Programa de Doutorado da Rede Nordeste de Biotecnologia

AÇÃO DE BIOSSURFACTANTES COMO COLETORES NATURAIS NA MELHORIA DA EFICIÊNCIA DO PROCESSO DE TRATAMENTO DE ÁGUAS OLEOSAS POR FLOTAÇÃO

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Ação de biossurfactantes como coletores naturais na melhoria da eficiência do processo de tratamento de águas oleosas por flotação

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

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2018

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Xote ecológico

Não posso respirar, não posso mais nadar
A terra está morrendo, não dá mais pra plantar
E se plantar não nasce, se nascer não dá
Até pinga da boa é difícil de encontrar

Não posso respirar, não posso mais nadar
A terra está morrendo, não dá mais pra plantar
E se plantar não nasce, se nascer não dá
Até pinga da boa é difícil de encontrar

Cadê a flor que estava aqui?
Poluição comeu
E o peixe que é do mar?
Poluição comeu
E o verde onde é que está?
Poluição comeu
Nem o Chico Mendes sobreviveu

Luiz Gonzaga

Chega um momento em sua vida, que você sabe: Quem é imprescindível para você, quem nunca foi, quem não é mais e quem será sempre!

Charles Chaplin

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RESUMO

O petróleo, embora seja a fonte propulsora do desenvolvimento econômico mundial, gera diversos tipos de efluentes e resíduos, muitas vezes complexos e de difícil tratamento. A produção de efluentes do tipo água oleosa tem provocado problemas ambientais para diversas indústrias. Processos físico-químicos como centrifugação e ultrafiltração podem ser eficazes quando usados para separar óleos e graxas, porém são ineficientes quando os óleos estão emulsionados. O processo de flotação por ar dissolvido (FAD), por outro lado, tem sido utilizado com sucesso no tratamento de águas residuárias. Essa técnica de separação físico-química utiliza pequenas quantidades de coletores que facilitam a adesão das partículas e, consequentemente, a separação dos poluentes, melhorando a eficiência do processo. Por outro lado, esses coletores químicos são tóxicos, fator que representa a geração de outros poluentes ambientais. Assim, os biossurfactantes, moléculas anfipáticas biodegradáveis e atóxicas produzidas por micro-organismos, apresentam-se como coletores promissores no aumento de eficiência da flotação. Nesse sentido, o presente trabalho teve como objetivo realizar um estudo experimental para investigar a separação gravitacional de emulsões oleosas utilizando-se o processo de FAD com a adição de biossurfactantes. Inicialmente, um biossurfactante foi produzido pela bactéria *Pseudomonas aeruginosa* UCP 0992 cultivada em 0,5% de milhocina e 4,0% de resíduo de óleo vegetal em biorreator, empregando um Delineamento Composto Central Rotacional (DCCR) com a finalidade de otimizar as condições de cultivo para obtenção do rendimento máximo. Os melhores resultados foram alcançados na condição de fermentação com aeração de 1,0 vvm, 3,0% do inóculo a 225 rpm durante 120 horas, resultando em uma tensão superficial de 26,5 mN/m e um rendimento de 26 g/L de biossurfactante. Em seguida, o biossurfactante foi caracterizado como um glicolipídeo com uma Concentração Micelar Crítica (CMC) de 600 mg/L. Testes com o biossurfactante sob variações de temperatura, tempo de aquecimento, pH e adição de sal demonstraram a estabilidade da biomolécula. Ensaios cinético e estático com óleo de motor adsorvido em areia demonstrarm remoções de 90 e 80%, respectivamente. Experimentos de degradação do óleo pela bactéria e pela combinação da bactéria e do biossurfactante também foram realizados em amostras de areia e água do mar. Em ambos os casos, a degradação de óleo alcançou níveis superiores a 90% na presença do biossurfactante e de sua espécie produtora. Após sua caracterização, o biossurfactante bruto foi aplicado como coletor a um protótipo de FAD de bancada construído em acrílico. Os experimentos de flotação seguiram um DCCR, tendo como variáveis independentes a vazão de água oleosa, a vazão de água de microbolhas, a vazão da solução aquosa de biossurfactante e a concentração de biossurfactante, e como variável resposta a eficiência de separação de óleo. O biossurfactante isolado e formulado com sorbato de potássio também foi testado no protótipo após otimização das condições operacionais. Os resultados demonstraram que o biossurfactante aumentou a eficiência de separação do óleo pelo processo de FAD de 65 para 95% e que não houve grandes diferenças entre as formas de biossurfactante utilizadas no sistema. Dois biossurfactantes produzidos pelas bacterias *Pseudomonas cepacia* CCT6659 e *Bacillus cereus*1615, foram aplicados no tratamento de efluente oleoso de uma usina termoelétrica, obtendo como resultados, percentuais de remoção de 94 e 80%, respectivamente. Concluiu-se que o uso do biossurfactante como auxiliar na

flotação constitui uma alternativa promissora no tratamento de águas oleosas geradas no ambiente industrial.

Palavras-chave: Biossurfactante; *Pseudomonas aeruginosa;* Água Oleosa; Flotação por ar dissolvido

ABSTRACT

Oil, although it is the propeller of world economic development, generates diverse types of effluents and wastes, often complex and difficult to treat. The production of oily water effluents has caused environmental problems for several industries. Physical-chemical processes such as centrifugation and ultrafiltration can be effective when used to separate oils and greases but are inefficient when oils are emulsified. The dissolved air flotation (DAF) process, on the other hand, has been successfully used in the treatment of wastewater. This physicochemical separation technique uses small amounts of collectors that facilitate the adhesion of the particles and, consequently, the separation of the pollutants, improving the efficiency of the process. On the other hand, these chemical collectors are toxic, a factor that represents the generation of other environmental pollutants. Thus, biosurfactants, biodegradable and non-toxic amphipathic molecules produced by microorganisms, present themselves as promising collectors in increasing flotation efficiency. In this sense, the present work had as objective of carrying out an experimental study to investigate the gravitational separation of oily emulsions using the DAF process with the addition of biosurfactants. Initially, a biosurfactant was produced by the bacterium *Pseudomonas aeruginosa* UCP 0992 grown in 0.5% corn steep liquor and 4.0% of vegetable oil residue in a bioreactor, using a Rotational Central Compound Design (RCCD) in order to optimize the cultivation conditions for maximum yield. The best results were achieved in the fermentation condition with 1.0 vvm aeration, 3.0% of the inoculum at 225 rpm for 120 hours, resulting in a surface tension of 26.5 mN/m and a yield of 26 g/L of biosurfactant. The biosurfactant was then characterized as a glycolipid with a Critical Micellar Concentration (CMC) of 600 mg/L. Tests with the biosurfactant under temperature variations, heating time, pH and addition of salt demonstrated the stability of the biomolecule. Kinetic and static tests with motor oil adsorbed on sand demonstrated removals of 90 and 80%, respectively. Experiments of oil degradation by the bacterium and by the combination of bacteria and biosurfactant were also carried out on samples of sand and sea water. In both cases, the oil degradation reached levels higher than 90% in the presence of the biosurfactant and its producing species. After its characterization, the crude biosurfactant was applied as a collector to a bench DAF prototype built in acrylic. The flotation experiments followed a DCCR, having as independent variables the oily water flow, the microbubble water flow, the aqueous biosurfactant solution flow and the biosurfactant concentration and as a variable response the oil separation efficiency. The isolated and potassium sorbate formulated biosurfactant was also tested in the prototype after optimization of the operating conditions. The results showed that the biosurfactant increased the separation efficiency of the oil by the DAF process from 65 to 95% and that there were no great differences between the biosurfactant forms used in the system. Two biosurfactants produced by the bacteria *Pseudomonas cepacia* CCT6659 and *Bacillus cereus* 1615 were applied in the treatment of oily effluent from a thermoelectric plant, obtaining, as results, percentages of removal of 94 and 80%, respectively. It was concluded that the use of biosurfactant as a collector in flotation is a promising alternative in the treatment of oily waters generated in the industrial environment.

Keywords: biosurfactant; *Pseudomonas aeruginosa*; oily water; Dissolved air flotation

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°C	Grau Celsius
G	Grama
L	Litro
rpm	Rotações por minuto
mg	Miligrama
m	Metro
N	Newton
mN/m	Mili Newton por metro
µm	Micrometro
$/mg	Valor por miligrama
$/kg	Valor por quilograma
mg/L	Miligrama por litro
Kg	Quilo ou Quilograma
mg/kg	Miligrama por quilograma
g/L	Grama por Litro
mL	Mililitro
Ton/ano	Toneladas por ano
C	Concentração
V	Volume
K	Constante cinética do processo de remoção
M	Molar

LISTA DE ABREVIATURAS

A/O	Água em óleo
AIP	Agência Internacional de Petróleo
ANP	Agência Nacional do Petróleo
BETX	Bezenos, etilbezenos, toluenos e xilenos
CMC	Concentração Micelar Crítica
CONAMA	Conselho Nacional de Meio Ambiente
DAM	Drenagem Ácida de Minas
DBO	Demanda bioquímica de oxigênio
DCCR	Delineamento Composto Central Rotacional
DCM	Diclometano
FAD	Flotação por ar dissolvido
HTP	Hidrocarbonetos totais de petróleo
IMO	International Maritime Organization
MARPOL	Conveção Internacional para a Prevenção da Poluição por Óleo
MTBE	Éter metil terc-butílico
NTU	Unidade Nefelométrica de Turbidez
O/A	Óleo em água
ONU	Organização das Nações Unidas
pH	Potencial Hidrogeniônico
ppb	Partes por bilhão
ppm	Partes por milhão
RSM	Metodologia de Superficíe de Resposta
SAO	Separadores Óleo Água
SST	Sólidos solúveis totais
TOG	Teor de óleos e graxas

SUMÁRIO

1. INTRODUÇÃO

As indústrias petrolíferas são as principais responsáveis pela produção de águas oleosas devido ao processo de perfuração e extração do petróleo (LIU et al., 2017) . A reutilização destes efluentes provenientes dos processos industriais se torna cada vez mais comum, tendo em vista o apelo ambiental e econômico desta prática, uma vez que há incentivos para reduzir custos de produção e agregar valor de sustentabilidade à empresa (MISHRA; KUMAR, 2015).

As águas oleosas surgem em consequência da mistura mecânica entre água e óleo, produzindo uma suspensão de gotículas de óleo em água ou uma emulsão estável. O que ocasiona a formação de uma emulsão é a grande tensão interfacial entre água e óleo, acompanhada pela existência de grandes áreas interfaciais, além do fornecimento de energia de Gibbs através da agitação (WANG et al., 2017). Devido à estabilidade dessas emulsões, o tratamento de uma água oleosa pode se tornar uma operação, por vezes, complexa e dependente de processos altamente eficientes.

Tecnologias disponíveis, como separação por gravidade, separação de ciclones, precipitação química, sorção, filtração de membrana e oxidação química são usados para remoção de óleo. Embora tenham sido relatadas muitas vantagens dessas tecnologias, algumas desvantagens específicas estão associadas a essas abordagens (isto é, baixa eficiência, longo tempo de processamento, poluição secundária e altos custos). Há também um aumento da demanda por água limpa, particularmente em áreas com estresse hídrico devido ao rápido crescimento da população e da economia (AN et al., 2017).

Nesse contexto, o processo de flotação tem mostrado bastante eficiência, pois é capaz de remover uma maior quantidade de óleo quando comparado com os outros métodos (ALBUQUERQUE et al., 2012; DA ROCHA E SILVA et al., 2015; MENEZES et al., 2011a). A flotação pode ser definida como um processo de separação de partículas via adesão de bolhas. A união partícula de óleo–bolha apresenta uma densidade menor que a do meio aquoso, flutuando até a superfície da célula de flotação de onde as partículas são removidas (ALIFF RADZUAN et al., 2016).

A flotação foi utilizada pela primeira vez no processamento de minerais e, como tal, tem sido utilizada há muito tempo em aplicações de separação sólido/

líquido que utilizam espumas estáveis para recuperar partículas minerais (PENG et al., 2009).

Os princípios básicos de funcionamento da Flotação por ar dissolvido (FAD) são bastante simples, pois se resumem ao contato das partículas sólidas com as bolhas de ar dissolvidas no líquido e no seu consequente arraste para a superfície do líquido; contudo, alguns parâmetros são essenciais para o êxito do processo, como o uso de coletores. A dosagem de coletores, normalmente com características coagulantes, está relacionada com o grau de clarificação do efluente final. Normalmente, os coletores possuem características específicas que visam promover a adesão à fase dispersa e facilitar a separação dos poluentes (MENEZES et al., 2011).

O uso de flotação como processo de separação, quer como parte do controle da poluição ou durante o tratamento da água, por vezes tem sido criticado devido à provável toxicidade dos coletores, considerando que surfactantes químicos derivados de petróleo são utilizados como coletores nesse processo (MENEZES et al*.,* 2011).

Os surfactantes são compostos constituídos por moléculas anfipáticas contendo porções hidrofílicas e hidrofóbicas que se particionam na interface óleo/água ou ar/água. A porção apolar é frequentemente uma cadeia hidrocarbonada, enquanto a porção polar pode ser iônica (catiônica ou aniônica), não-iônica ou anfotérica. Estas características permitem aos surfactantes reduzir a tensão superficial e interfacial e formar microemulsões onde os hidrocarbonetos possam se solubilizar em água ou onde a água possa se solubilizar em hidrocarbonetos (SILVA et al., 2014).

O uso de surfactantes biológicos ou naturais para a flotação é uma área de grande potencial. Uma série de estudos incluem surfactantes no grupo de contaminantes orgânicos emergentes (COEs) que são biologicamente ativos, mas ainda não estão regulados ou são poucos regulados. Por exemplo, os compostos de polifluoroalquilo (PFC) são conhecidos por serem persistentes e bioacumulativos no ambiente aquático e ter possíveis efeitos adversos sobre seres humanos e animais selvagens. Os surfactantes aniônicos derivados de ácidos naftênicos do petróleo bruto são extremamente letais para peixes e outros organismos aquáticos (VECINO et al., 2013). Neste contexto, surge a utilização de surfactantes biológicos como alternativa para o aumento da floculação.

Estudos recentes mostram que os surfactantes microbianos (conhecidos cientificamente como biossurfactantes ou comercialmente como biodetergentes), metabólitos produzidos por bactérias e leveduras, têm habilidade para solubilizar e mobilizar efetivamente compostos orgânicos e inorgânicos adsorvidos em solos e em águas contaminadas (ROCHA E SILVA et al., 2014). Os biossurfactantes apresentam excelentes vantagens em seu uso, como toxidade reduzida, alta solubilidade na presença de subustâncias orgânicas e inorgânicas, resistência a altas temperaturas, salinidade e pH (SARUBBO et al., 2015).

Os biossurfactantes, embora sejam bastante atrativos frente aos seus similares sintéticos, ainda não são competitivos no mercado devido a razões funcionais e custos da produção elevada. No entanto, o uso de substratos de baixo custo pode reduzir acentuadamente o custo da produção dos biossurfactantes. Nesse sentido, vários recursos renováveis como óleos vegetais, resíduos amiláceos e resíduos lácteos podem ser usados como substratos mais econômicos, e colaboram para a biodegradabilidade da molécula. A seleção de substratos de baixo custo é importante para a economia global do processo, uma vez que eles são responsáveis por 10-30% do custo final do produto (ALMEIDA et al., 2016).

Dessa forma, diante dos desafios apresentados e das necessidades de desenvolvimento e aperfeiçoamento das técnicas atualmente conhecidas de tratamento de efluentes, este trabalho teve como objetivo propor soluções eficientes para o tratamento e controle de águas oleosas industriais. O método utilizado como base tecnológica foi a flotação por ar dissolvido (FAD), utilizando como coletor biodegradável um novo biossurfactante produzido pela bácteria *Pseudomonas aeruginosa*, bem como outros biossurfactantes bacterianos com comprovada capacidade tensoativa.

2. OBJETIVOS

2.1. OBJETIVO GERAL

Produzir e caracterizar biossurfactantes, avaliar o seu potencial de aplicação na biorremediação, bem como, sua ação como coletores naturais no tratamento de águas oleosas a partir de um sistema de flotação por ar dissolvido.

2.2. OBJETIVOS ESPECÍFICOS

ETAPA I – PRODUÇÃO DO BIOSSURFACTANTE

- Produzir um biossurfactante em meio de baixo custo pela bactéria *Pseudomonas aeriginosa*.
- Aplicar a Metodologia de Superfície de Respostas (RSM), com auxílio de um planejamento fatorial como ferramenta para maximização do rendimento em biossurfactante a partir das variáveis agitação, aeração e tempo de cultivo em biorreator.

ETAPA II – ESTUDO DAS PROPRIEDADES DO BIOSSURFACTANTE

- Determinar a tensão superficial e atividade emulsificante do biossurfactante frente a condições específicas de pH, temperatura e adição de NaCl.
- Determinar a CMC do biossurfactante.
- Elucidar a estrutura química do biossurfactante.
- Testar a capacidade de remoção de derivado de petróleo adsorvido em areia pelo biossurfactante em ensaios cinético e estático.
- Testar a capacidade de degradação de derivado de petróleo em areia e água do mar pela bactéria e pela combinação da bactéria e seu biossurfactante.

ETAPA III – APLICAÇÃO DO BIOSSURFACTANTE EM SISTEMA DE FLOTAÇÃO POR AR DISSOLVIDO

- Utilizar um protótipo de bancada de flotação por ar dissolvido (FAD).
- Testar o biossurfactante bruto como coletor no tratamento de água oleosa no protótipo de FAD.
- Aplicar a Metodologia de Superfície de Respostas (RSM), com auxílio de um planejamento fatorial como ferramenta para maximização da separação do óleo a partir das variáveis vazão de água oleosa, vazão de água de microbolhas, vazão da solução aquosa de biossurfactante e concentração de biossurfactanteno protótipo de FAD.
- Testar o biossurfactante isolado e formulado no protótipo de FAD após otimzação das condições operacionais.

ETAPA IV – COMPROVAÇÃO DA VIABILIDADE DO SISTEMA FAD-BIOSSURFACTANTE POR APLICAÇÃO DE OUTROS AGENTES TENSOATIVOS NO TRATAMENTO DE EFLUENTE OLEOSO DE USINA

- Investigar o potencial de dois biossurfactantes previamente caracterizados, produzidos por *Pseudomonas cepacia* CCT6659 e *Bacillus cereus* UCP 1615*,* nas suas formas isolada e formulada*,* como coletores no protótipo de FAD operando sob condições otimizadas, no tratamento de efluente oleoso de uma usina termoelétrica.

3. REVISÃO DA LITERATURA

3.1. EFLUENTES OLEOSOS INDUSTRIAIS

O petróleo é considerado um dos principais produtos na sociedade moderna. Também referido como petróleo bruto, é composto por uma mistura de hidrocarbonetos e outros compostos com quantidades variáveis de enxofre, nitrogênio e oxigênio, o que pode modificar consideravelmente sua volatilidade, gravidade específica (densidade) e viscosidade (VECINO et al., 2013).

