is a complex emulsion of various petroleum hydrocarbons containing solid particles, water and heavy metals that effective treatment methods have become a highly sought after technology attracting widespread attention (Hu et al., 2013).

Different technological options have been adopted by petroleum refineries worldwide to manage generated wastes during crude refining and stocking (Joseph and Joseph, 2009). Typically, various physical and chemical processes such as solvent extraction, dewatering, and incineration, stabilization, pyrolysis, washing with hot water or surfactant, and biodegradation are among the most common oil sludge handling techniques. Such methods are often expensive and requires complex equipment increasing cost and complexity (Guolin et al., 2011). Biological methods may be considered more suitable due to their less hazardous and more selectivity to specific reactions (Assadi and Tabatabaee, 2010). Various investigations in laboratory, pilot and field scale have been carried out to use biosurfactants in oily sludge treatment and have reported obtaining higher oil recoveries using biosurfactants (Pornsunthorntawee et al., 2008; Hu et al., 2013).

Lima et al. (2011) evaluated the removal of oily sludge through the use of biosurfactants obtained from five bacterial isolates from oil contaminated sites. Biosurfactants use led to a reduction in viscosity and promoted the formation of oil-water emulsions leading to easier sludge pumping and emulsion breaking for better crude oil recovery. The process was highly efficient for oil recovery resulting in up to 95.0% reduction in sludge volume. In laboratory and pilot-scale experiments, Yan et al. (2012) investigated the use of a rhamnolipid produced by *Pseudomonas aeruginosa* F-2 to recover oil from refinery oily sludge reporting up to 91.5% oil recovery during field pilot-scale studies.

Petroleum industry unavoidably generates large volumes of oily wastewater which has become an urgent challenge for most oilfield and petroleum company focusing attention toward efficient treatment techniques (Yu et al., 2013). Separation technologies such as centrifugation, ultrafiltration, decantation, flotation, and flocculation are examples of physical/chemical processes effectively used for the separation of oil-water mixtures (Painmanakula et al., 2010). Coagulants of chemical origin are usually used to improve the efficiency of separation of oil-water (Liu et al., 2010). Biosurfactants however are promising coagulants and/or dispersants capable of increasing the efficiency of these techniques. For instance, Rocha e Silva et al. (2015)

investigated the removal of the emulsified oil products from water in a pilot scale by dissolved air flotation and reported increasing separation efficiency from 80.0 to 98.0% in the presence of biosurfactants.

It is important to note that although there are many reports on successful application applications of biosurfactants in such bioremediation processes, several cases of little or no effects of biosurfactant use in these activities have been reported (Franzetti et al., 2011). This may be mainly due the complex interactions occurring within this environment between the cell surfaces, the amphiphilic and the abiotic environment. A more detailed understanding of the natural roles and effects of biosurfactants on biological and abiotic compartments is therefore necessary to consider them as a fully reliable agents for enhancing bioremediation.

Biosurfactants as Demulsifying Agents

Oilfield emulsions represent one of the major problems for the petroleum industry and are generated at various stages of petroleum exploration, production and recovery. Such emulsions are often complex and are a result of the prevalence of amphiphilic molecules within the oil such as the resin fraction containing naphthenic acids and asphaltenes in addition to fine solids such as clays, scales, and wax crystals (Assadi and Tabatabaee, 2010; Reis et al., 2013). The water present in oil emulsions may originate from water or steam injected to improve oil recovery or water added during de-salting operations and need to be separated out by breaking the emulsion prior to refining. The presence of water can cause problems including corrosion, scale formation, sludge accumulation in storage tanks, reduced distillation efficiency, and altered viscosity and flow properties (Mohebali et al., 2012). Breaking the emulsion (de-emulsification) takes place through the disruption of the thermodynamic conditions at the interface leading to the disruption of the stable surfaces between the bulk and the internal phases. It is, therefore, an important process before downstream oil processing, as emulsifying agents can hinder the production processes (Satpute et al., 2010). De-emulsification is a challenging process that is usually carried out by physical treatment methods including centrifugation, heat treatment, electrical treatment and/or through chemicals and as such are capital intensive and constitute a disposal problem as most chemical de-emulsifier(s) have the potential to cause environmental problems (Assadi and Tabatabaee, 2010; Mohebali et al., 2012; Reis et al., 2013).

Microbial de-emulsifiers generally have low toxicity and are biodegradable and often have unique characteristics that cannot be matched by chemically synthesized alternatives (Mohebali et al., 2012). Biological de-emulsifiers also can replace the use of chemical de-emulsifiers *in situ* which reduces the need to transport oil emulsion for treatment and provides a more environmentally-friendly solution. They are also easier to remove and recover at the end of the process (Reis et al., 2013). Microorganisms exploit the hydrophilic/hydrophobic nature of biological surface active compounds to disrupt the emulsions. Glycoproteins, glycolipids, phospholipids and polysaccharides are such microbial metabolites capable of displacing emulsifiers from the oil-water interface. Some researchers have also reported that microbial de-emulsification abilities are phenomena associated with microbial whole cells including those of *Acinetobacter* sp., *Pseudomonas* sp., *Nocardia* sp., *Bacillus* sp., *Rhodococcus* sp. *Corynebacterium* sp., and *Micrococcus* sp. (Assadi and Tabatabaee, 2010; Mohebali et al., 2012; Reis et al., 2013).

Chirwa et al. (2013) compared the de-emulsification and separation ability of oil and sludge using either commercial sodium dodecyl sulfate (SDS) surfactant with that from a biosurfactant and reported a slower recovery with biosurfactant compared to SDS yet strong feasibility for using biosurfactants for removal and recovery of oil from waste sludge. Most of the literature testing demulsifying capabilities and many other biosurfactant related activities have used crude biosurfactant extractions which not only have some other components within, but contains mixtures of biosurfactant congeners that often has different characteristics and properties. One feature of microbially produced biosurfactants is that they are synthesized as a mixture of different congeners with varying bioactivity. For many applications this is a big disadvantage and considerable downstream processing would be required to produce a product that could be used in the formulation of a consumer product. The ability to purify the products and separate such congeners we expect would significantly improve our knowledge and outcomes in this regards.

Biosurfactants as Anti-Corrosive Agents

Corrosion represents a major problem for the petroleum industry. All equipment used in oil wells refineries, petrochemical plants and transport are susceptible to corrosion with consequent negative effects on investment within the petroleum sector (Kanicky et al., 2002; Abbasov et al., 2015; Noor El-Din et al., 2016). Corrosion often starts with the adsorption of protons on metallic surfaces and an irreversible electrochemical reaction with the metal atoms. The metallic cations either dissolve in the aqueous phase or react with anions such as sulfur therefore exposing more metallic surface for subsequent attacks (Kanicky et al., 2002). Such corrosion problems have been long known to be associated with naphthenic acid and sulfur compounds constituents of crude oil refining products (Saji, 2010).

Corrosion inhibitors have been the focus of research for many years as the most practical methods for prevention. Controlling corrosion in oil field is quite complicated and requires specialty inhibitors depending on the area of application such as wells, refineries, pipelines, recovery units, pipelines storage tanks, etc. Such inhibitors can be inorganic or organic chemicals surfactant or mixed components inhibitors (Saji, 2010; Malik et al., 2011). Synthetic surfactants are usually used to control corrosion due to their ability to affect the properties of surfaces and interface mostly through adsorption to the metal surface reducing the chance of corrosion initiation. Most such chemicals however, have risks and hazardous effects to people and the environment. An alternative is the use of biosurfactants to replace

the chemically synthesized surfactant compounds (Malik et al., 2011; Korenblum et al., 2012).

Most of the biosurfactants exhibit anti-corrosion properties and have a great potential for such use through conditioning metals surfaces to delay the corrosion process (Korenblum et al., 2012; Araujo and Freire, 2013). Metal corrosion leads to the formation of corrosion products and release of energy. The most protected surfaces against corrosion are those with lower free energy. When surfaces interact with H^+ ions they tend to become more hydrophilic which may initiate the corrosion process. When surfaces however are conditioned with biosurfactants a film of these molecules attach to the surface, orienting the hydrophobic tail to the external environment while hydrophilic head to the surface, maintaining the surface protected from interaction with O_2 and H^+ ions, reducing corrosion (Malik et al., 2011; Araujo and Freire, 2013). In a study of corrosion behavior of metal surface carried out by Dagbert et al. (2006), he reported that the presence of biosurfactant produced by *Pseudomonas fluorescens* significantly delayed the corrosion of the AISI 304 stainless steel surface.

OTHER APPLICATIONS FOR BIOSURFACTANTS IN THE OIL INDUSTRY

Biosurfactant for Control of Sulfate Reducing Bacteria (SRB)

SRB are a group of anaerobic bacteria that use sulfate (SO_4^-) as a final electron acceptor instead of oxygen during anaerobic respiration and are known to cause oil reservoir souring and microbial induced corrosion making them to be considered undesirable and harmful for the oilfield production process (Dinh et al., 2004; Hubert et al., 2005; Song et al., 2014). Oilfield souring occurs as a result of H_2S and sulfides ions production, which occurs when the reservoirs are subjected to water flooding during secondary oil recovery. H_2S can also accelerated corrosion rates (Gouda et al., 1993). SRBs' biomass and sulfide metals ions can also decrease the efficiency of secondary oil recovery due to reservoir plugging (Nemati et al., 2001), in addition to the toxic and explosive nature of hydrogen sulfide when mixed with air (Gaathaug et al., 2014).

Although SRB are mainly known to use different low molecular organic compounds such as simple organic acids or alcohols and often H_2 for growth while reducing SO_4^- to H_2S, recent studies have shown that hydrocarbons in petroleum may also serve as electron donors for SRBs (Nemati et al., 2001; Song et al., 2014). When seawater or other waters containing sulfate are introduced into oil reservoirs, SRBs intensify the souring process though sulfate reduction, to sulfide while oxidizing organic electron donors present in the crude oil (Korenblum et al., 2012). Naturally souring decreases the value of the produced oil and increases the corrosion risk, increasing, thus, the total cost of oil production (Nemati et al., 2001; Hubert et al., 2005). Microbial corrosion represents some 10% of all damages to metals and non-metals (Dinh et al., 2004). Severe microbial corrosion on petroleum reservoirs occurs under anaerobic conditions and *Desulfovibrio* species are conventionally regarded as the main culprits of corrosion to oil transport equipment, including pipelines (Korenblum et al., 2012; Song et al., 2014). This process often occurs within microbial biofilms which starts with the adhesion in which hydrophobic interactions between the abiotic surface and the microorganism and progress to maturation in time leading to metal pitting (Sherry et al., 2013).

Different approaches can be used to control SRBs proliferation mainly through the use of biocide among which glutaraldehydes cocodiamines and molybdates (Nemati et al., 2001). However, both the cost and the environmental impact of using these compounds are usually high (Korenblum et al., 2012) as they can lead to the emergence of biocide-resistant SRBs and do not effectively penetrate biofilms within reservoirs or on metal surfaces in addition to causing corrosion themselves at high concentrations (Hubert et al., 2005).

Therefore, the provision of alternative sources to chemical biocides is desired by the oil industry. Recently, biosurfactants have been shown to be potential alternatives to chemical biocides and as surface coating agents to prevent SRBs growth. Their antimicrobial activity and surfactant properties increase the osmotic pressure within the cell causing leakage of the intracellular contents (Korenblum et al., 2012). El-Sheshtawy et al. (2015) assessed the inhibitory potential of biosurfactant from *Bacillus licheniformis* to SRBs growth and reported some antimicrobial activity against the growth of different strains of SRB and a complete inhibition of SRB growth after 3 h exposure to 1.0% crude biosurfactant.

Biosurfactant for Extraction of Bitumen from Tar Sands

Tar sands are sedimentary rocks that contain bitumen and other heavy petroleum fractions and are usually the product of biodegradation and chemical changes due to bacteria degradation and water washing (Spirov et al., 2013). The largest tar sands deposits are in Canada, USA, Venezuela, Madagascar and Russia and the biggest producer of synthetic oil from tar sands is Canada. In 2010, 55% of its tar sands production was from mining operations with a maximum burial depth of 75 m while *in situ* operations produced, the other 45% had deeper depths. The proportion of non-upgraded bitumen exports is projected to increase from 42% of total production in 2009, to 52% by 2019 (Spirov et al., 2013; Rudyk and Spirov, 2014).

The recovery of bitumen from tar sand is a difficult process due to its high viscosity which is typically reduced by steam (300–340°C), solvents or caustic soda injections into the sands. These processes require more water and need larger amounts of energy than conventional extraction methods (Spirov et al., 2013). Biosurfactants have been tested for bitumen extraction from tar sands and have shown effectiveness in reducing the interfacial tension between oil and water *in situ* while acting on solid-liquid interfaces. These proprieties can be used for viscosity reduction of the oil, removing water from emulsions prior to processing and releasing bitumen from tar sands. Such process can be carried out at lower temperatures and without requiring the use of caustic soda both of which are considered advantageous (Duyvesteyn et al., 2000; Oliveira et al.,

2015). Moreover, bitumen froth can be extracted from tar sands using a water process which involves the biotreatment reducing waste by-products (Mulligan and Gibbs, 1993; Shete et al., 2006). The type of microorganisms used for this purpose included *Bacillus megaterium*, *Arthrobacter terregens*, *A. xerosis*, *Corynebacterium lepus*, *C. xerosis*, *Pseudomonas asphaltenicus*, *Nocardia petrophilia* and *Vibrio ficheri* (Shete et al., 2006).

Cooper and Paddock (1984) tested glycolipids produced by the yeast *Torulopsis bombicola* ATCC 22214 in the release of bitumen from tar sand and reported effects on liquid-liquid and solid-liquid interfaces which caused significant release of bitumen from the sand. Zajic and Gerson (1978) evaluated the performance of microbial surfactants for the recovery of bitumen from Athabasca tar sand, in northeastern Alberta, Canada. These surfactants were produced by hydrocarbon fermentations of five different strains (*Corynebacterium* sp. OSGBl, *Pseudomonas* sp. Aspha 1, *Candida lipolytica* GA, *Vibrio* sp. Chry-B and *Corynebacterium* sp. CD1). These microbial surfactants compared well with synthetic surfactants and proved to be effectives in tar sand separation by a cold-water extraction process to cause flotation of the bitumen or to cause removal of sand and clay from the bitumen.

FUTURE PROSPECTS FOR BIOSURFACTANTS IN THE PETROLEUM INDUSTRY

Biosurfactant for Fuels Formulation

One of the unexplored area for potential biosurfactant applications in the petroleum industry is possible use in the formulation of emulsified fuels (Youssef et al., 2009; Perfumo et al., 2010). Emulsified fuels are mixtures that includes surfactants that facilitates the formation of a stable emulsion of the water or other substances within the fuel phase and a variety of additives such as detergents, anti-foaming agents, lubricity enhancers, anti-rust agents, ignition improvers and metal deactivators (Coleman and Sibley, 2003; Dantas Neto et al., 2011).

Diesel fuel blended with water is a well-known emulsified fuel currently applied worldwide for public transport fleets, locomotives, marine engines and heat generators in industrial settings. In addition to cost saving such fuels improve combustion efficiency, do not need engine modification and effectively reduces carbon monoxide (CO), NO_x, unburned hydrocarbon, particulate matter emission and reduce exhaust gas temperatures and general pollutant emissions (Perfumo et al., 2010; Dantas Neto et al., 2011). Surfactants can stabilize the emulsion ensuring that the finely dispersed water droplets remain in suspension within the fuels preventing phase separation upon long-term storage.

Currently the most used surfactants includes non-ionic and polymeric surfactants such as alcohol ethoxylates, sugar esters of fatty acids, and fatty acids ethoxylates. However, investigations into the possibility of replacing traditional chemical compounds with microbial surfactants to formulate fuel or diesel emulsions have been carried out (Coleman and Sibley, 2003; Perfumo et al., 2010). Leng et al. (2015) successfully tested a biosurfactant rhamnolipids to obtain nano-scaled glycerol/water-in-diesel microemulsions, which can be formed spontaneously with low energy consumption. In addition, the physicochemical properties of glycerol/water-in-diesel microemulsion were similar to those of diesel.

Recombinant DNA Technology to Enhance Biosurfactant Production

Genetic engineering consists in modifying the genetic material of microorganisms of industrial importance to acquire new or enhanced capabilities through recombinant DNA technology. The construction of hyper producing microorganisms to increase the biosurfactant secretion to promote activity and decrease cost is a general aim (Assadi and Tabatabaee, 2010). However, industrial-scale usage of biosurfactants for MEOR still appears to be limited due to high production costs (Banat et al., 2010; Makkar et al., 2011). To reduce this cost it is important to develop mutant or recombinant strains with enhanced production yields (Bachmann et al., 2014), or with an ability to selectively produce particular effective congeners of biosurfactants which are often a mixture of closely related products. Biosurfactants producers could also be engineered to be resistant to process conditions generally found in the petroleum industry. An alternative is to isolate new gene sequences from extreme environments similar to ones that might be encountered in oil reservoirs such as high salt concentration, high temperatures, and extreme pH values. For example, alkaliphilic halophiles microorganisms can be found in hypersaline soda lakes such as Lake Magadi in Kenya, Wadi Natrum lakes in Egypt and Soda lakes in China; genes from such isolates may then be transferred into selected biosurfactant producers which can be active and effective under such extreme conditions. Other possibilities include the use genes that code for the production of biosurfactant that are particularly well evolved at elevated temperatures through isolation from high temperature oil reservoirs (Kohr, 2012; Kohr et al., 2016).

CONCLUDING REMARKS

It is concluded that advances in oil biotechnology are becoming increasingly evident in recent years and due to the versatility and efficiency demonstrated by many types of biosurfactants in the service of or in processes related to the petroleum industry, they are increasingly gaining recognition and appreciation. These compounds are not only providing supporting roles but are beginning to provide essential roles, making them necessary compounds in petroleum biotechnology. The one major advantage of biosurfactants would be their biodegradability which significantly reduces the environmental impact of these compounds compared to chemical surfactants. It is their other successful applications, however that are becoming recognized and we believe will lead to an expansion in their use within the petroleum industries.

AUTHOR CONTRIBUTIONS

All authors contributed in this work. DD, RS, VS, LS, IB, RR, and JL designed the project and wrote the manuscript. LS and IB carried out manuscript editing and final improvement.

FUNDING

Funding for this study was provided by the State of Pernambuco Foundation for the Assistance to Science and Technology (FACEPE), the Research and Development Program of the Brazilian National Electrical Energy Agency (ANEEL), the National Council for Scientific and Technological Development (CNPq) and the Federal Agency for the Support and Evaluation of Graduate Education (CAPES).

ACKNOWLEDGMENTS

The authors are grateful to the laboratories of the Centre for Science and Technology of the Catholic University of Pernambuco and the Centre for Technology and Innovation Management (CGTI), Brazil.

REFERENCES

Abbasov, V. M., Aliyeva, L. I., Abd El-Lateef, H. M., and Ismayilov, I. T. (2015). Some surfactants based on the vegetable oils as CO_2 corrosion inhibitors for mild steel in oilfield formation water. *Int. J. Corros. Scale Inhib.* 4, 162–175. doi: 10.17675/2305-6894-2015-4-2-162-175

Al-Bahry, S. N., Al-Wahaibi, Y. M., Elshafie, A. E., Al-Bemani, A. S., Joshi, S. J., Al-akhmari, H. S., et al. (2013). Biosurfactant production by *Bacillus subtilis* B20 using date molasses and its possible application in enhanced oil recovery. *Int. Biodeter. Biodegr.* 81, 141–146. doi: 10.1016/j.ibiod.2012.01.006

Amani, H., and Kariminezhad, H. (2016). Study on emulsification of crude oil in water using emulsan biosurfactant for pipeline transportation. *Petrol. Sci. Technol.* 34, 216–222. doi: 10.1080/10916466.2015.1118500

Araujo, L. V., and Freire, D. M. G. (2013). Biossurfactantes: propriedades anticorrosivas, antibiofilmes e antimicrobianas. *Quim. Nova.* 36, 848–858. doi: 10.1590/S0100-40422013000600001

Assadi, M., and Tabatabaee, M. S. (2010). Biosurfactants and their use in upgrading petroleum vacuum distillation residue: a review. *Int. J. Environ. Res.* 4, 549–572.

Bachmann, R. T., Johnson, A. C., and Edyvean, R. G. J. (2014). Biotechnology in the petroleum industry: an overview. *Int. Biodeter. Biodegr.* 86, 225–237. doi: 10.1016/j.ibiod.2013.09.011

Banat, I. M., Franzetti, A., Gandolfi, I., Bestetti, G., Martinotti, M. G., Fracchia, L., et al. (2010). Microbial biosurfactants production, applications and future potential. *Appl. Microbiol. Biotechnol.* 87, 427–444. doi: 10.1007/s00253-010-2589-0

Banat, I. M., Samarah, N., Murad, M., Horne, R., and Banerjee, S. (1991). Biosurfactant production and use in oil tank clean-up. *World J. Microbiol. Biotechnol.* 7, 80–88. doi: 10.1007/BF02310921

Brigmon, R. L., and Berry, C. J. (2009). *Biological Enhancement of Hydrocarbon Extraction*. U.S. Patent No 7,472,747. Washington, DC: U.S. Patent and Trademark Office.

Busche, F. D., Rollins, J. B., Noyes, H. J., and Bush, J. G. (2011). *System and Method for Preparing Near-Surface Heavy Oil for Extraction Using Microbial Degradation*. U.S. Patent No 7,922,893. Washington, DC: U.S. Patent and Trademark Office.

Cerón-Camacho, R., Martínez-Palou, R., Chávez-Gómez, B., Cuéllar, F., Bernal-Huicochea, C., Clavel, J. C., et al. (2013). Synergistic effect of alkyl-O-glucoside and -cellobioside biosurfactants as effective emulsifiers of crude oil in water. A proposal for the transport of heavy crude oil by pipeline. *Fuel.* 110, 310–317. doi: 10.1016/j.fuel.2014.01.023

Chirwa, E. M., Mampholo, T., and Fayemiwo, O. (2013). Biosurfactants as demulsifying agents for oil recovery from oily sludge-performance evaluation. *Water Sci. Technol.* 67, 2875–2881. doi: 10.2166/wst.2013.207

Clark, J. B., and Jenneman, G. E. (1992). *Nutrient injection method for subterranean microbial processes*. U.S. Patent No 5,083,611. Washington, DC: U.S. Patent and Trademark Office.

Coleman, G. N., and Sibley, J. E. (2003). *Constant Heating Value Aqueous Fuel Mixture and Method for Formulating the Same*. U.S. Patent No 6,656,236.

Converse, D. R., Hinton, S. M., Hieshima, G. B., Barnum, R. S., and Sowlay, M. R. (2003). *Process for Stimulating Microbial Activity in a Hydrocarbon-Bearing, Subterranean Formation*. U.S. Patent No 6,543,535. Washington, DC: U.S. Patent and Trademark Office.

Cooper, D. G., and Paddock, D. A. (1984). Production of a biosurfactant from *Torulopsis bombicola*. *Appl. Environ. Microbiol.* 47, 173–176.

Crews, J. B. (2006). *Bacteria-Based and Enzyme-Based Mechanisms and Products for Viscosity Reduction Breaking of Viscoelastic Fluids*. U.S. Patent No 7,052,901. Washington, DC: U.S. Patent and Trademark Office.

Dagbert, C., Meylheuc, T., and Bellon-Fontaine, M. (2006). Corrosion behaviour of AISI 304 stainless steel in presence of a biosurfactant produced by *Pseudomonas fluorescens*. *Electrochim. Acta.* 51, 5221–5227. doi: 10.1016/j.electacta.2006.03.063

Dantas Neto, A. A., Fernandes, M. R., Barros Neto, E. L., Castro Dantas, T. N., and Moura, M. C. P. A. (2011). Alternative fuels composed by blends of nonionic surfactant with diesel and water: engine performance and emissions. *Braz. J. Chem. Eng.* 28, 521–531. doi: 10.1590/S0104-66322011000300017

Devold, H. (2013). *Oil and Gas Production Handbook: An Introduction to Oil and Gas Production, Transport, Refining and Petrochemical Industry*. Oslo: ABB Oil and Gas.

Diab, A., and El Din, S. G. (2013). Application of the biosurfactants produced by *Bacillus* spp. (SH 20 and SH 26) and *Pseudomonas aeruginosa* SH 29 isolated from the rhizosphere soil of an Egyptian salt marsh plant for the cleaning of oil - contaminated vessels and enhancing the biodegradation of oily sludge. *Afr. J. Environ. Sci. Technol.* 7, 671–679. doi: 10.5897/AJEST2013.1451

Dinh, H. T., Kuever, J., Mußmann, M., Hassel, A. W., Stratmann, M., and Widdel, F. (2004). Iron corrosion by novel anaerobic microorganisms. *Nature.* 427, 829–832. doi: 10.1038/nature02321

Duyvesteyn, W. P. C., Budden, J. R., and Picavet, M. A. (2000). *Extraction of Bitumen from Bitumen Froth and Biotreatment of Bitumen Froth Tailings Generated from Tar Sands*. C.A. Patent No 2,350,907.

Dziegielewska, E., and Adamczak, M. (2013). Evaluation of wast products in the synthesis of surfactants by yeasts. *Chem. Pap.* 67, 1113–1122. doi: 10.2478/s11696-013-0349-1

Elraies, K. A., and Tan, I. M. (2012). "The application of a new polymeric surfactant for chemical EOR," in *Introduction to Enhanced Oil Recovery (EOR) Processes and Bioremediation of Oil-contaminated Sites*, ed L. Romero-Zerón (Rijeka: InTech), 45–70.

El-Sheshtawy, H. S., Aiad, I., Osman, M. E., Abo-ELnasr, A. A., and Kobisy, A. S. (2015). Production of biosurfactant from *Bacillus licheniformis* for microbial enhanced oil recovery and inhibition the growth of sulphate reducing bacteria. *Egypt J. Pet.* 24, 155–162. doi: 10.1016/j.ejpe.2015.05.005

Fallon, R. D. (2011). *Methods for Improved Hydrocarbon and Water Compatibility*. U.S. Patent No 7,992,639. Washington, DC: U.S. Patent and Trademark Office.

Franzetti, A., Gandolfi, I., Bestetti, G., and Banat, I. M. (2011). "(Bio)surfactant and bioremediation, successes and failures," in *Trends in Bioremediation and Phytoremediation*, ed G. Plaza (Kerala: Research Signpost), Chapter 9, 145–156.

Gaathaug, A. V., Bjerketvedt, D., Vaagsaether, K., and Nilsen, S. H. (2014). Experimental study of gas explosions in hydrogen sulphide-natural gas-air mixtures. *J. Combust.* 2014:905893. doi: 10.1155/2014/905893

Galabova, D., Sotirova, A., Karpenko, E., and Karpenko, O. (2014). "Role of microbial surface-active compounds in environmental protection," in *The Role of Colloidal Systems in Environmental Protection*, ed M. Fanun (Amsterdam: Elsevier), 41–83.

Gouda, V. K., Shalaby, H. M., and Banat, I. M. (1993). The effect of sulphate-reducing bacteria on the electrochemical behavior of corrosion-resistant alloys in seawater. *Corros. Sci.* 35, 689–691. doi: 10.1016/0010-938X(93)90204-T

Grand View Research (2016). *Biosurfactants Market by Product (Rhamnolipids, Sophorolipids, MES, APG, Sorbitan Esters, Sucrose Esters) Expected to Reach USD 2308.8 Million by 2020*. Available online at: http://www.grandviewresearch.com/industry-analysis/biosurfactants-industry (Accessed April 15, 2016).