Com o avanço da industrialização, a poluição da água está se tornando um problema cada vez mais sério. A água residuária, que é produzida principalmente pelos processos de produção industrial e pela vida diária, é prejudicial não apenas à saúde humana, mas também ao meio ambiente (CAI et al., 2018).

A descarga de águas residuáris contendo óleos e graxas (O & G) para o ambiente aumenta a cada ano devido à rápida urbanização e desenvolvimento industrial. As águas residuais emanam de refinarias de petróleo, metalúrgicas, usinagem, processadores de alimentos, eletrônicos e elétricos, óleo de palma e efluentes do moinho. Ao contrário do óleo livre ou do flutuante derramado no mar, a maioria das águas residuárias industriais contém emulsões óleo/água, o que pode ocasionar graves problemas nos diferentes estágios do processamento (CAI et al., 2018).

Segundo Rajasulochana e Preethy (2016), os métodos de tratamento das águas industriais variam de acordo com alguns fatores, tais como volumes envolvidos, constituição da água, limites da legislação ambiental vigente, entre outros. Esses tratamentos têm como finalidade a redução da concentração de óleo disperso na água para que depois elas possam ser descartadas nos corpos d'água ou reutilizadas no processo.

A presença de O & G em unidades de estação tratamento de águas causará incrustações nos equipamentos e problemas nas etapas do tratamento biológico, além de complicações no tocante ao seu descarte pelos requisitos impostos pelos órgãos ambientais (AFFANDI et al., 2014).

Nesse contexto, os efluentes do tipo água oleosa representam todos os tipos de água que apresentam quantidades variáveis de óleos e graxas, além de uma ampla variedade de materiais em suspensão, incluindo areia, argila e outras

substâncias coloidais e dissolvidas como detergentes, metais pesados etc (HU; LI; ZENG, 2013).

Com relação às concentrações de óleos e graxas livres, a EPA (Environment Protection Agency) dos EUA estabelece como limite a média de 29 mg/L e o maximo diário de 42 mg/L.

A legislação brasileira (Resolucao CONAMA n° 357 – Art. 21 e 34 de 17/03/2005) determina que o teor de óleo e graxas (TOG) máximo para o descarte de água produzida seja de 20 mg/L. Entretanto, é importante que também sejam analisados, além do TOG, outros contaminantes como fenóis, amônia e sulfetos, entre outros presentes na legislação para o enquadramento das águas.

3.2. EMULSÕES

Uma emulsão é definida como uma mistura de dois líquidos imiscíveis ou com miscibilidade limitada onde uma das fases encontra-se dispersa sob a forma de pequenas gotículas de diâmetro médio de 0,1 a 100 μm na outra (fase contínua). Isto só é possível na presença de um agente emulsificante e de energia (mecânica ou não) suficiente para que ocorra a dispersão. Dependendo da disposição do líquido na fase contínua, as emulsões são classificadas como água-em-óleo (A/O) ou óleo-em-água (O/A) (ZADYMOVA et al., 2017).

Rocha e Silva (2015) produziu uma emulsão óleo/água sintética com concentração de 50 mg/L utilizando um óleo lubrificante, por mecanismos de agitação, onde o afluente (água industrial) passa por uma bomba de recirculação em que o óleo é misturado com mesmo fluido de entrada, e através de agitadores mecânicos é simulada a formação de emulsão no processo. Rosa (2002) também gerou uma emulsão estável por meio do cisalhamento do óleo submetendo este a passagem por uma válvula de agulha, em que a passagem da mistura (óleo e água) pela válvula cisalha o óleo, dispersando-o em gotas pequenas, produzindo emulsões estáveis. Nunes (2009) obteve uma emulsão através da agitação em um agitador Ultraturrax, obtendo uma concentração de 80 mg/L que segundo o autor é um valor médio de referência da água de produção. Outro exemplo é a emulsão gerada por Santana (2009) por meio da agitação em um homogeneizador Ultra Turraz T50, a uma velocidade de 6.000 rpm por 15 min. Silva (2007) analisou

emulsões sintéticas, com concentração entre 50 a 400mg/L, produzidas por sistema de agitação mecânica com rotação de 2000 rpm.

Algumas pesquisas que estudaram a remoção de óleo em água de produção constataram que uma emulsão óleo/água sintética é muito mais estável do que uma emulsão não sintética, não perdendo suas características iniciais, e ainda diminuindo bastante a presença de alguns interferentes, como por exemplo, aditivos utilizados no processo, facilitando o estudo de remoção do óleo da água (CALVO et al., 2009).

3.2.1. Estabilização das emulsões

As emulsões podem ser estabilizadas física ou quimicamente. As emulsões estabilizadas fisicamente são aquelas formadas sem adição de substâncias surfactantes, ou seja, a estabilidade é mantida por cargas elétricas inerentes ao sistema ou outras forças diferentes à influência de agentes estabilizantes. Quando se mistura mecanicamente a água e óleo é possível produzir uma suspensão de gotículas de óleo em água, ou uma emulsão (WEN et al., 2016).

A desestabilização das emulsões é geralmente necessária em alguns processos químicos e é particularmente importante para o tratamento de águas residuais emulsionadas. Os métodos convencionais de desestabilização incluem a desestabilização química, desestabilização eletroquímica e separação por membrana de ultrafiltração (YUAN; TONG; WU, 2011).

O termo "estabilidade da emulsão" refere-se à capacidade de uma emulsão para resistir às mudanças nas suas propriedades ao longo do tempo. A instabilidade física resulta em uma alteração na distribuição espacial ou organização estrutural das moléculas. O creme, a floculação, a coalescência, a coagulação parcial, a inversão de fase e a maturação de Ostwald são exemplos de instabilidade física. O desenvolvimento de uma estratégia eficaz para evitar mudanças indesejáveis nas propriedades de uma emulsão alimentar específica depende dos mecanismos físico-químicos dominantes responsáveis pelas mudanças. Na prática, dois ou mais desses mecanismos podem operar em conjunto. Portanto, é importante que os pesquisadores identifiquem a importância relativa de cada mecanismo, a relação entre eles e os fatores que os influenciam,

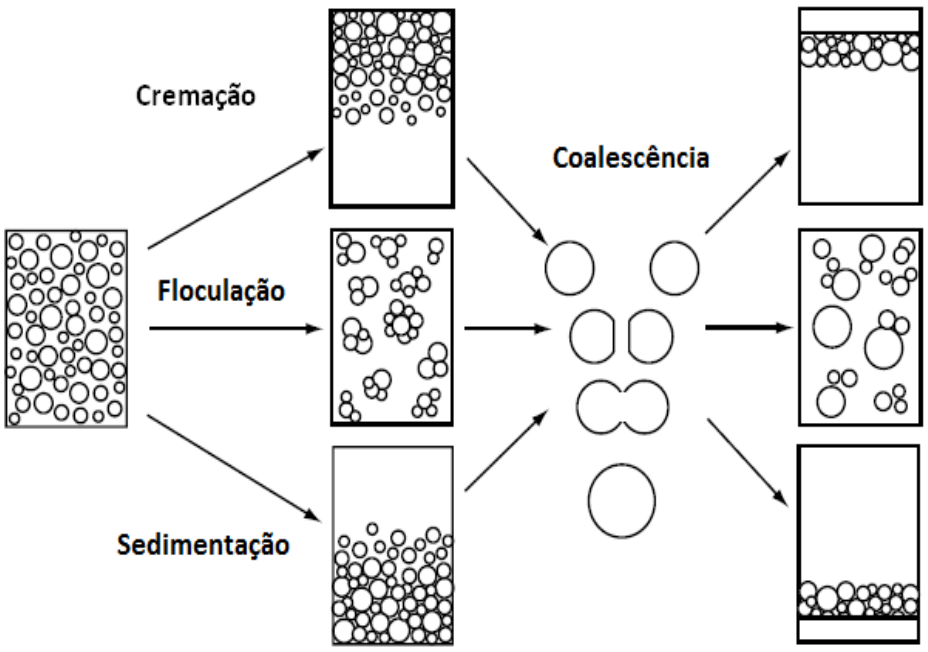
de modo que possam ser estabelecidos meios eficazes de controle da estabilidade e propriedades físico-químicas das emulsões (HUCK-IRIART et al., 2011).

Existem quatro mecanismos principais para a estabilização de emulsões (com casos em que ocorre uma combinação de mecanismos) (UMAR et al., 2018):

- Certas emulsões podem ser fracamente estabilizadas pela presença de íons adsorvidos e sais não ativos na superfície.
- A presença de sois coloidais parcialmente úmido em ambas as fases da emulsão pode formar uma barreira mecânica para a queda de contato e coalescência.
- Muitas emulsões são estabilizadas por moléculas de polímero adsorvido.
- Juntamente com polímeros, as moléculas de surfactante adsorvido representam o mecanismo de estabilização mais comum.

O processo de desestabilização de uma emulsão pode ser conduzido por quatro diferentes fenômenos: coagulação, floculação, sedimentação (*creaming*) e coalescência (MARTA-ALMEIDA et al., 2016) (Figura 1).

Figura 1. Mecanismos envolvidos na desestabilização de emulsões



Fonte: MARTA-ALMEIDA et al. (2016)

Coalescência significa a formação de uma fase de óleo imiscível após a floculação, que é um processo irreversível. Diminuir o potencial superficial das microgotas de óleo é essencial para diminuir a repulsão eletrostática e para melhorar a floculação. Reduzir o pH pode diminuir o potencial da superfície e o aumento da temperatura pode melhorar o movimento browniano e contribuir para a floculação (YUAN; TONG; WU, 2011).

O “creaming” caracteriza-se pelo deslocamento das gotículas de óleo para a superfície, baseado na diferença de densidade existente entre as fases. Mesmo existindo este deslocamento, a estabilidade das gotículas é mantida (AMARAL FILHO et al., 2016;)

A coagulação ocorre quando a interação repulsiva entre as duplas camadas elétricas é suficientemente reduzida, permitindo a aproximação das partículas até que a força de atração de Van der *Waals* predominem (WANG et al., 2015). O processo de coagulação corresponde à desestabilização da dispersão coloidal, obtida por redução das forças de repulsão entre as partículas com cargas negativas, por meio da adição de produtos químicos apropriados (TANSEL; PASCUAL, 2011). Ainda de acordo com Tansel e Pascual (2011), foi possível observar que a utilização da coagulação seguida do processo de flotação por ar dissolvido não apresentou efeitos significativos em relação à redução dos níveis de concentração de óleo abaixo de 5 mg/L.

3.3. FLOTAÇÃO COMO TÉCNICA EMPREGADA AO TRATAMENTO DE EFLUENTES OLEOSOS

As tecnologias de tratamento comumente usadas de efluentes oleosos incluem decantação por gravidade, flotação, separação centrífuga, método químico, separação por membrana, método biológico, mecânicos e elétricos, podendo ser empregadas de maneira conjunta em função do tipode efluente e do objetivo do tratamento. Dentre as tecnologias citadas acima, a flotação tem se mostrado uma alternativa promissora devido à alta capacidade de processamento e eficiência (RANGEL, 2008; CAI et al., 2017).

O tratamento convencional de águas oleosas é feito basicamente por separadores água-óleo (SAO), os quais utilizam o princípio da força gravitacional para a separação óleo/água. A água tratada alcança níveis de remoção do óleo na

faixa de 200 mg/L, devido principalmente à presença de óleo emulsionado, que dificilmente é removido por flutuação, necessitando assim de processos mais eficientes (YU; HAN, 2013), como a flotação. A flotação pode ser considerada como uma tecnologia limpa, uma vez que usa pequenas quantidades de coagulantes e ar para promover a separação (ROCHA E SILVA et al., 2015).

A separação do óleo por flotação consiste na melhor alternativa para o tratamento de águas oleosas, esteja o óleo livre ou emulsionado, pois é de baixo custo (equipamentos compactos), fácil operação e muito eficiente, garantindo o cumprimento das exigências ambientais e, muitas vezes, permitindo o reuso da água (ROCHA E SILVA et al., 2014).

O princípio de flotação de dispersões aquosas baseia-se na utilização de agentes com baixa densidade, geralmente bolhas de gás, que aderem na superfície das partículas da fase dispersa, aumentando o empuxo sobre elas e promovendo, por conseguinte, sua separação (ROCHA E SILVA et al., 2015).

3.3.2. Flotação por ar dissolvido (FAD)

O processo de Flotação por ar dissolvido caracteriza-se basicamente pela geração e utilização de microbolhas obtidas pelo processo de cavitação induzida através da passagem de água sobresaturada por constrições de fluxo, tipo válvula de venturi, placa de orifício ou válvula agulha. Neste processo, o ar é dissolvido em água à pressão elevada (3 a 5 kgf/cm²) em um saturador e essa água é injetada na célula de flotação através de uma constrição redutora de pressão, causando sua liberação sob a forma de microbolhas, cujo diâmetro situa-se entre 50 e 100 μm, como descrito anteriormente (MENEZES et al., 2011).

Como a densidade do ar é muito menor do que a densidade das partículas é de se esperar que as bolhas ascendam na massa líquida promovendo a ocorrência do contato (choque) bolha-partícula, formando um aglomerado bolha-partícula (MENEZES et al., 2011). Sendo as bolhas relativamente menores e em maior quantidade do que em outras técnicas de flotação, a probabilidade de colisão bolha-partícula é muito maior (PENG et al., 2009).

As bolhas de ar e as gotículas de óleo se juntam para formar flocos. O aglomerado em água tem uma diferença de densidade média maior do que a gota

de óleo em água. Esta característica aumenta a força flutuante e, assim, a velocidade de subida do óleo.

O projeto de um sistema específico de flotação por ar dissolvido (FAD) depende de fatores como o volume de águas residuais a serem tratadas, o grau e a natureza da contaminação, a extensão do tratamento necessário e qualquer tratamento subsequente que seja necessário para a concentração do produto recuperado. Esses fatores, por sua vez, indicam a pressão de dissolução, taxa de fluxo, tempo de retenção, taxa de reciclagem, pré-tratamento de coagulante e floculante apropriados e tamanho do tanque de flotação. Apesar da ampla aceitação e uso industrial da tecnologia FAD, muitas questões pertinentes à funcionalidade e otimização permanecem sem resposta (DASSEY; THEEGALA, 2012).

Após a formação dos flocos, as bolhas de ar aderem nas gotículas de óleo e flotam. As características dos flocos formados nas etapas de coagulação e floculação determinam juntamente com as características das micro-bolhas e as condições hidrodinâmicas, a eficiência do processo de FAD (MORUZZI et al., 2017).

Na FAD com microbolhas <100 μm, além da adesão normal bolha-partícula, ocorrem os processos de nucleação ou precipitação do ar dissolvido diretamente sobre a superfície das partículas, o aprisionamento das microbolhas no interior de agregados de partículas (flocos) e o simples arraste mecânico dos flocos por parte de uma frente (leito) de pequenas bolhas em ascensão (RUBIO; SMITH, 2002).

Alguns parâmetros operacionais fundamentais na FAD são o tempo de retenção, taxa de reciclo, tensão superficial e o contato bolha-partícula.

3.3.2.1. Tempo de retenção

A flotação consiste em duas etapas: a primeira é o acondicionamento, onde os reagentes se adsorvem sobre as superfícies da gota de óleo, somente sob agitação. Durante o acondicionamento ocorrem as transformações físico-químicas necessárias à adsorção dos componentes. A segunda etapa é a própria flotação, iniciada pela injeção de microbolhas na emulsão, havendo a interação do óleo com as bolhas de ar (ARRUDA et al., 2010).

O tempo de acondicionamento varia muito para cada sistema, podendo ser de 3 a 30 minutos para sistemas industriais. Contudo, o tempo de retenção pode variar de estudo para estudo (RODRIGUES; RUBIO, 2007) .

3.3.2.2. Taxa de reciclo

Em um sistema de tratamento, a taxa de reciclo possui geralmente um volume fixo, calculado a partir do efluente a tratar. Em um sistema contínuo, logo após o primeiro tratamento, uma parte da água ou do efluente tratado volta para o vaso de saturação onde é injetado novamente ar atmosférico, a partir do qual o processo recomeça (MENEZES et al., 2011b).

Um volume excessivo de água de reciclo resulta em uma desvantagem econômica do processo, principalmente quando o volume de efluente a ser tratado for alto, como por exemplo, em ecossistemas aquáticos contaminados (BARROS, 2011). Desta forma, o uso de grande volume de água no processo para se conseguir uma suposta eficiência que não significa necessariamente extração do poluente, constitui uma insustentabilidade ambiental para a sociedade e prejuízo econômico para a empresa. Barros (2011) verificou que a quantidade de água em excesso não participa do processo de extração ou ascensão da partícula, logo, não apresenta um papel importante, exceto no aumento de probabilidade de colisão entre bolha e partícula quando a distância espacial entre elas é tão elevada por baixa concentração do poluente.

Santana (2009) encontrou os melhores resultados utilizando taxa de reciclo de 40 e 60% no tratamento de água produzida. Já Zouboulis e Avranas (2000)

encontraram como sendo a melhor taxa de reciclo para remoção de óleo da água um valor de 30%.

3.3.2.3. Tensão superficial e o contato bolha-partícula.

A superfície pode ser pensada como um filme muito fino, o qual constitui a interface entre dois materiais diferentes, como por exemplo, entre um líquido e um gás ou um sólido, entre dois líquidos imiscíveis, entre outros. As superfícies possuem características diferentes daquelas dos corpos dos materiais. Há uma propriedade de superfície chamada de tensão superficial, que não está presente no interior do líquido. Isso acontece, por que no interior do material, átomos e moléculas estão rodeados pelo mesmo material. Já na superfície, átomos e moléculas possuem as mesmas características apenas de um lado, sendo que no outro lado possuem moléculas diferentes ou nenhuma molécula (BALL, 2006).

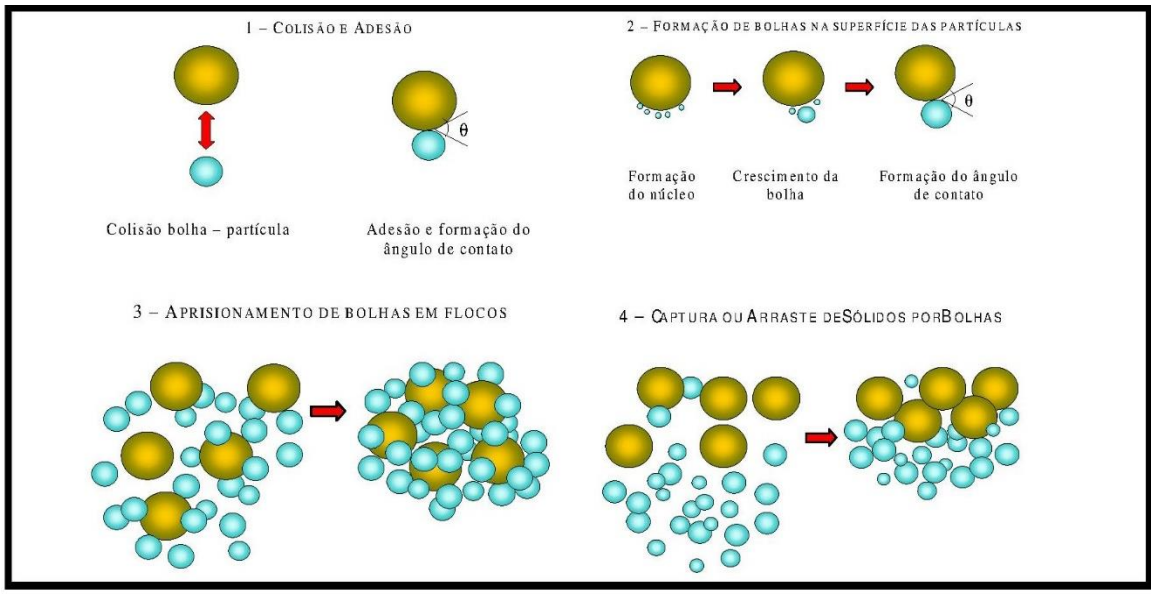
A tensão superficial é uma característica única da superfície, e a mesma é muito importante no que diz respeito ao comportamento dos líquidos. As superfícies podem reagir com certos reagentes, o que causa a aceleração da velocidade, ou catálise de algumas reações químicas (BALL, 2006).

É de extrema relevância o contato entre as superfícies da bolha de ar e gota de óleo. Contudo, esse contato deve ser efetivo, para que o aglomerado formado permaneça acoplado até que atinja o topo da célula de flotação (SILVA, 2008). Os poluentes que se encontram na forma de gotas finamente dispersas formam aglomerados de óleo de tamanhos maiores por meio da ação de tensoativos, devido à diminuição da tensão interfacial, facilitando o contato. As bolhas de ar e as gotas de óleo ligam-se para formar glóbulos. O aglomerado em água tem uma diferença de densidade média mais alta do que a gota de óleo em água. Esta característica aumenta a força de flutuação e, assim, a velocidade de subida do óleo. A flotação é bem-sucedida uma vez que as gotículas de óleo criam uma camada na superfície do tanque da FAD e a água é suficientemente clarificada. O contato da bolha de ar com uma gota de óleo não garante a subida da gota de óleo à superfície. A gotícula de óleo precisa espalhar-se sobre a bolha de ar para formar um aglomerado que é capaz de tolerar as forças de arraste e gravitacional sem quebrar-se à medida que se move (ALIFF RADZUAN; ABIA-BITEO BELOPE; THORPE, 2016).

As gotas de óleo em meio aquoso podem se aderir às bolhas de gás por meio de um dos seguintes fenômenos (Figura 2) (MARIA; MELO, 2017):

- Colisão entre bolha e gota resultando na adesão.
- Desenvolvimento da bolha diretamente na superfície da partícula com a formação de um ângulo de contato, seguida da formação de uma lente ou filme.
- Aprisionamento das bolhas na estrutura do floco.
- Capturas oo arraste das partículas por bolhas.

Figura 2. Fenômenos de colisão, adesão, nucleação, aprisionamento e captura de partículas e agregados por microbolhas



Fonte: MARIA; MELO, (2017)

Este processo de flotação é um dos mais econômicos e efetivos métodos de recuperação-remoção de sólidos, óleos emulsionados, micro-organismos, redução da DBO insolúvel e no espessamento de lodos. A crescente utilização da FAD, em todos os campos, deve-se às diversas vantagens em relação ao processo de sedimentação. Entre outras podem ser citadas as seguintes: a) emprego de menores concentrações de coagulantes e/ou floculantes, o que reduz custos operacionais; b) maior concentração de sólidos no produto separado (lodo) e, por conseguinte, menor custo de desidratação do mesmo; c) alta eficiência na remoção

de sólidos (elevada clarificação); d) elevada cinética de separação e portanto menor área requerida para instalação de os equipamentos: apenas uma fração da área ocupada pelas unidades de sedimentação para capacidades similares; e) maior eficiência na remoção de DBO de que outros processos de separação gravítica (MENEZES et al., 2011).

A eficiência da separação do processo de FAD depende fortemente das condições em que ocorrem os contatos entre as bolhas e as partículas, a etapa inicial é imprescindível para a flotação. A freqüência das colisões aumenta com a redução do tamanho das bolhas, devido ao aumento da área superficial disponível para o contato com as partículas (RUBIO; SMITH, 2002)

Neste sentido, o fenômeno da coalescência das bolhas é prejudicial já que implica em aumento do diâmetro médio das bolhas. Uma das funções do reagente espumante é a de dificultar a coalescência. Uma pequena adição do espumante tem forte influência no tamanho das bolhas, cujo diâmetro médio tende a diminuir até o tamanho original. A concentração correspondente a esse tamanho limite é denominada *concentração crítica de coalescência* (ccc) que é característica de cada sistema (AZGOMI; GOMEZ; FINCH, 2007)

Sendo assim, existe uma série de produtos químicos que podem induzir ou melhorar a separação seletiva das espécies. Estes reagentes são comumente classificados como segue (LUNA, 2004; ROCHA E SILVA et al., 2018):

- Coletores: substâncias químicas utilizadas com o objetivo de provocar uma hidrofobização seletiva nas partículas presentes na polpa de flotação, possibilitando sua aderência às bolhas de ar e aumentando a eficiência de coleta.
- Ativadores: Substâncias conhecidas como ativadores são adicionadas à polpa de flotação com o objetivo de propiciar uma melhor adsorção do coletor na superfície destas partículas.
- Depressores ou Inibidores: substâncias que evitam a adsorção do coletor a uma determinada espécie, permitindo uma coleta seletiva. Formam um dos mais importantes grupos de compostos químicos usados na flotação de minérios.

- Reguladores: a eficiência da maioria dos processos de separação por flotação depende consideravelmente do pH da suspensão. Compostos que modulam o ambiente da flotação através da regulação do pH são denominados de reguladores.
- Espumantes: substâncias tensoativas heteropolares que adsorvem na interface ar-água. Sua ação na fase líquida da polpa de flotação eleva a resistência mecânica das bolhas de ar, favorecendo a dispersão das bolhas e diminuindo a coalescência. Ocorre um aumento da superfície de aderência das partículas, permitindo a formação de uma espuma estável e consistente.
- Floculantes: atuam na aglomeração das partículas, possibilitando a formação de agregados mais susceptíveis a serem separados por flotação. Em geral, são substâncias de alta massa molar, sintéticas ou naturais.

3.3.2.4. Classificação da FAD de acordo com os métodos de dissolução do ar

Os sistemas de flotação por ar dissolvido podem ser classificados de acordo com os métodos de dissolução do ar utilizados em sistemas de compressão total do efluente (no qual todo o fluxo de alimentação é submetido à pressão), sistemas de compressão parcial do efluente, e sistemas de compressão do reciclo de parte do efluente clarificado (MENEZES et al., 2011).

O sistema de compressão total do efluente tem a vantagem de requerer pressões menores de saturação, pois a probabilidade de adesão bolha-gota é máxima neste sistema. Sua principal desvantagem é a compressão e o bombeamento, o que pode promover a emulsificação do óleo (ALBUQUERQUE et al., 2012).

3.3.2.5. Aplicações da FAD

A flotação por ar dissolvido (FAD) pode ser usada (ALBUQUERQUE et al., 2012):

- em operações de separação sólido-líquido e recirculação de água (como em espessamento);
- remoção de íons do processo presentes na água, o qual muitas partículas gangas ativas alimentam a planta de flotação mineral;

- tratamento da flotação de efluentes líquidos removendo poluentes líquidos, derramamentos de óleos ou emulsões, íons de metais pesados, precipitados coloidais, coletores orgânicos residuais e espumantes;
- tratamento das drenagens ácidas de mina (DAM), removendo sólidos gerados após a neutralização;
- tratamento e reuso da água da lavagem de equipamentos da mineração, veículos e maquinários grandes;
- tratamento da água filtrada proveniente da flotação de minérios concentrados;
- recuperação de íons valiosos (Au, Pd, Ag e Pt);
- tratamento de mineral fino associado com bolhas grosseiras;
- na otimização da unidade de tratamento do (DAM) da mina de carvão;
- remoção de íons sulfato e manganês por precipitação.

3.3.2.6. Viabilidade econômica no uso da FAD

A crescente utilização da FAD, em todos os campos, deve-se às diversas vantagens em relação ao processo de sedimentação. Entre outras podem ser citadas as seguintes (JAMALY; GIWA; HASAN, 2015):

- emprego de menores concentrações de coagulantes e / ou floculantes, o que reduz custos operacionais;
- maior concentração de sólidos no produto separado (lodo) e, por conseguinte, menor custo de desidratação do mesmo;
- alta eficiência na remoção de sólidos (elevada clarificação);
- elevada cinética de separação e, portanto, menor área requerida para instalação de equipamentos: apenas uma fração da área ocupada pelas unidades de sedimentação para capacidades similares;
- maior eficiência na remoção de DBO de que outros processos de separação gravitacional e;
- rápida retomada na operação.

A aplicação da FAD, em sistemas diluídos (< 4% em massa) é um dos mais econômicos e efetivos métodos de recuperação-remoção de sólidos, óleos

emulsionados, micro-organismos, redução da DBO insolúvel e no espessamento de lodos (JAMALY; GIWA; HASAN, 2015).

Atualmente, a flotação por ar dissolvido (FAD) está sendo bastante difundida na área de tratamento de água de abastecimento e, também, na remoção de óleos e graxas e/ou detergentes vindos das petroquímicas e termelétricas entre outras, que misturam óleo e água no seu processo (EDZWALD, 2010).

3.4. SURFACTANTES

Os surfactantes são compostos anfipáticos contendo porções hidrofílicas e hidrofóbicas que se particionam, preferencialmente, na interface entre fases fluidas com diferentes graus de polaridade e pontes de hidrogênio, como interfaces óleo/água ou ar/água (GUTNICK; BACH, 2017).

Essas características permitem aos surfactantes reduzir a tensão superficial e interfacial e com isso formar microemulsões onde os hidrocarbonetos possam se solubilizar em água ou onde a água possa se solubilizar em hidrocarbonetos (BURGHOFF, 2012).

Tais microemulsões ocorrem espontaneamente e dependem principalmente do tipo e estrutura do surfactante. Por exemplo, se o surfactante é iônico juntamente com uma única cadeia de hidrocarboneto, a formação de microemulsão ocorre na presença de co-surfactante e / ou eletrólito, enquanto que, para surfactantes iônicos não iônicos e de cadeia dupla, co-surfactante não é necessário (DOSHI; SILLANPÄÄ; KALLIOLA, 2018).

A propriedade de maior importância para os agentes tensoativos é a tensão superficial, que é a força de atração existente entre as moléculas dos líquidos. A tensão superficial diminui quando a concentração de surfactante no meio aquoso aumenta, ocorrendo a formação de micelas, que são moléculas anfipáticas agregadas com as porções hidrofílicas posicionadas para a parte externa da molécula e as porções hidrofóbicas para a parte interna (BURGHOFF, 2012).

A eficiência e a efetividade são características básicas essenciais que determinam um bom surfactante. A eficiência é medida através da CMC, enquanto que a efetividade está relacionada com as tensões superficiais e interfaciais (DE FRANC¸A et al., 2015).

A maioria dos surfactantes disponíveis comercialmente é produzida a partir de derivados de petróleo. Entretanto, o crescimento e a preocupação ambiental

entre os consumidores, combinados a novas legislações de controle do meio ambiente levaram à procura por surfactantes naturais como alternativa aos produtos existentes (SANTOS et al., 2016).

A concentração micelar crítica (CMC) é a mínima concentração de surfactante necessária para reduzir a tensão superficial até o grau máximo após o qual os surfactantes adicionais não têm mais efeito. Quando o CMC é alcançado, um número de micelas são formadas (LUNA; RUFINO; SARUBBO, 2016).

3.5. BIOSSURFACTANTES

Os estudos relacionados aos biossurfactantes iniciaram-se em 1960 e a utilização desses compostos se estendeu nas últimas décadas, surgindo como alternativa aos surfactantes sintéticos, especialmente em indústrias farmacêuticas, alimentícias e na petrolífera (MARCHANT; BANAT, 2012a).

Tendo em vista a crescente conscientização em relação às salvaguardas ambientais, políticas rígidas, preços voláteis do petróleo e aumento simultâneo na demanda do consumidor, nos últimos anos, o foco tem sido direcionado ao uso de compostos anfifílicos ativos produzidos na superfície microbianos conhecidos como biossurfactantes. São substitutos promissores para os surfactantes quimicamente sintetizados devido às suas propriedades únicas, como maior biodegradabilidade, baixa toxicidade, aceitabilidade ecológica, aumento das atividades superficiais, maior formação de espuma, baixa concentração de micelar crítica (CMC), alta seletividade e especificidade, resistência a condições extremas de temperaturas, faixas de pH e salinidade (JOY; RAHMAN; SHARMA, 2017).

A razão desta notoriedade está relacionada à baixa toxicidade, biodegradabilidade, habilidade de produção a partir de fontes renováveis, capacidade de ação em ambientes extremos, como pH, temperatura e salinidade, além de estruturas químicas variadas (MUTHUSAMY et al., 2008)

A maioria dos surfactantes disponíveis comercialmente é produzida a partir de derivados de petróleo. Entretanto, o crescimento e a preocupação ambiental entre os consumidores, combinados a novas legislações de controle do meio ambiente levaram à procura por surfactantes naturais como alternativa aos produtos existentes (SANTOS et al., 2013).

Vários compostos com propriedades tensoativas são sintetizados por organismos vivos, desde plantas (saponinas) até micro-organismos (glicolipídeos)

e também no organismo humano (sais biliares), sendo considerados surfactantes naturais (MANEERAT, 2005).

Os compostos de origem microbiana que exibem propriedades surfactantes são denominados biossurfactantes e consistem em subprodutos metabólicos de bactérias, leveduras e fungos filamentosos (ROCHA E SILVA et al., 2015; SANTOS et al., 2013; SINGH; VAN HAMME; WARD, 2007).

Os biossurfactantes são classificados como glicolípidos, lipoproteínas ou lipopépitidos, fosfolípidos, ácidos graxos ou lípideos naturais, surfactantes poliméricos e surfactantes em partículas (HOŠKOVÁ et al., 2013).

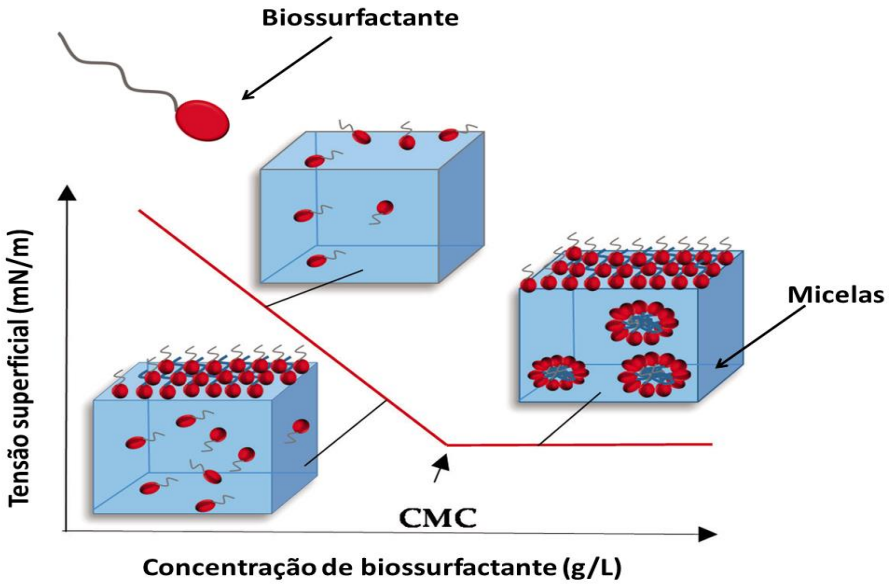
A maioria dos biossurfactantes conhecidos é produzida em substratos insolúveis em água como hidrocarbonetos sólidos e líquidos, óleos e gorduras, embora muitos tenham sido obtidos a partir de substratos solúveis, ou pela combinação destes (BANAT et al., 2010).

3.5.1. Micelação e Concentração Micelar Crítica (CMC)

Em soluções aquosas, o tensoativo atua na forma de monômeros, orientando-se preferencialmente nas interfaces (CAMPOS et al., 2013), de modo que as cabeças polares estejam direcionadas para a solução e as caudas apolares orientadas para o ar, reduzindo a tensão interfacial da água (ALMEIDA et al., 2016).

A tensão superficial de um líquido é reduzida com o aumento da concentração do tensoativo no meio, até um valor determinado (Figura 3). Contudo, a partir de certo momento, a tensão permanecerá constante, mesmo com o acréscimo de mais tensoativo. Isto ocorre devido à saturação da interface (SILVA, 2008). Em consequência desse excesso, as moléculas formam agregados, denominadas micelas, cujo aparecimento se dará em uma concentração conhecida como Concentração Micelar Critica (CMC) (CAMPOS et al., 2013).

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



Fonte: SANTOS et al. (2016)

A comparação entre os valores de CMC de biossurfactantes e de seus equivalentes químicos está apresentada na Tabela 1 e mostra CMCs muito mais baixas no caso dos biossurfactantes. Em princípio, quanto menor a CMC, mais eficaz o surfactante e mais favorável, do ponto de vista econômico, a sua utilização em processos industriais (MARCHANT; BANAT, 2012).

Tabela 1. Exemplos de Concentração Micelar Crítica de biossurfactantes e surfactantes químicos

Agente surfactante	***CMC (mg/L)***
Fosfatidiletanolaminas	30
Ácidos fosfatídicos	70
Raminolipídeo	20
Surfactina	11
Lipopeptídeo	60
Glicolipídeo	30
Alquil benzeno sulfonato	590
Produzido por *C. shaerica*	92
Produzido por *Bacillus sp.*	30
Tween 80	45
Triton X-100	70
Lauril sulfato de sódio	2 000 – 2 900

Fonte: adaptado de CHAPRÃO et al. (2015)

3.5.2. 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 (SANTOS et al., 2014). 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).

Alguns micro-organismos podem produzir biossurfactantes quando crescem em diferentes substratos, cariando desde carboidratos até hidrocarbonetos. O uso de diferentes fontes de carbono altera a estrutura dos biossurfactantes produzidos e, consequentemente, suas propriedades emulsificantes. Estas mudanças podem ser benéficas quando se deseja propriedades específicas para uma aplicação direcionada (ALMEIDA et al., 2016).