Gudiña, E. J., Rodrigues, A. I., Alves, E. M., Domingues, R., Teixeira, J. A., and Rodrigues, L. R. (2015). Bioconversion of agro-industrial by-products in rhamnolipids toward applications in enhanced oil recovery and bioremediation. *Bioresour. Technol.* 177, 87–93. doi: 10.1016/j.biortech.2014.11.069

Guolin, J., Mingming, L., and Tingting, C. (2011). Prospects for development of oily sludge treatment. *Chem. Technol. Fuels Oil.* 47, 312–326. doi: 10.1007/s10553-011-0301-4

Gutnick, D., and Rosenberg, E. (1981). *Cleaning Oil-Contaminated Vessels With α-Emulsans*. U.S. Patent No 4,276,094. Patent No 6,090,051. Washington, DC: U.S. Patent and Trademark Office.

Hayes, M. E., Hrebenar, K. R., and Murphy, P. L. (1988). *Bioemulsifier Stabilized Hydrocarbons*. A.U. Patent No 574,403.

Hazra, C., Kundu, D., and Chaudhari, A. (2012). "Biosurfactant-assisted bioaugmentation in bioremediation," in *Microorganisms in Environmental Management: Microbes and Environment*, eds T. Satyanarayana, B. N. Johri, and A. Prakash (New York, NY: Springer), 631–664.

Helmy, Q., Kardena, E., and Funamizu, N., Wisjnuprapto. (2011). Strategies toward commercial scale of biosurfactant production as potential substitute for it's chemically counterparts. *Int. J. Biotechnology.* 12, 66–86. doi: 10.1504/IJBT.2011.042682

Hitzman, D. O. (1984). *Enhanced Oil Recovery Process Using Microorganisms*. U.S. Patent No 4,450,908. Patent No 6,090,051. Washington, DC: U.S. Patent and Trademark Office.

Holmager, M. (2010). *Offshore Book — An Introduction to the Off Shore Industry*. Esbjerg: Offshore Center Denmark.

Hu, G., Li, J., and Zeng, G. (2013). Recent development in the treatment of oily sludge from petroleum industry: a review. *J. Hazard. Mater.* 261, 470–490. doi: 10.1016/j.jhazmat.2013.07.069

Hubert, C., Nemati, M., Jenneman, G., and Voordouw, G. (2005). Corrosion risk associated with microbial souring control using nitrate or nitrite. *Appl. Microbiol. Biotechnol.* 68, 272–282. doi: 10.1007/s00253-005-1897-2

Joseph, P. J., and Joseph, A. (2009). Microbial enhanced separation of oil from a petroleum refinery sludge. *J. Hazard. Mater.* 161, 522–525. doi: 10.1016/j.jhazmat.2008.03.131

Joshi, S. J., and Desai, A. J. (2010). Biosurfactant's role in bioremediation of NAPL and fermentative production. *Adv. Exp. Med. Biol.* 672, 222–235. doi: 10.1007/978-1-4419-5979-9_17

Kanicky, J. R., Lopez-Montilla, J., Pandey, S., and Shah, D. O. (2002). "Surface chemistry in the petroleum industry," in *Handbook of Applied Surface and Colloid Chemistry*, ed K. Holmberg (Chichester: John Wiley & Sons), 251–266.

Kapadia, S. G., and Yagnik, B. N. (2013). Current trend and potential for microbial biosurfactants. *Asian J. Exp. Biol. Sci.* 4, 1–8.

Kohr, W. J. (2012). *Microbial Enhanced Oil Recovery Methods*. U.S. Patent No 8,316,933. Washington, DC: U.S. Patent and Trademark Office.

Kohr, W. J., Zhang, Z., and Galgoczy, D. J. (2016). *Alkaline Microbial Enhanced Oil Recovery*. U.S. Patent no 9,290,688. Washington, DC: U.S. Patent and Trademark Office.

Korenblum, E., Araujo, L. V., Guimarães, C. R., Souza, L. M., Sassaki, G., Abreu, F., et al. (2012). Purification and characterization of a surfactin-like molecule produced by *Bacillus* sp. H2O-1 and its antagonistic effect against sulphate reducing bacteria. *BMC Microbiol.* 12:252. doi: 10.1186/1471-2180-12-252

Ławniczak, L., Marecik, R., and Chrzanowski, L. (2013). Contributions of biosurfactants to natural or induced bioremediation. *Appl. Microbiol. Biotechnol.* 97, 2327–2339. doi: 10.1007/s00253-013-4740-1

Leng, L., Yuan, X., Zeng, G., Chen, X., Wang, H., Li, H., et al. (2015). Rhamnolipid based glycerol-in-diesel microemulsion fuel: formation and characterization. *Fuel* 147, 76–81. doi: 10.1016/j.fuel.2015.01.052

Lima, T. M. S., Fonseca, A. F., Leão, B. A., Mounteer, A. H., and Tótola, M. R. (2011). Oil recovery from fuel oil storage tank sludge using biosurfactants. *J. Bioremed. Biodegrad.* 2:12. doi: 10.4172/2155-6199.1000125

Liu, J., Jiang, X., and Han, X. (2011). Devolatilization of oil sludge in a lab-scale bubbling fluidized bed. *J. Hazard. Mater.* 185, 1205–1213. doi: 10.1016/j.jhazmat.2010.10.032

Liu, S. H., Guo, D., and Xie, G. X. (2010). Water film confined in a nanoscale gap: surface polarity and hydration effects. *J. Appl. Phys.* 108, 084315–084322. doi: 10.1063/1.3499695

Luna, J. M., Rufino, R. D., Campos-Takakia, G. M., and Sarubbo, L. A. (2012). Properties of the biosurfactant produced by *Candida sphaerica* cultivated in low-cost substrates. *Chem. Eng. Trans.* 27, 67–72. doi: 10.3303/CET1227012

Luna, J. M., Rufino, R. D., Sarubbo, L. A., and Campos–Takaki, G. M. (2013). Characterisation, surface properties and biological activity of a biosurfactant produced from industrial waste by *Candida sphaerica* UCP0995 for application in the petroleum industry. *Colloids Surf. B. Biointerfaces.* 2, 202–209. doi: 10.1016/j.colsurfb.2012.08.008

Makkar, R. S., Cameotra, S. S., and Banat, I. M. (2011). Advances in utilization of renewable substrates for biosurfactant production. *Appl. Microbiol. Biotechnol.* 1, 1–5. doi: 10.1186/2191-0855-1-5

Malik, M. A., Hashim, M. A., Nabi, F., A. L.-, and Thabaiti, S. A., Khan, Z. (2011). Anti-corrosion ability of surfactants: a review. *Int. J. Electrochem. Sci.* 6, 1927–1948.

Marchant, R., and Banat, I. M. (2012). Microbial biosurfactants: challenges and opportunities for future exploitation. *Trends Biotechnol.* 11, 558–565. doi: 10.1016/j.tibtech.2012.07.003

Matsui, T., Namihira, T., Mitsuta, T., and Saeki, H. (2012). Removal of oil tank bottom sludge by novel biosurfactant, JE1058BS. *J. Jpn. Pet. Inst.* 55, 138–141. doi: 10.1627/jpi.55.138

McInerney, M. J., Jenneman, G. E., Knapp, R. M., and Menzie, D. E. (1985). *Biosurfactant and Enhanced Oil Recovery*. U.S. Patent No 4,522,261. Washington, DC: U.S. Patent and Trademark Office.

Mohebali, G., Kaytash, A., and Narges, E. (2012). Efficient breaking of water/oil emulsions by a newly isolated de-emulsifying bacterium, *Ochrobactrum anthropi* strain RIPI5-1. *Colloids Surf. B. Biointerfaces.* 98, 120–128. doi: 10.1016/j.colsurfb.2012.04.037

Montiel, C., Quintero, R., and Aburto, J. (2009). Petroleum biotechnology: Technology trends for the future. *Afr. J. Biotechnol.* 8, 2653–2666.

Müller, M. M., Kügler, J. H., Henkel, M., Gerlitzki, M., Hörmann, B., Pöhnlein, M., et al. (2012). Rhamnolipids—next generation surfactants? *J. Biotechnol.* 162, 366–380. doi: 10.1016/j.jbiotec.2012.05.022

Mulligan, C. N., and Gibbs, B. F. (1993). "Factors influencing the economics of biosurfactants," in *Biosurfactants: Production, Properties, Applications*, eds N. Kosaric and F. V. Sukan (New York, NY: CRC Press), 329–371.

Mulligan, C. N., Sharma, S. K., Mudhoo, A., and Makhijani, K. (2014). "Green chemistry and biosurfactant research," in *Biosurfactants: Research Trends and Applications*, eds C. N. Mulligan, S. K. Sharma and A. Mudhoo (Boca Raton, FL: CRC Press), 1–30.

Nemati, M., Jenneman, G. E., and Voordouw, G. (2001). Mechanistic study of microbial control of hydrogen sulphide production in oil reservoirs. *Biotechnol. Bioeng.* 74, 424–434. doi: 10.1002/bit.1133

Noor El-Din, M. R., Farag, R. K., and Elazbawy, O. E. (2016). Utilization of new anionic polymeric surfactants for corrosion inhibition enhancement in petroleum industries. *Int. J. Electrochem. Sci.* 11, 815–835.

Okoliegbe, I. N., and Agarry, O. O. (2012). Application of microbial surfactant (a review). *Scholarly J. Biotechnol.* 1, 15–23.

Oliveira, M. R., Magri, A., Baldo, C., Camilios-Neto, D., Minucelli, T., and Celligoi, M. A. P. (2015). Review: sophorolipids a promising biosurfactant and its applications. *Int. J. Adv. Biotechnol. Res.* 6, 161–174. doi: 10.1016/j.colsurfb.2012.06.011

Painmanakula, P., Sastaraveta, P., Lersjintanakarnb, S., and Khaodhiara, S. (2010). Effect of bubble hydrodynamic and chemical dosage on treatment of oily wastewater by induced air flotation (IAF) process. *Chem. Eng. Res. Des.* 88, 693–702. doi: 10.1016/j.cherd.2009.10.009

Perfumo, A., Rancich, I., and Banat, I. M. (2010). "Possibilities and challenges for biosurfactants use in petroleum industry," in *Biosurfactants Advances in Experimental Medicine and Biology*, ed R. Sen, (New York, NY: *Springer*), 135–145.

Pornsunthorntawee, O., Arttaweeporn, N., Paisanjit, S., Somboonthanate, P., Abe, M., Rujiravanit, R., et al. (2008). Isolation and comparison of biosurfactants produced by *Bacillus subtilis* PT2 and *Pseudomonas aeruginosa* SP4 for microbial surfactant-enhanced oil recovery. *Biochem. Eng. J.* 42, 172–179. doi: 10.1016/j.bej.2008.06.016

Powell, J. E. Jr. (2000a). *System and process for in tank treatment of crude oil sludges to recover hydrocarbons and aid in materials separation*. U.S. Patent No 6,033,901. Washington, DC: U.S. Patent and Trademark Office.

Powell, J. E. Jr. (2000b). *System and process for in Tank Treatment of Crude Oil Sludges to recover hydrocarbons and aid in materials separation*. U.S. Patent No 6,069,002. Washington, DC: U.S. Patent and Trademark Office.

Randhawa, K. K. S., and Rahman, P. K. S. M. (2014). Rhamnolipid biosurfactants—past, present, and future scenario of global market. *Front. Microbiol.* 5:454. doi: 10.3389/fmicb.2014.00454

Reis, R. S., Pacheco, G. J., Pereira, A. G., and Freire, D. M. G. (2013). "Biosurfactants: production and applications," in *Biodegradation — Life of Science*, eds R. Chamy and F. Rosenkranz (Rijeka: InTech), 31–61.

Rocha e Silva, F. C. P., Rocha e Silva, N. M. P., Moura, A. E., Galdino, R. A., Luna, J. M., Rufino, R. D., et al. (2015). Effect of biosurfactant addition in a pilot scale dissolved air flotation system. *Separ. Sci. Technol.* 50, 618–625. doi: 10.1080/01496395.2014.957319

Rocha e Silva, N. M. P., Rufino, R. D., Luna, J. M., Santos, V. A., and Sarubbo, L. A. (2013). Screening of *Pseudomonas* species for biosurfactant production using low-cost substrates. *Biocatal. Agric. Biotechnol.* 3, 132–139. doi: 10.1016/j.bcab.2013.09.005

Rudyk, S., and Spirov, P. (2014). Upgrading and extraction of bitumen from Nigerian tar sand by supercritical carbon dioxide. *Appl. Energy.* 113, 1397–1404. doi: 10.1016/j.apenergy.2013.08.076

Rufino, R. D., Luna, J. M., Campos-Takaki, G. M., and Sarubbo, L. A. (2014). Characterization and properties of the biosurfactant produced by *Candida lipolytica* UCP 0988. *Electron. J. Biotechnol.* 17, 34–38. doi: 10.1016/j.ejbt.2013.12.006

Sachdev, D. P., and Cameotra, S. S. (2013). Biosurfactants in agriculture. *Appl. Microbiol. Biotechnol.* 97, 1005–1016. doi: 10.1007/s00253-012-4641-8

Saji, V. S. (2010). A review on recent patents in corrosion inhibitors. *Recent Patents Corr. Sci.* 2, 6–12. doi: 10.2174/1877610801002010006

Sajna, K. V., Höfer, R., Sukumaran, R. K., Gottumukkala, L. D., and Pandey, A. (2015). "White biotechnology in biosurfactants," in *Industrial Biorefineries & White Biotechnology*, eds A. Pandey, R. Höfer, M. Taherzadeh, M. Nampoothiri, and C. Larroche (Amsterdam: Elsevier), 499–521.

Santos, D. K. F., Rufino, R. D., Luna, J. M., Santos, V. A., Salgueiro, A. A., and Sarubbo, L. A. (2013). Synthesis and evaluation of biosurfactant produced by *Candida lipolytica* using animal fat and corn steep liquor. *J. Pet. Sci. Eng.* 105, 43–50. doi: 10.1016/j.petrol.2013.03.028

Santos, D. K. F., Rufino, R. D., Luna, J. M., Santos, V. A., and Sarubbo, L. A. (2016). Biosurfactants: multifunctional biomolecules of the 21st century. *Int. J. Mol. Sci.* 17, 401. doi: 10.3390/ijms17030401

Sarafzadeh, P., Niazi, A., Oboodi, V., Ravanbakhsh, M., Hezave, A. Z., and Shahab Ayatollahi, S. (2014). Investigating the efficiency of MEOR processes using *Enterobacter cloacae* and *Bacillus stearothermophilus* SUCPM#14 (biosurfactant-producing strains) in carbonated reservoirs. *J. Pet. Sci. Eng.* 113, 46–53. doi: 10.1016/j.petrol.2013.11.029

Sarubbo, L. A., Luna, J. M., and Rufino, R. D. (2015). Application of a biosurfactant produced in low-cost substrates in the removal of hydrophobic contaminants. *Chem. Eng. Trans.* 43, 295–300. doi: 10.3303/CET1543050

Satpute, S. K., Banpurkar, A. G., Dhakephalkar, P. K., Banat, I. M., and Chopade, B. A. (2010). Methods for investigating biosurfactants and bioemulsifiers: a review. *Crit. Rev. Biotechnol.* 20, 1–18. doi: 10.3109/07388550903427280

Sekhon, K. K., Khanna, S., and Cameotra, S. S. (2012). Biosurfactant production and potential correlation with Esterase activity. *J. Pet. Environ. Biotechnol.* 3:7. doi: 10.4172/2157-7463.1000133

Sen, R. (2008). Biotechnology in petroleum recovery: the microbial EOR. *Prog. Energy Combust. Sci.* 34, 714–724. doi: 10.1016/j.pecs.2008.05.001

Sheehy, A. (1992). *Recovery of Oil From Oil Reservoirs*. U.S. Patent No 5,083,610. Washington, DC: U.S. Patent and Trademark Office.

Sherry, A., Gray, N. D., Ditchfield, A. K., Aitken, C. M., Jones, D. M., Röling, W. F. M., et al. (2013). Anaerobic biodegradation of crude oil under sulphate-reducing conditions leads to only modest enrichment of recognized sulphate-reducing taxa. *Int. Biodeter. Biodegr.* 81, 105–113. doi: 10.1016/j.ibiod.2012.04.009

Shete, A. M., Wadhawa, G., Banat, I. M., and Chopade, B. A. (2006). Mapping of patents on bioemulsifier and biosurfactant: a review. *J. Sci. Ind. Res.* 65, 91–115.

Silva, R. C. F. S., Almeida, D. G., Rufino, R. D., Luna, J. M., Santos, V. A., and Sarubbo, L. A. (2014). Applications of biosurfactants in the petroleum industry and the remediation of oil spills. *Int. J. Mol. Sci.* 15, 12523–12542. doi: 10.3390/ijms150712523

Singh, A., Singh, B., and Ward, O. (2012). Potential applications of bioprocess technology in petroleum industry. *Biodegradation* 23, 865–880. doi: 10.1007/s10532-012-9577-2

Sobrinho, H. B., Luna, J. M., Rufino, R. D., Porto, A. L. F., and Sarubbo, L. A. (2013). "Biosurfactants: classification, properties and environmental applications," in *Recent Developments in Biotechnology*, ed J. N. Govil (Houston, TX: Studium Press LLC), 1–29.

Song, W., Ma, D., Zhu, Y., Wei, X., Wu, J., Li, S., et al. (2014). The role of sulphate-reducing bacteria in oil recovery. *Int. J. Curr. Microbiol. Appl. Sci.* 7, 385–398.

Spirov, P., Rudyk, S., Tyrovolas, A., and Jimoh, I. (2013). The bitumen extraction from Nigerian tar sand using dense carbon dioxide. *Chem. Eng. Trans.* 32, 283–288. doi: 10.3303/CET1332048

Thomas, J. E. (2004). *Fundamentos de Engenharia de Petróleo*. Rio de Janeiro: Interciência.

Wagner, F., Rapp, P., Bock, H., Lindorfer, W., Schulz, W., and Gebetsberger, W. (1982). *Method and Installation for Flooding Petroleum Wells and Oil-Sands*. C.A. Patent No 1,119,794.

Yan, P., Lu, M., Yang, Q., Zhang, H. L., Zhang, Z. Z., and Chen, R. (2012). Oil recovery from refinery oily sludge using a rhamnolipid biosurfactant-producing *Pseudomonas*. *Bioresour. Technol.* 116, 24–28. doi: 10.1016/j.biortech.2012.04.024

Youssef, N., Elshahed, M. S., and McInerney, M. J. (2009). "Microbial processes in oil fields: culprits, problems, and opportunities," in *Advances in Applied Microbiology*, eds A. I. Laskin, S. Sariaslani, G. M. Gadd (Burlington: Academic Press), 141–251.

Yu, L., Han, M., and He, F. (2013). A review of treating oily wastewater. *Arabian J. Chem.* doi: 10.1016/j.arabjc.2013.07.020. [Epub ahead of print].

Zajic, J. E., and Gerson, D. F. (1981). *Hydrocarbon Extraction Agents and Microbiological Processes for their Production*. C.A. Patent No 1,114,759.

Zajic, J. E., and Gerson, D. F. (1978). "Microbial extraction of bitumen from Athabasca oil sand," in *Oil Sands and Oil Shale*, ed O. Strausz (New York, NY: Verlag Chemie), 145–161.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5.2. CAPÍTULO 2

Response Surface Methodology for Optimizing the Production of Biosurfactant by *Candida tropicalis* on Industrial Waste Substrates

Artigo publicado na revista *Frontiers in Microbiology*.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 07 February 2017
doi: 10.3389/fmicb.2017.00157

Response Surface Methodology for Optimizing the Production of Biosurfactant by *Candida tropicalis* on Industrial Waste Substrates

Darne G. Almeida[1,2], *Rita de Cássia F. Soares da Silva*[1,2], *Juliana M. Luna*[2,3], *Raquel D. Rufino*[2,3], *Valdemir A. Santos*[2,3] *and Leonie A. Sarubbo*[1,2,3]*

[1] Northeast Biotechnology Network, Federal Rural University of Pernambuco, Recife, Brazil, [2] Advanced Institute of Technology and Innovation, Recife, Brazil, [3] Center of Sciences and Technology, Catholic University of Pernambuco, Recife, Brazil

OPEN ACCESS

Edited by:
Sanket J. Joshi,
Sultan Qaboos University, Oman

Reviewed by:
Seung Gu Shin,
Pohang University of Science and Technology, South Korea
Palashpriya Das,
Indian Institute of Chemical Biology (CSIR), India

***Correspondence:**
Leonie A. Sarubbo
leonie@unicap.br

Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: 21 October 2016
Accepted: 20 January 2017
Published: 07 February 2017

Citation:
Almeida DG, Soares da Silva RCF, Luna JM, Rufino RD, Santos VA and Sarubbo LA (2017) Response Surface Methodology for Optimizing the Production of Biosurfactant by Candida tropicalis on Industrial Waste Substrates. Front. Microbiol. 8:157. doi: 10.3389/fmicb.2017.00157

Biosurfactant production optimization by *Candida tropicalis* UCP0996 was studied combining central composite rotational design (CCRD) and response surface methodology (RSM). The factors selected for optimization of the culture conditions were sugarcane molasses, corn steep liquor, waste frying oil concentrations and inoculum size. The response variables were surface tension and biosurfactant yield. All factors studied were important within the ranges investigated. The two empirical forecast models developed through RSM were found to be adequate for describing biosurfactant production with regard to surface tension (R^2 = 0.99833) and biosurfactant yield (R^2 = 0.98927) and a very strong, negative, linear correlation was found between the two response variables studied (r = −0.95). The maximum reduction in surface tension and the highest biosurfactant yield were 29.98 mNm^{-1} and 4.19 gL^{-1}, respectively, which were simultaneously obtained under the optimum conditions of 2.5% waste frying oil, 2.5%, corn steep liquor, 2.5% molasses, and 2% inoculum size. To validate the efficiency of the statistically optimized variables, biosurfactant production was also carried out in 2 and 50 L bioreactors, with yields of 5.87 and 7.36 gL^{-1}, respectively. Finally, the biosurfactant was applied in motor oil dispersion, reaching up to 75% dispersion. Results demonstrated that the CCRD was suitable for identifying the optimum production conditions and that the new biosurfactant is a promising dispersant for application in the oil industry.

Keywords: biosurfactant, *Candida tropicalis*, optimization, scale up, oil dispersion

INTRODUCTION

Surfactants are amphipathic molecules that reduce the surface and interfacial tensions of liquids (Santos et al., 2013; Silva et al., 2014a). Such compounds have a predilection for interfaces of dissimilar polarities (liquid–air or liquid–liquid) and are soluble in both organic (non-polar) and aqueous (polar) solvents (Luna et al., 2013). Due to these properties, surfactants have a wide variety of applications in medicine, household products, agriculture, food products, cosmetics, pharmaceuticals, and the petroleum industry (Rufino et al., 2014).

Biosurfactants are surfactants of biological origin. Microorganisms (bacteria, yeasts and fungi) are known to produce biosurfactants (Luna et al., 2013), which are classified as glycolipids, lipopeptides, fatty acids, polymers, or particulate compounds (Rufino et al., 2014). Although the majority of biosurfactants have been reported in bacteria, the pathogenic nature of some producers restricts the wide application of these compounds (Toribio et al., 2010; Sharma et al., 2016). Given the industrial importance of yeasts and their potential to biosurfactant production, a growing number of aspects related to the production of biosurfactants from yeasts have been the topic of research during the last decade (Amaral et al., 2010). Some species of *Candida*, such as *Candida bombicola* (Roelants et al., 2013; Luna et al., 2016), *Candida glabrata* (Luna et al., 2009; Gusmão et al., 2010), *Candida lipolytica* (Santos et al., 2013; Rufino et al., 2014), *Candida sphaerica* (Sobrinho et al., 2013a; Luna et al., 2015), *Candida utilis* (Campos et al., 2013), *Candida guilliermondii* (Sitohy et al., 2010), *Candida antarctica* (Kim et al., 2002; Hua et al., 2003), and *Candida tropicalis* (Batista et al., 2010; Priji et al., 2013) are known to produce biosurfactant.

Biosurfactants offer a number of advantages over chemical surfactants, such as better biodegradability, environmental compatibility, low toxicity and highly specific activity under extreme conditions of temperature, pH and salinity (Banat et al., 2010). Despite the advantages, biosurfactants are not yet competitive with their synthetic counterparts due to the high production costs. Therefore, most commercially available surfactants are synthesized from the petrochemical industry, which currently accounts for 70–75% of all surfactants used in industrialized nations (Campos et al., 2013). However, with the increased awareness among consumers for environmentally friendly compounds, industries are presently seeking to replace some or all chemical surfactants with sustainable biosurfactants (Marchant and Banat, 2012; Sekhon et al., 2012). As a result, the global market for biosurfactants has been growing in recent years. For instance, in 2011, the worldwide biosurfactant market was worth approximately US$ 1.7 billion, which is expected to reach US$ 2.2 billion by the year 2018 (Sekhon et al., 2012).

The development of economical processes for biosurfactant production has become the key factor to reducing costs and increasing competitiveness. Industrial wastes have attracted considerable interest from researchers as low-cost substrates for this purpose, as the substrate generally accounts for up to 50% of the final production cost (Das et al., 2009; Rufino et al., 2014). Residues, such as corn steep liquor (Silva et al., 2013), glycerol (Silva et al., 2010), clarified cashew apple juice (Oliveira and Garcia-Cruz, 2013), vinasse (Oliveira et al., 2013), cassava wastewater (Barros et al., 2008), soybean oil refinery residue (Luna et al., 2011a), ground-nut oil refinery residue (Sobrinho et al., 2008), animal fat (Santos et al., 2013), vegetable fat (Gusmão et al., 2010), waste frying oil (Batista et al., 2010), and molasses (Santos et al., 2010) have demonstrated excellent results when used for biosurfactant production by microorganisms.

Response surface methodology (RSM) has been effectively employed to reduce the production cost of biosurfactants through the selection of balanced proportions of the constituents of the culture medium and the optimization of culture conditions (Najafi et al., 2011; Kim and Kim, 2013; Silva et al., 2013; Kumar et al., 2015). RSM constitutes a collection of statistical techniques for designing experiments, building models, simultaneously evaluating the effects of factors and establishing optimum conditions. A central composite rotational design (CCRD) is used with RSM to examine the relationship between one or more response variables and set of quantitative experimental factors (Kim and Kim, 2013). As the correlation between the response and independent variables is generally unknown at the onset of a process, the first step in RSM is to approximate the function (response) by analyzing the factors (independent variables) (Najafi et al., 2010; Silva et al., 2013).

Large-scale biosurfactant production seems to be an effective strategy to overcome the competitiveness with their synthetic counterparts. There is a lack of fundamental knowledge about biosurfactant production scaling up. 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. The use of bioreactors becomes an alternative even more attractive and promising when associated to previous optimization of the culture condition using shake flasks to reduce the cost and process time. Then the scale up in bioreactors will lead to an operational facilitation in the implementation of the industrial-scale production (Mukherjee et al., 2006; Amani et al., 2010; Chikere et al., 2012; Luna et al., 2015).

The aim of the present study was to optimize biosurfactant production by the yeast *C. tropicalis* UCP0996 in flask experiments using a combination of CCRD and RSM and test the best response in large scale conditions. The biosurfactant ability as an oil dispersant was also evaluated.

MATERIALS AND METHODS

Materials

All chemicals were reagent grade. Growth media were purchased from Difco Laboratories (USA). 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. Sugarcane molasses was obtained from a local sugar mill in the municipality of Vitória de Santo Antão, state of Pernambuco, Brazil. Corn steep liquor is 21–45% protein, 20–26% lactic acid, 8% ash (containing Ca^{2+}, Mg^{2+}, K^{+}), 3% sugar and has low fat content (0.9–1.2%), while sugarcane molasses is 75% dry matter, 9–12% non-sugar organic matter, 2.5% protein, 1.5–5.0% potassium and 1% magnesium, phosphorus, and calcium (Santos et al., 2016). Canola waste frying oil is composed by the following fatty acids: 53–70% oleic acid, 15–36% linoleic acid, and, 5–13% linolenic acid. Seawater was collected near the Suape Port, located in the municipality of Cabo de Santo Agostinho, in Pernambuco state, Brazil. Water samples were collected and stored in plastic bottles of 5 L.

Yeast Strain and Preparation of Inoculum

A strain of *C. tropicalis* UCP0996 was provided from the culture collection of the Catholic University of Pernambuco, Recife city, Pernambuco, Brazil. The microorganism was maintained at 5°C on yeast mold agar (YMA) slants containing (w/v) yeast extract (0.3%), malt extract (0.3%), tryptone (0.5%), D-glucose (1.0%), and agar (5.0%). Transfers were made to fresh agar slants each month to maintain viability. Inoculum was prepared by transferring cells grown on a slant to 250-ml Erlenmeyer flasks containing 50 ml of yeast mold broth (YMB). The initial pH of YMA and YMB media was adjusted to 5.5. The cultivation conditions for the seed culture were 28°C, 200 rpm and 24 h of incubation.

Production Medium

The composition of the production medium varied according to the experimental design described below. Sugarcane molasses, corn steep liquor and canola waste frying oil were dissolved in distilled water and the pH was adjusted to 5.5 by the addition of 1 M NaOH solution or 1 M HCl solution. The medium was sterilized by autoclaving at 121°C for 20 min. Aliquots of the YMB suspension containing 10^8 cells/mL of *C. tropicalis* UCP0996 (varying in accordance with the experimental design) were used to inoculate 500-mL Erlenmeyer flasks containing 100-mL of production medium. The surface tension of the production medium before oil addition and inoculation was 55 mNm^{-1}. Cultivation was carried out at 28°C with agitation at 200 rpm for 120 h in a Marconi MA832 shaker (Marconi LTDA, Brazil). No adjustment of pH was performed during cultivation. At the end of fermentation, samples were taken from the liquid culture to determine the surface tension and biosurfactant yield, as described below.

Optimization of Biosurfactant Production Using RSM

A CCRD was used to determine the effects and interactions of four factors for biosurfactant production. Sugarcane molasses, corn steep liquor and waste frying oil concentrations and inoculum size were the independent variables. Surface tension and biosurfactant yield were the response variables. In this design, a set of 30 experiments was performed, with four replicates at the central points. The statistical analysis of the four replicates gives an indication of the experimental error of the production technique. The range and levels of the components (factors or independent variables) are given in **Table 1**. Each factor in the design was studied on five levels (−2.0,−1.0, 0,+1, and +2), with zero as the central coded value. These levels were based on results obtained in preliminary experiments. The optimum values from the CCRD were obtained by solving the regression equation and analyzing the response surface contour plots. Analysis of variance (ANOVA) with 95% confidence intervals was used to determine the significance of the effects. ANOVA, the determination of regression coefficients and the construction of graphs were performed with the aid of the Statistica® program, version 12.0.

TABLE 1 | Experimental ranges and levels of independent variables for central composite rotational design used in optimization of biosurfactant production by *C. tropicalis* UCP 0996.

Variables	Range and levels				
	−2	−1	0	+1	+2
Sugarcane molasses (%), X_1	2	2.5	3	3.5	4
Corn steep liquor (%), X_2	2	2.5	3	3.5	4
Waste frying oil (%), X_3	2	2.5	3	3.5	4
Inoculum size (%), X_4	1	2	3	4	5

Determination of Surface Tension

Surface tension was determined in the cell-free broth obtained by centrifuging the cultures at 5000 × g for 20 min. Surface tension was determined with a Tensiometer (Sigma 700, KSV Instruments Ltd., Finland), using the Du Nouy ring method at room temperature.

Scale up of Biosurfactant Production

Scale up of biosurfactant production was carried out in both 2.0 L bioreactor TEC-BIO (TECNAL, Brazil) and 50 L bioreactor MA 502/50 L (Marconi LTDA, Brazil), containing 1.0 and 25 L production medium, respectively. The substrates used as carbon and nitrogen sources, as well as the inoculum size employed were selected from results obtained by the previous experimental planning. The culture medium was aseptically inoculated with a 24 h inoculum. Initial pH was adjusted to 5.5. Both bioreactors were kept under controlled conditions: aeration rate 1.0 $vv^{-1}m^{-1}$ and 200 rpm mechanical agitation for 120 h at 28°C. At the end of fermentation, samples were collected from the liquid culture to check surface tension and biosurfactant yield.

Isolation of Biosurfactant

Biosurfactant was extracted from the cell-free broth following cell removal by centrifugation at 5000 × g for 20 min and filtered through Whatman no.1 filter paper. Any trace of remaining oil in the metabolic broth was removed before the extraction process. An equal volume of $CHCl_3/CH_3OH$ (2:1) was placed with 50 mL of the cell-free broth in a separatory funnel at 28°C. 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).

Evaluation of the Biosurfactant as Dispersant

A visual test was used for determination of the dispersant effectiveness of the biosurfactant. Motor oil samples of 5, 10, 20, 40, 80, and 100 μL were carefully added to the surface of seawater (20 mL) contained in a beaker until create 1 cm of deep vortex by slow magnetic stirring. Then 5.0 μL of the cell-free broth or isolated biosurfactant was added to the center of the vortex and stirred to a maximum rate of 2000 rpm over a 1 min period. Deep

TABLE 2 | Experimental deign matrix for optimization of biosurfactant optimization produced by *C. tropicalis* UCP0996 according to CCRD.

Runs	Sugarcane molasses (%), X_1	Corn steep liquor (%), X_2	Waste frying oil (%), X_3	Inoculum size (%), X_4	Surface tension (mNm^{-1}), Y_1	Biosurfactant yield (gL^{-1}), Y_2
1	−1.0	−1.0	−1.0	−1.0	29.98	4.19
2	−1.0	−1.0	−1.0	1.0	35.07	2.43
3	−1.0	−1.0	1.0	−1.0	32.53	2.90
4	−1.0	−1.0	1.0	1.0	34.20	2.63
5	−1.0	1.0	−1.0	−1.0	30.66	3.21
6	−1.0	1.0	−1.0	1.0	34.28	2.33
7	−1.0	1.0	1.0	−1.0	31.49	3.03
8	−1.0	1.0	1.0	1.0	31.23	3.06
9	1.0	−1.0	−1.0	−1.0	31.76	3.05
10	1.0	−1.0	−1.0	1.0	36.76	1.55
11	1.0	−1.0	1.0	−1.0	34.63	2.44
12	1.0	−1.0	1.0	1.0	35.31	2.31
13	1.0	1.0	−1.0	−1.0	32.35	2.88
14	1.0	1.0	−1.0	1.0	35.87	1.68
15	1.0	1.0	1.0	−1.0	33.04	2.70
16	1.0	1.0	1.0	1.0	32.66	2.95
17	−2.0	0.0	0.0	0.0	32.12	3.13
18	2.0	0.0	0.0	0.0	35.35	2.10
19	0.0	−2.0	0.0	0.0	33.86	2.77
20	0.0	2.0	0.0	0.0	31.96	2.89
21	0.0	0.0	−2.0	0.0	32.95	2.76
22	0.0	0.0	2.0	0.0	32.28	2.90
23	0.0	0.0	0.0	−2.0	31.16	3.18
24	0.0	0.0	0.0	2.0	35.84	1.84
25	0.0	0.0	0.0	0.0	30.10	3.42
26	0.0	0.0	0.0	0.0	30.12	3.44
27	0.0	0.0	0.0	0.0	30.13	3.39
28	0.0	0.0	0.0	0.0	30.14	3.40
29	0.0	0.0	0.0	0.0	30.09	3.38
30	0.0	0.0	0.0	0.0	30.11	3.41

Mean and standard deviation for replicate experiments at the central point for: Surface tension, 30.12 ± 0.02 mNm^{-1} and Biosurfactant yield, 3.41 ± 0.02 gL^{-1}.

vortex was created as follows: a magnetic bar was placed inside the beaker containing sea water. The beaker was then placed on a stirring plate, connecting the equipment and increasing stirring of the magnetic bar that when rotating, creates the vortex. The proportions of motor oil added and biosurfactant resulted in biosurfactant/oil ratios of 1/1, 1/2, 1/4, 1/8, 1/16, and 1/20 (v/v). Oil dispersion level was visually estimated after 1 min rest and classified as A, B, C, D, and E to 100% oil dispersion (without any oil surface layer), 75% oil dispersion (with discrete layer on the seawater surface), 50% oil dispersion, 25% oil dispersion and without dispersion, respectively (Sobrinho et al., 2013a).

RESULTS AND DISCUSSION

Optimization of Biosurfactant Production Using RSM

The CCRD matrix and corresponding results are given in **Table 2**. Multiple regression analysis using RSM was performed to fit the response function to the experimental data and investigate the simultaneous influence of the four variables selected. The best condition for biosurfactant production was found in Run 1 for both response variables, as the lowest surface tension coincided with the highest biosurfactant yield. This occurred using the minimum values of the independent variables X_1, X_2, X_3, and X_4.

The application of RSM for the estimation of the optimum parameters resulted in an empirical relationship between surface tension and the process variables. The following quadratic polynomial equation best fit the data:

$$\begin{aligned} Y_1 = {} & 30.115 + 0.80833X_1 - 0.51917X_2 - 0.12417X_3 \\ & + 1.17917X_4 - 0.02625X_1X_2 - 0.035X_1X_3 - 0.08125X_1X_4 \\ & - 0.49X_2X_3 - 0.37125X_2X_4 - 0.97X_3X_4 + 0.91313X_1^2 \\ & + 0.70688X_2^2 + 0.63313X_3^2 + 0.85438X_4^2, \end{aligned} \quad (1)$$

in which Y_1 is surface tension (mNm^{-1}) and X_1, X_2, X_3, and X_4 are coded values for sugarcane molasses, corn steep liquor, waste frying oil and inoculum size, respectively.

TABLE 3 | Analysis of variance for response surface quadratic model regarding surface tension achieved with biosurfactant produced by *C. tropicalis* UCP0996[a].

Factor	Sum of squares	Degrees of freedom	Mean square	F-ratio	*p*-value[b]
X_1 (L) [c]	15.6817	1	15.68167	44804.76	0.000000
X_1 (Q) [d]	22.8699	1	22.86987	65342.48	0.000000
X_2 (L)	6.4688	1	6.46882	18482.33	0.000000
X_2 (Q)	13.7053	1	13.70530	39157.99	0.000000
X_3 (L)	0.3700	1	0.37002	1057.19	0.000001
X_3 (Q)	10.9947	1	10.99467	31413.34	0.000000
X_4 (L)	33.3704	1	33.37042	95344.05	0.000000
X_4 (Q)	20.0217	1	20.02167	57204.77	0.000000
X_1 (L) × X_2 (L)	0.0110	1	0.01103	31.50	0.002484
X_1 (L) × X_3 (L)	0.0196	1	0.01960	56.00	0.000673
X_1 (L) × X_4 (L)	0.1056	1	0.10563	301.79	0.000012
X_2 (L) × X_3 (L)	3.8416	1	3.84160	10976.00	0.000000
X_2 (L) × X_4 (L)	2.2052	1	2.20523	6300.64	0.000000
X_3 (L) × X_4 (L)	15.0544	1	15.05440	43012.57	0.000000
Lack of Fit	0.2074	10	0.02074	59.25	0.000147
Pure Error	0.0018	5	0.00035	–	–
Total square sum	125.2933	29	–	–	–

[a] R^2, 0.99833; adjusted R^2 = 0.99677.
[b] $p \leq 0.05$–significant at 5% level.
[c] (L), linear effect.
[d] (Q), quadratic effect.

ANOVA was performed to validate the quadratic model (**Table 3**). ANOVA is crucial to testing the significance and acceptability of a model. As can be seen from the table, linear and quadratic terms as well as their interactions were all statistically significant ($p < 0.05$) and the F-value (with a 95% confidence interval) was much larger than 4 for each variable and respective interaction. The very low pure error (0.0018) indicated excellent reproducibility of the experimental data. The concentration of the inoculum was the most important factor to the reduction in the surface tension, followed by quadratic term of sugarcane molasses concentration. The correlation between inoculum size and surface tension demonstrates that a minor amount of inoculum is more suitable for reducing surface tension within the experimental limits chosen. Moreover, the correlation coefficient ($R^2 = 0.99833$) indicates that <1% of the total variation could not be explained by the empirical model. Therefore, the regression model was significant and could adequately be used to describe biosurfactant production.

The predicted vs. the actual plot for surface tension determined by the model equation demonstrated that observed values were distributed near the straight line (**Figure 1**), which indicates that such values were very close to the predicted values ($R^2 = 0.99833$). Hence, the model proved to be suitable for the prediction of biosurfactant production under the experimental conditions.

Figure 2 shows the 3D plots for minimum surface tension (i.e., maximum biosurfactant production) to visualize the interactions of the independent variables, two by two. **Figure 2A** indicates that a decrease in sugarcane molasses and corn steep liquor near the middle level (with a weak increase from this point) led to a decrease in surface tension. **Figure 2B** shows that the lowest surface tension was obtained when sugarcane molasses was decreased from the middle level to the minimum level and waste frying oil value was near the middle level. **Figure 2C** shows that the combination of minimum sugarcane molasses and inoculum size led to a reduction in surface tension. **Figure 2D** displays a very well-delimited region reflecting the optimized conditions of biosurfactant production with corn steep liquor near the middle level (with a weak increase from this point) and waste frying oil at the middle level. **Figure 2E** shows that the lowest surface tension was reached when the inoculum size was at the middle level with a weak decreased from this point and corn steep liquor was around the middle level. The combination of minimum inoculum size and waste frying oil concentration (**Figure 2F**) led to a decrease in surface tension. The elliptical nature of the contour plots presented in **Figures 2C–F** indicates a high degree of interaction among the factors in each surface response plot analyzed, i.e., it is not possible to predict the biosurfactant properties by modifying only one of the factors studied in each surface response plot. On the other hand, the level curves in **Figures 2A,B** demonstrate considerable parallelism among the factors and, consequently, a weak interaction, i.e., it is possible to predict surface tension from variations in only of one of the factors in each response surface plot.

Response surface methodology (RSM) was also applied to construct an empirical model for modeling biosurfactant yield and the process variables. The following quadratic polynomial equation best fit the data:

$$\begin{aligned} Y_2 = {} & 3.406667 - 0.261667X_1 + 0.024167X_2 + 0.040833X_3 \\ & - 0.339167X_4 + 0.08625X_1X_2 + 0.11125X_1X_3 \\ & + 0.01875X_1X_4 + 0.16125X_2X_3 + 0.11625X_2X_4 \\ & + 0.32625X_3X_4 - 0.195833X_1^2 - 0.142083X_2^2 \\ & - 0.142083X_3^2 - 0.222083X_4^2, \end{aligned} \quad (2)$$

in which Y_2 is biosurfactant yield (gL^{-1}) and X_1, X_2, X_3, and X_4 are coded values for sugarcane molasses, corn steep liquor, waste frying oil and inoculum size, respectively.

The evaluation of the empirical model was performed using ANOVA (**Table 4**). The p- and F-value (with 95% confidence interval) indicate that all terms were statistically significant ($p < 0.05$; $F > 4$). Reproducibility of the experimental data was once again proven by the relatively low pure error (0.002333). Inoculum concentration was the most important factor to the increase in biosurfactant yield, followed by sugarcane molasses concentration. The explained variance ($R^2 = 0.98927$) ensured adequate fit ($R = 0.97925$) and predicted vs. actual values were distributed very close to the straight line (**Figure 3**), thereby validating the forecast model.

Figure 4 displays the three-dimensional plots for biosurfactant yield. The interactions between the variables

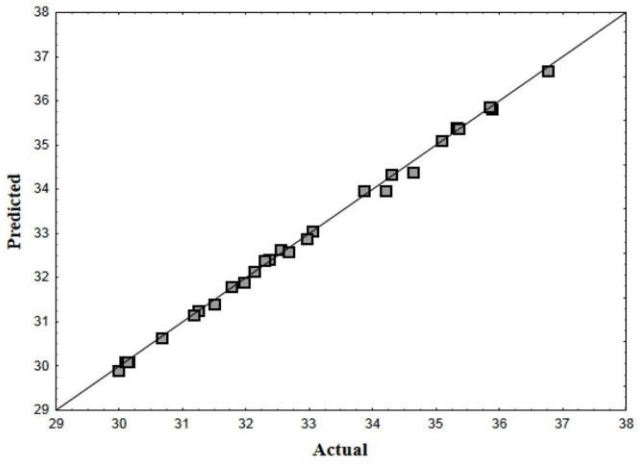


FIGURE 1 | Plot of predicted vs. actual surface tension achieved using biosurfactant produced by *C. tropicalis* UCP 0996.

shown in **Figures 4A–F** are very similar to those found in **Figures 2A–F** for surface tension. Biosurfactant yield increased when sugarcane molasses decreased and corn steep liquor remained near the middle level (**Figure 4A**). The curves in this figure demonstrate considerable parallelism between the factors and, consequently, a weak interaction. A similar effect was found in the relationship between sugarcane molasses and waste frying oil (**Figure 4B**). The combination of minimum sugarcane molasses concentration and inoculum size (**Figure 4C**) led to an increase in biosurfactant yield. The curves indicate a reasonable interaction. Around middle level corn steep liquor and inoculum size with a weak decreased from this point produced satisfactory biosurfactant yield, with a strong interaction between the variables (**Figure 4D**). **Figure 4E** shows that biosurfactant yield increased when corn steep liquor and inoculum size decreased tending toward the minimum levels. The elliptical nature of the contour plots indicates a high degree of interaction between the factors. **Figure 4F** shows that the combination of minimum inoculum size and minimum waste frying oil led to the best biosurfactant yield under the conditions tested (local maximum in the region studied). The very sinuous curves indicate the high degree of interaction between these factors. It is important to consider that higher substrate concentrations did not necessarily stimulated the biosurfactant production. This can be explained by the fact that when the substrate is present in high concentrations it can be directed to the production of cellular biomass. RSM demonstrated that the interaction between the three substrates (sugarcane molasses, waste frying oil and corn steep liquor) should be considered simultaneously, rather than individually. Regarding the inoculum size, the results showed that the minimum inoculum size favored the production of the biosurfactant. The higher number of cells may increase the competition for the nutrients for cell maintenance rather than the production of biosurfactant (Ghribi and Ellouze-Chaabouni, 2011; Onwosi and Odibo, 2013; Nalini and Parthasarathi, 2014). Inoculum size has demonstrated high influence on biosurfactant production in previous studies and can have a definite effect on economics of a microbial process (Mnif et al., 2013; Onwosi and Odibo, 2013). The CCRD conducted by Lima and Souza (2014) showed that the inoculum concentration was the factor that had the greatest effect on biosurfactant production by *Bacillus subtilis* PC, providing the best surface tension values of the 32 mNm^{-1}. In another study, Ghribi and Ellouze-Chaabouni (2011) studied the effect of inoculum size on biosurfactants production by *B. subtilis* SPB1 where adequate inoculum size reached lipopeptide biosurfactants production of 2.04 gL^{-1}. Kiran et al. (2010) also found that a new glycolipid biosurfactant from marine *Nocardiopsis lucentensis* MSA04 was critically controlled by inoculum size by either independently and/or interactively with others studied variables.

Few studies have been conducted using sugarcane molasses as the carbon source in the cultivation of microorganisms for biosurfactant production. In a study conducted by Santos et al. (2010), biosurfactants were synthesized by *Pseudomonas aeruginosa* (P.A.) using sugarcane molasses as carbon and energy source in a concentration of 3.0%. The results showed a maximum rhamnolipid production of 4.47 gL^{-1}. Daverey and Pakshirajan (2009) obtained minimum surface tension of 34.15 mNm^{-1} by production of sophorolipid when cultivated the yeast *C. bombicola* in a cheap fermentative medium containing sugarcane molasses. Joshi et al. (2008) also tested the biosurfactant production using sugarcane molasses for cultivation of *Bacillus licheniformis* K51, *B. subtilis* 20B, *B. subtilis* R1, and *Bacillus* strain HS3, which had surface

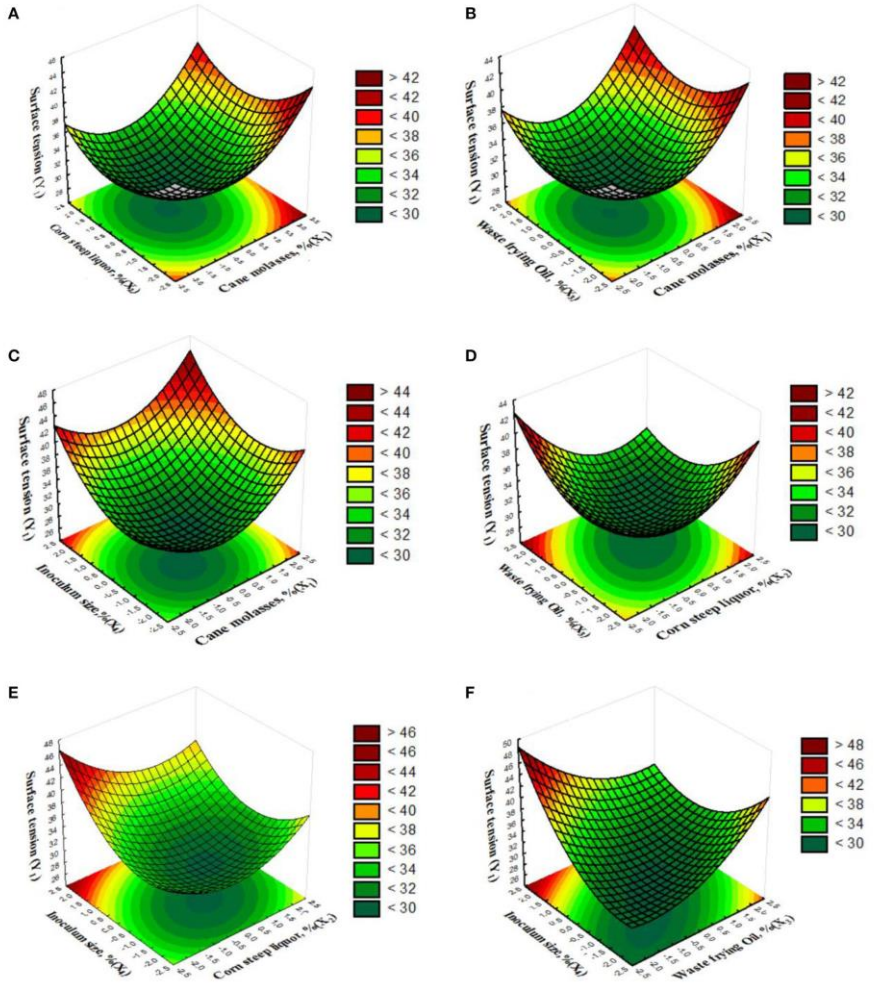


FIGURE 2 | Response surface plots and contour plots for minimum surface tension generated using data in Table 3. Inputs, 30 experimental runs carried out under conditions established by CCRD; reduction in surface tension as function of **(A)** sugarcane molasses and corn steep liquor; **(B)** sugarcane molasses and waste frying oil; **(C)** sugarcane molasses and inoculum size; **(D)** corn steep liquor and waste frying oil; **(E)** corn steep liquor and inoculum size; **(F)** waste frying oil and inoculum size.

tension values of 29.67, 29.33, 30.33, and 30.67 mNm^{-1}, respectively.

On the other hand, the use of corn steep liquor as the nitrogen source for biosurfactant produced by microorganisms has been widely studied (Luna et al., 2013; Silva et al., 2013, 2014a; Campos et al., 2014; Santos et al., 2014). In a study by Luna et al. (2013), the utilization of corn steep liquor as low cost constituent to *C. sphaerica* growth resulted in reducing the surface tension of cell-free broth to 25 mNm^{-1} and biosurfactant yield was 9 gL^{-1}. Corn steep liquor also was used by Santos et al. (2013) as a constituent of low cost for the cultivation of *C. lipolytica* UCP0988, obtaining a reduction in surface tension from 50 to 28 mNm^{-1}. Rocha e Silva et al. (2014) used 2.0% corn steep liquor as nitrogen source on *Pseudomonas cepacia* medium growth and as a result,

TABLE 4 | Analysis of variance (ANOVA) for response surface quadratic model of biosurfactant yield by *C. tropicalis* UCP0996[a].

Factor	Sum of squares	Degrees of freedom	Mean square	F-ratio	*p*-value[b]
X_1 (L)[c]	1.643267	1	1.643267	3521.286	0.000000
X_1 (Q)[d]	1.051905	1	1.051905	2254.082	0.000000
X_2 (L)	0.014017	1	0.014017	30.036	0.002758
X_2 (Q)	0.553719	1	0.553719	1186.541	0.000000
X_3 (L)	0.040017	1	0.040017	85.750	0.000247
X_3 (Q)	0.553719	1	0.553719	1186.541	0.000000
X_4 (L)	2.760817	1	2.760817	5916.036	0.000000
X_4 (Q)	1.352805	1	1.352805	2898.867	0.000000
X_1 (L) × X_2 (L)	0.119025	1	0.119025	255.054	0.000018
X_1 (L) × X_3 (L)	0.198025	1	0.198025	424.339	0.000005
X_1 (L) × X_4 (L)	0.005625	1	0.005625	12.054	0.017814
X_2 (L) × X_3 (L)	0.416025	1	0.416025	891.482	0.000001
X_2 (L) × X_4 (L)	0.216225	1	0.216225	463.339	0.000004
X_3 (L) × X_4 (L)	1.703025	1	1.703025	3649.339	0.000000
Lack of Fit	0.102192	10	0.010219	21.898	0.001638
Pure Error	0.002333	5	0.000467	–	–
Total square sum	9.740750	29	–	–	–

[a] R^2, 0.98927; adjusted R^2, 0.97925.
[b] $p \leq 0.05$–significant at 5% level.
[c] (L), linear effect.
[d] (Q), quadratic effect.

it was obtained surface tension of 27.57 mNm^{-1} at the end of the cultivation e yield of 5.2 gL^{-1} of isolated biosurfactant.

Microorganisms have been also studied using waste frying oil as the carbon source. Batista et al. (2010) report a maximum biosurfactant yield of 3.61 gL^{-1} by *C. tropicalis* using waste frying oil as substrate, with a 33.66 mNm^{-1} reduction in surface tension. Oliveira and Garcia-Cruz (2013) tested different concentrations waste frying oil as alternative carbon sources on *Bacillus pumilus* cultivation, which obtained surface tension reduction of 45 mNm^{-1} and maximum crude biosurfactant production of 5.7 gL^{-1} for waste frying oil, in concentration of 5%. Using waste frying oil as substrate for biosurfactant production by *C. tropicalis* CECT 1440, Haba et al. (2000) found a reduction of 35 mNm^{-1} in mean surface tension.