Diversos são os estudos realizados por vários autores utilizando espécies do gênero *Candida*, incluindo *Candida sphaerica*,(LUNA et al., 2015; SOBRINHO et al., 2013) *Candida glabrata* (GUSMÃO et al., 2010; LUNA et al., 2009), *Candida lipolytica* (RUFINO et al., 2014; SANTOS et al., 2013), *Candida utilis* (CAMPOS et al., 2013), *Candida guilliermondii* (SITOHY et al., 2010), *Candida antarctica* (HUA et al., 2003) e *Candida tropicalis* (BATISTA et al., 2010; PRIJI et al., 2013; ALMEIDA et al., 2017) 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., 2014b). 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., 2016a). 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). 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 (SAMAL; DAS; MOHANTY, 2017).

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; LIN; CHANG, 2015).

3.5.3. Propriedades dos biossurfactantes

As propriedades físicas e químicas dos biossurfactantes, como redução da tensão superficial, capacidade espumante, capacidade emulsificante e estabilizante, concentrações micelares críticas baixas, solubilidade e poder detergente são muito importantes na avaliação de seu desempenho e na seleção de micro-organismos com potencial de produção destes agentes (DELEU; PAQUOT, 2004).

Biossurfactantes de glicolipídeos possuem várias propriedades funcionais (emulsionantes, espumantes, molhantes, anti-adesivos e anti-biofilmes) e propriedades biológicas (atividade antibacteriana), permitindo seu uso em indústrias de alimentos como aditivos e conservantes. Realmente, a melhoria da qualidade dos alimentos e sua preservação tornaram-se de grande interesse nas últimas décadas (MNIF; GHRIBI, 2016).

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

- Atividade superficial e interfacial: os biossurfactantes são mais eficientes e mais efetivos do que os surfactantes convencionais, pois produzem menor tensão superficial a menores concentrações. A CMC dos biossurfactantes (medida de sua eficiência) varia entre 1-2000 mg/L, enquanto que a tensão interfacial (óleo/água) e superficial fica em torno de 1 e 30 mN/m respectivamente (SANTOS et al., 2016).
- Tolerância à temperatura, pH e força iônica: muitos biossurfactantes podem ser utilizados sob condições extremas. 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)
- Biodegradabilidade: os biossurfactantes são facilmente degradados por bactérias e outros micro-organismos microscópicos na água e no solo, o que os torna adequados para aplicações na biorremediação e tratamento de resíduos (SANTOS et al., 2016).
- Baixa toxicidade: os biossurfactantes têm recebido maior atenção devido à crescente preocupação da população com os efeitos alérgicos dos produtos artificiais; além disso, sua baixa toxicidade permite o uso em alimentos, em cosméticos e em produtos farmacêuticos (CAMPOS et al., 2013).
- Disponibilidade: biossurfactantes podem ser produzidos a partir de matérias-primas largamente disponíveis, além da possibilidade de serem produzidos a partir de resíduos industriais (SANTOS et al., 2016; SILVA et al., 2014).
- Especificidade: biossurfactantes, sendo moléculas orgânicas complexas com grupos funcionais específicos também serão específicos em suas ações. Essa propriedade pode ser de grande interesse da detoxificação de poluentes específicos ou em determinadas aplicações nas indústrias farmacêutica, cosmética ou alimentícia.
- Biocompatibilidade e digestibilidade, o que garante a aplicação dessas biomoléculas nos mais deviersos setores industriais, destacando as indústrias farmacêutica, cosmética e alimentícia.

A despeito das vantagens, alguns pontos desfavoráveis devem ser citados, como (JOY; RAHMAN; SHARMA, 2017):

- A produção em grande escala de biossurfactantes pode ser dispendiosa. Esse problema, entretanto, pode ser resolvido pela combinação de substratos de baixo custo.
- A obtenção de produtos com elevado grau de pureza, que se torna difícil em virtude da necessidade de etapas consecutivas de purificação do líquido metabólico.
- A existência de espécies super produtoras é rara e as conhecidas não são capazes de produzir altos rendimentos em surfactantes, além de necessitarem de meios de cultivo complexos (SILVA et al., 2014)
- A regulação da síntese de biossurfactantes não está totalmente compreendida, uma vez que essas biomoléculas podem ser produzidas como metabólitos secundários ou em associação ao crescimento microbiano (ALMAEIDA et al., 2016)
- O aumento da produtividade é muitas vezes prejudicado pela formação de espuma, o que requer a utilização de meios diluídos.

3.5.4. Aplicações ambientais dos biossurfactantes

As aplicações industriais dos surfactantes são classificadas de acordo com seus usos: 54 % como detergentes, 13 % nas indústrias têxteis, de couro e de papel, 10 % em processos químicos, outros 10 % nas indústrias farmacêuticas e de cosméticos, 3 % na indústria de alimentos, 2 % na agricultura e os 2 % restantes em outras aplicações (MUTHUSAMY et al., 2008).

A atividade superficial única, juntamente com o baixo custo de produção e a compatibilidade com outros os co-tensoativos conduziram à exploração dos surfactantes em larga escala em várias aplicações industriais. Por exemplo, as formulações de surfactantes são amplamente utilizadas como detergentes, emulsionantes, desemulsionantes, dispersantes, agentes molhantes, retardadores de espuma, estabilizadores, agentes gelificantes, etc. Em 2014, a produção mundial de surfactantes foi cerca de 17,5 mil toneladas por ano, com vendas brutas no valor de cerca de US $ 29 bilhões, revelando um crescimento projetado e estável de 3-5% ao ano (EDSER, 2016).

Os biossurfactantes, assim como os surfactantes sintéticos, devido às diversas estruturas e propriedades, apresentam aplicações em vários processos industriais, além da possibilidade de novas aplicações para estas biomoléculas. Acredita-se que os biossurfactantes ficarão conhecidos como os “materiais multifuncionais” do novo século (MUTHUSAMY et al., 2008).

Os biossurfactantes receberam atenção considerável no campo de processos de remediação ambiental. Essas substâncias influenciam tais processos devido à sua eficácia como agentes de dispersão e remediação, bem como suas características ecologicamente corretas, como baixa toxicidade e alta biodegradabilidade (SILVA et al., 2014).

Essas características contribuem para a relevância dos biossurfactantes para diferentes indústrias, especialmente na indústria do petróleo, que possui muitas condições de processos adversos (SILVA et al., 2014). A maioria das aplicações bem-sucedidas de biossurfactantes que conseguiram chegar ao mercado foi impulsionada principalmente pelo processo econômico de produção e pela eficácia de custos (BANAT et al., 2010). Isso foi facilitado pelas especificações de pureza mais baixa exigidas para tais aplicações, eliminando as etapas de processamento a jusante de purificação que frequentemente representam quase 60% dos custos totais de produção (SARUBBO et al., 2015).

3.5.4.1. Aplicação na biorremediação

Biorremediação é a habilidade de organismos vivos em transformar ou mineralizar contaminantes orgânicos gerando substâncias menos nocivas, que possam ser integradas ao ciclo biogeoquímico natural. Contudo, a biodegradabilidade desses contaminantes é influencidade por fatores como oxigênio, pH, presença de macro e micronutrientes, características físico-químicas do histórico da poluição ambiental e das partículas de solo ou outras às quais os organismos e contaminantes possam estar adsorvidos (PACWA-PŁOCINICZAK et al., 2011; PŁAZA et al., 2011).

A biorremediação utilizando micro-organismos ou processos microbianos em ambientes contaminados tem inúmeras aplicações incluindo a limpeza de águas subterrâneas, solos, lagos e processos de tratamento de esgotos. Essa é uma tecnologia bem aceita pela opinião pública na recuperação de ambientes poluídos não afetando o equilíbrio ecológico, já que as bactérias, os fungos filamentosos e

as leveduras são agentes transformadores eficazes, face as suas habilidades em degradar uma ampla diversidade de substâncias orgânicas (PERFUMO; BANAT; MARCHANT, 2018).

Os novos biossurfactantes ainda não caracterizados de diferentes micro-organismos mostraram potenciais aplicações na biorremediação reduzindo os solos contaminados por hidrocarbonetos. Por exemplo, um biossurfactante extraído de *Lactobacillus pentosus* foi mais eficiente do que o detergente SDS na remoção de octano de solos contaminados, enquanto um HMB produzido pela bactéria *Variovorax paradoxus* removeu o petróleo bruto de solos contaminados artificialmente (GUTNICK; BACH, 2017).

Como os biossurfactantes aumentam a interação água/óleo, aceleram a degradação de vários óleos por micro-organismos e promovem a biorremediação de águas e solos contaminados (MULLIGAN, 2005).

A capacidade dos surfactantes em emulsificar e dispersar hidrocarbonetos em água aumenta a degradação desses compostos no ambiente. Os biossurfactantes também são úteis na biorremediação de locais contaminados com metais pesados tóxicos como urânio, cádmio e chumbo e na remoção de piche após a introdução de *Pseudomonas, Arhtrobacter,* e *Bacillussubtilis,* demonstrando resultados promissores (PACWA-PŁOCINICZAK et al., 2011).

Pesquisas com consórcios microbianos e raminolipídeos demonstraram o potencial de biorremediação de hidrocarbonetos de petróleo. A aplicação do raminolipídeo de *Pseudomonas aeruginosa* DS10-129 aumentou a biorremediação de gasolina adsorvida em solo (JOY; RAHMAN; SHARMA, 2017).

Alguns estudos demonstraram o aumento da biodisponibilidade de compostos aromáticos pouco solúveis como os hidrocarbonetos aromáticos policíclicos (HPAS) pelo uso de biossurfactantes (MULLIGAN, 2009; SHARMA; SINGH; UPADHYAY, 2008).

3.5.4.2. Aplicação na limpeza de reservatórios de óleos

A aplicação de biossurfactantes no tratamento de resíduos oleosos torna-se um dos pré-requisitos importantes para que ocorram interações entre os resíduos e a célula microbiana, devido à redução da tensão superficial existente entre o óleo e a fase aquosa (CALVO et al., 2009; HUANG et al., 2016).

A utilização de biossurfactantes para a limpeza de tanques, em substituição aos surfactantes convencionais, promoveu a limpeza e recuperação de 90 % dos hidrocarbonetos presentes no resíduo (PACWA-PŁOCINICZAK et al., 2011).

O cenário atual de um mercado tão volátil para os preços do petróleo é um caminho bastante árduo para as indústrias de petróleo e países altamente dependentes da renda baseada no petróleo. No entanto, a indústria de petróleo enfrentando esse cenário em tempos desesperadores, sempre trouxe espaço para melhorias e modernização de tecnologias para garantir resultados mais econômicos e eficazes. Essas tecnologias incluem a recuperação avançada do petróleo (MEOR) desenvolvida para melhorar economicamente a extração e recuperação de óleo. Existem três estágios na recuperação de petróleo: primário, secundário e terciário. As operações de MEOR são geralmente empregadas em estágios secundários ou terciários (GEETHA; BANAT; JOSHI, 2018).

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).

A remoção de resíduos e frações de óleos pesados requer lavagens com solventes ou mesmo manuais, ambas perigosas, demoradas, e caras já que os resíduos e as frações de óleos pesados que sedimentam no fundo dos tanques são altamente viscosos e podem não ser removidos através de bombeamento convencional. Um processo alternativo a esta limpeza é o uso de biossurfactantes que promovem a diminuição na viscosidade e a formação de emulsões óleo/água, facilitando o bombeamento dos resíduos e a recuperação do óleo cru, após quebra da emulsão (SHARMA; SINGH; UPADHYAY, 2008).

3.5.4.3. Aplicação na dispersão de manchas de petróleo

O derramamento de óleos ocorridos durante o seu transporte ou na construção de oleodutos afeta drasticamente as regiões costeiras e praias, sendo hoje uma das maiores causas de catástrofes ecológicas e sociais no mundo (MUTHUSAMY et al., 2008).

Uma das técnicas de remediação de derramamentos de óleo é a aplicação de dispersantes de manchas de óleo. Os dispersantes utilizados para este fim são compostos de misturas complexas de surfactantes, solventes e aditivos que aumentam a taxa de dispersão natural do óleo e sua retirada da superfície contaminada (PERFUMO; BANAT; MARCHANT, 2018). Além disso, o uso de dispersantes minimiza o impacto de derramamento de óleo sobre os recursos sensíveis na orla costeira, reduzindo a quantidade de óleo derramado.

A aplicação de dispersantes minimiza o impacto do derramamento de óleo em aves e mamíferos marinhos, pois remove o óleo da superfície da água. Além disso, o uso de dispersantes minimiza o impacto de derramamento de óleo sobre os recursos sensíveis na orla costeira, reduzindo a quantidade de óleo derramado. O aumento da área superficial do petróleo como resultado da sua dispersão em pequenas gotículas facilita também sua biodegradação através da atividade de micro-organismos de ocorrência natural (PACWA-PŁOCINICZAK et al., 2011).

Os biossurfactantes exercem influência sobre os processos de remediação através de sua eficácia como agentes dispersantes. A literatura descreve o uso de biossurfactantes no aumento da dispersão e biodegradação de hidrocarbonetos No entanto, poucos estudos têm investigado na prática a aplicação de biossurfactantes como dispersantes de derramamento de óleo (SAEKI et al., 2009).

3.5.4.4. Aplicação na flotação de metais pesados e petroderivados

Nos últimos anos, os estudos voltados para a aplicação de biossurfactantes na flotação têm se intensificado em função das características desses compostos como biodegradabilidade, baixa toxicidade, especificidade e estabilidade sob condições ambientais extremas de temperatura, pH e salinidade (SANTOS et al., 2016)

Diante deste panorama, Menezes et al. (2011) demonstraram que a operação com FAD utilizando surfactantes sintéticos e biológicos testados nas

mesmas condições obtiveram valores de turbidez inferiores ao limite da legislação brasileira vigente que é de 5 NTU (Unidades Nefelométricas de Turbidez), tendo o biossurfactante produzido por *Candida lipolytica* apresentado resultado superior ao do oleato de sódio, um surfactante químico, para experimentos de remoção de metais pesados.

A maioria dos biossurfactantes utilizados na biorremediação do solo são produzidos por bactérias, especialmente espécies de *Pseudomonas e Bacillus* e algumas utilizados na remoção de metais pesados (MENEZES et al., 2011a).

Albuquerque et al. (2012) também observaram resultados para biossurfactantes semelhantes aos do oleato de sódio, na remoção de metais pesados. Com esses métodos, pretende-se desenvolver processos de reciclagem para os biossurfactantes tornarem-se substitutos atrativos aos coletores sintéticos, no tratamento de efluentes por flotação, reduzindo o impacto ambiental deste tipo de atividade.

Uma quantidade excepcionalmente alta de contaminação por metais pesados é encontrada na mineração, na fundição ou no aterro de resíduos industriais, onde os metais mais freqüentemente identificados são Pb, Cu, Zn, Cd, Ni, Cr (LUNA; RUFINO; SARUBBO, 2016).

Rocha e Silva et al. (2015) analisaram o uso de biotensoativos na eficiência de separação do sistema FAD em escala piloto. O processo foi eficaz com e sem o uso de biotensoativos. No entanto, a utilização do biossurfactante produzido por *Candida sphaerica* potencializou a eficiência de separação de 80 para 95%. Sendo assim, observa-se que a flotação é uma tecnologia limpa e eficaz na separação de óleo-água, mas ainda necessita de maiores estudos quanto ao uso de coletores no seu processo, devido ao uso não sustentável de surfactantes químicos.

Em estudos realizados para verificar a cinética de sorção de sedimentos utilizando o biossurfactante produzido por *Lactobacillus pentosus*e dois surfactantes sintéticos, os resultados demostraram que o biossurfactante apresentou resultados muito promissores e que a natureza da molécula é o mais importante para a obtenção de resultados satisfatórios. O SDS não foi bem adsorvido pelos sedimentos, enquanto que o Tween 20 e o biossurfactante devido as suas naturezas aniônica e não iônica, respectivamente, apresentaram resultados satisfatórios (ZOUBOULIS et al., 2003).

Alguns trabalhos também apresentam estudos sobre a aplicação dos processos de flotação para remoção de íons metálicos dos efluentes. Dois agentes tensoativos produzidos por micro-organismos (Surfactin-105 e Lichenysin-A) foram aplicados como coletores de flotação para separação dos agentes metálicos dos efluentes. Os testes demonstraram que os biossurfactantes apresentaram resultados mais promissores do que os surfactantes sintéticos (SDS e dodecilamina) (VECINO et al., 2013).

3.5.5. Utilização de resíduos Industriais na produção de biossurfactantes

A sociedade atual caracteriza-se pelo aumento das despesas, a necessidade de reutilizar materiais e com a preocupação ambiental, consequentemente, vem dando uma ênfase maior a recuperação, reciclagem e reutilização de diversos resíduos (MARCHANT; BANAT, 2012; SANTOS et al., 2016).

A necessidade de preservação ambiental leva à reutilização de diversos resíduos industriais. Isto particularmente é válido para os alimentos e as indústrias de produção de alimentos cujos resíduos, efluentes e co-produtos podem ser reutilizados. Estas indústrias produzem grandes volumes de resíduos sólidos e líquidos, resultantes da produção, preparação e consumo dos alimentos e quando descartados geram poluição e representam uma grande perda de nutrientes, particularmente das indústrias de alimentos, vêm sendo utilizados na bioconversão e chamando mais atenção devido à possibilidade de aplicação na produção de bioadsorventes (BANAT et al., 2010; MAKKAR; CAMEOTRA; BANAT, 2011; MARCHANT; BANAT, 2012b).

O uso dos substratos mais baratos, como o agroindustrial resíduos é uma das estratégias desejáveis para a produção econômica de biossurfactantes, pois os substratos podem representar 10-30% do produto final custo (BANAT et al., 2014).

Várias alternativas mais baratas são relatadas para a produção de biossurfactantes, tais como: melaço e resíduos de amido, resíduos da indústria de laticínios, resíduos da indústria de óleo vegetal, resíduos óleos de fritura e agricultura e resíduos lignocelulósicos (AL-BAHRY et al., 2013).

A escolha do substrato mais barato depende da sua fácil disponibilidade e pode variar muito de país para país, como o melaço da cana será mais barato nos

países que produzem mais cana de açúcar, em comparação com outras partes do mundo (GEETHA; BANAT; JOSHI, 2018).

A seleção do substrato depende da escolha de um resíduo com um certo balanço de nutrientes para crescimento e produção. Os resíduos industriais com elevado valor de carboidratos ou lipídeos encontrados são muito atrativos como substratos para produção de biossurfactantes (MARCHANT; BANAT, 2012).

Barros et al. (2007) descreveram a importância da variedade de resíduos industriais como matéria-prima para diversos bioprocessos. Segundo os autores desse trabalho, a utilização de resíduos agroindustriais para produção de biossurfactantes é um dos passos para viabilização e implantação desses processos em escala industrial, sendo necessário um balanço de nutrientes para desenvolver condições adequadas no desenvolvimento e produção. Os efluentes do processamento de batata foram evidenciados como substitutos atrativos dos substratos convencionais, uma vez que são fontes de carboidratos na forma de amido e açúcar, de nitrogênio e de carbono, considerando que a composição do meio interfere na redução da tensão superficial.