A statistical correlation test was used to determine the association between surface tension (Y_1) and biosurfactant yield (Y_2) in the CCRD (**Table 2**). A strong negative correlation was found between Y_1 and Y_2 (Pearson's bivariate correlation coefficient: $r = -0.95$, $p < 0.0001$) (**Figure 5**), i.e., an increase in biosurfactant concentration resulted in a decrease in surface tension in all experiments performed. Determination coefficient (R^2) for both models (**Tables 3, 4**) was very close to 1, which means that either model can be applied to explain the variability between experimental and predicted values in its respective responses (Y_1 or Y_2). The very low coefficient of variation (CV) for surface tension (CV = 6.38%) and reasonably low CV for biosurfactant yield (CV = 20.37%) indicate a high degree of precision and adequate reliability of the experimental

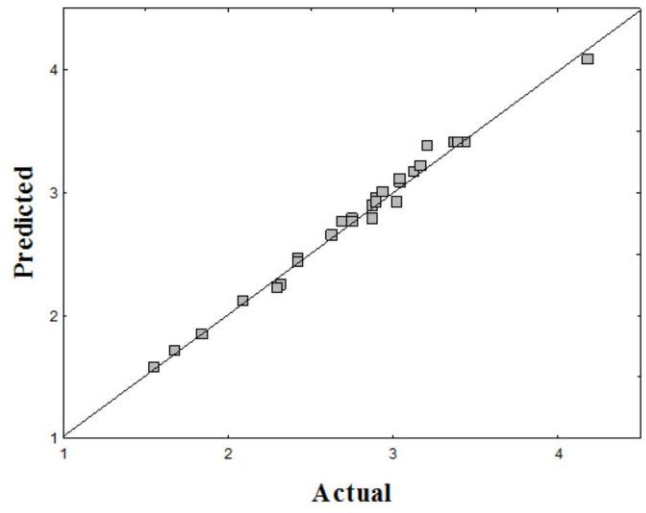


FIGURE 3 | Predicted vs. actual biosurfactant yield by *C. tropicalis* UCP 0996.

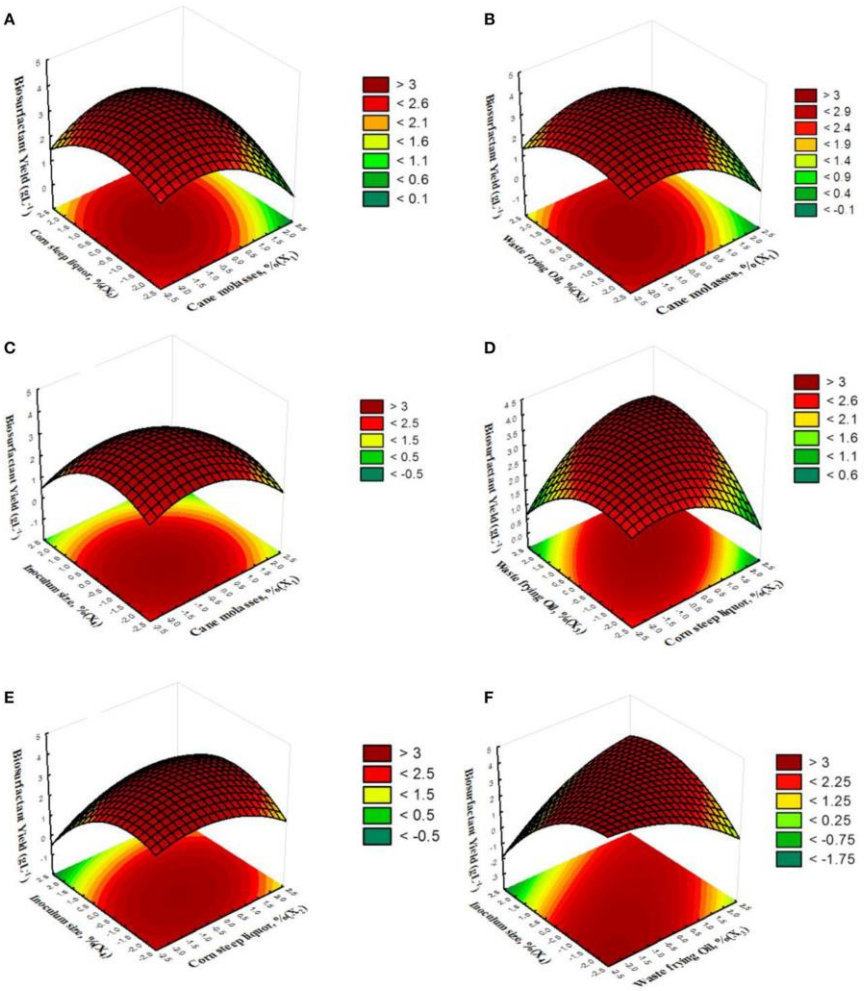


FIGURE 4 | 3D response surface curve of interaction of sugarcane molasses and corn steep liquor (A) , sugarcane molasses and waste frying oil **(B)**, sugarcane molasses and inoculum size **(C)**, corn steep liquor and waste frying oil **(D)**, corn steep liquor and inoculum size **(E)**, and waste frying oil and inoculum size **(F)** on biosurfactant yield.

values for both Y_1 and Y_2, although the CV for Y_2 was much smaller. The use of statistical models to enhance the fermentation process has been increasing in current biotechnology, due to the applicability and suitability of this approach (Zhu et al., 2013).

As the high production cost is the major drawback regarding biosurfactants, economical processes that employ low-cost materials at the key to successful production (Rufino et al., 2014; Silva et al., 2014b). Analyzing the effectiveness of the process with respect to production costs, the conditions established in Run 1 of the present study proved to be the most suitable, since the constituents of the medium (sugarcane molasses, corn steep liquor, waste frying oil and inoculum) were used at their lowest concentrations (−1). Therefore, the use of industrial byproducts

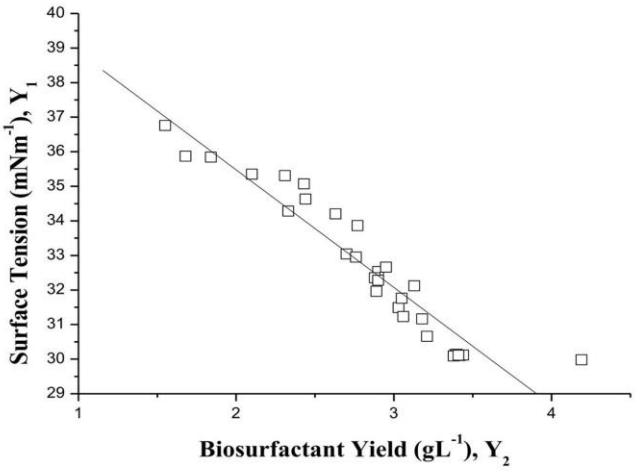


FIGURE 5 | Comparative parity plot of surface tension (Y_1) and biosurfactant yield (Y_2) based on correlation coefficients.

proved to be a promising way to reduce biosurfactant production costs.

Previous studies have already demonstrated the efficiency of the dual application of industrial wastes and RSM in enhancing of economic production of biosurfactant by microorganisms. High yields in biosurfactants have been reached through the combination between a vegetable oil and carbohydrate as substrate (Santos et al., 2016). Regarding the use of waste frying vegetal oils, isolated or combined with a soluble substrate, promising results have been obtained in the last decades for *Candida* species in our laboratories (Sarubbo et al., 1999, 2001, 2006, 2007; Coimbra et al., 2009; Campos et al., 2014). Thus, waste frying oil and molasses were used as the insoluble and soluble carbon sources, respectively, while corn steep liquor was used as the nitrogen source. Luna et al. (2011b) reported the utilization of a statistical experimental design and RSM to optimize the concentrations of two agro-industrial residues, soybean oil and corn steep liquor, for biosurfactant production by *C. sphaerica* UCP0995, reaching a minimum surface tension of 25.25 mNm^{-1}. Similarly, Silva et al. (2013) also reported the utilization of a CCRD and RSM to biosurfactant production from a new strain *Pseudomonas cepacia* CCT6659 cultivated in a low cost medium formulated with 2% waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$ allowed to achieve a maximal reduction in surface tension of 26 mNm^{-1} and biosurfactant yield of 8.0 gL^{-1}. In another study conducted by Mnif et al. (2012), a central composite design and RSM were employed to increase the yield of a lipopeptide biosurfactant produced by *B. subtilis* SPB1 using also low-cost substrates, where was obtained biosurfactant yield about 4.5 gL^{-1} with an optimal medium composed of sesame peel flour (33 gL^{-1}) and diluted tuna fish cooking residue (40%).

TABLE 5 | Surface tension and biosurfactant yield evaluation of the biosurfactant from *C. tropicalis* UCP0996 grown in distillated water supplemented with 2.5% sugarcane molasses, 2.5% waste frying oil, and 2.5% corn steep liquor in bioreactors.

Vessel type	Biosurfactant yield (gl^{-1})	Surface tension (mnm^{-1})
2-L bioreactor	5.87 ± 0.21	34.12 ± 0.07
50-L bioreactor	7.36 ± 0.34	35.6 ± 0.05

Results are expressed as means ± standard deviations of values obtained from triplicate experiments.

Based on the results of the CCRD (ANOVA, **Tables 3, 4**), the condition performed with 2.5% sugarcane molasses, 2.5% corn steep liquor, 2.5% waste frying oil, and 2% inoculum size (Run 1) was selected for biosurfactant production. Among the variables studied, the most significant in both models were inoculum size and sugarcane molasses, respectively.

Scale Up of Biosurfactant Production

To confirm the efficiency of the statistically optimized variables, experiments in bench-scale and pilot bioreactors were carried out using the optimum concentrations of the variables in Run 1. **Table 5** displays the results of the biosurfactant produced in bioreactors. As can be seen, the yield of the biosurfactant produced by *C. tropicalis* had an increase of 40 and 75% in values when produced in 2- and 50-L bioreactors, respectively, compared to the best results found in flasks fermentation (Run 1). This excellent result is probably due to the better control of aeration, agitation and temperature, once bioreactors are systems completely closed, favoring cell growth and a greater biosurfactant yield. In addition, the cultivation conducted in

TABLE 6 | Evaluation of the biosurfactant from *C.tropicalis* UCP0996 grown in distillated water supplemented with 2.5% sugarcane molasses, 2.5% waste frying oil, and 2.5% corn steep liquor as an oil dispersant.

Cell-free broth		Isolated biosurfactant	
Biosurfactant/ oil ratio	Dispersion classification	Biosurfactant/ oil ratio	Dispersion classification
1/1	C (50%)	1/1	B (75%)
1/2	C (50%)	1/2	C (50%)
1/4	D (25%)	1/4	D (50%)
1/8	D (25%)	1/8	D (25%)
1/16	D (25%)	1/16	D (25%)
1/20	E	1/2	E

shaker uses orbital shaking while the bioreactor uses mechanical agitation, where the oxygen supply is continuous, allowing better yields of the biosurfactant (Luna et al., 2015). In a similar study performed by Sobrinho et al. (2013b) *C. sphaerica* UCP 0995 grown in distilled water supplemented with 5.0% soybean oil refinery residue and 2.5% corn steep liquor presented a biosurfactant yield of 6.36 gL^{-1} after 144 h of culture in a 5-L bioreactor, whereas with the same culture medium Sobrinho et al. (2008) reported a yield of 4.5 gL^{-1} after 144 h of a culture of *C. sphaerica* UCP 0995 using flasks.

Biosurfactant produced by *C. tropicalis* decreased the surface tension of water from 72 to a minimum of 29. 98 mNm^{-1} when produced in shaker (Run 1). However, it had a remarkable variation in the surface tension when produced in bioreactors (**Table 5**). This same phenomenon was also observed on a recent study carried out by Luna et al. (2015) for *C. sphaerica* cultivated in a medium with 9.0% of refinery residue of soybean oil and 9% of corn steep liquor during 144 h. Shake-flask method reported a surface tension of 25 mNm^{-1} while in the bioreactor the surface tension was 27.48 mNm^{-1}. This may be related to the intrinsic effects of the scaling up, since the increase in volume promotes a consequent increase in the total surface area for complete saturation with biosurfactants, which explains this small variation observed in surface tension. When the issue is the production in large scale of biosurfactants, the use of bioreactors becomes the most promising alternative that will make the microbial production more favorable from the technical and economic points of view, compared to the limitations in relation to benchtop techniques (Marti et al., 2014; Luna et al., 2015).

Evaluation of the Biosurfactant as Dispersant

Many processes of the oil industry are carried out in the marine environment. Eventually, a part of the process oil reaches accidentally the seawater and, in turn, surfactants must be used in conjunction with other containment measures. Dispersion is a process by which a hydrocarbon is dispersed into the aqueous phase as very small emulsions. Dispersion is related to both the interfacial tension and surfactant concentration, and differs from displacement in that displacement process is only related to the interfacial tension between aqueous and hydrophobic phases and no emulsion formation (Almeida et al., 2016). In this study, the biosurfactant from *C. tropicalis* UCP0996 was tested as an oil dispersant in seawater. As a result, the maximum motor oil dispersion achieved by the biosurfactant produced under optimized conditions at a biosurfactant/oil ratio of 1/1 (v/v) was 50 and 75% for the cell-free broth and isolated biosurfactant, respectively (**Table 6**). Similar results were obtained by Sobrinho et al. (2013a) for the biosurfactant produced by *C. sphaerica* cultivated in 5% vegetal oil refinery waste and 2.5% corn steep liquor that showed an oil spreading efficiency of 75%.

CONCLUSION

The present study demonstrated the effectiveness of using a central composite rotational design to identify the optimum culture conditions for the production of biosurfactant from *C. tropicalis* UCP0996. The feasibility of the application of bioreactors and its combination with the use of industrial and agricultural wastes proved to be important tools toward a high yield and low cost for biosurfactant production, especially if the process is going to be implemented on an industrial scale and commercial application of this promising biosurfactant. The biomolecule exhibited considerable potential regarding the dispersion of oil from water surface demonstrating its potential application in the oil industry.

AUTHOR CONTRIBUTIONS

All authors contributed in this work. DA carried out the experiments. VS analyzed the data. LS designed the project. DA, RS, JL, RR, and LS wrote the manuscript. LS performed manuscript editing and final improvement.

FUNDING

This study received funding from the Brazilian fostering agencies the State of Pernambuco Foundation for the Assistance to Science and Technology (FACEPE); the Research and Development Program of the Brazilian National Electrical Energy Agency (ANEEL); the National Council for Scientific and Technological Development (CNPq), and the Federal Agency for the Support and Evaluation of Graduate Education (CAPES).

ACKNOWLEDGMENTS

The authors are grateful to the laboratories of the Centre for Science and Technology of the Catholic University of Pernambuco and the Advanced Institute of Technology and Innovation (IATI), Brazil.

REFERENCES

Almeida, D. G., Soares da Silva, R. C. F., Luna, J. M., Rufino, R. D., Santos, V. A., Banat, I. M., et al. (2016). Biosurfactants: promising molecules for petroleum biotechnology advances. *Front. Microbiol.* 7:1718. doi: 10.3389/fmicb.2016.01718

Amani, H., Mehrnia, M. R., Sarrafzadeh, M. H., Haghighi, M., and Soudi, M. R. (2010). Scale up and application of biosurfactant from *Bacillus subtilis* in enhanced oil recovery. *Appl. Biochem. Biotechnol.* 162, 510–512. doi: 10.1007/s12010-009-8889-0

Amaral, P. F., Coelho, M. A., Marrucho, I. M., and Coutinho, J. A. (2010). Biosurfactants from yeasts: characteristics, production and application. *Adv. Exp. Med. Biol.* 672, 236–249. doi: 10.1007/978-1-4419-5979-9_18

Banat, I. M., Franzetti, A., Gandolfi, I., Bestetti, G., Martinotti, M. G., Fracchia, L., et al. (2010). Microbial biosurfactants production, applications and future potential. *Appl. Microbiol. Biotechnol.* 87, 427–444. doi: 10.1007/s00253-010-2589-0

Barros, F. F. C., Quadros, C. P., and Pastore, G. M. (2008). Propriedades emulsificantes e estabilidade do biossurfactante produzido por *Bacillus subtilis* em manipueira. *Ciênc. Tecnol. Aliment.* 28, 979–985. doi: 10.1590/S0101-20612008000400034

Batista, R. M., Rufino, R. D., Luna, J. M., Souza, J. E. G., and Sarubbo, L. A. (2010). Effect of medium components on the production of a biosurfactant from *Candida tropicalis* applied to the removal of hydrophobic contaminants in soil. *Water Environ. Res.* 82, 418–425. doi: 10.2175/106143009X1248709 5237279

Campos, J. M., Stamford, T. L. M., and Sarubbo, L. A. (2014). Production of a bioemulsifier with potential application in the food industry. *Appl. Biochem. Biotechnol.* 172, 3234–3252. doi: 10.1007/s12010-014-0761-1

Campos, J. M., Stamford, T. L., Sarubbo, L. A., Luna, J. M., Rufino, R. D., and Banat, I. M. (2013). Microbial biosurfactants as additives for food industries. *Biotechnol. Prog.* 29, 1097–1108. doi: 10.1002/ btpr.1796

Chikere, C. B., Chikere, B. O., and Okpokwasili, G. C. (2012). Bioreactor-based bioremediation of hydrocarbon-polluted Niger Delta marine sediment, Nigeria. *3 Biotech.* 2, 53–66. doi: 10.1007/s13205-011-0030-8

Coimbra, C. D., Rufino, R. D., Luna, J. M., and Sarubbo, L. A. (2009). Studies of the cell surface properties of *Candida* species and relation with the production of biosurfactants for environmental applications. *Curr. Microbiol.* 58, 245–251. doi: 10.1007/s00284-008-9315-5

Das, P., Mukherjee, S., and Sen, R. (2009). Substrate dependent production of extracellular biosurfactant by a marine bacterium. *Bioresour. Technol.* 100, 1015–1019. doi: 10.1016/j.biortech.2008.07.015

Daverey, A., and Pakshirajan, K. (2009). Production, characterization, and properties of sophorolipids from the yeast *Candida bombicola* using a low-cost fermentative medium. *Appl. Biochem. Biotechnol.* 158, 663–674. doi: 10.1007/s12010-008-8449-z

Ghribi, D., and Ellouze-Chaabouni, S. (2011). Enhancement of *Bacillus subtilis* lipopeptide biosurfactants production through optimization of medium composition and adequate control of aeration. *Biotechnol. Res. Int.* 2011, 1–6. doi: 10.4061/2011/653654

Gusmão, C. A. B., Rufino, R. D., and Sarubbo, L. A. (2010). Laboratory production and characterization of a new biosurfactant from *Candida glabrata* UCP1002 cultivated in vegetable fat waste applied to the removal of hydrophobic contaminant. *World J. Microbiol. Biotechnol.* 26, 1683–1692. doi: 10.1007/s11274-010-0346-2

Haba, E., Espuny, M. J., Busquets, M., and Manresa, A. (2000). Screening and production of rhamnolipids *Pseudomonas aeruginosa* 47T2 NCIB 40044 from waste frying oils. *J. Appl. Microbiol.* 88, 379–387. doi: 10.1046/j.1365-2672.2000.00961.x

Hua, Z., Chena, J., Luna, S., and Wang, X. (2003). Influence of biosurfactants produced by *Candida antarctica* on surface properties of microorganism and biodegradation of n-alkanes. *Water Res.* 37, 4143–4150. doi: 10.1016/S0043-1354(03)00380-4

Joshi, S., Bharucha, C., Jhas, S., Yadav, S., Nerurkar, A., and Desai, A. J. (2008). Biosurfactant production using molasses and whey under thermophilic conditions. *Bioresour. Technol.* 99, 195–199. doi: 10.1016/j.biortech.2006.12.010

Kim, B., and Kim, J. (2013). Optimization of culture conditions for the production of biosurfactant by *Bacillus subtilis* JK-1 using response surface methodology. *J. Korean Soc. Appl. Biol. Chem.* 56, 279–287. doi: 10.1007/s13765-013-3044-6

Kim, H., Jeon, J., Lee, H., Park, Y., Seo, W., Oh, H., et al. (2002). Extracellular production of a glycolipid biosurfactant, mannosylerythritol lipid, from *Candida antarctica*. *Biotechnol. Lett.* 24, 225–229. doi: 10.1023/A:1014100422857

Kiran, G. S., Thomas, T. A., and Selvin, J. (2010). Production of a new glycolipid biosurfactant from marine *Nocardiopsis lucentensis* MSA04 in solid-state cultivation. *Colloids Surf. B Biointerfaces.* 78, 8–16. doi: 10.1016/j.colsurfb.2010.01.028

Kumar, A. P., Janardhan, A., Radha, S., Viswanath, B., and Narasimha, G. (2015). Statistical approach to optimize production of biosurfactant by *Pseudomonas aeruginosa* 2297. *3 Biotech.* 5, 71–79. doi: 10.1007/s13205-014-0203-3

Lima, A. M., and Souza, R. R. (2014). Use of sugar cane vinasse as substrate for biosurfactant production using *Bacillus subtilis* PC. *Chem. Eng. Trans.* 37, 673–678. doi: 10.3303/CET1437113

Luna, J. M., Raquel, D. R., Jara, A. A. T., Brasileiro, P. P. F., and Sarubbo, L. A. (2015). Environmental applications of the biosurfactant produced by *Candida sphaerica* cultivated in low-cost substrates. *Colloids Surf. A Physicochem. Eng. Asp.* 480, 413–418. doi: 10.1016/j.colsurfa.2014.12.014

Luna, J. M., Rufino, R. D., Albuquerque, C. D. C., Sarubbo, L. A., and Campos-Takaki, G. M. (2011b). Economic optimized medium for tensio-active agent production by *Candida sphaerica* UCP0995 and application in the removal of hydrophobic contaminant from sand. *Int. J. Mol. Sci.* 12, 2463–2476. doi: 10.3390/ijms12042463

Luna, J. M., Rufino, R. D., Sarubbo, L. A., and Campos–Takaki, G. M. (2013). Characterisation, surface properties and biological activity of a biosurfactant produced from industrial waste by *Candida sphaerica* UCP0995 for application in the petroleum industry. *Colloids Surf. B Biointerfaces.* 102, 202–209. doi: 10.1016/j.colsurfb.2012.08.008

Luna, J. M., Rufino, R. D., Sarubbo, L. A., Rodrigues, L. R. M., Teixeira, J. A. C., and Campos-Takaki, G. M. (2011a). Evaluation antimicrobial and antiadhesive properties of the biosurfactant lunasan produced by *Candida sphaerica* UCP0995. *Curr. Microbiol.* 62, 1527–1534. doi: 10.1007/s00284-011-9889-1

Luna, J. M., Santos, F. A., Rufino, R. D., and Sarubbo, L. A. (2016). Production of a biosurfactant from *Candida bombicola* URM 3718 for environmental applications. *Chem. Eng. Trans.* 49, 583–588. doi: 10.3303/CET1649098

Luna, J. M., Sarubbo, L. A., and Campos-Takaki, G. M. (2009). A new biosurfactant produced by *Candida glabrata* UCP 1002: characteristics of stability and application in oil recovery. *Braz. Arch. Biol. Technol.* 52, 785–793. doi: 10.1590/S1516-89132009000400001

Marchant, R., and Banat, I. M. (2012). Microbial biosurfactants: challenges and opportunities for future exploitation. *Trends Biotechnol.* 11, 558–565. doi: 10.1016/j.tibtech.2012.07.003

Marti, M. E., Colonna, W. J., Patra, P., Zhang, H., Green, C., Reznik, G., et al. (2014). Production and characterization of microbial biosurfactants forpotential use in oil-spill remediation. *Enzyme Microb. Tech.* 55, 31–39. doi: 10.1016/j.enzmictec.2013.12.001

Mnif, I., Ellouze-Chaabouni, S., and Ghribi, D. (2012). Economic production of *Bacillus subtilis* SPB1 biosurfactant using local agro-industrial wastes and its application in enhancing solubility of Diesel. *J. Chem. Tech. Biotech.* 88, 779–787. doi: 10.1002/jctb.3894

Mnif, I., Ellouze-Chaabouni, S., and Ghribi, D. (2013). Optimization of inocula conditions for enhanced biosurfactant production by *Bacillus subtilis* SPB1, in submerged culture, using Box–Behnken design. *Probiotics Antimicrob Proteins* 5, 92–98. doi: 10.1007/s12602-012-9113-z

Mukherjee, S., Das, P., and Sen, R. (2006). Towards commercial production of microbial surfactants. *Trends Biotechnol.* 24, 509–514. doi: 10.1016/j.tibtech.2006.09.005

Najafi, A. R., Rahimpour, M. R., Jahanmiri, A. H., Roostaazad, R., Arabian, D., and Ghobadi, Z. (2010). Enhancing biosurfactant production from an indigenous strain of *Bacillus mycoides* by optimizing the growth conditions using a response surface methodology. *Chem. Eng. J.* 163, 188–194. doi: 10.1016/j.cej.2010.06.044

Najafi, A. R., Rahimpour, M. R., Jahanmiri, A. H., Roostaazad, R., Arabian, D., Soleimani, M., et al. (2011). Interactive optimization of biosurfactant production by *Paenibacillus alvei* ARN63 isolated from an Iranian oil well. *Colloids Surf. B Biointerfaces.* 82, 33–39. doi: 10.1016/j.colsurfb.2010.08.010

Nalini, S., and Parthasarathi, R. (2014). Production and characterization of rhamnolipids produced by Serratia rubidaea SNAU02 under solid-state fermentation and its application as biocontrol agent. *Bioresour. Technol.* 173, 231–238. doi: 10.1016/j.biortech.2014.09.051

Oliveira, D. W. F., Franc, I. W. L., Félix, A. K. N., Martins, J. J. L., Giroa, M. E. A., Melo, V. M. M., et al. (2013). Kinetic study of biosurfactant production by *Bacillus subtilis* LAMI005 grown in clarified cashew apple juice. *Colloids Surf. B Biointerfaces.* 101, 34–43. doi: 10.1016/j.colsurfb.2012.06.011

Oliveira, J. G., and Garcia-Cruz, C. H. (2013). Properties of a biosurfactant produced by *Bacillus pumilus* using vinasse and waste frying oil as alternative carbon sources. *Braz. Arch. Biol. Technol.* 56, 155–160. doi: 10.1590/S1516-89132013000100020

Onwosi, C. O., and Odibo, F. J. C. (2013). Use of response surface design in the optimization of starter cultures for enhanced rhamnolipid production by *Pseudomonas nitroreducens*. *Afr. J. Biotechnol.* 12, 2611–2617. doi: 10.5897/AJB12.2635

Priji, P., Unni, K. N., Sajith, S., and Benjamin, S. (2013). *Candida tropicalis* BPU1, a novel isolate from the rumen of the Malabari goat, is a dual producer of biosurfactant and polyhydroxybutyrate. *Yeast* 30, 103–110. doi: 10.1002/yea.2944

Rocha e Silva, N. M. P., Rufino, R. D., Luna, J. M., Santos, V. A., and Sarubbo, L. A. (2014). Screening of *Pseudomonas* species for biosurfactant production using low-cost substrates. *Biocatal. Agric. Biotechnol.* 3, 132–139. doi: 10.1016/j.bcab.2013.09.005

Roelants, S. L., Saerens, K. M., Derycke, T., Li, B., Lin, Y. C., Van de Peer, Y., et al. (2013). *Candida bombicola* as a platform organism for the production of tailor-made biomolecules. *Biotechnol. Bioeng.* 110, 2494–2503. doi: 10.1002/bit.24895

Rufino, R. D., Luna, J. M., Campos-Takaki, G. M., and Sarubbo, L. A. (2014). Characterization and properties of the biosurfactant produced by *Candida lipolytica* UCP 0988. *Electronic J. Biotech.* 17, 34–38. doi: 10.1016/j.ejbt.2013.12.006

Santos, A. C., Bezerra, M. S., Pereira, H. S., Santos, E. S., and Macedo, G. R. (2010). Production and recovery of rhamnolipids using sugar cane molasses as carbon source. *J. Chem. Eng. Chem. Eng.* 4, 1–27.