Nitschke et al. (2007) selecionaram micro-organismos para a produção de biopolímeros utilizando resíduos agroindustriais. Utilizaram melaço, soro de leite e manipueira obtendo valores de tensão superficial em torno de 26 mN/m. Lang (2002) investigou a produção de biossurfactantes usando óleo vegetal doméstico como substrato da bactéria *Tsukamurellaspec* DSM 44370, conseguindo reduzir a tensão da água de 70 mN/m para 35 mN/m com CMC de 10 mg/L. Haba et al., (2014) compararam o uso de óleo de oliva e girassol para a produção de biopolímeros usando valores de tensão superficial até 40 mN/m como critério de seleção de micro-organismos potencialmente produtores. Rufino et al. (2007) utilizaram um resíduo de refinaria na produção de biossurfactante por *Candida lipolytica* obtendo resultados satisfatórios em termos de tensão superficial.

A milhocina e um resíduo de refinaria de petróleo foram relatados como nutrientes de baixo custo para a produção de um biossurfactante glicolipídico de *C. sphaerica* (UCP 0995). O biossurfactante recuperou cerca de 95% de óleo de motor adsorvido em amostra de areia e apresentou vastas aplicações em processos de biorremediação (LUNA et al, 2011a; 2013; 2015). Silva et al. (2014b) mostraram também a produção de um novo biossurfactante de *P. cepacia* cultivada

em meio mineral suplementado com 2,0% de milhocina e 2,0% de óleo de fritura de soja.

Mukherjee et al. (2006) descreveram o uso de substratos de baixo custo como alternativa econômica e promissora para a produção de biossurfactantes. Derivados de óleo vegetal, substâncias a base de amido, soro de leite, óleo de babaçu e girassol, melaço e efluente de arroz foram utilizados com eficiência na produção de raminolipídeos e soforolipídeos por vários micro-organismos.

Diferentes elementos encontrados nos efluentes dos processos industriais também são fontes de nutrientes. Nitrogênio e ferro foram utilizados para aumentar o rendimento de biossurfactantes de *Pseudomonas aeruginosa* BS-2 e *Ustilagomaydis* (JUWARKAR et al., 2007).

Amézcua-Vega et al. (2007) descreveram a importância da relação entre diferentes elementos como C e N, C e P, C e Fe ou C e Mg na produção de biopolímeros e na otimização de seus processos de obtenção.

A borra oleosa do fundo de tanques da Petrobrás, contendo querosene, óleo diesel e petróleo, foi utilizado como matéria-prima de baixo custo para a produção de biossurfactante pela bactéria *Pseudomonas aeruginosa* isolada de solo contaminado (PIRÔLLO, 2006).

A gordura animal e o sebo podem ser obtidos em grandes quantidades nas indústrias de processamento de carne e têm sido usados como meio para cozinhar alimentos. Recentemente, Santos et al. (2013; 2014) reportaram a produção máxima de um glicolipídeo usando gordura animal e milocina em comparação com outras fontes de carbono usando a levedura *C. lipolytica* UCP0988.

A Tabela 2 mostra um resumo de algumas matérias-primas de baixo custo e os respectivos micro-organismos utilizados na produção de biossurfactantes.

Tabela 2. Matérias-primas de baixo custo e respectivos micro-organismos utilizados na produção de biossurfactante

Matéria-prima de baixo custo ou resíduos	Tipo de biossurfactante	Espécie microbiana produtora	Rendimento máximo (g/L)
Óleo de babaçu	Glicolipídeo	*Candida lipolytica* IA 1055	---
Óleo de milho	Glicolipídeo	*Candida bombicola* ATCC 22214	400
Óleo de girassol e óleo de soja	Raminolipídeo Lipídeo manosileritritol	*Pseudomonas aeruginosa* DS10-129 *Candida* sp. SY16	4,31 / 2,98 95
Óleo residual de fritura (óleos de oliva e girassol)	Raminolipídeo	*Pseudomonas aeruginosa* 47T2 NCIB 40044	2,7
Óleo residual de fritura (óleos de oliva e girassol)	Raminolipídeo	*Pseudomonas aeruginosa* 47T2 NCIB 40044	2,7
Resíduo de refinaria de óleo vegetal	Raminolipídeo	*Pseudomonas aeruginosa* LBI	11,72
Resíduo de refinaria de óleo de girassol	Raminolipídeo	*Pseudomonas aeruginosa* LBI	16
Resíduo de refinaria de óleo vegetal	Glicolipídeo	*Candida antactica* e/ou *Candida apícola*	10,5 / 13,4
Solo e resíduo de refinaria de óleo vegetal	Raminolipídeo	*Pseudomonas aeruginosa* AT10	0,92
Efluentes do processamento de Batatas	Lipopeptídeo	*Bacillu subtilis*	---
Manipueira	Lipopeptídeo	*Bacillus subtilis* ATCC 21332 e *Bacillus subtilis* LB5a	2,2 – 3,0
Resíduo de refinaria de óleo de amendoim e licor de maceração de milho.	Glicolipídeo	*Candida sphaerica* UCP0995	9,0
Resíduo de refinaria de óleo de soja e ácido glutâmico	Lipopeptídeo	*Candida lipolytica* UCP0998	8,0

Fonte: adaptado de LUNA et al. (2013; 2014)

3.5.6. Desenvolvimento de bioprocessos para a produção e recuperação de biossurfactantes

Um processo eficiente e econômico constitui a base de qualquer indústria biotecnológica com fins lucrativos; assim, o desenvolvimento de bioprocessos é o primeiro passo para a comercialização de todos os produtos biotecnológicos, inclusive os biossurfactantes. Qualquer tentativa de aumentar o rendimento de um biossurfactante exige a adição ótima de componentes do meio e a seleção das condições ótimas que conduzam à produtividade máxima ou ótima (MARCHANT; BANAT, 2012b).

De maneira semelhante, técnicas e métodos de processamento eficazes são necessários para a máxima recuperação do produto.

Vários elementos, componentes do meio e precursores são mencionados como capazes de afetar o processo de produção dos biossurfactantes e a quantidade e a qualidade finais. Segundo a literatura, elementos como o nitrogênio, o ferro e o manganês afetam o rendimento dos biossurfactantes. Da mesma maneira, as proporções entre diferentes elementos como C:N, C:P, C:Fe ou C:Mg afetam a produção de biossurfactantes e a sua otimização intensifica a produção (MAKKAR et al., 2011; BANAT et al., 2010). A maximização da produtividade ou a minimização dos custos de produção exigem o uso de estratégias de otimização do processo, envolvendo múltiplos fatores.

Mesmo que se obtenha uma produção ótima utilizando-se meios e condições de cultivo adequados, o processo de produção ainda requer métodos eficazes e econômicos de recuperação dos produtos. Assim, um fator importante na determinação da viabilidade de um processo de produção em escala comercial é a disponibilidade de procedimentos de recuperação e “downstream” econômicos. No caso de muitos produtos biotecnológicos, os custos do processamento correspondem a 60% dos custos totais de produção. Vários métodos convencionais para a recuperação de biossurfactantes como precipitação com ácidos, extração com solventes, cristalização, precipitação com sulfato de amônio e centrifugação têm sido amplamente mencionados na literatura (MUTHUSAMY et al., 2008).

Alguns métodos de recuperação não-convencionais foram utilizados nos últimos anos. Esses procedimentos tiram vantagem de algumas propriedades dos biossurfactantes – como a atividade superficial ou a capacidade de formar micelas

– e são particularmente adequados à recuperação contínua em larga escala de biossurfactantes extracelulares do líquido metabólico. Alguns exemplos dessas estratégias de recuperação de biossurfactantes incluem fracionamento de espuma (SEN; SWAMINATHAN, 2005), ultrafiltração, adsorção-dessorção em resinas de poliestireno e cromatografia de troca iônica. Uma das principais vantagens desses métodos é a capacidade de operar de modo contínuo na recuperação de biossurfactantes com um alto nível de pureza (SAEKI et al., 2009).

Biossurfactantes com alto teor de pureza são exigidos em indústrias como a farmacêutica, alimentícia e cosmética, as quais irão exigir a aplicação dessas técnicas de recuperação (JOY; RAHMAN; SHARMA, 2017).

Novas pesquisas são necessárias para aperfeiçoar as fases de processamento já existentes, tornando-as mais competitivas em termos de custos. Muitas vezes, uma só técnica de processamento não é suficiente para a recuperação e purificação do produto. A Tabela 3 descreve os procedimentos de recuperação dos biossurfactantes e suas vantagens.

Tabela 3. Propriedades físico-químicas dos métodos de recuperação de biossurfactantes e suas vantagens relativas

Processo de recuperação	Propriedade responsável pela seleção do método de separação	Instrumentação necessária	Vantagens
Precipitação ácida	Biossurfactantes se tornam insolúveis a baixos pH	Não requer equipamentos	Baixo custo; eficiente na recuperação do surfactante bruto
Extração com solventes orgânicos	Biossurfactantes são solúveis em solventes orgânicos devido à presença da cadeia hidrofóbica	Não requer equipamentos	Eficiente na recuperação do surfactante bruto e na purificação parcial; natureza reutilizável
Precipitação por sulfato de amônio	Exclusão da fase saturada em sal pelo biossurfactante polimérico rico em proteínas	Não requer equipamentos	Efetiva no isolamento de determinados tipos de biossurfact. Poliméricos
Centrifugação	Biossurfactantes insolúveis precipitam em função da força centrífuga	Necessidade de Centrífuga	Reutilizável; efetiva na recuperação do surfactante bruto
Fracionamento de espuma	Biossurfactantes, devido à atividade surfactante, formam e se particionam na espuma	Construção de biorreatores especiais que facilitem a recuperação da espuma durante a fermentação	Utilizado em processos contínuos de recuperação; alta pureza do produto
Ultrafiltração em membrana	Biossurfactantes formam micelas acima da CMC, as quais são retidas em membranas poliméricas	Unidades de ultrafiltração com membrana polimérica porosa	Rápido; recuperação em apenas uma etapa; alto grau de pureza
Adsorção em resinas de poliestireno	Biossurfactantes são adsorvidos em resinas poliméricas e podem ser desorvidos usando solvente orgânico	Resina de poliestireno empacotada em colunas de vidro	Rápido; recuperação em apenas uma etapa; alto grau de pureza; reutilizável

Adsorção em carbono ativo	Biossurfactante são adsorvidos em carvão ativo e podem ser desorvidos usando solvente orgânico	Não requer equipamentos; pode ser adicionado ao meio de cultivo; também pode ser empregado em colunas de vidro	Pureza elevada do biossurfact.; baixo custo; reutilizável; recuperação em cultura contínua
Cromatografia de troca iônica	Biossurfactantes carregados se ligam a resinas trocadoras de íons e podem ser eluídos com um tampão específico	Resinas trocadoras de íons empregadas em colunas	Alta pureza, reutilização, rápida recuperação do produto
Extração por solvente (com MTBE)	Biossurfactantes são solúveis em solventes orgânicos devido à presença de cadeia hidrofóbica	Não requer equipamentos	Menos tóxico do que os solventes convencionais; baixo custo

Fonte: MUKHERJEE et al. (2006)

No processo de recuperação de múltiplas etapas dos biossurfactantes, será possível obter o produto a qualquer grau de pureza desejado. Biossurfactantes brutos ou impuros obtidos nas fases iniciais do processo de recuperação podem ser utilizados em aplicações ambientais e também na recuperação de petróleo e nas indústrias de tintas e têxtil e obtidas a custos mais baixos (MARCHANT; BANAT, 2012). Em alternativa, os biossurfactantes de elevado grau de pureza exigido pelas indústrias farmacêutica, alimentícia e cosmética podem ser obtidos por meio de novos estágios de purificação. Este tipo de recuperação de fases múltiplas deverá ser útil nas indústrias que produzem biossurfactantes para uma vasta gama de aplicações (SANTOS et al., 2016a).

3.5.7. Aspectos econômicos da produção de biossurfactantes

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

produção junto à necessidade de aplicação pretendida (BANAT et al., 2010; COIMBRA et al., 2009; RUFINO et al., 2008; MUTHUSAMY, 2008).

Embora os biossurfactantes tenham demonstrado alguns traços superiores sobre os surfactantes químicos, as aplicações industriais generalizadas são bastante limitadas, principalmente devido à competitividade dos custos. Os surfactantes químicos ainda são comparativamente mais baratos do que os biossurfactantes. A produção industrial em grande escala de biossurfactantes ainda é um desafio, principalmente por causa de problemas associados à ampliação (processo e substratos), downstream (GEETHA; BANAT; JOSHI, 2018).

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

A produção de biossurfactante é afetada por vários fatores que dependem do isolamento, do meio (fonte de carbono, nitrogênio e salinidade) e condições operacionais (pH, temperatura e velocidade de agitação). Esses fatores influenciam a quantidade e o tipo de biossurfactante produzido (FADHILE ALMANSOORY et al., 2017).

Várias alternativas mais baratas são relatadas para a produção de biossurfactantes, tais como: o uso do melaço e milhocina que são desperdiçados pelas indústrias de laticínios e de óleos vegetais, óleos de fritura e resíduos da agricultura e lignocelulósicos. A escolha do substrato mais barato depende da sua fácil disponibilidade e pode variar muito de país para país, como o melaço da cana será mais barato nos países que têm uma produção excedente do açúcar, em comparação com outras partes do mundo. Otimização adicional do meio de cultura, condições de crescimento, parâmetros de processo, processos downstream, usando modelos estatísticos e processos de separação-purificação econômicos para maximizar a recuperação, também podem aumentar significativamente o rendimento e reduzir o custo total (GEETHA; BANAT; JOSHI, 2018).

A produção de biossurfactantes a um baixo custo é dificultada quando se faz necessário uma refinação extensiva. Para o desenvolvimento de processos deve-se usar biossurfactantes capazes de serem recuperados por técnicas simples e

baratas. O processo mais comum de recuperação dos biossurfactantes é a extração com solventes (clorofórmio-metanol, diclorometano-metanol, butanol, acetato de etila, pentano, hexano e ácido acético, entre outros). Todavia, tem sido reportada técnicas de precipitação dos produtos como a precipitação por sulfato de amônia, centrifugação por cristalização, adsorção, fermentação fracionária, etc.

Shah et al. (2016), descreveram métodos práticos e econômicos de recuperação de ramnolipídeos produzidos por *Pseudomonas aeruginosa*. Esses métodos incluem a precipitação ácida, precipitação com sulfato de amônio, precipitação com sulfato de zinco e extração com solventes. Dentre os quais, a extração com solvente orgânico foi a melhor técnica de recuperação, com rendimento de 7,5g/L de biossurfactante, enquanto a precipitação ácida obteve 3,5g/L. Vários processos de recuperação de biossurfactantes estão demonstrados na Tabela 4 (MAKKAR; CAMEOTRA; BANAT, 2011)

Tabela 4. Processos de recuperação de biossurfactante

Processo	**Recuperação de biossurfactante**
Precipitação do sulfato de amônia	Emulsan Biodispersan Bioemulsifier
Precipitação de acetona	Bioemulsificantes Glicolipídios
Precipitação de ácido	Surfactin Trealoselipídios
Extração de solvente	Sophorolipídios Liposan Celobiolipídios
Cristalização	Glicolipídios
Centrifugação	Glicolipídios Ramnolipídios
Adsorção	Lipopeptídeos Glicolipídios
Fermentação Fracionada	Surfactin
Filtração de fluxo tangencial	Biossurfactante misto
Precipitação	Glicolipídios
Ultrafiltração	Glicolipídios

Fonte: MAKKAR; CAMEOTRA; BANAT (2011)

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5. ARTIGOS DERIVADOS DA TESE

5.1 CAPÍTULO 1 - Recovery of contaminated marine environments by biosurfactant-enhanced bioremediation

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Recovery of contaminated marine environments by biosurfactant-enhanced bioremediation

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ABSTRACT

The need to remediate areas contaminated by petroleum products has led to the development of novel technologies for treating such contaminants in a non-conventional manner, that is, without the use of chemical or physical methods. Biosurfactants are amphipathic biomolecules produced by microorganisms that can be used in bioremediation processes in environments contaminated by petroleum products due to their excellent tensioactive properties. The aim of the present study was to produce a biosurfactant from *Pseudomonas aeruginosa* UCP 0992 cultivated in 0.5% corn steep liquor and 4.0% vegetable oil residue in a 1.2-L bioreactor employing a central composite rotatable design to optimize the cultivation conditions for maximum yield. The best results were achieved with aeration rate of 1.0 vvm and 3.0% inoculum at 225 rpm for 120 h, resulting in a surface tension of 26.5 mN/m and a biosurfactant yield of 26 g/L. Kinetic and static assays were then performed with the biosurfactant for the removal of motor oil adsorbed to sand, with removal rates around 90% and 80%, respectively, after 24 h. Oil degradation experiments with the bacterium and the combination of the bacterium and biosurfactant were also conducted to simulate the bioremediation process in sand and seawater samples (duration: 75 and 30 days, respectively). In both cases, oil degradation rates were higher than 90% in the presence of the biosurfactant and the producing species, indicating the potential of the biomolecule as an adjuvant in petroleum decontamination processes in the marine environment.

1. Introduction

Petroleum is one of the most important resources of the modern industrial world. However, the extraction, transport, storage and use of petroleum pose a constant risk of an oil spill, which is a serious threat to the environment [1,2]. In recent years, petroleum leaks and spills have occurred with greater frequency, such as the leaking of four million barrels in the Gulf of Mexico in 2010 and approximately 140 thousand liters of oil in Campos Bay in the state of Rio de Janeiro, Brazil, in 2011 [3].

Like other large-scale industrial processes, oil refineries are potential sources of environmental pollution. A report from a mixed commission to analyze the accidents at Petrobrás/Repar (CREA-PR) cites 33 accidents involving the spilling of petroleum and petroleum products in Brazil between 1975 and 2001, totaling millions of liters of contaminants in soil, rivers and the ocean [4,5]. The sudden exposure of a large volume of petroleum affects the majority of populations of native organisms. Although the government and non-governmental organizations have initiated necessary measures to safeguard lives, the impact of these spills is still felt today [2]. The release of hydrocarbons into the environment – either accidentally or due to human activities – is one of the main causes of water and soil pollution and has disastrous consequences for ecosystems [6].

The treatment of areas contaminated by hydrocarbons, especially the ocean, has been performed using physical containment methods and the mechanical removal of contaminants [7,8]. Chemical methods involving the application of chemical dispersants, such as Corexit, which was used on the leak in the Gulf of Mexico, have also been largely employed. However, besides being inefficient, depending on the type of dispersants used, these methods can constitute another source of contamination in the form of the accumulation of other toxic compounds in the environment [9,10].

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The need to remediate contaminated areas has led to the development of novel technologies for the detoxification of these contaminants in a non-conventional manner, that is, without the use of chemical or physical methods. One of these novel technologies is known as bioremediation, which involves the use of microorganisms or microbial metabolites to degrade pollutants [3,8]. Although bioremediation is effective and environmentally compatible, the time and costs necessary for the treatment of a large quantity of contaminant make this process unviable. Thus, surfactant compounds can be used to increase the solubility of oil and make bioremediation viable [12].