Santos, D. K. F., Brandão, Y. B., Rufino, R. D., Luna, J. M., Salgueiro, A. A., Santos, V. A., et al. (2014). Optimization of cultural conditions for biosurfactant production from *Candida lipolytica*. *Biocatalysis Agric. Biotech.* 3, 48–57. doi: 10.1016/j.bcab.2014.02.004

Santos, D. K. F., Rufino, R. D., Luna, J. M., Santos, V. A., Salgueiro, A. A., and Sarubbo, L. A. (2013). Synthesis and evaluation of biosurfactant produced by *Candida lipolytica* using animal fat and corn steep liquor. *J. Petroleum Sci. Eng.* 105, 4–50. doi: 10.1016/j.petrol.2013.03.028

Santos, D. K. F., Rufino, R. D., Luna, J. M., Santos, V.A., and Sarubbo, L. A. (2016).Biosurfactants: multifunctional biomolecules of the 21st century. *Int. J. Mol. Sci.* 17:401. doi: 10.3390/ijms17030401

Sarubbo, L. A., Farias, C. B. B., and Campos-Takaki, G. M. (2007). Co-utilization of canola oil and glucose on the production of a surfactant by *Candida lipolytica*. *Curr. Microbiol.* 54, 68–73. doi: 10.1007/s00284-006-0412-z

Sarubbo, L. A., Luna, J. M., and Campos-Takaki, G. M. (2006). Production and stability studies of the bioemulsifier obtained from a new strain of *Candida glabrata* UCP 1002. *Electron. J. Biotechnol.* 9, 400–406. doi: 10.2225/vol9-issue4-fulltext-6

Sarubbo, L. A., Marçal, M. C. R., Neves, M. L. C., Silva, M. P. C., Porto, A. L. F., and Campos-Takaki, G. M. (2001). Bioemulsifier production in batch culture using glucose as carbon source by *Candida lipolytica*. *Appl. Biochem. Biotechnol.* 95, 59–67. doi: 10.1385/ABAB:95:1:59

Sarubbo, L. A., Porto, A. L. F., and Campos-Takaki, G. M. (1999). The use of babassu oil as substrate to produce bioemulsifiers by *Candida lipolytica*. *Can. J. Microbiol.* 45, 423–426. doi: 10.1139/w99-025

Sekhon, K. K., Khanna, S., and Cameotra, S. S. (2012). Biosurfactant production and potential correlation with esterase activity. *J. Pet. Environ. Biotechnol.* 3:133. doi: 10.4172/2157-7463.1000133

Sharma, D., Saharan, B. S., and Kapil, S. (eds.). (2016). "Biosurfactants of lactic acid bacteria," in *SpringerBriefs in Microbiology* (New York, NY: Springer), 87.

Silva, E. J., Rocha e Silva, N. M. P., Rufino, R. D., Luna, J. M., Silva, R. O., and Sarubbo, L. A. (2014a). Characterization of a biosurfactant produced by *Pseudomonas cepacia* CCT6659 in the presence of industrial wastes and its application in the biodegradation of hydrophobic compounds in soil. *Colloids Surf. B Biointerfaces.* 117, 36–41. doi: 10.1016/j.colsurfb.2014.02.012

Silva, R. C. F. S., Almeida, D. G., Rufino, R. D., Luna, J. M., Santos, V. A., and Sarubbo, L. A. (2014b). Applications of biosurfactants in the petroleum industry and the remediation of oil spills. *Int. J. Mol. Sci.* 15, 12523–12542. doi: 10.3390/ijms150712523

Silva, R. C. F. S., Rufino, R. D., Luna, J. M., Farias, C. B. B., Filho, H. J. B., Santos, V. A., et al. (2013). Enhancement of biosurfactant production from *Pseudomonas cepacia* CCT6659 through optimisation of nutritional parameters using response surface methodology. *Tenside Surf. Det.* 50, 137–142. doi: 10.3139/113.110241

Silva, S. N. R. L., Farias, C. B. B., Rufino, R. D., Luna, J. M., and Sarubbo, L. A. (2010). Glycerol as substrate for the production of biosurfactant by *Pseudomonas aeruginosa* UCP0992. *Colloids Surf. B Biointerfaces.* 79, 174–183. doi: 10.1016/j.colsurfb.2010.03.050

Sitohy, M. Z., Rashad, M. M., Sharobeem, S. F., Mahmoud, A. E., Nooman, M. U., and Kashef, A. S. A. (2010). Bioconversion of soy processing waste for production of surfactants. *Afr. J. Microbiol. Res.* 4, 2811–2821.

Sobrinho, H. B. S., Luna, J. M., Rufino, R. D., Porto, A. L. F., and Sarubbo, L. A. (2013a). Application of biosurfactant from *Candida sphaerica* UCP 0995 in removal of petroleum derivative from soil and sea water. *Life Sci.* 7, 559–569.

Sobrinho, H. B. S., Luna, J. M., Rufino, R. D., Porto, A. L. F., and Sarubbo, L. A. (2013b). Assessment of toxicity of a biosurfactant from *Candida sphaerica* UCP 0995 cultivated with industrial residues in a bioreactor. *Electron. J. Biotechnol.* 16, 1–11. doi: 10.2225/vol16-issue4-fulltext-4

Sobrinho, H. B. S., Rufino, R. D., Luna, J. M., Salgueiro, A. A., Campos-Takaki, G. M., Leite, L. F. C., et al. (2008). Utilization of two agroindustrial by-products for the production of asurfactant by *Candida sphaerica* UCP0995. *Process Biochem.* 43, 912–917. doi: 10.1016/j.procbio.2008.04.013

Toribio, J., Escalante, A. E., and Soberón-Chávez, G. (2010). Rhamnolipids: production in bacteria other than *Pseudomonas aeruginosa*. *Eur. J. Lipid Sci. Technol.* 112, 1082–1087. doi: 10.1002/ejlt.200900256

Zhu, Z., Zhang, F., Wei, Z., Ran, W., and Shen, Q. (2013). The usage of rice straw as a major substrate for the production of surfactin by *Bacillus amyloliquefaciens* XZ-173 in solid-state fermentation. *J. Environ. Manage.* 127, 96–102. doi: 10.1016/j.jenvman.2013.04.017

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5.3. CAPÍTULO 3

Production, characterization and commercial formulation of a biosurfactant from *Candida tropicalis* UCP 0996 and its application in decontamination of pollutants generated by the petroleum industry

Artigo a ser submetido na revista *International Journal of Molecular Sciences*.

International Journal of
Molecular Sciences

Production, characterization and commercial formulation of a biosurfactant from *Candida tropicalis* UCP 0996 and its application in decontamination of pollutants generated by the petroleum industry

Darne Germano de Almeida[a,c]; Rita de Cássia Freire Soares da Silva[a,c] ; Hugo M. Meira[b,c]; Pedro Pinto Ferreira Brasileiro[b,c]; Elias J. Silva[b,c]; Juliana Moura de Luna[b,c] ; Raquel Diniz Rufino[b,c] ; Leonie Asfora Sarubbo[a,b,c]

[a]*Northeast Biotechnology Network, Federal Rural University of Pernambuco, Rua Dom Manoel de Medeiros, s/n, Dois Irmãos. CEP: 52171-900, Recife – Pernambuco, Brazil.*
[b]*Centre of Sciences and Technology, Catholic University of Pernambuco, Rua do Príncipe, n. 526, Boa Vista, CEP: 50050-900, Recife – Pernambuco, Brazil.*
[c] *Advanced Institute of Technology and Innovation (IATI), Rua Carlos Porto Carreiro, n.70, Derby, CEP: 50000-000, Recife, Pernambuco, Brazil.*

Leonie Asfora Sarubbo (Corresponding author)
Tel.: +55 81 21194084; fax: +55 81 21194000.
E-mail: leonie@unicap.br

Abstract

In the present study, production of the biosurfactant from *Candida tropicalis* UCP 0996 cultivated in the low cost-medium formulated with molasses, residual frying oil and corn steep liquor for 120 h at 28 °C and 200 rpm and its characterization, toxicity, formulation and application in process of removal and biodegradation of oil were investigated. In the studies of *C. topicalis* growth and biosurfactant production, the surface tension of the medium was reduced to a minimum of 30.4 mN/m, yielding 4.11 g/L of isolated biosurfactant. Tests under extreme conditions of pH, temperature and NaCl indicated the stability of the biosurfactant. Chemical characterization by thin layer chromatography (TLC), Fourier transform infrared spectroscopy (FTIR), proton nuclear magnetic resonance (^{1}H NMR) and gas chromatography and mass spectroscopy (CG-MS) revealed the glycolipidic nature of the biosurfactant. Isolated biosurfactant was not toxic to the microcrustacean *Artemia salina*. Tensoactive properties of the formulated biosurfactant remained stable through 120 tests days. Biosurfactant demonstrated 70.95 % oil spreading efficiency in seawater and removed 66.18

% of motor oil from marine stones. Biosurfactant stimulated around 70 % motor oil degradation by the seawater indigenous microorganisms, demonstrating great potential to be applied as future commercial product in the bioremediation of oil spills.

Keywords Bioremediation; *Candida tropicalis* UCP 0996; Surface tension; Formulation; Oil spill

1. Introduction

Petroleum and byproducts release into environment is one of the global pollution main causes and has become a focus of great concern both in industrialized and developing countries once oil pollution can have dramatic detrimental effects to the environment and cause significant damages to resident organisms. 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 (Almeida et al., 2017; Silva et al., 2014).

The need for remediating polluted areas has paved the way for development of new technologies to detoxify contaminants not only through chemical or physical methods, but through biological techniques as well. Bioremediation is a set of technologies that make the removal of contaminants possible, or failing that, make a number of contaminants less harmful by means of biological activity (Silva et al., 2015). For the success of bioremediation technologies, microorganisms employment with the appropriate metabolic abilities for biodegradation and able to transform contaminants into less toxic substances is the most important requisite on oil spill bioremediation (Al-Wasify and Hamed, 2014; Cerqueira et al., 2012).

The *Candida tropicalis* yeast has been widely studied by several researchers as a hydrocarbons degrading potent agent (Almeida et al., 2017; Chandran and Das, 2011; Farag and Soliman, 2011). Further recent studies have reported that this species has also the metabolic capacity to produce biosurfactant when cultivated on water-immiscible substrates (Batista et al., 2010; Chandran and Das, 2012; Verma et al., 2015).

Biosurfactants are 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 hydrocarbons enhancing mobility, bioavailability, and biodegradation of such compounds (Silva et al., 2014). The considerable interest in these compounds is related to their properties, as biodegradability, production from renewable substrates, low toxicity,

biocompatibility, diversity for chemical structure and properties, effectiveness even at extreme conditions of temperature, pH and salinity (Sarubbo et al., 2015).

These features have led to the intensification of scientific studies on a wide range of industrial applications for biosurfactants in the field of bioremediation. Besides exerting a strong positive impact on the main global problems, biosurfactant production has considerable importance to the implantation of sustainable industrial processes, such as the use of renewable resources and "green" products (Santos et al., 2016b).

In the present study, the biosurfactant produced from *Candida tropicalis* UCP 0996 in industrial waste was evaluated. It has also been described the growth and production curves, surface active properties, characterization and toxicity. A further aim was to formulate the biosurfactant and evaluated it as a dispersing agent in the spilled oil removal and bioremediation processes.

2. MATERIALS AND METHODS

2.1. Materials

All chemicals were reagent grade. Growth media were purchased from Difco Laboratories (USA). 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 Ingredion Brasil, Cabo de Santo Agostinho-PE, Brazil. Cane molasses was obtained from a local sugar mill in the municipality of Vitória de Santo Antão, state of Pernambuco, Brazil. Seawater was collected near the Thermoelectric TERMOPE, located in the municipality of Cabo de Santo Agostinho, in Pernambuco state, Brazil. Seawater samples were collected and stored in plastic bottles of 5 L.

2.2. Yeast strain and preparation of inoculum

A strain of *Candida tropicalis* UCP0996 was provided from the culture collection of the Catholic University of Pernambuco, Recife city, Pernambuco, Brazil. The microorganism was maintained at 5 °C on yeast mold agar slants containing (w/v) yeast extract (0.3 %), malt extract (0.3 %), tryptone (0.5 %), D-glucose (1.0 %) and agar (5.0 %). Transfers were made to fresh agar slants each month to maintain viability. Inoculum was prepared by transferring

cells grown on a slant to 250-ml Erlenmeyer flasks containing 50 ml of yeast mold broth (YMB). YMA and YMB media initial pH was adjusted to 5.5. The cultivation conditions for the seed culture were 28 °C, 200 rpm and 24 h of incubation.

2.3. Growth curve and biosurfactant production

The production of biosurfactant was performed in a basal medium composed of 2.5 % cane molasses, 2.5 % waste frying oil and 2.5 % corn steep liquor. Initial pH was adjusted to 5.5. 500-mL shake flasks were kept under 200 rpm orbital agitation for 120 h at 28 °C. At 0, 2, 4, 6, 8, 16, 24, 32, 48, 72, 96 and 120 hours of fermentation, samples were taken from the liquid culture to determine the growth profile (biomass), pH, surface tension and biosurfactant yield.

2.4. Determination of biosurfactant properties

Surface tension was determined in the cell-free broth obtained by centrifuging the cultures at 10,000 × g for 15 min. Surface tension was determined with a Tensiometer (Sigma 700, KSV Instruments Ltd., Finland), using the Du Nouy ring method at room temperature (Silva et al., 2014a). The emulsification index was determined using the method described by Cooper and Goldenberg (1987). The ionic charge of the biosurfactant was determined using the agar double diffusion technique (Meylheuc et al., 2001).

2.5. Stability studies

Stability studies of the biosurfactant were performed in the cell-free broth. 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. The effect of NaCl concentration (2.0–12.0 %) was also determined after addition of the salt to the samples. The cell-free broth was also heated at 0, 5, 28, 70, 100 and 120 °C during 1h and cooled to room temperature. After this, cell-free broth was used for the measurement of surface tension and emulsification. The assays were carried out in triplicate and did not vary more than 5 %.

2.6. Determination of cell hydrophobicity

Cell hydrophobicity was determined by cell adhesion to hydrocarbons. Cells were washed twice in sterile deionised water and re-suspended in saline buffer (16.9 g/L K_2HPO_4; 7.3 g/L KH_2PO_4) to provide an optical density (OD) of 0.5–600 nm. One hundred ml of n-hexadecane were added to 2.0 ml of the cell suspension in a test tube (10 mm ×100 mm) and stirred in a vortex for 3 min. The contents were left to rest for 1 h for the separation of the aqueous and hexadecane phases. In the aqueous phase, OD was measured at 600 nm. Hydrophobicity was expressed as the percentage of adhesion to hexadecane and calculated as follows: 100×(1−aqueousphase OD/initial cell suspension OD). Three determinations were performed for each sample. Cells were considered very hydrophobic when rates exceeded 60 % and poorly hydrophobic when the rate was under 10 % (Bouchez-Naitali et al., 1999).

2.7. Isolation of biosurfactant

After cultivation (120 h), bio-surfactant was extracted from the cell-free broth following cell removal by centrifugation at 5000 × g for 20 min and filtered through Whatman n°.1 filter paper. An equal volume of $CHCl_3$/CH_3OH (2:1) was placed with 50 ml of the cell-free broth in a separatory funnel at 28 °C. 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). After extraction, the product was treated with a base and crystallized for maximum removal of impurities.

2.8. Critical micelle concentration

The critical micelle concentration (CMC) was determined by measuring the surface tension of the dilutions of the isolated biosurfactant in distilled water up to a constant surface tension value. Stabilisation was allowed to occur until the standard deviation of 10 successive measurements was less than 0.4 mN/m. Each result was the mean of 10 determinations after stabilisation. The CMC was obtained by plotting surface tension against surfactant concentration and expressed as g/L of biosurfactant.

2.9. Characterization

2.9.1 Biosurfactant characterisation by thin-layer chromatography

After isolating the biosurfactant, a sample of 0.1 g 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., Rf 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).

2.9.2 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).

2.9.3 Fourier transform infrared spectroscopy

The biosurfactant extract recovered from the supernatant of the *C. tropicalis* UCP 0996 isolate was characterized by Fourier transform infrared spectroscopy (FTIR). The FTIR spectrum 400 Perkin Elmer with a resolution of 4 cm^{-1} was collected from 400 to 4000 wavenumbers (cm^{-1}).

2.9.4 Gas chromatography and mass spectroscopy (GC-MS)

The 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.

2.10. Toxicity against Artemia salina as indicator

A modified toxicity assay previously described by Meyer et al. (1982) was performed using microcrustacean *Artemia salina* as toxicity indicator. Shrimp eggs were incubated in seawater for 24 h. Assays were conducted with 10 microcrustacean larvae in penicillin tubes of 10 ml

capacity containing solutions with 5 ml of seawater with isolated biosurfactant to give concentrations based on the CMC until LC_{50} (lowest concentration that kills 50 % of tested brine shrimp) and cell-free broth. They were observed for 24 h to calculate mortality. Each test was run in triplicate, and seawater was used as the control.

2.11. 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 (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).

2.12. Application of the biosurfactant in hydrophobic contaminant spreading

Oil displacement test was carried out slowly by dropping of 15 µl of motor oil onto the surface of 40 ml of seawater 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 formulated biosurfactant and aqueous solutions containing the isolated surfactant at ½xCMC, at 1xCMC, 2xCMC and 5xCMC 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).

2.13. Washing of hydrophobic compound adsorbed to porous surface

The removal of motor oil adsorbed to rock was carried out 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, at 1xCMC, 2xCMC and 5xCMC concentration. After the culture process, the percentage of removal through washing 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 rotoevaporated at 50 ºC and the amount of oil removed was determined by gravimetry (Sarubbo et al., 2012).

2.14. Swirling bottle test

A 1-L cylindrical open bottle (diameter: 10 cm) with an outlet valve at the bottom to take samples was used in the dispersion experiment. Samples of 200 mL of seawater were added to the bottle and 2 mL of oil was gently added to the surface of the water with a pipette. The formulated biosurfactant was dispensed in the center of the oil slick in the following proportions of biosurfactant-to-oil: 1:1, 1:2, 1:10 and 1:20 (v/v). The bottle was placed on an orbital shaker table at 28 ºC to induce a swirling motion in the water content of the bottle. The shaking speed was 150 rpm for a period of 10 min, followed by 1 to 2 min settling time to allow the larger droplets to return to the surface. Samples were taken at 0 min, 5 min and 10 min. The first 2 mL of the sample was removed through the stopcock and discarded and 30 mL of the sample was collected. This sample was extracted three times with hexane, as the biosurfactant is insoluble in hexane. The extract was adjusted. Efficacy was calculated by dividing the concentration of dispersed oil in the water (determined by analysing the hexane extract) by the total concentration of oil, which depended on the total volume of oil added to the flask (Sobrinho et al., 2013; Jain et al., 2012).

2.15.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 ½ 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).

2.16. Statistical analysis

All presented experiments results were the average data of three independent replicates and were calculated using STATISTICA ® program, version 10.0 (Statsoft Inc, USA).

3. RESULTS AND DISCUSSION

3.1. Growth curve and biosurfactant production

The growth curve (Figure 1) demonstrated an exponential phase at 5 h–15 h of cultivation. Maximum biomass production was 23.75 g/L after 120 h of cultivation. Maximum biosurfactant production was 4.11 g/L and occurred in the stationary growth phase, after 120 h of cultivation. Surface tension of the medium was reduced from 55 mN/m to 30.4 mN/m after 96 h and the pH decreased in the first 30 h and after that it remains around 5.5 after 72 h. According to the literature, most *Candida* species have demonstrated the capacity to produce efficient biosurfactants. Rufino et al. (2014) evaluated *C. lipolytica* UCP 0988 growth in mineral medium supplemented with 6 % soybean oil refinery residue and 1 % glutamic acid for biosurfactant procuction. After 72 h of cultivation, the surface tension of the cell-free broth was reduced from 55 to 25 mN/m and the yield of biosurfactant was 8.0 g/L. In another work, Luna et al. (2013) studied a biosurfactant produced by *C. sphaerica* using a medium containing 9 % ground nut oil refinery residue and 9 % corn steep liquor. They obtained a biosurfactant yield of 9 g/L and a surface tension of 25 mN/m after 144 h.

Input Figure 1

3.2. Stability studies

Biosurfactant produced by *C. tropicalis* UCP 0996 showed surface tension practically unchanged at a wide range of salinity, pH, temperature and heating time at 90 °C (Figure 2A – 2D). The resistance of the *C. tropicalis* UCP 0996 biosurfactant to salinity was also investigated by the emulsification index (Figure 3A). As can be seen, the biosurfactant emulsification activity proved stable for the three oils types tested, independent of the salt concentration added. This salinity resistance of the *C. tropicalis* UCP 0996 biosurfactant is an advantage for its application in marine environment where salt concentrations are high. Biosurfactants produced by the yeasts *C. glabrata* (Gusmão et al., 2010) and *C. lipolytica* (Santos et al., 2013) also have shown excellent stability in the presence of salt. The corn and soybean oil emulsification remained practically stable with the change in pH values with

some improvement throughout pH values of 10–12. On the other hand, the motor oil had outstanding emulsification indexes, reaching maximum values above 70 %, decreasing only 10 % in more extremes alkaline pH (Figure 3B). The biosurfactant stability in a large pH range has been also reported for other surfactants from the *Candida* species (Rufino et al., 2007; Luna et al., 2013). The emulsification indexes of the biosurfactant were quite stable at the investigated temperature, with the maximum activity at 100 °C for motor oil and had its emulsification capacity conserved for 120 h at 90 °C (Figures 3C – 3D). These results were very promising, showing the potential for using the biosurfactant from *C. tropicalis* in a broad spectrum of industrial applications at extreme temperatures. Therefore, it is important to study the influence of these parameters when considering specific applications for these compounds (Sobrinho et al., 2008).

Input Figure 2 and Figure 3

3.3. Biosurfactant properties

Surface tensions versus the isolated biosurfactant concentrations were plotted in Figure 4. The water surface tension decreased gradually with increasing biosurfactant concentration from 70 to 25.6 mN/m with a biosurfactant concentration of 0.06 %, and then remained constant. Further increase in the biosurfactant concentration did not reduce the surface tension of water, indicating that the CMC was reached at this concentration. The CMC of the *C. tropicalis* biosurfactant is within the CMC values reported by the literature for different types of biosurfactants produced by other *Candida* species, as the biosurfactants from *C. lipolytica* UCP 0988 (0.03 %) (Rufino et al., 2014), from *C. glabrata* (2.5 %) (Luna et al., 2009), from *C. antarctica* (0.6%) (Adamczak and Bednarski, 2000) and from *C. sphaerica* UCP 0995 (0.025%) (Luna et al., 2013).

C. tropicalis biosurfactant showed an anionic nature, being in accordance with other data reported in the literature (Rufino et al. 2014; Luna et al., 2016). The low hydrophobicity found (3.73 %) revealed that the biomolecule is not attached to the membrane, and it is extracellular (Bouchez- Naitali et al., 1999).

Input Figure 4

3.4. Characterization

The TLC analysis of the biosurfactant isolated from *C. tropicalis* revealed two values of Rf (retention factor) (Figure 5). With Molish reagents, the spots showed positive reactions for sugars and with iodine vapours for lipids, but negative reactions with ninhydrin for amino groups.

Input Figure 5

The FT-IR spectrum is illustrated in Figure 6, which shows extending vibration at 3300–3500 cm^{-1} which is characteristic of O–H stretching vibrations. The band peak at 3000–2800 cm^{-1} was characteristic of aliphatic chains; around 1710 cm^{-1} the presence of C=O group is evidenced; and the appearance of band peaks at 1550–1400 cm^{-1} may be due to C double bond and at ~ 1260 cm^{-1} shows the presence of ketone group.

Input Figure 6

^{1}H NMR spectrum is shown in the Figure 7. Signals of the biosurfactant from *C. tropicalis* between δ 0.60 and 1.6 ppm suggests the presence of aliphatic and methyl groups in biosurfactant; signals between δ 2.0 and 2.2 ppm indicate the presence of aldehyde group; signals at δ 3.5 ppm and between δ 4.6 and 4.8 ppm were attributed to hydroxyl groups and those between δ 5.0 and 5.4 ppm corresponds to double bounds.

Input Figure 7

The biosurfactant was analyzed by GC-MS and compared with the library data. The Chromatogram (Figure 8) showed two very evident peaks probably related to cyclical structures. The first peak (45.53 %) is related to a cyclic structure with carbonyl group. The second peak (28.21 %) also points to a cyclic structure containing a hydroxyl group. The structure showed molar mass between 150 and 200 (m/z).

Input Figure 8

Results obtained by ^{1}H NMR, FTIR spectroscopy, TLC and GC-MS analysis to the biosurfactant studied indicate its glycolipid nature. Yeasts biosurfactants characterization

utilising ^{1}H NMR, FTIR spectroscopy, TLC and GC-MS analysis has also been described in the literature (Santos et al., 2013; Chandran and Das, 2011; Fukuoka et al., 2007; Kim et al., 1999).

3.5. Toxicity against Artemia salina as indicator

C. tropicalis UCP 0996 biosurfactant was tested for its toxicity in a short term bioassay using brine shrimp. Results indicated an absence toxicity of the biosurfactant from *C. tropicalis* UCP 0996, which only reached the LC_{50} at a concentration of 10xCMC (Table 1). Similar results were found by Sobrinho et al. (2013) for the biosurfactant produced by *C. sphaerica* UCP 0995, which demonstrated low degree of toxicity at concentrations between 200 and 400 mg/L due to the low percentage of morality (less than 50 %).

Input Table 1

3.6. Formulation

One of the main requirements for byproduct formulation is that it should be stable over time and their properties should not significantly change with variations of pH, temperature, salinity, etc. In this study, it was observed through the long-term stability and accelerated stability studies that, in general, biosurfactant preserved its tensoative, emulsifying and dispersing properties (Figures 9 – 11). It was found that the reduction of the surface tension remained practically constant around 30 mN/m over the 120 day test through the three methods employed (Figures 9A – 9H), with the best results presented by the addition of potassium sorbate. With respect to the emulsification property it was observed that, generally, biosurfactant submitted to the addition of potassium sorbate promoted a high rate of motor oil emulsification (above 90 %) in practically all conditions tested, while for fluent vaporization and fractionated tyndallization methods changes in emulsification indexes were observed (Figures 10A – 10H). The biosurfactant showed a motor oil variable dispersion rate, with the best results found after addition of potassium sorbate, for which maximum values were above 60 % (Figures 11A – 11H). Based on the results found, potassium sorbate addition proved to be the most efficient preservation method. Furthermore, potassium sorbate use eliminates the additional costs of thermal treatment procedure, which make the process more economical from an industrial point of view.

Input Figure 9, Figure 10 and Figure 11

Freitas et al. (2016) formulated a biodegradable commercial biosurfactant form *C. bombicola* URM 3718 cultivated in industrial waste for application as a dispersant in oil spills. Results obtained by these authors were also promising for the biosurfactant formulated with the preservative, which demonstrated stability through 120 days. For other applications, Campos et al. (2015) tested six different formulations of mayonnaise with the addition of a bioemulsifier isolated from *C. utilis*. As a result, the most stable formulation with the best quality was obtained with combination of guar gum and the isolated biosurfactant with an absence of pathogenic microorganisms. In addition, the biosurfactant from *C. utilis* proved to be safe use in food emulsions. In another study, Bafghi et al. (2012) studied the application of rhamnolipid in the formulation of a detergent. The results showed that the biosurfactant was effective in oil removal from the samples and the formulation presented was comparable to commercial powders in terms of the stain removal. Nguyen and Sabatini (2009) evaluated the formulating of alcohol-free micro-emulsions using a rhamnolipid biosurfactant. They reported that the formulations obtained proved to be viable for a variety of applications. In another study, Youssef et al. (2007) tested biosurfactant and synthetic surfactant mixtures in mobilizing entrapped hydrocarbons. As a result, they obtained formulating biosurfactant mixtures that provided ultralow interfacial tension values against hydrocarbons. There are few studies describing the use of biosurfactant formulations in several purposes, which makes this work a more valuable contribution.

3.7. Application of the biosurfactant in hydrophobic contaminant spreading

Many processes carried out by the oil industry contaminate the marine environment. Eventually, a part of the process, oil accidentally reaches the seawater and, in turn, surfactants must be used together with other containment measures. In this study, it was evaluated the dispersing ability of the formulated biosurfactant from *C. tropicalis* (Table 2). As can be seen, the best dispersion indices found were 70.95 % and 57.14 % for the isolated and formulated biosurfactant, respectively. Luna et al. (2016) evaluated the dispersion capacity of the biosurfactant produced by *C. bombicola*. As a result, the isolated biosurfactant showed a maximum dispersion rate 50 % of motor oil. Freitas et al. (2016) evaluated the dispersion capacity of a formulated biosurfactant also from the yeast *C. bombicola* URM 3718. They

observed that the formulation of the biosurfactant with addition of potassium sorbate allowed dispersion ratios between 30 and 50 % after 30 days of storage.

Input Table 2

3.8. Washing of hydrophobic compound adsorbed to porous surface

Few methods are suitable for cleaning contaminants in coral reefs, which are very delicate and difficult to access. However, the use of dispersants is an attractive method when an ecosystem is exposed to an oil spill (Sobrinho et al., 2013). The literature has little to offer about oil removal on porous surfaces. However, Luna et al. (2016) evaluated the removal capacity of the biosurfactant from *C. bombicola* showing a removal of 70 % of the motor oil adsorbed on a porous surface. In another study, the rate of removal of 60 % of motor oil on porous surfaces with a crude biosurfactant was reported, demonstrating the potential of the biosurfactant from *C. sphaerica* as a dispersant (Sobrinho et al., 2013). In the present study, a removal of about 66.18 % was found to be the best result for the isolated biosurfactant from *C. tropicalis* (Table 3), showing the feasibility of its application as a biological dispersant to remove hydrophobic pollutants in sensitive ecosystems such as coral reefs.

Input Table 3

3.9. Swirling bottle test

Table 4 shows the results for simulation of the formulated biosurfactant in the dispersion of oil spill in a seawater sample. As can be seen, the best results for dispersion occurred with a biosurfactant/oil ratio of 1:1 (v/v) for all times tested (with dispersion index above 50 %). This kind of test is extremely important since the application of dispersants reduces the effects of the oil spills as it removes the oil from the surface of water reducing the amount of spilled oil. The dispersion of oil into tiny droplets also increase the surface area of exposure, which stimulates biodegradation by indigenous microorganisms (NRC, 2005; Sobrinho et al., 2013).

Input Table 4

3.10. Bioremediation test

Biosurfactant effect on the motor oil biodegradation through the activity of indigenous marine bacteria and fungi was evaluated during 28 days (Figure 12). As can be seen, the results related to the addition of biosurfactants were superior to the control (biosurfactant absence) as predicted since the formulated and isolated biosurfactant from *C. tropicalis* had the ability to stimulate growth of indigenous microorganisms and consequent motor oil biodegradation. Similar results were obtained by Santos et al. (2016a). They observed that the presence of the biosurfactant from *C. lipolytica* favored the growth of indigenous microorganisms in seawater at the concentrations of ½xCMC, CMC and 2×CMC throughout 30 days of cultivation.

Input Figure 12

4. Conclusions

The biosurfactant produced by *C. tropicalis* cultivated in a low-cost medium demonstrated a great potential of application as an oil spill remediation agent in marine environments. The biosurfactant showed no toxicity and presented not only good surface tension reduction and biosurfactant yield, but also an excellent stability under extreme conditions, making this biomolecule effective for application in the bioremediation processes as a commercial stable dispersant.

Acknowledgments

This work received financial supporting from the Brazilian fostering agencies State of Pernambuco Support Foundation for Science and Technology (FACEPE), Thermoelectric of Pernambuco (TERMOPE), National Agency of Electric Energy (ANEEL), National Council for Scientific and Technological Development (CNPq) and Coordination for the Improvement of Higher Level Education Personnel (CAPES). The authors would like to express their gratitude to the laboratories of the Center for Sciences and Technology of the Catholic University of Pernambuco, Brazil.

References

ADAMCZAK, M.; BEDNARSKI, W. Influence of medium composition and aeration on the synthesis of biosurfactants produced by *Candida antarctica*. **Biotechnology Letters**. vol.22, 4, p. 313–316, 2000.

ALMEIDA, D.G.; SILVA, M.G.C.; BARBOSA, R.N.; SILVA, D.S.P.; SILVA, R.O.; LIMA, G. M.S.; GUSMÃO, N. B.; SOUSA, M.F.V.Q. Biodegradation of marine fuel MF-380 by microbial consortium isolated from seawater near the petrochemical Suape Port, Brazil. **International Biodeterioration & Biodegradation.** v.116, p.73–82, 2017.

AL-WASIFY, R.S.; HAMED, S.R. Bacterial biodegradation of crude oil using local isolates. **International Journal of Bacteriology**. p.1–8, 2014.

AMERICAN Public Health Association. Standard Methods For The. Examination of Water and Wastewater. 21 ed. Washington: APHA, 2005.

BAFGHI, M.K.; FAZAELIPOOR, M.H. Application of rhamnolipid in the formulation of a detergent. **Journal of Surfactants and Detergents**. vol.15, 6, p. 679–684, 2012.

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

BOUCHEZ-NAITALI, M.; RAKATOZAFY, H.; MARCHAL, R.; LEVEAU, J.Y.; VANDECASTEELE, K. Diversity of bacterial strains degrading hexadecane in relation to the mode of substrate uptake. **Journal of Applied Microbiology.** vol. 3, p.42–428, 1999.

CAMPOS, J.M.; STAMFORD, T. L.M.; SARUBBO, L.A.; LUNA, J.M.; RUFINO, R.D.; BANAT, I.M. Microbial biosurfactants as additives for food industries. **Biotechnology Progress**. vol.29, p.1097–108, 2013. doi: 10.1002/btpr.1796

CERQUEIRA, V.S.; HOLLENBACH, E.B.; MABONI, F.; CAMARGO, F.A.O.; PERALBA, M.C.R.; BENTO, F.M. Bioprospection and selection of bacteria isolated from environments contaminated with petrochemical residues for application in bioremediation. **World Journal of Microbiology and Biotechnology**. vol. 28, p.1203–1222, 2012.

CHANDRAN, P.; DAS, N. Degradation of diesel oil by immobilized *Candida tropicalis* and biofilm formed on gravels. **Biodegradation**. vol.22, p.1181–1189, 2011. doi:10.1007/s10532-011-9473-1

COOPER, D.G.; GOLDENBERG, B.G. Surface active agents from two *Bacillus* species. **Applied Environmental Microbiology**. v.53, p.224–229, 1987.

DESHPANDE, M.; DANIELS, L. Evaluation of sophorolipid biosurfactant production by Candida bombicola using animal fat. **Bioresource Technology**. vol.54, p.143–150, 1995. doi.org/10.1016/0960-8524(95)00116-6

FARAG, S.; SOLIMAN, N.A. Biodegradation of crude petroleum oil and environmental pollutants by *Candida tropicalis* strain. **Brazilian Archives of Biology and Technology**. vol. 54, p. 821-830, 2011. doi: 10.1590/S1516-89132011000400023

FREITAS, B.G.; MOREIRA, J.G.B.; BRASILEIRO, P.P.F.; RUFINO, R.D.; LUNA, J. M.; SANTOS, V. A.; SARUBBO, L.A. Formulation of a commercial biosurfactant for application as a dispersant of petroleum and by-products spilled in oceans. **Frontiers in Microbiology**. v.7, p.1646, 2016.

FUKUOKA, T.; MORITA, T.; KONISHI, M.; IMURA, T.; KITAMOTO, D. Characterization of new glycolipid biosurfactants, tri-acylated mannosylerythritol lipids, produced by *Pseudozyma* yeasts. **Biotechnology Letters**. vol.29, 7, p.1111–1118, 2007.

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

JAIN, R.M.; MODY, K.; MISHRA, A.; JHA, B. Physicochemical characterization of 520 biosurfactant and its potential to remove oil from soil and cotton cloth. **Carbohydrate Polymers**. vol.89, p.1110–1116, 2012. http://dx.doi.org/10.1016/j

KIM, H.S.; YOON, B.D.; CHOUNG, D.H.; OH H.M.; KATSURAGI T.; TANI Y. Characterization of a biosurfactant, mannosylerythritol lipid produced from *Candida* sp. SY16. **Applied Microbiology and Biotechnology.** vol.52, 5, p.713-21, 1999.

LUNA J. M; SARUBBO L. A; CAMPOS-TAKAKI G. M. A new biosurfactant produced by *Candida glabrata* UCP1002: characteristics of stability and application in oil recovery. **Brazilian Archives Biology and Technology**. v.52, p.785, 2009.

LUNA, J.M.; RUFINO, R.D.; SARUBBO, L.A. Biosurfactant from *Candida sphaerica* UCP0995 exhibiting heavy metal remediation properties. **Process Safety and Environmental Protection**. vol.102, p.558–566, 2016.

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

MEYER, B.N.N.R.; FERRIGNI, J.E.; PUTNAM, L.B.; JACOBSEN, D.E.; NICHOLS, D.E., MCLAUGHLIN, J.L. Brine shrimp: a convenient general bioassay for active plant constituents. **Journal of Medicinal Plants Research**. vol.45, p.31–34, 1982. doi.org/10.1055/s-2007-971236

MEYLHEUC, T.; VAN, O.S.S.; BELLON-FONTAINE, C.J. Adsorption of biosurfactants on solid surfaces and consequences regarding the bioahesion of *Listeria monocytogenes* LO28. **Journal of Applied Microbiology**. vol.91, p.822–832, 2001. doi.org/10.1046/j.1365-2672.2001.01455.x

NGUYEN, T.T.; SABATINI, D.A. Formulating alcohol-free microemulsions using rhamnolipid biosurfactant and rhamnolipid mixtures. **Journal of Surfactants and Detergents**. Vol.12, 2, p. 109-115, 2009.

OHNO, A.; TAKASHI, A.; SHODA, N. Production of antifungal peptide antibiotics iturin by *Bacillus subtilis* NB22 in solid state fermentation. **Journal of Fermentation and Bioengineering**. v. 75, p. 23–27, 1993.

ROCHA E SILVA, F.C.P.; ROCHA E SILVA, N.M.P.; MOURA, A.E.; GALDINO, R.A.; LUNA, J.M.; RUFINO, R.D.; SANTOS, V.A.; SARUBBO, L.A. Effect of biosurfactant addition in a pilot scale dissolved air flotation system. **Separation Science and Technology**. vol. 50, p.618-625, 2015.

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

RUFINO, R.D.; SARUBBO, L.A. CAMPOS-TAKAKI, G.M. Enhancement of stability of biosurfactant produced by *Candida lipolytica* using industrial residue as substrate. **World J Microbiol Biotechnol**. vol.23, p.729–734, 2007. doi.org/10.1007/S11274-006-9278-2

SANTOS, A.S.; SAMPAIO, A.P.W.; VASQUEZ, G.S.; ANNA, L.M.S.; PEREIRA JR, N.; FREIRE, D.M.G. Evaluation of different carbon and nitrogen sources in production of

rhamnolipids by a strain of *Pseudomonas aeruginosa*. **Applied Biochemistry and Biotechnology**. vol.98, p.1025-1035, 2002. doi.org/10.1385/ABAB:98-100:1-9:1025

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

SANTOS, D.K.F.; MEIRA, H.M.; RUFINO, R.D.; LUNA, J.M.; SANTOS, V.A.; SARUBBO, L.A. Biosurfactant production from *Candida lipolytica* in bioreactor and evaluation of its toxicity for application as a bioremediation agent. **Process Biochemistry**, 2016a. 10.1016/j.procbio.2016.12.020

SANTOS, D.K.F.; RUFINO, R.D.; LUNA, J.M.; SANTOS, V.A.; SARUBBO, L.A. Biosurfactants: multifunctional biomolecules of the 21st century. **International Journal of Molecular Science**, v.17, p. 401, 2016b.

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

SARUBBO, L.A.; RUFINO, R.D.; LUNA, J.M.; FARIAS, C.B.B.; SANTOS, V.A. Production of biosurfactants for application in the removal of hydrophobic contaminants originated by the petroleum industry. **Chemical Engineering Transactions**. vol.27, p.7–12, 2012.

SILVA, E.J.; ROCHA E SILVA, N.M.P.; RUFINO, R.D.; LUNA, J.M.; SILVA, R.O.; SARUBBO, L.A. Characterization of a biosurfactant produced by *Pseudomonas cepacia* CCT6659 in the presence of industrial wastes and its application in the biodegradation of hydrophobic compounds in soil. **Colloids and Surfaces B: Biointerfaces**, v.117, p. 36–41, 2014a.

SILVA, R.C.F.S.; ALMEIDA, D.G.; RUFINO, R.D.; LUNA, J.M.; SANTOS, V.A.; SARUBBO, L.A. Applications of biosurfactants in the petroleum industry and the remediation of oil spills. **International Journal of Molecular Sciences.** vol.15, p.12523–12542, 2014. doi: 10.3390/ijms150712523

SILVA, R.C.F.S.; ALMEIDA, D.G.; RUFINO, R.D.; LUNA, J.M.; SANTOS, V.A.; SARUBBO, L.A. Applications of biosurfactants in the petroleum industry and the

remediation of oil spills. **International Journal of Molecular Sciences**. vol.15, p.12523–12542, 2014.

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

SOBRINHO, H.B.S.; LUNA, J.M.; RUFINO, R.D.; PORTO, A.L.F.; SARUBBO, L.A. Application of biosurfactant from *Candida sphaerica* UCP 0995 in removal of petroleum derivative from soil and sea water. **Life Sciences.** vol.7, p.559–569, 2013.

SOBRINHO, H.B.S.; RUFINO, R.D.; LUNA, J.M.; SALGUEIRO, A.A.; CAMPOS-TAKAKI, G.M.; LEITE, L.F.C.; SARUBBO, L.A. Utilization of two agroindustrial by-products for the production of a surfactant by *Candida sphaerica* UCP0995. **Process Biochemistry**. vol.43, 9, p. 912–917, 2008.

VERMA, A.; GUPTA, N.; VERMA, S.K.; DAS, M.D.J. Multifactorial approach to biosurfactant production by adaptive strain *Candida tropicalis* MTCC 230 in the Presence of Hydrocarbons. **Journal of Surfactants and Detergents**. vol.18, 1, p.145–153, 2015. doi:10.1007/s11743-014-1608-z

YOUSSEF, N.H.; NGUYEN, T.; SABATINI, D.A.; MCINERNEY, M.J. Basis for formulating biosurfactant mixtures to achieve ultra low interfacial tension values against hydrocarbons. **Journal of Industrial Microbiology and Biotechnology**. vol.34, 7, p. 497–507, 2007.

Figure captions

Figure 1. Growth, pH, surface tension and biosurfactant concentration profiles of *C.tropicalis* UCP 0996 grown in medium supplemented with 2.5% waste frying oil, 2.5% corn steep liquor and 2.5% molasses during 120 h at 200 rpm and 28ºC.

Figure 2. Influences of salt concentration (A), pH (B), temperature (C) and heating time at 90°C (D) on surface-tension-reducing activity of cell-free broth containing biosurfactant from *C.tropicalis* cultivated in distilled water supplemented with 2,5% cane molasses, 2,5% waste frying oil and 2.5% corn steep liquor for 120 h at 200 rpm and 28°C.

Figure 3. Influences of salt concentration (A), pH (B), temperature (C) and heating time at 90°C (D) on emulsifying activity of cell-free broth containing biosurfactant from *C.tropicalis* cultivated in distilled water supplemented with 2,5% cane molasses, 2,5% waste frying oil and 2.5% corn steep liquor for 120 h at 200 rpm and 28°C.

Figure 4. Surface tension versus concentration of the isolated biosurfactant from *C.tropicalis* UCP 0996 cultivated in mineral medium supplemented with 2,5% cane molasses, 2,5% waste frying oil and 2.5% corn steep liquor for 120 h at 200 rpm and 28°C.

Figure 5. TLC of the biosurfactant from *C.tropicalis* UCP 0996 cultivated in mineral medium supplemented with 2,5% cane molasses, 2,5% waste frying oil and 2.5% corn steep liquor for 120 h at 200 rpm and 28°C.

Figure 6. FTIR spectrum for biosurfactant extract produced by *C.tropicalis* UCP 0996 cultivated in mineral medium supplemented with 2,5% cane molasses, 2,5% waste frying oil and 2.5% corn steep liquor.

Figure 7. ^{1}H NMR spectrum (CD_3OD, 300 MHz) of the isolated biosurfactant from *C.tropicalis* UCP 0996 cultivated in mineral medium supplemented with 2,5% cane molasses, 2,5% waste frying oil and 2.5% corn steep liquor.

Figure 8. Chromatogram of the GC-MS separation of the biosurfactant produced by the *C.tropicalis* UCP 0996 showing peaks for cyclic structures containing carbonyl and hydroxyl groups.

Figure 9. Surface tension of biosurfactant formulated with sorbate 0.2% for 120 days under varying pH (A – C), temperature (D – E), and NaCl (F – H) after each storage period.

Fig. 10. Emulsification index of biosurfactant formulated with sorbate 0.2% for 120 days under varying pH (A – C), temperature (D – E), and NaCl (F – H) after each storage period.

Fig. 11. Dispersion index of biosurfactant formulated with sorbate 0.2% for 120 days under varying pH (A – C), temperature (D – E), and NaCl (F – H) after each storage period.

Fig. 12. Influence of biosurfactant from *C.tropicalis* UCP 0996 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.

Table 1. Toxicity of the biosurfactant from *C. tropicalis* UCP 0996 cultivated in medium supplemented with 2.5 % waste frying oil, 2.5 % corn steep liquor and 2.5 % cane molasses on brine shrimp larvae

Concentrations (CMC)	**Shrimp larvae mortality (%)**
Cell-free broth	No mortality
1/2xCMC	No mortality
1xCMC	No mortality
2xCMC	No mortality
5xCMC	10 ± 0.13
10xCMC (LC_{50})	50 ± 0.19

Table 2. Motor oil dispersion by biosurfactant from *C. tropicalis* UCP 0996 cultivated in distilled water supplemented with 2.5 % waste frying oil, 2.5 % corn steep liquor and 2.5 % cane molasses

Removal agent	Dispersion (%)
Formulated biosurfactant	57.14 ± 0.12
1/2 x CMC	38.09 ± 0.2
1 x CMC	45.23 ± 0.11
2 x CMC	53.8 ± 0.12
5 x CMC	70.95 ± 0.3

Table 3. Removal of motor oil adsorbed on marine stones by the formulated and isolated biosurfactant produced by *C. tropicalis* UCP 0996

Removal agent	Removal (%)
Formulated biosurfactant	41.89 ± 0.5
1/2 x CMC	28.37 ± 0.3
1 x CMC	42.01 ± 0.12
2 x CMC	56.02± 0.21
5 x CMC	66.18± 0.4
Control (distilled water)	2.35± 0.1

Table 4. Evaluation of the biosurfactant from *C. tropicalis* UCP 0996 cultured in a medium containing 2.5 % waste frying oil, 2.5 % corn steep liquor and 2.5 % cane molasses as an oil spill dispersant

Biosurfactant/oil ratio	Resting (min)	Dispersion (%)
1/1	0	65.03 ± 0.5
	5	59.45± 0.1
	10	50.23± 0.3
1/2	0	41.13± 0.11
	5	31.50± 0.6
	10	27.11± 0.23
1/8	0	20.47± 0.2
	5	17.31± 0.14
	10	14,34± 0.16
1/20	0	11.26± 0.5
	5	7.92± 0.2
	10	4.15± 0.1

Figure 1

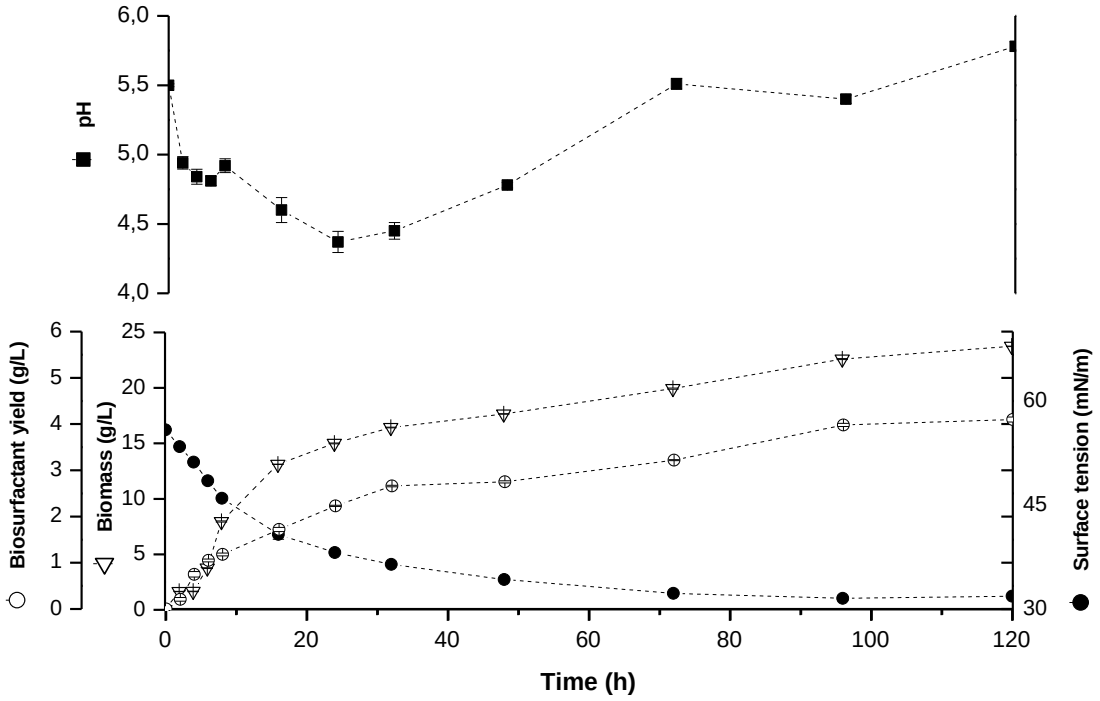

pH
6,0
5,5
5,0
4,5
4,0
Biosurfactant yield (g/L)
Biomass (g/L)
Surface tension (mN/m)
0
1
2
3
4
5
6
0
5
10
15
20
25
30
45
60
0
20
40
60
80
100
120
Time (h)

Figure 2

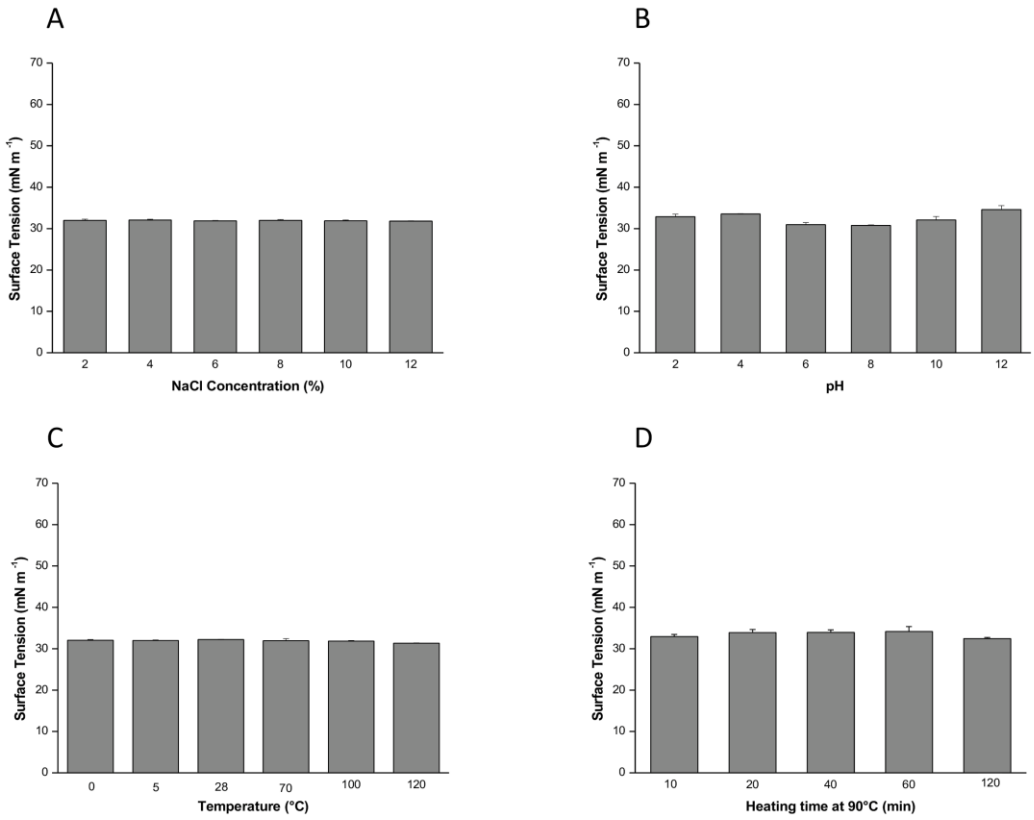

A
Surface Tension (mN m-1)
NaCl Concentration (%)
B
Surface Tension (mN m-1)
pH
C
Surface Tension (mN m-1)
Temperature (°C)
D
Surface Tension (mN m-1)
Heating time at 90°C (min)