Recent studies have demonstrated that microbial surfactants, which are metabolites produced by bacteria and yeasts, have the ability to solubilize and mobilize organic compounds adsorbed to soil [13]. Some synthetic surfactants, such as Triton X-100 and Tween 80, also have the ability to increase the concentration of non-polar compounds in the aqueous phase [13,14]. However, synthetic surfactants largely used in oil spills, as Corexit, are associated with toxic effects and resistance to biodegradation [7,12]. Compared to their synthetic counterparts, biosurfactants generally exhibit strong environmental compatibility, greater surface activity, low toxicity and high biodegradability [5]. Therefore, biosurfactants are strong candidates for use in the bioremediation of contaminated soil and aquatic environments [17], along with being produced by renewable sources, such as microbial fermentation, which is a chemical advantage over their synthetic counterparts.

The first studies on biosurfactants emerged in the 1980s and research has since has led to the development and commercialization of two products: Surfactin, which is a lipoprotein produced by the bacterium *Bacillus subtilis* [18], and rhamnolipids [19,20], which are a group of glycolipids produced by the bacterium *Pseudomonas aeruginosa*. Although extremely efficient, these two biosurfactants are sold at a high price due to the substrates used for production and the level of purity required for pharmaceutical and medical applications [16–18]. Although the bacterium *Pseudomonas aeruginosa* is considered an opportunistic pathogen, its biosurfactants are especially capable of reducing the surface tension of liquids to 29 mN/m and can be produced from various soluble and insoluble substrates and have been successfully used in remediation processes, especially after evaluation of toxicity that ensures the safety of these biomolecules [5].

One of the great advantages of using biosurfactants is the possibility of producing these natural compounds from different substrates, especially renewable sources, such as vegetable oil, distillery and milk product residues. The choice of low-cost substrates is important to the overall economy of the process, as substrates represent 50% of the final cost of the product. Indeed, successful biosurfactant production depends on the choice of renewable substrates that lead to high productivity and quality for specific biosurfactants [24]. The petroleum and petrochemical industries stand out as the largest fields for biosurfactant application, as such industries use these compounds in their crude form, that is, without the need for purification, which leads to an accentuated reduction in the cost of using a biotechnological agent [1].

Response surface methodology (RSM) has been effectively employed to reduce the biosurfactants production cost through the balanced proportions selection of the culture medium constituents and the culture conditions optimization. RSM constitutes a statistical techniques collection for designing experiments, building models, simultaneously evaluating the factors effects 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 [25].

The aim of the present study was to optimize the production of a biosurfactant by the bacterium *Pseudomonas aeruginosa* UCP 0992 cultivated in a low-cost culture medium in a bioreactor using a CCRD. The capacity of the biosurfactant as a bioremediation agent in decontamination processes of marine environments polluted with petroleum products was also evaluated.

2. Materials and methods

2.1. Microorganism

The bacterium *P. aeruginosa* UCP 0992, which belongs to the Culture Bank of the Environmental Science Research Center of the *Universidade Católica de Pernambuco*, was used as the biosurfactant-producing microorganism. This bacterium was previously isolated from a port area contaminated with complex hydrocarbons stemming from nautical activities in the city of Recife, Brazil and further submitted to biochemical and molecular identification. The strain was sub-cultured every 30 days and kept in slanted test tubes with solid nutrient agar under refrigeration (5 °C).

2.2. Maintenance, inoculum growth and biosurfactant production media

A nutrient agar medium with the following composition was used for the maintenance of the bacterium: meat extract (5.0 g), peptone (10.0 g), NaCl (5.0 g), agar (5.0 g) and distilled water (1000.0 ml). The constituents were solubilized and sterilized in an autoclave at 121 °C for 20 min. Nutritive broth, which has the same composition as nutrient agar, but without the agar, was used for the growth of the inoculum. The production medium was composed of distilled water containing 0.5% corn steep liquor and 4% residue from a vegetable oil refinery [20,21].

2.3. Preparation of inoculum

Cultures of the 24-h growing bacterium obtained from cultivation in the nutrient agar medium were transferred to Erlenmeyer flasks containing 50 mL of nutritive broth. The flasks were submitted to orbital shaking at 200 rpm for 24 h at 28 °C until achieving an optical density of 0.7 (corresponding to an inoculum of 10^7 colony-forming units [CFUs)/mL] at 600 nm). This reading was made with the inoculum at a concentration of 1% (v/v).

2.4. Optimization of biosurfactant production using CCRD

Biosurfactant production was performed in a 2-L bioreactor (Tec-Bio-Plus, Tecnal Ltda., Brazil) with a working volume of 1.2 L in batch mode with controlled pH (7.0 ± 0.2) and temperature (28 °C). The culture medium was inoculated with a 24-h inoculum and the fermentation conditions conformed a CCRD for the optimization of the production. The agitation velocity, cultivation time, aeration rate and inoculum size were the independent variables and the yield of the isolated biosurfactant was the response variable. A set of 26 experiments was performed with two repetitions at the central point. Table 1 displays the range and levels of the independent variables. Each variable was studied on five levels (−2.0, −1.0, 0, + 1.0 and +2.0), which were based on results obtained during preliminary experiments. The optimum values of the CCRD were determined by solving the regression equation and analyzing the response surface contours. Analysis of variance (ANOVA) with 95% confidence intervals was used to determine the significance of the effects. ANOVA, the determination of the

Table 1
Experimental range and levels of independent variables for biosurfactant production.

Variables	Range and levels				
	−2.0	−1.0	0.0	+1.0	+2.0
Inoculum size (%), X_1	1.0	2.0	3.0	4.0	5.0
Cultivation time (hours), X_2	72	84	96	108	120
Aeration rate (vvm), X_3	0.0	0.5	1.0	1.5	2.0
Agitation (rpm), X_4	175	200	225	250	275

regression coefficients and the construction of the graphs were performed with the aid of the Statistica® program, version 12.0. At the end of the culture, the samples were centrifuged and filtered for the determination of the biosurfactant yield, which was used as the criterion for the selection of the best production conditions.

2.5. Determination of surface tension and critical micelle concentration (CMC)

Surface tension in the cell-free broth, obtained obtained by centrifuging cultures at 5 000 g for 20 min, was measured in an automatic tensiometer (KSV Sigma 700, Finland) using the NUOY ring. The ring was immersed in the broth and the force required to pull it through the air-liquid interface was recorded. For determination of the CMC, the surface tension of dilutions of isolated biosurfactant in distilled water was measured until reaching to a constant value (standard deviation less than 0.4 mN/m during 10 successive measurements). The CMC was obtained by plotting surface tension against surfactant concentration and expressed as g/L of biosurfactant.

2.6. Isolation of biosurfactant

The biosurfactant produced by the bacterium *P. aeruginosa* was isolated. For such, the pH of the cell-free broth was adjusted to 2 with a solution of 6 M of HCl. The same volume of chloroform/methanol (2:1) was added to the broth. The mixture was shaken vigorously for 15 min and left to rest until the separation of the phases. The organic phase was removed and the operation was repeated two more times. The product was concentrated from the organic phases using a rotary evaporator at 45 °C until reaching a constant weight. The material obtained at the end of this procedure was checked for biosurfactant activity and the yield of the isolated biosurfactant was expressed as g/L [28].

2.7. Hydrophobic compound dispersion test in seawater

The oil dispersion capacity was simulated in the laboratory with the contamination of samples of seawater with motor oil. The seawater samples were collected from the proximities of the catchment system of the Pernambuco Thermoelectric Energy Generating System (TERMOPE). Motor oil was placed on the surface of 40 mL of seawater in a Petri dish measuring 15 cm in diameter, followed by the placement of the cell-free broth resulting from the best fermentative conditions selected in the CCRD/RSM (crude biosurfactant), containing 26 g/L of biosurfactant, on the layer of oil at biosurfactant/oil proportions of 1:2, 1:8 and 1:25 (v/v). The effect of different temperatures (5 °C, 70 °C and 120 °C), NaCl concentrations (2.0, 5.0 and 8.0%), pHs values (4.0, 8.0 and 10.0) and heating times to 90 °C (20, 40 and 60 min) on the dispersion power of the biosurfactant were evaluated. The results were observed visually and the mean diameter of the dispersion zone was measured in triplicate and expressed as a percentage value in relation to the diameter of the Petri dish [29].

2.8. Removal of petroleum product adsorbed to sand by biosurfactant – kinetic assay

The removal of the contaminant was performed according to the methods described by Luna et al. [30] and Lai et al. [31]. Standard sand for NBR 7214 cement (ABNT, 1982) was used in the experiments. Fifty g of standard sand contaminated with 10% motor oil were transferred to 250-mL Erlenmeyer flasks, to which 100 mL of the isolated biosurfactant solution was added at a concentration lower than the critical micelle concentration (1/2 CMC, i.e., 0.3 g/L) as well as at the CMC (0.6 g/L) and twice the CMC (1.2 g/L). The same quantity (100 mL) of the cell-free broth (crude biosurfactant), with 26 g/L of biosurfactant, was also tested and a flask with contaminated sand and 100 mL of water (without the addition of the biosurfactant) was used as the control. The flasks were shaken at 150 rpm for 5, 10 and 20 min as well as after 24 h at 28 °C. The treated sand and washing solution were then separated for analysis.

2.9. Removal of petroleum product adsorbed to sand by biosurfactant in packed columns – static assay

Glass columns (55 × 6 cm) were filled with 200 g of standard sand contaminated with 10% of the hydrophobic contaminant and the surface was then flooded with 200 mL of the cell-free broth (crude biosurfactant). A column with sand and 200 mL of water (without the biosurfactant) was used as the control. The percolation of the surfactant solutions was monitored for 24 h. At the end of each washing, the removed liquid was set to rest in Erlenmeyer flasks for five minutes [32].

2.10. Analysis of petroleum product removed from sand

The initial and final quantities of the hydrophobic contaminant were determined in the treated sand. One hundred mL of n-hexane was added to the sand and placed in a shaker at 200 rpm for two hours. The final extract of hexane and oil was placed in a hothouse at 68–70 °C. The removal efficiency was evaluated by gravimetric analysis after washing the sand containing the remaining contaminant with hexane. The percentage of degradation was calculated as follows: % of degradation = (amount of degraded oil/amount of oil added to the medium) x 100 [33].

2.11. Bioremediation experiments of petroleum product adsorbed to sand

Ten-gram samples of sand contaminated with motor oil were added to 100 mL of potable water and the mixture was enriched with 1 mL of sugarcane molasses donated by a local sugar processing plant. The mixture was submitted to free flowing steam sterilization and constituted the control condition. Next, solutions of the isolated biosurfactant at the CMC (0.6 g/L) and twice the CMC (1.2 g/L) and 15% of the producing microbial specie (15% of inoculum containing 10^7 CFUs/mL [optical density of 0.7 at 600 nm]) previously cultivated in its respective inoculum preparation medium (nutritive broth) were added. One of the mixtures consisted only of sand contaminated with oil and the microorganism inoculum. All mixtures were incubated at 28 °C at 150 rpm for 75 days. At 15-day intervals (Days 15, 30, 45 and 60), 1% molasses was added to the mixtures. Samples were withdrawn at 15-day intervals (15, 30, 45, 60 and 75 days – total of five samples) for analysis of the petroleum product. The percentage of oil degradation was calculated as the concentration of oil removed, determined by gravimetric analysis [34].

2.12. Bioremediation experiments of petroleum product spilled in seawater

The motor oil biodegradation experiments were performed in 250-mL Erlenmeyer flasks containing 50 mL of seawater collected from the Suape Port (state of Pernambuco, Brazil) and 1% motor oil (control condition). The medium was sterilized and inoculated with 5% inoculum (5% of inoculum containing 10^7 CFUs/mL [optical density of 0.7 at 600 nm]) of the biosurfactant-producing bacterium. The experiments were conducted under three different conditions, one of which consisted of seawater + motor oil + *P. aeruginosa* UCP 0992; the second was composed by seawater + motor oil + *P. aeruginosa* UCP 0992 + biosurfactant at CMC (0.6 g/L); and the third consisted of seawater + motor oil + *P. aeruginosa* UCP 0992 + biosurfactant at twice the CMC (1.2 g/L). The flasks were incubated in a shaker at 150 rpm for 30 days. Samples were removed every ten days (total: three samples).

Table 2
Central composite design matrix and experimental values of observed factors for biosurfactant production in bioreactor.

Run	Inoculum size (%), X_1	Cultivation time (hours), X_2	Aeration rate (vvm), X_3	Agitation (rpm), X_4	Biosurfactant yield (g/L), Y
1	−1.0	−1.0	−1.0	−1.0	3.788
2	−1.0	−1.0	−1.0	1.0	10.65
3	−1.0	−1.0	1.0	−1.0	11.85
4	−1.0	−1.0	1.0	1.0	12.88
5	−1.0	1.0	−1.0	−1.0	8.33
6	−1.0	1.0	−1.0	1.0	17.42
7	−1.0	1.0	1.0	−1.0	16.67
8	−1.0	1.0	1.0	1.0	18.76
9	1.0	−1.0	−1.0	−1.0	8.09
10	1.0	−1.0	−1.0	1.0	16.86
11	1.0	−1.0	1.0	−1.0	18.39
12	1.0	−1.0	1.0	1.0	12.50
13	1.0	1.0	−1.0	−1.0	20.17
14	1.0	1.0	−1.0	1.0	25.35
15	1.0	1.0	1.0	−1.0	21.94
16	1.0	1.0	1.0	1.0	23.34
17	−2.0	0.0	0.0	0.0	18.18
18	2.0	0.0	0.0	0.0	23.28
19	0.0	−2.0	0.0	0.0	4.02
20	0.0	2.0	0.0	0.0	26.00
21	0.0	0.0	−2.0	0.0	17.52
22	0.0	0.0	2.0	0.0	24.00
23	0.0	0.0	0.0	−2.0	8.27
24	0.0	0.0	0.0	2.0	18.74
25	0.0	0.0	0.0	0.0	25.72
26	0.0	0.0	0.0	0.0	25.69

2.13. Calculation of removal efficiency of petroleum product from seawater

The degraded oil was quantified in the samples and control medium after extraction with the same volume n-hexane, as described in Section 2.10.

2.14. Statistical analyses

Measurements were determined in triplicate experiments. Microsoft Office Excel 2007 was used for the calculation of mean and standard error values. Tukey's test was used to determine significant differences ($p < 0.05$).

3. Results and discussion

3.1. Optimization of biosurfactant production using CCRD/RSM

Table 2 displays the results corresponding to the CCRD matrix. The multiple regression analysis using the response surface methodology (RSM) was performed to adjust the response function to the experimental data and investigate the simultaneous influence of the four variables selected. The best conditions for biosurfactant production were found in Run 20.

The RSM to estimate the optimal variables resulted in an empirical relationship between the variables of the process and biosurfactant yield. The following quadratic polynomial equation best fit the data:

$$Y = 25.705 + 2.35325X_1 - 4.20633X_2 + 1.609X_3 + 2.06092X_4 + 0.80887X_1X_2 - 0.89175X_1X_3 - 0.60125X_1X_4 - 0.424625X_2X_3 + 0.437125X_2X_4 - 1.9545X_3X_4 - 1.58756X_1^2 - 3.01831X_2^2 - 1.58018X_3^2 - 3.39481X_4^2$$

in which Y is biosurfactant yield (g/L) and X_1, X_2, X_3 and X_4 are the coded values for the inoculum size, cultivation time, aeration rate and agitation, respectively.

The evaluation of the empirical model was performed using ANOVA (Table 3). The p and F (with 95% confidence interval) values indicate that all terms were statistically significant ($p < 0.05$, $F > 4$). The reproducibility of the experimental data was demonstrated by the extremely low pure error (0.0000). Cultivation time was the most important factor to the increase in biosurfactant yield, followed by the quadratic term of the agitation variable. The correlation coefficient ($R^2 = 0.93127$) indicates that only 6.873% of the total variation in the data can be explained by the empirical model. Thus, the regression model is adequate for describing the production of biosurfactant by *P. aeruginosa* UCP0992.

Table 3
Analysis of variance (ANOVA) for response surface quadratic model regarding biosurfactant yield achieved with biosurfactant produced by *P.aeruginosa* UCP0992.[a]

Factor	Sum of squares	Degrees of freedom	Mean square	F-ratio	p-value[b]
X_1 (L)[c]	132.9070	1	132.9069	295348.6	0.001171
X_1 (Q)[d]	43.9920	1	43.9916	97759.2	0.002036
X_2 (L)	424.6380	1	424.6378	943639.5	0.000655
X_2 (Q)	159.0150	1	159.0146	353365.7	0.001071
X_3 (L)	62.1330	1	62.1331	138073.7	0.001713
X_3 (Q)	43.5840	1	43.5839	96853	0.002046
X_4 (L)	101.9370	1	101.9371	226526.8	0.001338
X_4 (Q)	201.1590	1	201.1593	447020.7	0.000952
X_1 (L) x X_2 (L)	10.4680	1	10.4685	23263.2	0.004174
X_1 (L) x X_3 (L)	12.7230	1	12.7235	28274.4	0.003786
X_1 (L) x X_4 (L)	5.7840	1	5.7840	12853.4	0.005615
X_2 (L) x X_3 (L)	2.8850	1	2.8849	6410.9	0.007951
X_2 (L) x X_4 (L)	3.0570	1	3.0573	6793.9	0.007723
X_3 (L) x X_4 (L)	61.1210	1	61.1211	135824.7	0.001727
Lack of Fit	79.2340	10	7.9234	17607.5	0.005865
Pure Error	0.0000	1	0.0004		
Total square sum	1152.8810	25			

[a] $R^2 = 0.93127$; adjusted $R^2 = 0.8438$.
[b] $p \leq 0.05$ – significant at 5% level.
[c] (L) = linear effect.
[d] (Q) = quadratic effect.

Fig. 1 shows the three-dimensional plots. Biosurfactant yield increased with the increase in the cultivation time and inoculum size (Fig. 1A). The elliptical nature of the contour graph indicates a high degree of interaction among the factors, i.e., it is not possible to predict the biosurfactant yield by variating only one of these factors. The combination of aeration rate slightly above the central point and the increase in inoculum size (Fig. 1B) raised the biosurfactant yield and the curves indicate a moderate interaction. Agitation slightly above the mid-level and an increase in inoculum led to an increase in biosurfactant yield (Fig. 1C) and the curves indicate a high degree of interaction. Fig. 1D shows that biosurfactant yield increased when the aeration rate and cultivation time were increased, tending toward maximum levels. However, the curves demonstrate considerable parallelism between the factors and, consequently, a weak interaction. Thus, it is possible to make predictions regarding biosurfactant yield from variations in the values of only one of these factors. A similar effect was found with regard to the relationship between agitation and cultivation time (Fig. 1E): the curves indicate a high degree of parallelism and a very weak interaction between these factors. Fig. 1F shows that the combination of agitation and aeration rate a little above the mid-level increased the biosurfactant yield under the conditions tested. The bending curves indicate a high degree of interaction between these factors.