Figure 3

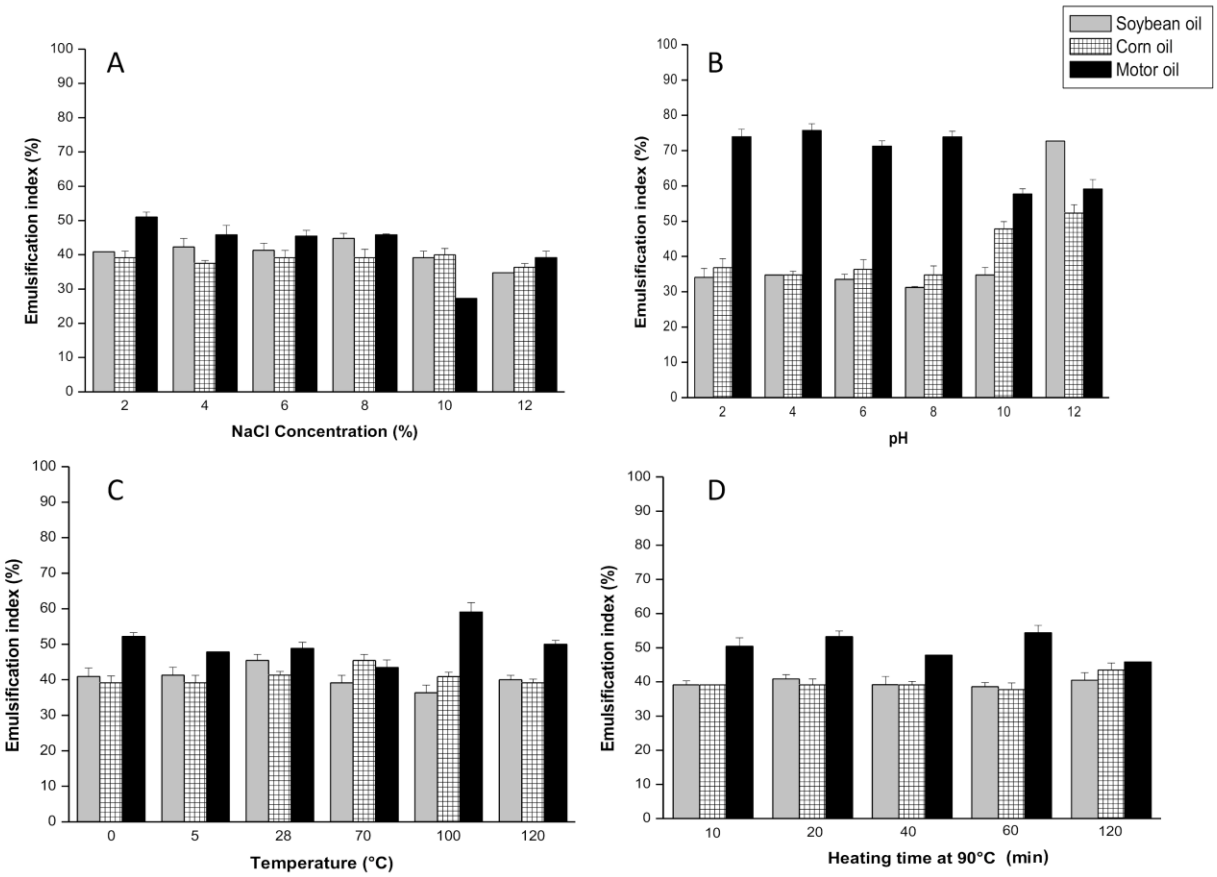

A
B
C
D
Soybean oil
Corn oil
Motor oil
Emulsification index (%)
NaCl Concentration (%)
pH
Temperature (°C)
Heating time at 90°C (min)

Figure 4

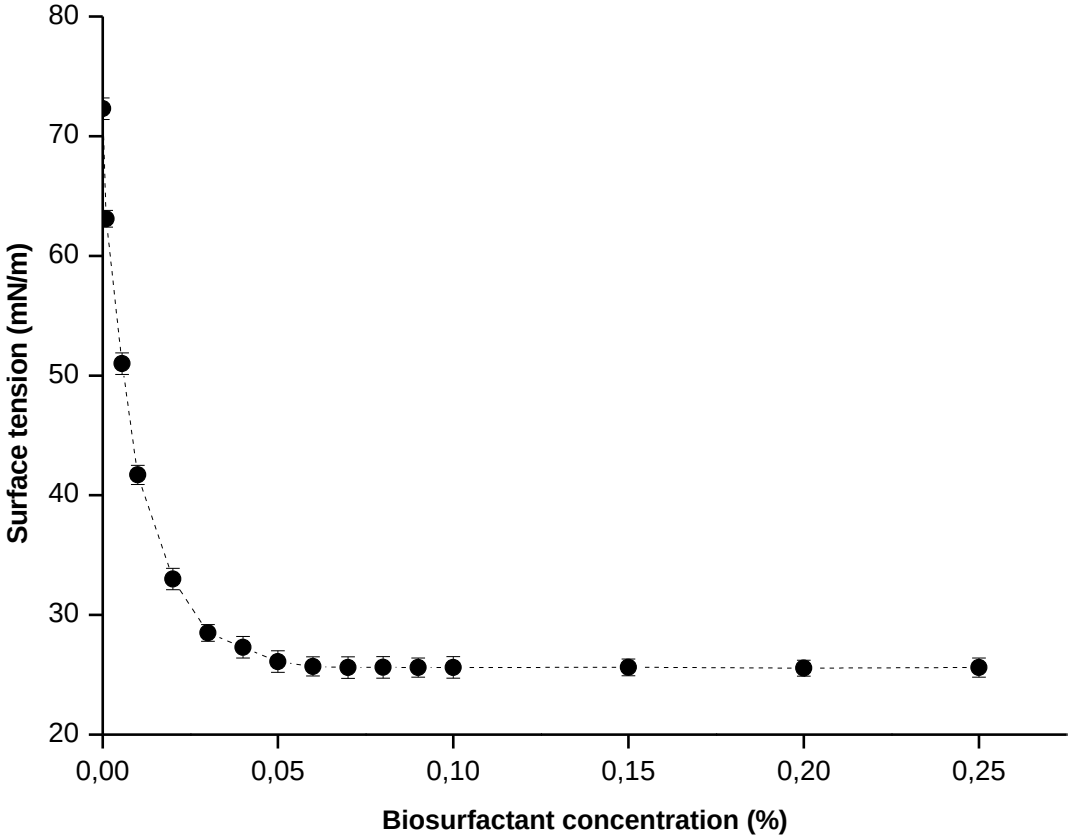

80
70
60
50
40
30
20
Surface tension (mN/m)
0,00
0,05
0,10
0,15
0,20
0,25
Biosurfactant concentration (%)

Figure 5

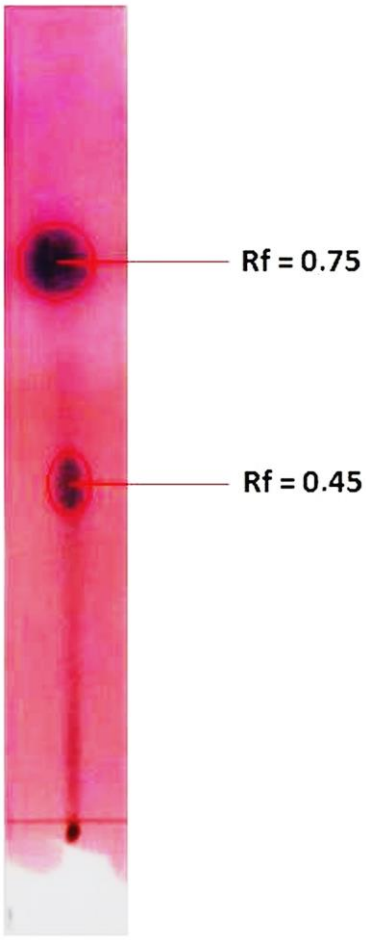


Figure 6

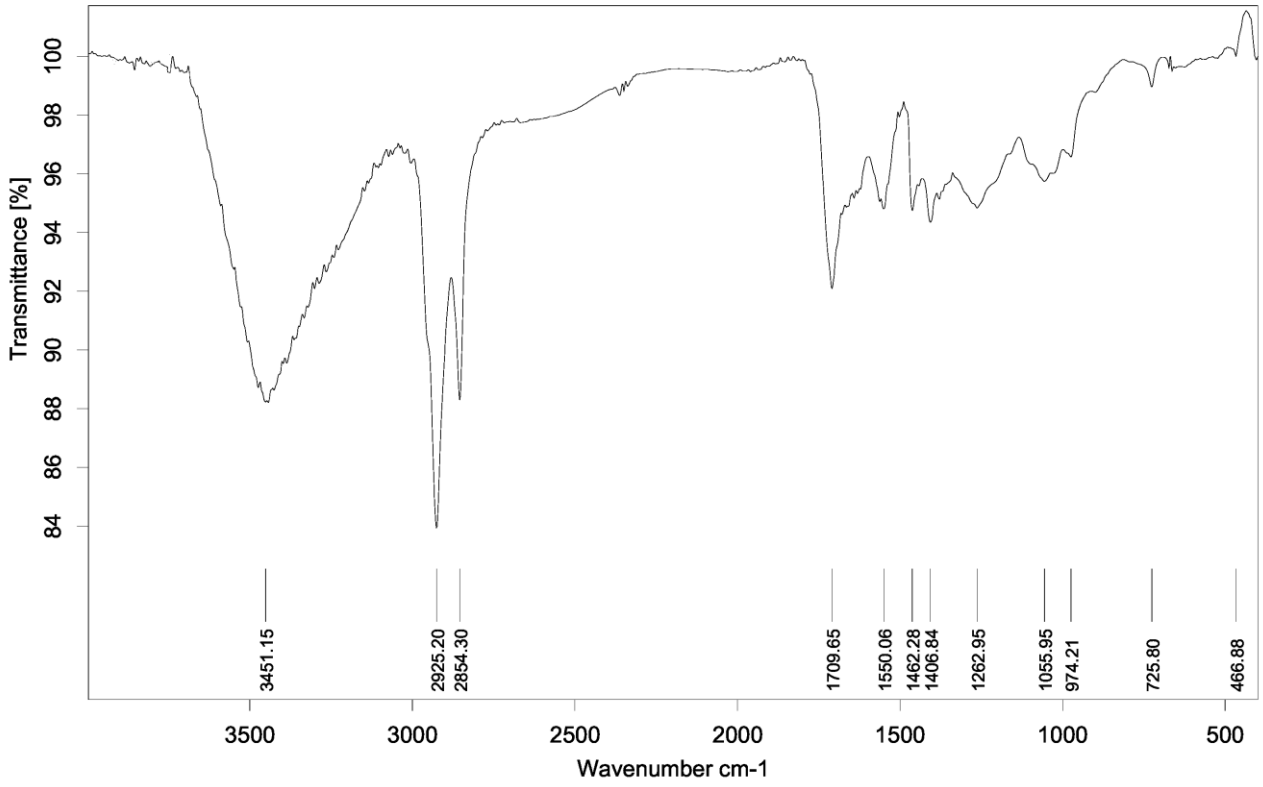

Transmittance [%]
100
98
96
94
92
90
88
86
84
3451.15
2925.20
2854.30
1709.65
1550.06
1462.28
1406.84
1262.95
1055.95
974.21
725.80
466.88
3500
3000
2500
2000
1500
1000
500
Wavenumber cm-1

Figure 7

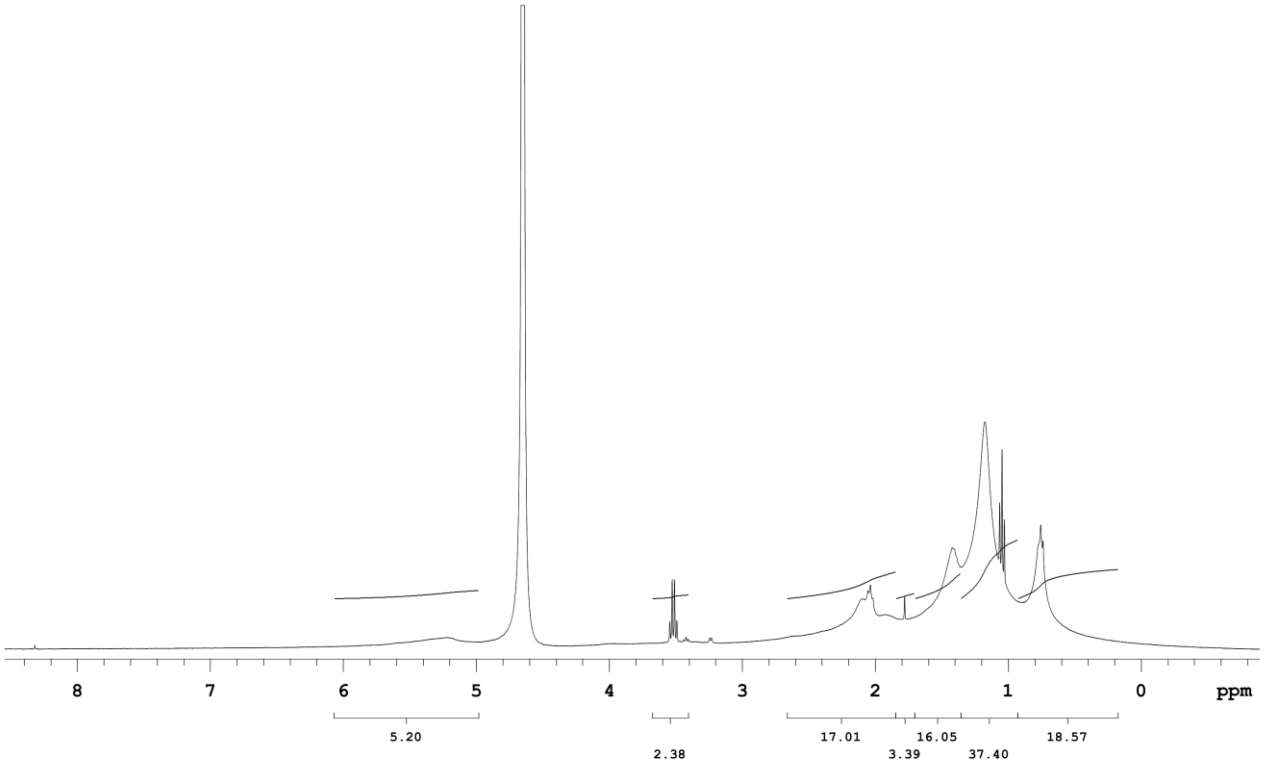


Figure 8

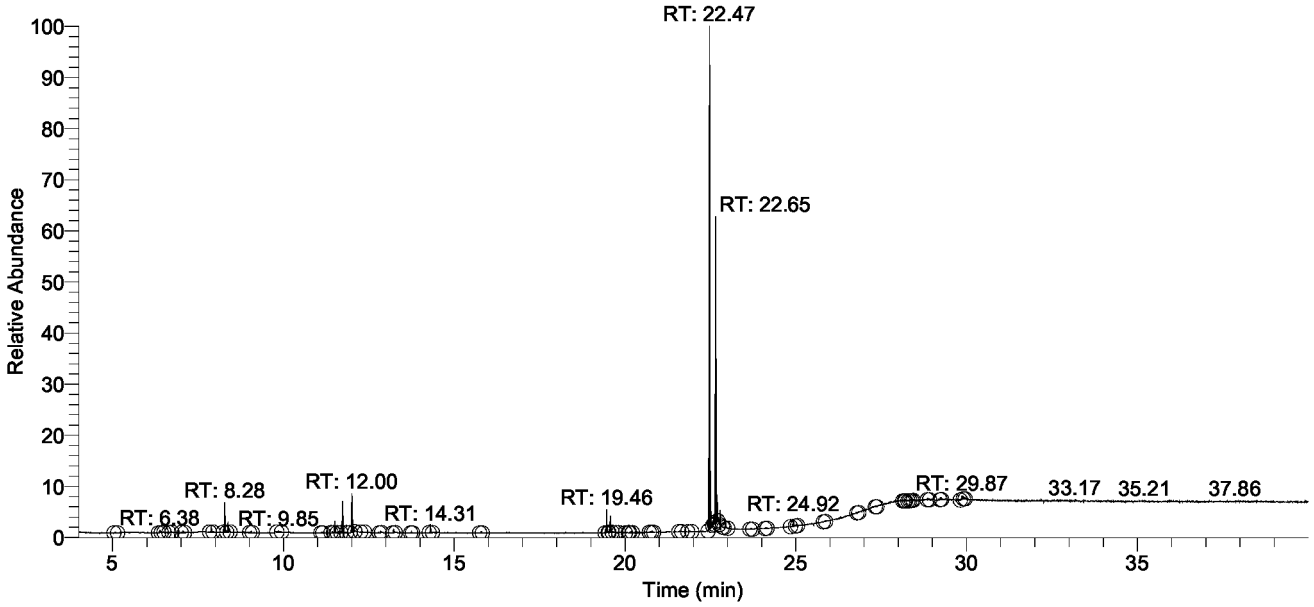

RT: 22.47
RT: 22.65
RT: 8.28
RT: 12.00
RT: 6.38
RT: 9.85
RT: 14.31
RT: 19.46
RT: 24.92
RT: 29.87
33.17
35.21
37.86
Relative Abundance
Time (min)
100
90
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30
35

Figure 9

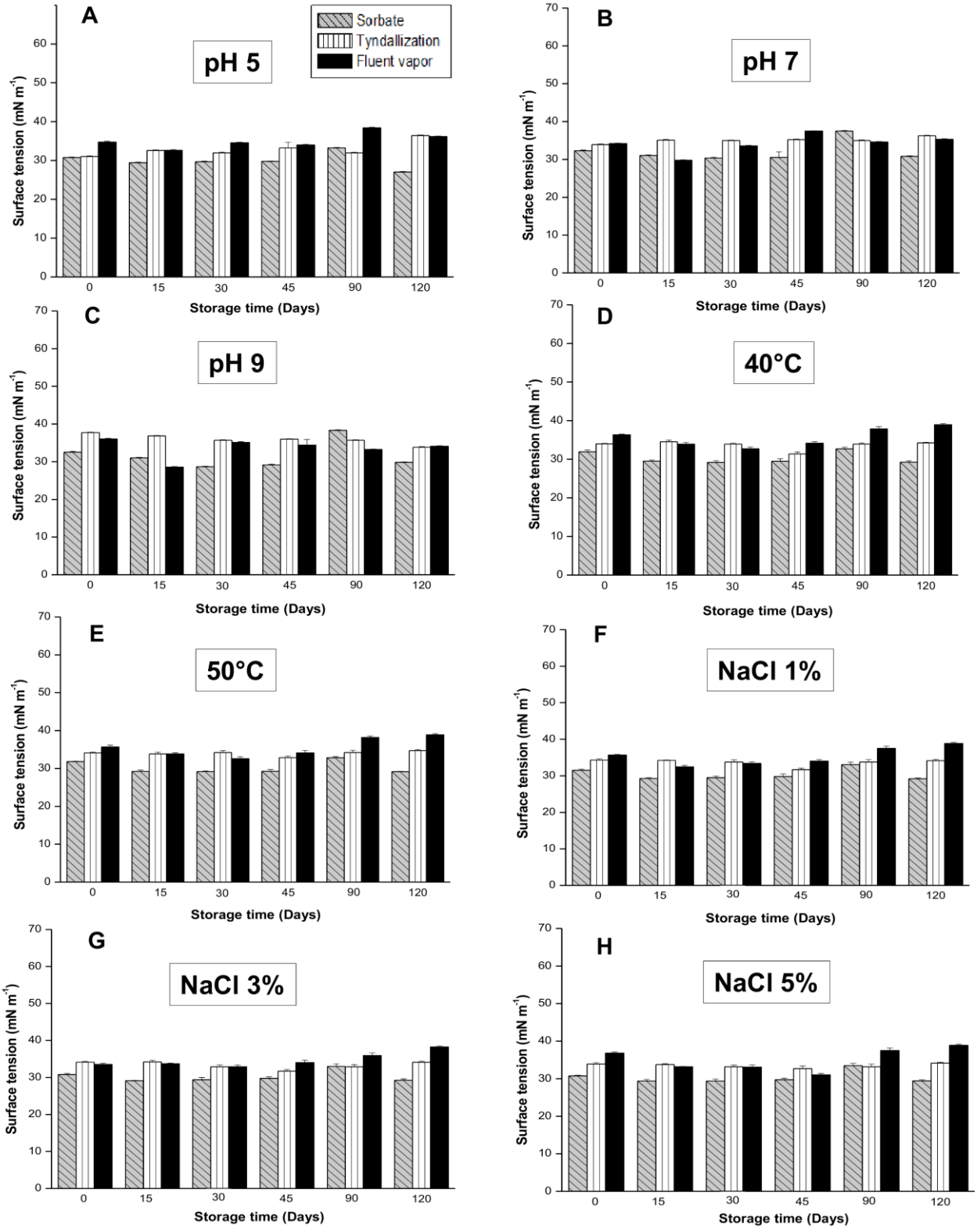

A
pH 5
Sorbate
Tyndallization
Fluent vapor
B
pH 7
C
pH 9
D
40°C
E
50°C
F
NaCl 1%
G
NaCl 3%
H
NaCl 5%
Surface tension (mN m-1)
Storage time (Days)
0
15
30
45
90
120

Figure 10

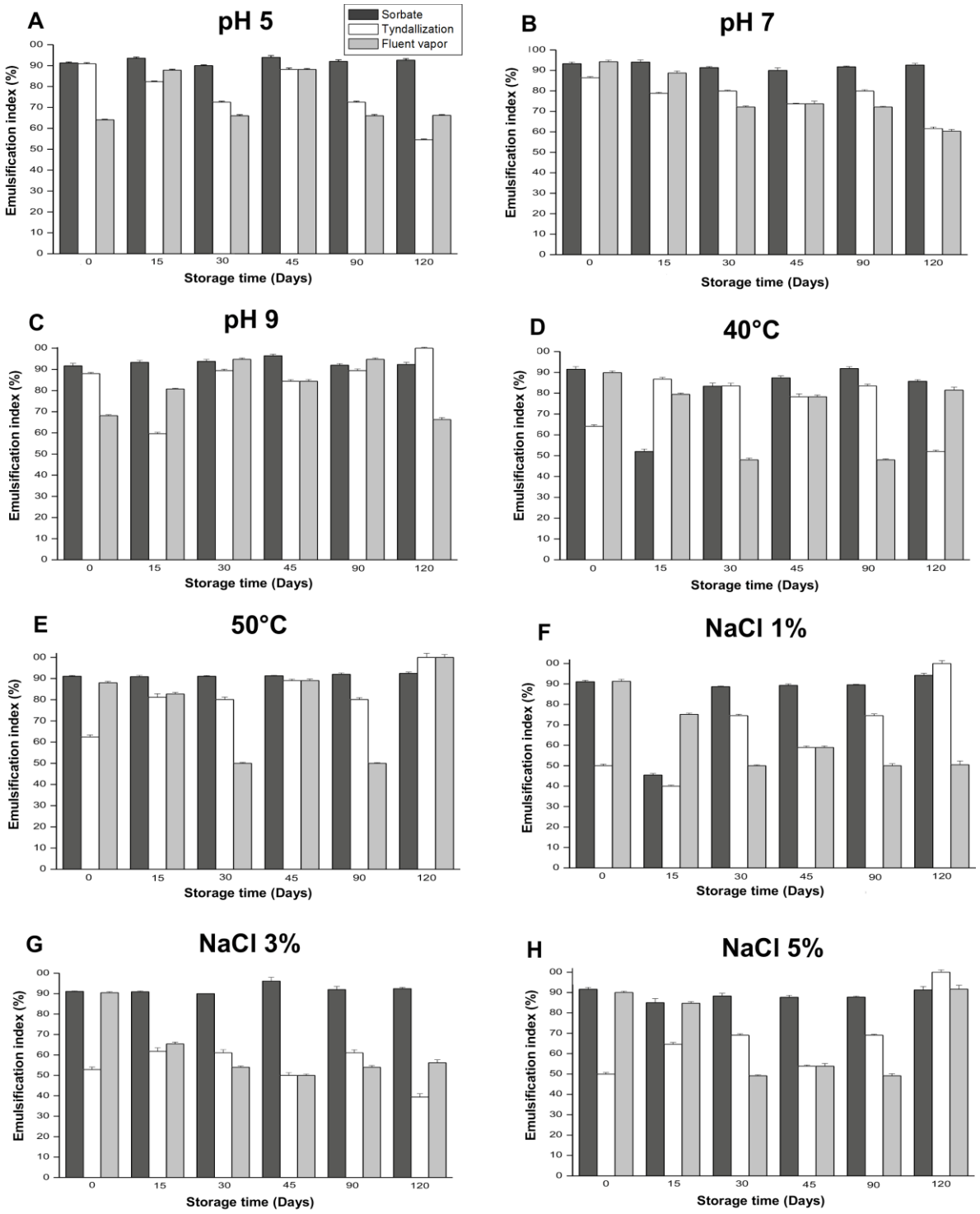

A
pH 5
Sorbate
Tyndallization
Fluent vapor
B
pH 7
C
pH 9
D
40°C
E
50°C
F
NaCl 1%
G
NaCl 3%
H
NaCl 5%
Emulsification index (%)
Storage time (Days)
0
15
30
45
90
120