The best results were achieved using a 3% inoculum, 120-h

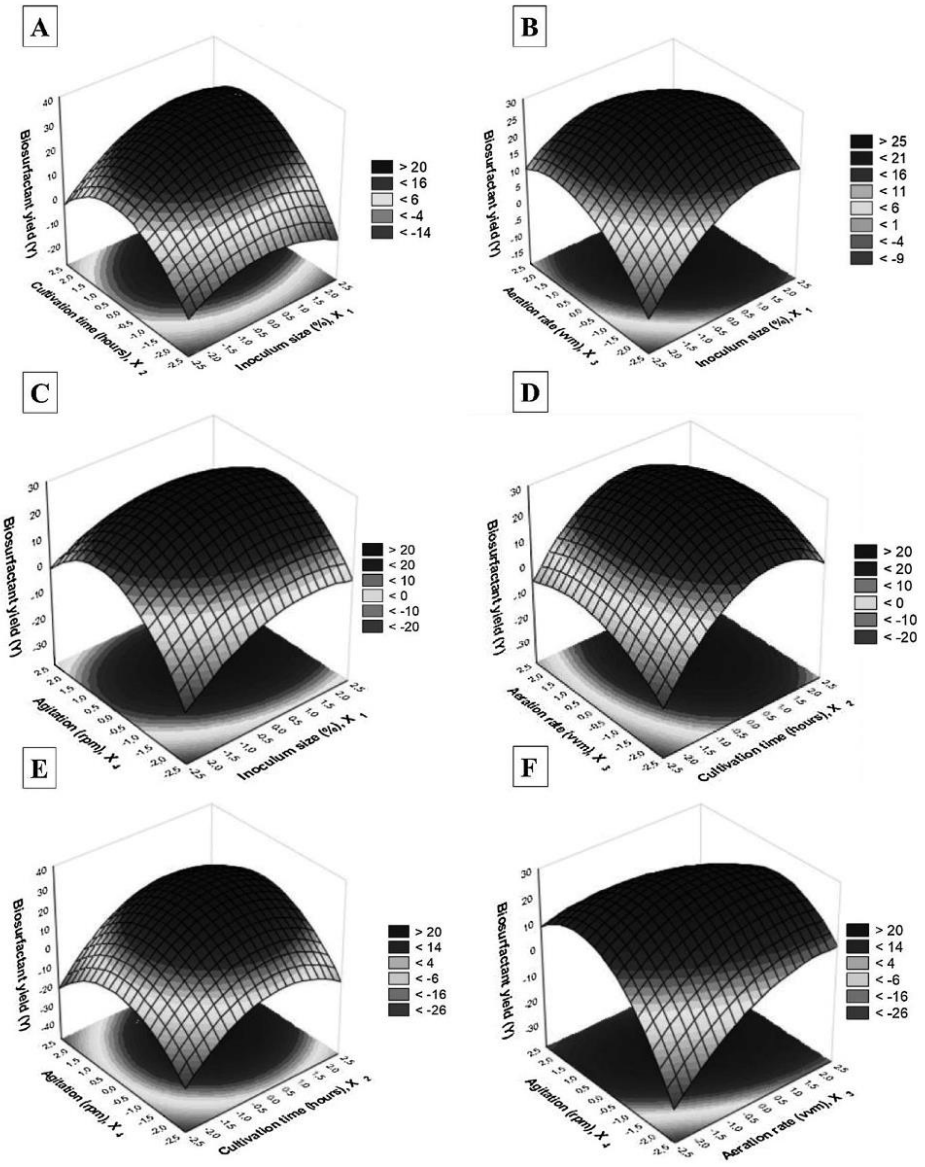

Fig. 1. Response surface plots and contour plots for maximum biosurfactants yield generated using data in Table 2. Inputs, 26 experimental runs carried out under conditions established by CCRD; biosurfactant yield as function of (A) time cultivation and inoculum size; (B) aeration rate and inoculum size; (C) agitation and inoculum size; (D) aeration rate and inoculum size; (E) agitation and time cultivation; (F) agitation and aeration rate.

cultivation time, aeration rate of 1 vvm and agitation of 225 rpm, leading to a biosurfactant yield of 26 g/L and corresponding to a reduction in the surface tension of the medium from 55.55 mN/m to 26.54 mN/m. The high yield under this condition may be related to better control of the aeration rate and agitation, optimized using CCRD/RSM, as well as the fact that the fermentation process was conducted in a bioreactor, which is a completely closed system with a continuous oxygen supply, favoring cell growth and greater biosurfactant yield [29,30]. The results are comparable to those described by Lima et al. [36], who used a strain of *Pseudomonas aeruginosa* PACL to investigate the efficiency of biosurfactant production when growing the bacterium in a medium supplemented with 22.0 g/L of soybean oil residue and applying a complete factorial experimental design with the aim of optimizing the aeration rate and agitation velocity; at optimum levels (aeration rate of 0.5 vvm and agitation at 550 rpm), the biosurfactant concentration was 3.3 g/L and the minimum surface tension was 26.0 dynes/cm.

Oluwaseum et al. [37] found that biosurfactant production by *P. aeruginosa* C1501 cultivated in a medium containing 3% glycerol as the carbon source led to a reduction in surface tension from 61.2 to

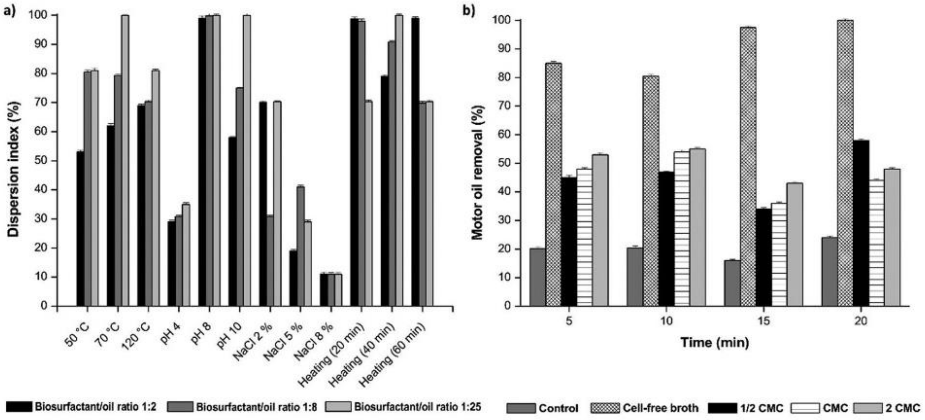

Fig. 2. Motor oil dispersion in sea water (a) and motor oil adsorbed to sand removal (b) by the biosurfactant from *P. aeruginosa* UCP 0992 cultivated in distilled water supplemented with 4% vegetable oil residue and 0.5% corn steep liquor. Error bars illustrate experimental errors (standard deviations), calculated from three independent experiments.

30 dynes/cm and a yield of 1300 mg dm^{-3}. Zhao et al. [38] found that a biosurfactant produced by *P. aeruginosa* DQ3 cultivated in a bioreactor with a medium containing 46.5% glycerol led to a surface tension of 33.8 mN/m. Aparna et al. [19] found that a biosurfactant produced by *Pseudomonas* sp 2B cultivated in a medium containing 1% molasses led to a surface tension of 30.14 mN/m and a yield of 4.97 g/L. Silva et al. [39] found that a biosurfactant produced by *P. aeruginosa* cultivated in a medium containing 3% glycerol led to a surface tension of 27.6 mN/m and a yield of 6.5 g/L. Thus, the majority of studies involving the bacterium *Pseudomonas aeruginosa* present satisfactory results with regard to biosurfactant production and the reduction in surface tension. The yield in the present study, which was optimized using CCRD/RSM, was comparatively much higher.

Regarding the CMC, the isolated biosurfactant led to a reduction in the surface tension of water from 70 to 26 mN/m, which was achieved at a concentration of 0.6 g/L, with no further reduction occurring thereafter.

3.2. Hydrophobic compound dispersion test in seawater

Many processes in the petroleum industry are performed in the marine environment and part of the oil generated during these processes is occasionally spilled into the sea, requiring substances with surfactant properties used in conjunction with other containment measures. Dispersion is a process by which a hydrocarbon is dispersed in the aqueous phase in the form of emulsions. The dispersion of oil in small droplets increases the surface area, which stimulates biodegradation by autochthonous microorganisms [1,35].

Fig. 2a displays the results of dispersion of motor oil by the biosurfactant from *P. aeruginosa* at different proportions (crude surfactant to oil: 1:2, 1:8 and 1:25 vol/vol) in seawater samples with variations in pH, temperature, NaCl concentration and heating time. The biosurfactant demonstrated better dispersion capacity at pH 8, reaching a nearly 100% dispersion index. This is a positive point for the application of this product in bioremediation process in the marine environment, as seawater has the same pH. On the other hand, the biosurfactant was very sensitive to high concentrations of salt, but quite resistant to the different thermal treatments, tending toward dispersion greater than 70%. The best dispersion results were achieved at a biosurfactant-to-oil proportion of 1:25 (v/v) for all conditions tested. Luna et al. [41] found that a biosurfactant produced by *Candida bombicola* demonstrated high motor oil dispersion activity, with a dispersion index of 80% relative to the diameter of the oil slick. In another study, a biosurfactant produced by *Candida tropicalis* demonstrated up to a 75% dispersion index of motor oil in seawater [25]. Therefore, the present results demonstrate the potential of the biosurfactant from *P. aeruginosa* with regard to the mobilization and solubilization of oil slicks in the marine environment.

3.3. Removal of petroleum product adsorbed to sand – kinetic assay

The low availability of hydrocarbons (low solubility in water, high fixation to the soil matrix and little transference of adsorbed pollutants from the solid to the aqueous phase) is one of the limiting factors in the bioremediation of contaminated soil. The use of surfactants is a way to increase the availability of hydrocarbons through microbial action [32].

Two mechanisms control the removal of hydrophobic contaminants from soil through surfactant agents. The first occurs below the CMC, in which surfactant monomers increase the angle of contract between the soil and hydrophobic contaminant, enabling the separation of the contaminant from the soil particles and consequent displacement of the oil. The second mechanism is known as solubilization and occurs above the CMC, in which the contaminant is partitioned in the center of the surfactant micelles [37,38]. In the present study, hydrophobic pollutant removal assays were performed in flasks (kinetic removal) and packed columns (static removal) to determine the effectiveness of the biosurfactant produced by *Pseudomonas aeruginosa* UCP0992. Both assays were performed with the cell-free broth (crude biosurfactant).

Fig. 2b displays the results of the kinetic assay of the removal of oil adsorbed to soil. The best results were achieved with the cell-free both, with removal rates ranging from 80 to 100%. Moreover, the biosurfactant remained effective when used at a concentration of ½ the CMC, with removal rates ranging from 45 to 65% throughout the experiment. The assays using the biosurfactant at the CMC and 2 × the CMC demonstrated better removal rates.

Using a biosurfactant produced by *P. cepacia*, Soares da Silva et al. [28] found removal rates higher than 70%, with maximum removal of 96% achieved using the isolated biosurfactant at 2 × the CMC. Other isolated biosurfactants produced by strains of *P. aeruginosa* led to crude oil removal rates of 49–54% at room temperature, 52–57% at 70 °C and 58–62% at 90 °C [44].

3.4. Removal of petroleum product adsorbed to sand – static assay

The potential of the biosurfactant from *P. aeruginosa* UCP0992 with regard to the removal of oil was estimated by measuring the quantity of oil removed from sand in glass columns after flushing with the aqueous

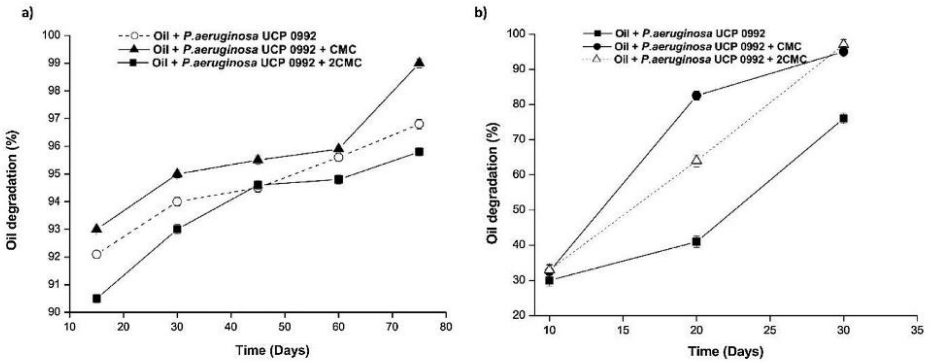

Fig. 3. Enhanced bioremediation by the biosurfactant from *Pseudomonas aeruginosa* UCP 0992. (a) Degradation of motor oil adsorbed to sand and (b) degradation of motor oil in seawater. Data calculated from three independent experiments.

solution containing the biosurfactant. In this case, the biosurfactant was used to simulate the oil removal power in a vertical section through percolation and demonstrated satisfactory results. The broth (crude biosurfactant) was capable of removing 79.55% of the motor oil in 1440 min. In contrast, the removal rate in the control experiment was 21.8%, which is likely related to gravitational action and the mechanical action of flushing.

The literature describes other results from studies involving the use of biosurfactants for the removal of hydrocarbons in compacted columns. Biosurfactants produced by *P. aeruginosa* MTCC7815, MTCC7812 and MTCC8165 in a medium containing 2% glycerol removed 49 to 54% of crude oil contained in packed columns [44]. High concentrations (2.5 and 5.0 g/L) of a biosurfactant isolated from *P. aeruginosa* 57SJ were required to remove 70% of pyrene adsorbed to soil (particle size: 2 mm) [45]. Bai et al. [46] found that an anionic rhamnolipid isolated from *P. aeruginosa* was capable of removing 84% of hexadecane adsorbed to sand with a particle size of 0.6–0.85 mm, whereas a 22% removal rate was found for sand with a particle size of 0.3–0.42 mm.

Lai et al. [31] found an approximately 35% removal rate of oil using a 0.2% Tween 80 solution. Jain et al. [47] investigated the potential of two biosurfactants in removing oil in glass columns in comparison to synthetic surfactants. Cameotra and Makkar [48] found that a biosurfactant isolated from *P. aeruginosa* was able to recover 56% of oil adsorbed to sand in a column. Ibrahim et al. [49] report a biosurfactant that achieved a 76% oil recovery rate in two hours in a column compared to a 30% recovery rate in the control experiment in the same period of time. Investigating a biosurfactant produced by *Bacillus* sp. (MTCC 5514), Kavitha et al. [2] found removal rates higher than 70% of oil adsorbed to standard sand and sandy soil, whereas the rates achieved with synthetic surfactants were much lower. Fernandes et al. [50] report a 69% residual oil recovery rate in a column using 600 mg l^{-1} of a biosurfactant produced by *Bacillus subtilis* RI 4114.

3.5. Bioremediation of petroleum product adsorbed to sand

Tests for the determination of the potential of the biomolecule in bioremediation processes were also performed. Fig. 3a displays the degradation percentages of motor oil adsorbed to sand by the biosurfactants from *P. aeruginosa* UCP0992.

The degradation of motor oil was generally above 90%, with higher percentages when the microorganism was used together with the biosurfactant in the bioremediation process, reaching nearly 100% removal with the concentration at the CMC after 75 days. It should be pointed out that the addition of molasses may have contributed to the excellent removal rates.

Su et al. [51] found that the biodegradation of motor oil by *P. aeruginosa* SU-1 was accelerated from 55.9% to 64.4% after adding yeast extract. Cerqueira et al. [52] analyzed the biodegradation of aliphatic and aromatic hydrocarbons from an oily petrochemical sludge in a liquid medium by a consortium of bacteria as well as five cultures of pure bacteria; the consortium demonstrated an excellent degradation capacity, reducing 90.7% of the aliphatic fraction and 51.8% of the aromatic fraction of oily sludge.

The effects of the addition of the biosurfactant from *P. cepacia*alone and with cells of the bacteria on the biodegradation of hydrophobic organic compounds adsorbed to soil were studied for 60 days. The results demonstrate the efficiency of the biosurfactant and its producing species in degrading high percentage of the compounds adsorbed to soil samples [9]. In a recent study, Pi et al. [53] evaluated the biodegradation of oil by *Pseudomona* sp. LSH-7 and found that rhamnolipids increase the bioremediation of petroleum hydrocarbons.

3.6. Bioremediation of petroleum product spilled in seawater

The effect of the biosurfactant on the biodegradation of motor oil through the bioactivity of the bacterium *P. aeruginosa* UCP 0992 was evaluated for 30 days (Fig. 3b). The results with the addition of the biosurfactant were better than in the absence of the biosurfactant, likely due to the greater stimulus, favoring microbial growth and the consequent biodegradation of the motor oil. Moreover, the addition of the biosurfactant at its CMC was more effective than at twice the CMC. The increase in the bioavailability of contaminants, such as petroleum products, can release toxic byproducts resulting from the microbial metabolism of the oil constituents, thereby increasing the toxicity of the environment [3,7]. Santos et al. [54] report similar results, as the authors found that the presence of a biosurfactant from *Candida lipolytica* favored the growth of autochthonous microorganisms in seawater at concentrations of ½ the CMC, the CMC and twice the CMC during 30 days of cultivation. Rocha e Silva et al. [55] found the same effect on the growth of autochthonous marine bacteria and fungi in the region of the Suape Port (state of Pernambuco, Brazil). Thus, the biosurfactant isolated from *Pseudomonas aeruginosa* promoted the accelerated growth of these microorganisms throughout the 30 days of cultivation and served as a solubilizing agent of the motor oil, thereby facilitating its biodegradation.

4. Conclusions

In the present study, the production of a biosurfactant by *Pseudomonas aeruginosa* UCP0992 cultivated in a low-cost medium in a bioreactor using a CCRD favored the reduction in surface tension to 26.5 mN/m and an increase in the biosurfactant yield to 26 g/L. The

application of the biosurfactant in the removal of hydrophobic contaminants in soil under kinetic and static conditions showed removal rates around 90% and 80%, respectively. Oil degradation experiments in sand and seawater showed degradation rates higher than 90% in the presence of the biosurfactant and the producing species, demonstrating its potential as a low-costadjuvant in environmental applications. This biosurfactant can also be used in the recovery of oil and cleaning of storage tanks as well as for the bioremediation of contaminated soil and seawater.