Figure 11

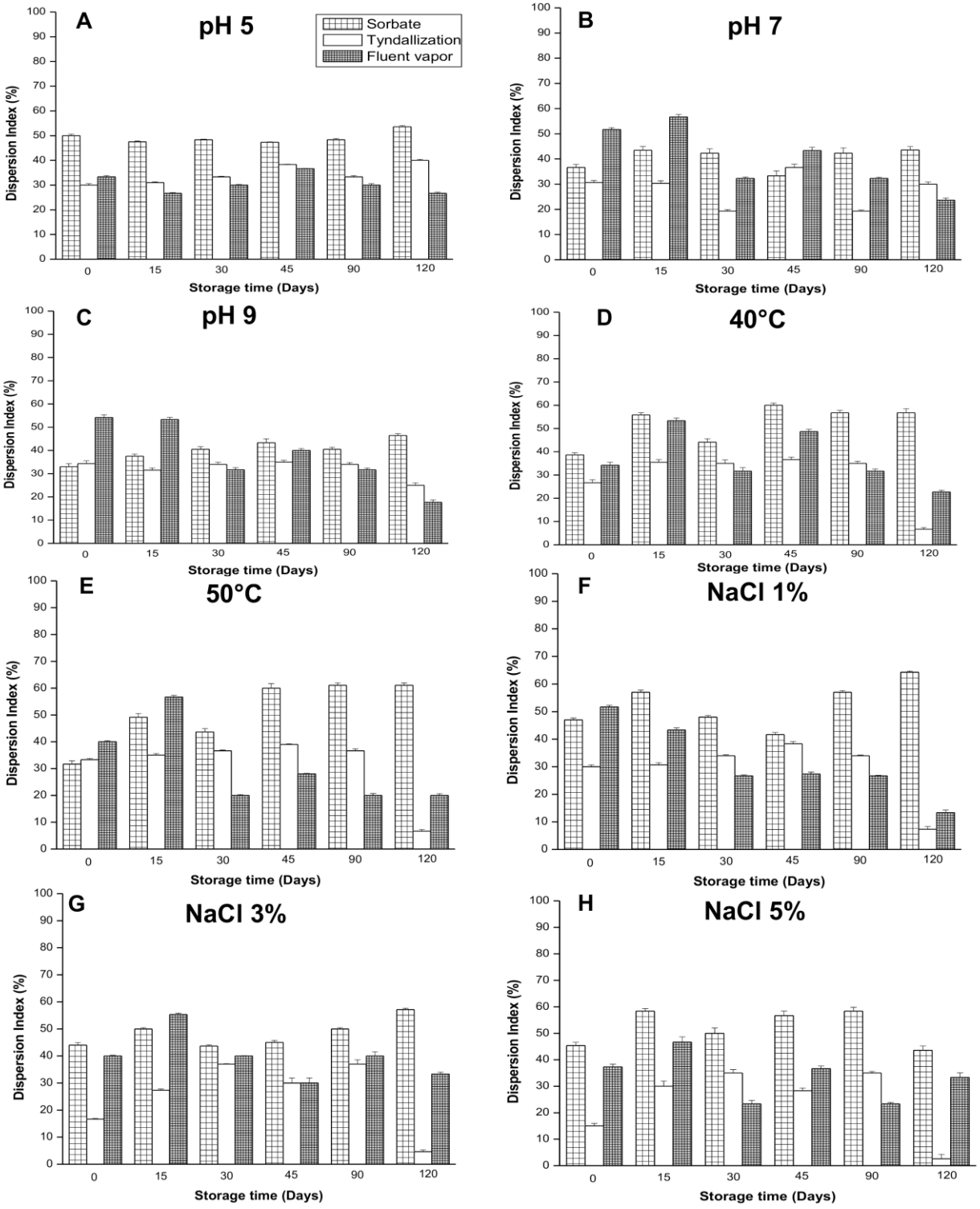
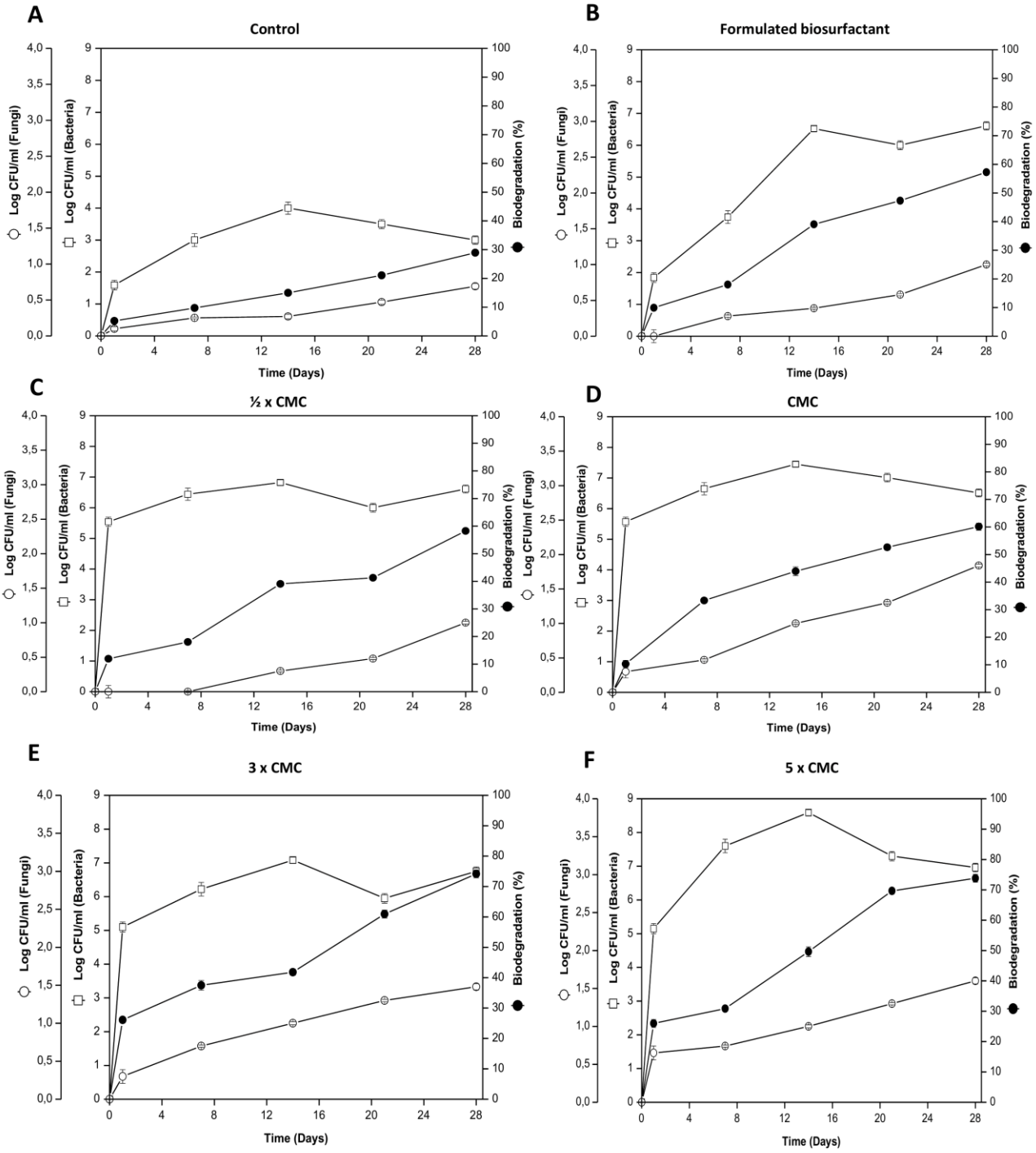

A
pH 5
Sorbate
Tyndallization
Fluent vapor
B
pH 7
C
pH 9
D
40°C
E
50°C
F
NaCl 1%
G
NaCl 3%
H
NaCl 5%
Dispersion Index (%)
Storage time (Days)
0
15
30
45
90
120

Figure 12


A
Control
B
Formulated biosurfactant
C
½ x CMC
D
CMC
E
3 x CMC
F
5 x CMC
Log CFU/ml (Fungi)
Log CFU/ml (Bacteria)
Biodegradation (%)
Time (Days)

5.4. CAPÍTULO 4

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

Patente depositada no *Instituto Nacional da Propriedade Industrial*.

< Uso exclusivo do INPI >

INPI INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL PROTOCOLO GERAL
AUGUSTO 018140013678
22/07/2014 12:32 DESP
BR 10 2014 017963 1

Espaço reservado para o protocolo | Espaço reservado para a etiqueta | Espaço reservado para o código QR

INPI INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

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) + certidão 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 e Tecnologia e Inovação - CGTI

Folha Anexa do Formulário FQ001

6. Inventor (72):

6.1 Nome: Leonie Asfora Sarubbo
6.2 Qualificação: química industrial
6.3 CPF: 735.801.634-34
6.4 Endereço completo: Rua Arthur Muniz, n. 147, Apto. 2001, bairro Boa Viagem, cidade Recife, estado Pernambuco.
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

6.1 Nome: Raquel Diniz Rufino
6.2 Qualificação: bióloga
6.3 CPF: 037.938.884-79
6.4 Endereço completo: Rua 24 de junho, n. 279, Apto. 1402, bairro Encruzilhada, cidade Recife, estado Pernambuco
6.5 CEP: 52.030-100
6.6 Telefone: (81) 3031-7248
6.7 Fax: (81) 3031-7248
6.8 E-mail: raqueldrufino@yahoo.com.br

6.1 Nome: Juliana Moura de Luna
6.2 Qualificação: bióloga
6.3 CPF: 041.451.344-43
6.4 Endereço completo: Rua José Nunes da Cunha, n. 370, Apto. 301, bairro Piedade, cidade Jaboatão dos Guararapes, estado Pernambuco.
6.5 CEP: 54.410-280
6.6 Telefone: (81) 3474-0623
6.7 Fax: (81) 3474-0623
6.8 E-mail: julianamouraluna@gmail.com.br

6.1 Nome: Valdemir Alexandre dos Santos
6.2 Qualificação: químico industrial
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.
6.5 CEP: 51.190-350
6.6 Telefone: (81) 3339-0523
6.7 Fax: (81) 3339-0523
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

- A aplicação de um planejamento fatorial como ferramenta estatística foi eficaz na identificação e seleção das condições mais favoráveis para a produção do biossurfactante de *Candida tropicalis* UCP 0996.
- A utilização de resíduos industriais como substratos de baixo custo foi favorável ao crescimento de *Candida tropicalis* UCP 0996 e à produção do biossurfactante.
- A produção do biossurfactante em escala semi industrial proporcionou um aumento expressivo do rendimento sem alterações significativas de sua propriedade tensoativa.
- O biossurfactante produzido reduziu consideravelmente a tensão superficial da água, demonstrando elevada capacidade emulsionante e dispersante de compostos hidrofóbicos.
- O biossurfactante demonstrou uma excelente estabilidade em condições ambientais extremas de salinidade, temperatura e variações de pH.
- O biossurfactante obtido foi caracterizado como um tensoativo de natureza aniônica e glicolipídica.
- O biossurfactante não apresentou toxicidade a organismos marinhos nas condições testadas.
- A biomolécula formulada exibiu uma vida de prateleira prolongada, apresentando potencial considerável para aplicação como um dispersante comercialmente estável e os testes com o biossurfactante formulado revelaram a viabilidade de aplicação sem a necessidade de purificação do produto.
- Os testes de lavagem de composto hidrofóbico adsorvido em pedras marinhas com o biossurfactante formulado demonstraram a capacidade de desta biomolécula para aplicação na remoção de óleo.
- Os testes de deslocamento (dispersão/agregação) de composto hidrofóbico em água do mar pelo biossurfactante formulado revelaram a viabilidade de aplicação desta biomolécula como biodispersante.
- O biossurfactante produzido apresentou grande potencial de aplicação em processos de biorremediação de derrames de óleo em água do mar em ambientes marinhos.

ANEXOS

renorbio
rede nordeste de biotecnologia

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 **Darne Germano de Almeida**, ingresso no doutorado do Programa de Pós-Graduação em Biotecnologia da Rede Nordeste de Biotecnologia (RENORBIO) em março de 2013, sob o número de matrícula 13023045, defendeu a tese intitulada **"Produção, caracterização e aplicação de biossurfactante como agente de remediação em ambiente marinho"**, desenvolvida na Instituição Associada Universidade Católica de Pernambuco, sob a orientação da Profa. Dra. Leonie Asfora Sarubbo, no dia 22 de fevereiro de 2017, a qual foi aprovado 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, Valdemir Alexandre dos Santos da Universidade Católica de Pernambuco, como titular, Adalberto Pessoa Júnior – USP, como titular, e Raquel Diniz Rufino bolsista PNPD da Universidade Católica de Pernambuco, estando apto a receber o Diploma de Doutor em Biotecnologia, Área de Concentração em Biotecnologia Industrial, após a entrega da versão final da tese.

Recife 22 de fevereiro de 2017.

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

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) 3320.6079 - E-mail: coordenacao.renorbio@ufrpe.br

DECLARAÇÃO

Declaramos, para os devidos fins, que a Qualificação intitulada "Produção, caracterização de biossurfactante como agente de remediação em ambiente marinho", de autoria do Doutorando **DARNE GERMANO DE ALMEIDA**, sob a orientação da Profa. Dra. Leonie Asfora Sarubbo- UNICAP, foi defendida em 20 de setembro de 2016, tendo como Banca Examinadora os seguintes membros:

Profa. Dra. Ana Lúcia Figueiredo Porto – UFRPE **1º Examinador**
Prof. Dr. Valdemir Alexandre dos Santos – UNICAP **2º Examinador**
Dra. Alícia Maria Andrade Torres Jara – Pós-Doc/UNICAP **3º Examinador**
Profa. Dra. Ester Ribeiro Gouveia - UFPE **Suplente**
Profa. Dra. Raquel Diniz Rufino – UNICAP **Suplente**

Recife, 20 de setembro de 2016

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

Coordenação Geral do Programa de Pós-Graduação em Biotecnologia
Universidade Estadual do Ceará
Av. Paranjana, 1.700 – Campus do Itaperi – 60740-000 Fortaleza, Ce
Telefone (85) 3101.9645 Fax: (85) 3101.9650 E-mail: renorbio@uece.br
Homepage: http://www.renorbio.org.br

COMPROVAÇÃO DE ESTÁGIO DE DOCÊNCIA

Nome do Aluno:	Nº Matrícula
DARNE GERMANO DE ALMEIDA	**13023045**

Nome do Orientador:
LEONIE ASFORA SARUBBO

Nome do Professor do Estágio:
LEONIE ASFORA SARUBBO – Disciplina: Processos de Separação

Curso	Ponto Focal
Doutorado	**UFRPE**

Atividades Desenvolvidas:

Aulas Teóricas:

- Liofilização (4h);
- Separação por solventes orgânicos (4h);
- Ultrafiltração (4h);

Aulas Práticas:

- Separação por processos físicos (3,5h);
- Separação por solventes orgânicos (3,5h);

Avaliação:

- Seminários, primeiro dia (2,5h);
- Seminários, segundo dia (2,5h);

Atividades extraclasse:

- Resolução de exercícios e tira-dúvidas, 1h por semana (10h)

Carga horária total: 34h

Período do Estágio:

De Agosto à Dezembro de 2014

renorbio
rede nordeste de biotecnologia

Avaliação do Professor do Estágio:

"O aluno participou ativamente das atividades sugeridas, desenvolvendo excelente atuação e extremo comprometimento".

Data: 10/12/14

Ivonii Assis Sanhlo

Professor do Estágio

Ivonii Assis Sanhlo

Orientador

Representante Estadual

renorbio
rede nordeste de biotecnologia

Coordenação Geral do Programa de Pós-Graduação em Biotecnologia
Universidade Estadual do Ceará
Av. Paranjana, 1.700 – Campus do Itaperi – 60740-000 Fortaleza, Ce
Telefone (85) 3101.9645 Fax: (85) 3101.9650 E-mail: renorbio@uece.br
Homepage: http://www.renorbio.org.br

COMPROVAÇÃO DE ESTÁGIO DE Ddocência

Nome do Aluno:	Nº Matrícula
Darne Germano de Almeida	13023045

Nome do Orientador:
Bronie Aspora Sarubbo

Nome do Professor do Estágio:
Roziana Jordão - Disciplina: Bioquímica

Curso	Ponto Focal
Doutorado	UFRPE

Atividades Desenvolvidas:

Aulas práticas
- Caracterização de proteínas (3h)
- Determinação quantitativa de proteínas (3h)
- Identificação de carboidratos (3 h)
- Determinação de glicose pelo método do DNS (3h)
- Cinética enzimática - aplicação de software educativo (3h)

Aula Teórica:
- Ciclo de Krebs - Produção de ácido cítrico (estudo dirigido; regulação enzimática) - (3h)
- Engenharia genética - Conceitos; ferramentas e aplicações - estudo dirigido - (3h)

Atividades extraclasse:
- Tira dúvidas: 1 h por semana para esclarecer eventuais dúvidas dos alunos (Total: 12 h)

Período do Estágio:
Total da carga horária: 33h

Avaliação do Professor do Estágio:
O aluno participou ativamente das atividades propostas, demonstrando excelente desempenho e comprometimento.

Data: 20/12/13

Professor do Estágio

Bronie Aspora Sarubbo
Orientador

Representante Estadual

APÊNDICES

Int. J. Mol. Sci. **2014**, *15*, 12523-12542; doi:10.3390/ijms150712523

OPEN ACCESS
International Journal of
Molecular Sciences
ISSN 1422-0067
www.mdpi.com/journal/ijms

Review

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

Rita de Cássia F. S. Silva [1,2], Darne G. Almeida [1,2], Raquel D. Rufino [2,3], Juliana M. Luna [2,3], Valdemir A. Santos [2,3] and Leonie Asfora Sarubbo [2,3,*]

[1] Post-Graduate Program in Biotechnology, Federal Rural University of Pernambuco, CEP 52.171-900 Recife, PE, Brazil; E-Mails: ritaf78@gmail.com (R.C.F.S.S.); darne@buenomak.com.br (D.G.A.)

[2] Center for Management of Technology and Innovation—CGTI, Rua da Praia, n.11, São José, CEP 50.020-550 Recife, PE, Brazil; E-Mails: raqueldrufino@yahoo.com.br (R.D.R.); julianamouraluna@gmail.com (J.M.L.); valdemir.alexandre@hotmail.com (V.A.S.)

[3] Center for Sciences and Technology, Catholic University of Pernambuco, Rua do Príncipe, n. 526, Boa Vista, CEP 50.050-900 Recife, PE, Brazil

* Author to whom correspondence should be addressed; E-Mail: leonie@unicap.br; Tel.: +55-81-2119-4084; Fax: +55-81-2119-4000.

Received: 16 May 2014; in revised form: 26 June 2014 / Accepted: 7 July 2014 / Published: 15 July 2014

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

1411

CHEMICAL ENGINEERING TRANSACTIONS
VOL. 43, 2015

A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet

Chief Editors: Sauro Pierucci, Jiří J. Klemeš

ISBN 978-88-95608-34-1; **ISSN** 2283-9216

DOI: 10.3303/CET1543236

Optimization of biosurfactant production from *Candida guiliermondii* using a Rotate Central Composed Design

Pedro P. F. Brasileiro*[a,b], Darne G. de Almeida[b], Juliana M.de Luna[a,b], Raquel D. Rufino[a,b], Valdemir A. dos Santos[a,b], Leonie A. Sarubbo[a,b]

[a]Centre of Science and Technology, Catholic University of Pernambuco, Rua do Príncipe, n. 526, Boa Vista, Cep: 50050-900, Recife, Pernambuco, Brazil
[b]Centre from Management of Technology and Information, CGTI, Rua da Praia, n. 11, São José, Cep: 50020-550, Recife-PE, Brazil
ppfbrasileiro@hotmail.com

Accidents in petroleum platforms happen because of the absence of an effective fluid control inside the pipes, as occurred in 2010, when the scientists were not prepared to treat the oil spill in the Deepwater Horizon platform. In such environmental accident, non-biodegradable chemical dispersants were applied. As a safe alternative for the environment, the combination of the biotechnological potential of the microorganisms and the low cost waste materials results in the production of biosurfactants, known as the petroleum bioremediators. Thus, the optimization of operational parameters for biosurfactant production by *Candida guilliermondii* UCP 0992 grown in a low-cost medium and formulated with 4.0 % of corn steep, 2.5 % of molasses and 2.5 % of soybean residual oil was carried out in a 1.2 L bioreactor using response-surface methodology. The application of a Rotate Central Composed Design (RCCD) led to the identification of agitation speed, aeration, time and inoculum size as significant variables affecting the fermentation process. The optimal levels of the aforementioned variables were 250 rpm agitation speed, 132 h of cultivation time, 0.5 L/min of filtrated air and 4 % inoculum size. The experimental verifications allowed a maximum relative surface tension reduction to 31.45 mN/m and interface tension reduction to 9.04 mN/m, which was found to be equivalent to about 30.2 g/L isolated biosurfactant as estimated gravimetrically, thereby resulting in an improved production. Besides the optimization of operational parameters, the economic cost of € 22.37 was estimated to the biosurfactant produced according to the local price of the kWh. This work, therefore, showed that the fermentation time spent in flasks (144 h), could be reduced in 12 hours, increasing 3.6 times the yield and keeping the surface and interface tensions at the lowest level. Moreover, the biosurfactant produced by *C. guilliermondii* shows potential to be applied in oil spills.

1. Introduction

Each year, over 5 million t petroleum are transported through the oceans, promoting the exchange desired by the capitalists. However, the soaring gain with the mixture of hydrocarbons and oils becomes a true loss when the control technologies do not act as predicted, spilling the oil into the sea and causing economic and environmental disasters during a long time (Al-Majed et al., 2012).

Among the most severe accidents, the oil spill from the Deepwater Horizon platform cost at least U$ 40 billion for the company, which was managing the giant structure of more than 1.5 km of depth. Besides, U$ 20 billion were destined to a victim compensation fund. All this money was spent because the block valve control mechanisms did not work inside the oil pipes, being considered a project mistake. Therefore, 997 birds, 400 marine turtles and 47 mammals died simply because a group of engineers did not planned the correct control of the petroleum (Bozeman, 2011).

After 20 days from the disaster, the scientists spilled 7,000,000 L of chemical dispersants to control the oil. On the other hand, these compounds are derivated from the petroleum, raising the toxicity in the lethal zone and killing more animals. The solution to substitute the chemical surfactants is to utilize microorganisms with a

Please cite this article as: Brasileiro P., Almeida D., Luna J., Rufino R., Santos V., Sarubbo L., 2015, Optimization of the biosurfactant production from candida guiliermondii using a central composite rotatable design, Chemical Engineering Transactions, 43, 1411-1416 DOI: 10.3303/CET1543236

frontiers in Microbiology

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

Danyelle Khadydja F. Santos[2], Ana Helena M. Resende[1], DARNE G. DE ALMEIDA[2], Rita D. Soares da Silva[2], Raquel D. Rufino[1], Juliana M. Luna[1], Ibrahim M. Banat[3], Leonie A. Sarubbo[1*]

[1]Catholic University of Pernambuco, Brazil, [2]Biotechnology, Federal Rural University of Pernambuco, Brazil, [3]University of Ulster, United Kingdom

Submitted to Journal:
Frontiers in Microbiology

Specialty Section:
Microbiotechnology, Ecotoxicology and Bioremediation

ISSN:
1664-302X

Article type:
Original Research Article

Received on:
20 Oct 2016

Accepted on:
13 Apr 2017

Provisional PDF published on:
13 Apr 2017

Frontiers website link:
www.frontiersin.org

Citation:
Santos DF, Resende AM, De_almeida DG, Soares_da_silva RD, Rufino RD, Luna JM, Banat IM and Sarubbo LA(2017) Candida lipolytica UCP0988 Biosurfactant: Potential as a Bioremediation Agent and in Formulating a Commercial Related Product. *Front. Microbiol.* 8:767. doi:10.3389/fmicb.2017.00767

CHEMICAL ENGINEERING TRANSACTIONS
VOL. 57, 2017
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Editors in Chief: Sauro Pierucci, Jiří Jaromír Klemeš

ISBN 978-88-95608- 48-8; **ISSN** 2283-9216

Biosurfactant Formulation of *Pseudomonas cepacia* and Application in the Removal of Oil from Coral Reef

Rita de Cássia F. Soares da Silva[a,c], Darne G. Almeida[a,c], Pedro P. F. Brasileiro[b,c], Raquel D. Rufino[b,c], Juliana M. Luna[b,c], Leonie A. Sarubbo*[a,b,c]

[a]Northeast Biotechnology Network, Federal Rural University of Pernambuco – RENORBIO/UFRPE, Rua Dom Manoel de Medeiros, s/n, Dois Irmãos. CEP: 52171-900, Recife – Pernambuco, Brazil
[b]Centre of Sciences and Technology, Catholic University of Pernambuco – UNICAP, Rua do Príncipe, n. 526, Boa Vista, CEP: 50050-900, Recife – Pernambuco, Brazil
[c]Advanced Institute of Technology and Innovation – IATI, Rua Carlos Porto Carreiro, n. 70, Boa Vista, CEP: 50070-090, Recife, Pernambuco, Brazil
*leonie@unicap.br

The innovative biosurfactants arisen to replace chemical surfactants in bioremediation processes because of their effectiveness as dispersants, greater stability and biodegradability. Currently, the major market for biosurfactant is the petroleum industry, where these compounds can be used in the cleanup of oil spills, the removal of oil waste from storage tanks, enhanced oil recovery and bioremediation of soil and water. In this sense, this study investigated the stability of the biosurfactant produced by *Pseudomonas cepacia* CCT6659 growing in culture medium containing 2 % residual canola oil, 3% corn steep liquor and 0.2% $NaNO_3$ for 60 hours at stirring 250 rpm and 28 °C. In the cell-free metabolic liquid was added a salt of potassium sorbate (0.2 %) to the conservation of their surfactant properties. Heating, pH and salinity conditions were applied to evaluate the best application results of the biosurfactant for 120 days. The biosurfactant was also used in the engine lubricating oil removal impregnated in samples of coral reefs so as to evaluate its potential in removing petroleum compounds. The most significant results found for biosurfactant were surface tension of 25.92 mN/m, engine-oil emulsifying capacity of 99 % and dispersion 53.5 % oil in seawater. Moreover, the biosurfactant removed 80 % of the hydrophobic compound of the coral reefs samples. Based on the presented conservation economical method and in the proven resistance to extreme conditions, the biosurfactant demonstrated its potential for applications in the oil industry and in the environmental decontamination processes.

1. Introduction

Contaminants release, such as petroleum and its derived, into the environment is one of the main causes of global pollution. A large number of contaminants are toxic and carcinogenic, placing both human and animal health at risk. Through capillarity, hydrocarbons are adsorb to surfaces and became trapped in a water immiscible phase, making difficult to remove these compounds from contaminated environments (Luna et al., 2013). Petroleum industry is the largest market for surfactants, where they can be used for removal/mobilization of oils encrusted in soil and storage tanks and bioremediation/dispersion of oily spots in the sea and oils removal from rocks and sand of the sea, increasing the recovery of the areas of environmental protection (Almeida et al., 2016; Silva et al., 2014).
Surfactants are amphipathic molecules capable of reducing surface and interfacial tensions between liquids, Solids and gases. All surfactants have two ends, one of which is hydrophobic and the other hydrophilic. A hydrocarbon part usually comprises the hydrophobic end, which is less soluble in water, whereas the water-soluble hydrophilic end may be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol (Luna et al., 2016).
Currently, most compounds with surfactant properties on the market are mainly of synthetic origin. The main factor that restricts biosurfactants use in the market is their production cost of when compared to their

CHEMICAL ENGINEERING TRANSACTIONS
VOL. 57, 2017
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Editors in Chief: Sauro Pierucci, Jiří Jaromír Klemeš

ISBN 978-88-95608- 48-8; **ISSN** 2283-9216

Commercial Formulation of Biosurfactant from Yeast and its Evaluation to Use in the Petroleum Industry

Darne G. Almeida[a,c], Rita de Cássia F. Soares da Silva[a,c], Pedro P. F. Brasileiro[b,c], Juliana M. Luna[b,c], Raquel D. Rufino[b,c], Leonie A. Sarubbo*[a,b,c]

[a]Northeast Biotechnology Network, Federal Rural University of Pernambuco – RENORBIO/UFRPE, Rua Dom Manoel de Medeiros, s/n, Dois Irmãos. CEP: 52171-900, Recife – Pernambuco, Brazil
[b]Centre of Sciences and Technology, Catholic University of Pernambuco – UNICAP, Rua do Príncipe, n. 526, Boa Vista, CEP: 50050-900, Recife – Pernambuco, Brazil
[c]Advanced Institute of Technology and Innovation – IATI, Rua Carlos Porto Carreiro, n. 70, Boa Vista, CEP: 50070-090, Recife, Pernambuco, Brazil
*leonie@unicap.br

Surfactants are amphipathic molecules capable of forming microemulsions of oil in water. Currently, the major market for surfactants 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. However, the limitations of these molecules to the extreme conditions encountered in these steps have opened space for application of so-called biosurfactants, which are molecules more resistant, biodegradable and non-toxic. Biosurfactants are mainly produced by aerobic microorganisms which can be obtained in an aqueous medium containing industrial waste as carbon and nitrogen source. 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 and bioavailability, and decreasing the viscosity. In this research, the application of a biosurfactant produced by *Candida tropicalis* UCP 0996 in the formulation of a biodispersant was investigated. Cell-free metabolic liquid obtained from *C. tropicalis* UCP0996 cultivated in industrial waste was mixed with an inexpensive and non-toxic preservative (0.2% potassium sorbate). The mixture was subjected to a long-term stability study to verify expiry date and recommend storage conditions, and an accelerated stability study to assess the impact of short exposure to adverse conditions outside those idealized for activity of the bioproduct. Properties of the formulated biosurfactant such as surface tension and emulsification were checked at 0, 15, 30, 45, 90 and 120 days. Then, formulated biosurfactant was examined about their dispersing efficiency against motor oil in seawater. As a result, formulated biosurfactant remained stable over time and under extreme conditions of pH, temperature and salinity. Moreover, the use of the formulated biosurfactant as biodispersant allowed reach levels of dispersion above 60%. Therefore, this research allowed the formulation of a low cost biotechnological product with high durability, that maintain the initial properties for many days, demonstrating its potential application in the oil industry as biodispersant.

1. Introduction

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. It is therefore important to develop technologies that allow the efficient use of this resource (Bachmann et al., 2014; CNI, 2007; EMBRAPA, 2006). Petroleum production is therefore also steadily moving toward unconventional crude oils including heavy/extra-heavy oils rather than medium to light oils, according to the International Energy Agency. In countries such as Canada, China, Mexico, Venezuela and the USA; the heavy and extra-heavy crude oils represents approximately half