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5.2 CAPÍTULO 2 - Treatment of oily effluent using a low-cost biosurfactant in a flotation system

TRABALHO SUBMETIDO PARA PUBLICAÇÃO NO PERIÓDICO

BIODEGRADATION (F.I. = 2,01)

TREATMENT OF OILY EFFLUENT USING A LOW-COST BIOSURFACTANT IN A FLOTATION SYSTEM

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Abstract

Fuel and lubricating oil leaks produce an oily wastewater that creates an environmental problem for industries. Dissolved air flotation (DAF) has been successfully employed for the separation of oily contaminants. Collectors constitute an auxiliary tool in the DAF process that enhances the separation efficiency by facilitating the adhesion of the contaminant particles. The use of biosurfactants as collectors is a promising technology in flotation processes, as these biomolecules are biodegradable and non-toxic. In the present study, a biosurfactant was produced from the bacteria *Pseudomonas aeruginosa* UCP 0992 cultivated in 0.5% corn steep liquor and 4.0% vegetable oil residue in a bioreactor at 200 rpm for 120 hours, resulting in a surface tension of 26.5 mN/m and a yield of 26 g/L. The biosurfactant demonstrated stability when exposed to different temperatures, heating times, pH values and salt and was characterised as a glycolipid with a critical micelle concentration of 600 mg/L. A central composite rotatable design was used to evaluate the effect of the crude biosurfactant added to a laboratory DAF prototype on the removal efficiency of motor oil. The isolated and formulated forms of the biosurfactant were also tested in the prototype after the optimisation of the operational conditions. The results demonstrated that all forms of the biosurfactant increased the oil separation efficiency of the DAF process by 65 to 95%. In conclusion, the use of biosurfactants is a promising alternative as an auxiliary tool in flotation processes for the treatment of oily waters generated by industrial activities.

Keywords Biosurfactant. *Pseudomonas aeruginosa*. Industrial residues. Dissolved air flotation. Oily water. CCRD.

1. Introduction

Drilling and petroleum extraction processes in the oil industry generates oily water, the disposal or reuse of which is only permitted after the removal of the oil and suspended solids to acceptable levels (Almeida et al. 2016). The reuse of effluents from industrial processes has become increasingly commonplace due to the economic and environmental appeal of this practice in the form of reduced production costs and aggregated value to the company in terms of sustainability (Yu and Han 2013; Rocha e Silva et al. 2015).

Gravitational separation is one of the main treatment methods for oily water and is performed by sedimenters, centrifuges, hydrocyclones, etc. However, oil removal levels only reach around 200 mg/L due mainly to the presence of emulsions, which are difficult to remove by simple gravitational methods and require auxiliary techniques, such as the addition of coagulants and surfactants (Rubio and Smith 2002; Yu and Han 2013). In this context, the flotation process has proven to be quite efficient, with the capability of removing a larger amount of oil in comparison to other methods (ALBUQUERQUE et al., 2012).

Flotation is a particle separation process based on adhesion to bubbles. The oil particle-bubble union has less density than the aqueous medium and floats to the surface of the flotation chamber, where the oil particles are removed (Bahadori et al. 2013). Flotation was first used in mineral processing and has long been employed in solid/liquid separation processes that involve the use of stable foams to recover mineral particles (PENG et al., 2009). With the development of the industrial sector, the application of the flotation process was improved, leading to the emergence of dissolved air flotation (DAF), which involves the removal of a solute through adsorption, co-precipitation or occlusion in a floc transporter and subsequent release by the addition of an adequate tensioactive agent (BENEVENTI

et al., 2009). With DAF, the water is saturated with pressurised (greater than 3 atm) air through a nozzle, forming bubbles that that reach the flotation chamber, which is at atmospheric pressure. The air becomes supersaturated and precipitates from the solution in the form of small bubbles (Babaahmadi, 2010; Rocha e Silva et al. 2015).

The use of flotation as a separation method has been criticised due to the probable toxicity of the synthetic surfactants used as collectors in this process (MENEZES et al., 2011a). Surfactants are compounds composed of amphipathic molecules with a hydrophilic portion and a hydrophobic portion that partition at the oil/water or air/water interface. The apolar portion is often a hydrocarbon chain, whereas the polar portion may be ionic (cationic or anionic), non-ionic or amphoteric. These characteristics enable surfactants to reduce surface and interfacial tension and form microemulsions, in which hydrocarbons can be solubilised in water or vice versa (ALMEIDA et al., 2016). The development of completely biodegradable surfactants could alleviate concerns with regard to toxicity and increase the acceptance of this separation technology (ROCHA E SILVA et al., 2015). Thus, surfactants of a biological origin (biosurfactants) could be a viable option for increasing the use of flotation processes.

Recent studies show that microbial surfactants, which are metabolites produced by bacteria, yeasts and fungi, have the ability to solubilise and effectively mobilise adsorbed organic and inorganic compounds in contaminated soil and water (SANTOS et al., 2016b). Biosurfactants offer excellent advantages, such as low toxicity, high solubility in the presence of organic and inorganic substances as well as stability at high temperatures, in a wide pH range, and in the presence of salt.

Biosurfactants have diverse chemical structures and are mainly classified as glycolipids and lipopeptides. These natural compounds can be produced from different substrates, especially renewable resources, such as vegetable oils and

agricultural waste products (Santos et al. 2016; Soares da Silva et al. 2014). The selectionoflow-costsubstratesisimportanttothe overall economyoftheprocess, as substratesaccount for 10 to 30% ofthe final costoftheproduct(ALMEIDA et al., 2016). According to the literature, *P. aeruginosa* is one of the most widely studied microorganisms for biosurfactant production. Most biosurfactants produced by this bacterium have demonstrated the ability to reduce surface tension to around 28 mN/m. These compounds have also been applied in the remediation of water and soil contaminated with hydrocarbons due to their potential as environmental decontamination agents (WITTGENS et al., 2017).

Considering the challenges described above and the need to improve currently known effluent treatment methods, the aim of the present study was to propose clean, efficient solutions for the treatment and control of oily water generated by industrial activities. For such, flotation with the use of a biosurfactant as a biodegradable collector was tested in a laboratory-scale DAF prototype.

2. Materials and Methods

2.1. Materials

All reagents were of the highest purity available. Soybean oil waste was obtained from a restaurant in the city of Recife, Brazil, stored following the supplier's recommendations, and used without any further processing. Corn steep liquor was obtained from Ingredion Brasil in the city of Cabo de Santo Agostinho, Brazil. A synthetic effluent was formulated with motor oil at a concentration of 15 g/L. The motor oil is commercially available for use in flex engines (gasoline, VNG and alcohol), type SAE 20W-50, with a synthetic guard (PETROBRAS) paraffin-based

lubricating oil (complex mixture of hydrocarbons) with performance enhancing additives.

2.2. Bacterial strain and preparation of seed culture

P. aeruginosa UCP0992 was obtained from the culture collection of the Centre for Research in Environmental Sciences of the Catholic University of Pernambuco, Brazil, which is registered with the World Federation of Culture Collections. Cultures were maintained in nutrient agar slants at 4°C. The strain from a 24-hour culture was transferred to 50 ml of nutrient broth and the seed culture was prepared at a temperature of 28°C, stirring at 150 rpm, and incubation for 10-14 h.

2.3. Fermentation media and conditions

Liquid fermentation was performed with a 3% cell suspension (optical density: 0.7) at 600 nm, corresponding to an inoculum of 10^7 colony-forming units/mL inoculated in a 2-L bioreactor (Tec-Bio-Plus, Tecnal Ltda., Brazil) with a working volume of 1.2 l, operating in a batch mode with controlled pH (6.8) and temperature (28°C). The culture medium was composed of distilled water containing 4% soybean frying oil and 0.5% corn steep liquor as substrates. Fermentation was conducted at 200 rpm with aeration (2 vvm) for 120 h. Samples were collected at the end of the fermentation period to determine surface tension and the concentration of the surfactant.

2.4.Surface tension and critical micelle concentration

Surface tension was determined in the cell-free broth obtained by centrifuging cultures at 5 000 g for 20 minutes, using the ring method with a Sigma 700 Tensiometer (KSV Instruments LTD, Finland) at room temperature. The surface tension of the dilutions of isolated biosurfactant in distilled water was measured until reaching to a constant value (standard deviation less than 0.4 mN/m during 10

successive measurements), which was considered the critical micelle concentration (CMC). The CMC was obtained by plotting surface tension against surfactant concentration and expressed as g/l of biosurfactant.

2.5. Isolation of biosurfactant

Cells were removed through centrifugation (5 000 g) for 30 min and the biosurfactant was then extracted from the culture media. PH of the supernatant was adjusted to 2.0 using HCl (6.0 M) with the addition of an equal volume of $CHCl_3$/CH_3OH (2:1). The mixture was shaken vigorously for 15 min and allowed to set. After separation into phases, the organic phase was removed and the operation was repeated two more times. A rotary evaporator was used to concentrate the biosurfactant from pooled organic phases. A yellowish viscous product was obtained, dissolved in methanol and further concentrated by evaporation of the solvent at 45°C (Silva et al. 2013).

2.6. Biosurfactant formulation

The broth was fermented and cells were removed through centrifugation (5000 *g* for 30 min). Following the addition of 0.2% potassium sorbate, the cell-free broth was stored at room temperature (28°C) for 120 days (FREITAS et al., 2016).

2.7 Emulsifying activity with different hydrophobic compounds

The method described by Cooper and Goldenberg (1987) was used for the determination of the emulsification index (EI). An aliquot (2 mL) of a liquid hydrophobic compound (soybean oil, cotton seed oil and motor oil) was added to 2 ml of the cell-free broth in a test tube and vortexed at high speed for 2 min. The stability of the emulsion was determined after 24 h. The EI was calculated as the height of the emulsion layer divided by the total height of the mixture multiplied by 100.

2.8 Biosurfactant stability

The stability of the biosurfactant was investigated in different experiments using the cell-free broth. NaCl was added at concentrations of 2, 5 and 8% (w/v) and surface tension and emulsification activity were determined as described above. Surface tension and emulsification activity were also measured using the broth after being submitted to different temperatures (5, 70 and 120°C) for 60 min. The effect of heating time on biosurfactant activity was investigated by maintaining the broth at 90ºC for 20, 40 and 60 min. The effect of pH (4, 8 and 10) on surface tension and emulsification was investigated by adjusting the broth with 6.0 M of either NaOH or HCl.

2.9 Characterisation of biosurfactant

Following re-dissolution of the extracted biosurfactant in deuterated chloroform ($CDCl_3$), the respective ^1H NMR spectra were recorded at 25 °C using an Agilent 300 Mz spectrometer operating at 300.13 MHz. Chemical shifts (δ) were given on the ppm scale relative to tetramethylsilane (TMS). Fourier transform infrared

spectroscopy (FTIR) was also used to characterise the biosurfactant extract recovered from the supernatant of the *P. aeruginosa* UCP 0992 isolate. The FTIR spectrum (400 Perkin Elmer) with a resolution of 4 cm $^{-1}$ was recorded from 400 to 4000 wavenumbers (cm $^{-1}$). The hydrophobic portion was analysed using gas chromatography-mass spectrometry (Thermo Scientific Trace 1300 - ISQ Single Quadrupole) in a TGMS-5 column (30 m x 0.25 mm; film thickness: 0.25 um). The column temperature was 60°C for 3 min, ramped at 10°C min^{-1} to 300 °C and held for 15 min. A one-µL sample was injected. Helium was the carrier gas. The injector temperature was 300°C and the detector temperature was 280 °C.

2.10 Dissolved Air Flotation System

Three flotation tests were performed in an acrylic 15-l laboratory-scale DAF unit (Fig. 1). Fifty g of motor oil were mixed in 10 l of water with the aid of a pump (1) in the storage tank (2) for one hour with the aim of obtaining good oil in water saturation. An aquarium (3) was filled with 15 l of clean distilled water. After recirculation in the storage tank, the oily effluent was fed into the aquarium with the aid of the same pump (1), entering through a valve (4) [flow rate was monitored using an Arduino sensor (5)] and contaminating the clean water. The contaminated water came into contact with microbubbles formed by the injection of a controlled amount of air in the aspiration line of the pump (6). The interaction between the microbubbles and oil droplets dispersed at the base of the DAF gave rise to flocs composed of oil and air, which floated due to the lower density in comparison to the water, forming a layer of oily foam, which was collected in the collection section (7). To enhance the efficiency of the process, quantities of biosurfactant were dosed using a burette (8). A return pump (9) connected to the treated water section (10) promoted

recirculation, returning the treated effluent to the storage tank, from which it could be collected (11) for subsequent analysis without coming into contact with the initial oily effluent contained in the storage tank. Each run of the DAF system lasted five minutes.

Insert Figure 1

2.11 Experimental Factorial Design and Response Surface Methodology

The effect of the biosurfactant on the separation efficiency of the DAF system was evaluated in experiments conducted following a central composite rotatable design (CCRD) and response surface methodology (RSM). A 2^4 CCRD was used to define the operating conditions of the prototype. The independent variables were the oily water flow rate, flow rate of water microbubbles, aqueous biosurfactant solution flow rate and biosurfactant concentration. The response variable was oil separation efficiency. The independent variables were coded on five levels. The complete design consisted of 28 experimental points, with four replications of the central points. Table 1 lists the coded levels of the independent variables used in the RSM design.

Separation efficiency was calculated according to Eq. (1).

$$\eta = \frac{C_I - C_O}{C_I} \cdot 100\% \quad (1)$$

in which η is the percentage of separation efficiency, while C_I and C_O are the oil concentrations in the inlet and outlet flow, respectively.

The experimental design was applied to the crude biosurfactant (cell-free broth obtained through centrifugation). After the determination of the optimum operating

conditions, solutions of the isolated biosurfactant at concentrations established based on the CMC and solutions of the formulated biosurfactant were also tested to compare the efficiency of the different forms of the biosurfactant. Fifty-ml samples were collected from the flotation system and placed in glass flasks for the measurement of residual oil. Control experiments without the biosurfactant were conducted for the purposes of comparison.

Statistical analysis involved ANOVA, the determination of regression coefficients and the construction of graphs using the Statistica® program, version 8.0 (Statsoft Inc., USA).

Insert Table 1

2.12 Quantification of hydrophobic contaminant

Residual oil was extracted from the samples of synthetic oily effluent using the same volume of hexane (1:1, v/v). The mixture was shaken for 15 min and left at rest for the separation of the phases. The organic phase was removed and the operation was repeated two more times. The product contained in this phase was submitted to analysis in a spectrophotometer. The oil extracted from the water was analysed for its concentration after direct sampling by measuring absorbance at a wavelength of 330 nm using quartz cuvettes with a 10-mm path length in an UV-vis spectrophotometer (SP-22- BIOSPECTRO). For the determination of the calibration curve, a standard solution of residual motor oil (5000 mg/L) was prepared in a 100-ml volumetric balloon. The solutions were diluted in n-hexane at concentrations ranging from 1 to 1000 mg/L obtained from the initial standard solution. N-hexane was used as the blank to calibrate the device. The solvent was analytical grade and adequate for the spectrophotometric equipment (EMMANDI; SASTRY; PATEL,

2014). All experiments were performed in triplicate at room temperature (27°C) and mean values are reported.

3 Results and Discussion

3.7 Biosurfactant production

Considering the potential of *Pseudomonas aeruginosa* as a biosurfactant producer when cultivated in soluble and/or insoluble substrates, the bacterium was initially cultivated in a bioreactor containing a medium formulated with 4% soybean oil residue and 0.5% corn steep liquor as the carbon and nitrogen sources, respectively. The biosurfactant produced was able to reduce the surface tension of water from 70 mN/m to around 26.5 mN/m and the yield was 26 g/L.

Silva et al. (2010) produced a biosurfactant from *P. aeruginosa* cultivated in a medium containing 3% glycerol that lowered surface tension to 27.6 mN/m and a yielded 6.5 g/L. In subsequent studies, Silva et al. (2013) found that *P. cepacia* cultivated in a medium with 2% waste frying oil and 3% corn steep liquor produced a biosurfactant that lowered surface tension to 26 mN/m and yielded 8 g/L. Aparna et al. (2012) produced a biosurfactant from *Pseudomonas* sp. 2B cultivated in a medium containing 1% molasses and found a surface tension of 30.14 mN/m and a yield of 4.97 g/L. Oliveira et al. (2009) produced a biopolymer from *P. alcaligenes*PCL cultivated in a medium supplemented with mineral salts and palm oil, which lowered surface tension to 28 mN/m and yielded 2.3 g/L. Monteiro et al. (2007) produced a biosurfactant from *P. aeruginosa* DAUPE 614 cultivated in a medium with glycerol and ammonium nitrate that lowered surface tension to 27.3

mN/m and yielded 3.9 g/L. Deepika et al. (2015) report the production of a biosurfactant in a salt medium supplemented with molasses that lowered surface tension to 33.03 mN/m and had a yield of 5.26 g/L. In another study, Deepika et al. (2016) produced a rhamnolipid from *P. aeruginosa* KVD-HR42 cultivated in a medium containing karanja oil and sodium nitrate that lowered surface tension to 30.14 mN/m, with a yield of 5.9 g/L. Nicolò et al. (2017) report a biosurfactant produced by *P. aeruginosa* L05 cultivated in a medium supplemented with mineral salts and 0.4% *B. carinata*oil that had a yield of 5.0 g/L. In comparison to these studies, the yield in the present investigation was substantially greater, demonstrating that the biopolymer obtained under the conditions specified herein is a promising agent for use on an industrial scale.

3.8 Biosurfactant stability

The use of biosurfactants as coadjutants in the treatment of oily waters requires products that remain stable under the necessary operational and environmental conditions (ALMEIDA et al., 2016). Moreover, due to economic considerations in the oil industry, most biosurfactants require either whole-cell culture broths or crude preparations (ALMEIDA et al., 2016). Therefore, the application of the biosurfactant from *P. aeruginosa* UCP0992 in its crude form without costly extraction and purification steps was investigated in stability studies as well as the subsequent flotation experiments. Table 2 displays the results of the stability tests using the cell-free broth containing the crude biosurfactant produced from *P. aeruginosa* when exposed to variations in temperature, pH, salinity and heating time.

Insert Table 2

Satisfactory surface tension values were found independently of the pH, temperature or salinity to which the biosurfactant was submitted. Lower surface tension values were found under alkaline conditions, with a slight increase in tension when the biosurfactant was submitted to acidic conditions. Similarly, emulsification indices were higher in alkaline media. Glycolipids exhibit optimum aqueous solubility at neutral to alkaline pH, which is attributed to their acidic nature (pKa = 5.6). The single free carboxylic acid group corresponding to the β-hydroxy fatty acid moiety makes glycolipids anionic. When the pH is increased from 5 to 8, the negative charge of the polar head increases, which is reflected greater aqueous solubility. If high concentrations of surface-active molecules are found, an increase of pH can change the morphology of the micelle structure formed above the CMC from lamellar to vesicular and finally to micelles (ABDEL-MAWGOUD; ABOULWAFA; HASSOUNA, 2009).

Despite the relationship between surface tension and the emulsification index, the ability of a molecule to form a stable emulsion is not always associated with surface tension activity. Thus, a good biosurfactant is not necessarily a good emulsifier (SANTOS et al., 2016b). Among the different oils tested, the best emulsification results were achieved with motor oil.

Emulsification indices ranged from 50 to 60% in the temperature and heating time tests, with a slight reduction in emulsifying capacity at higher temperatures and with a longer heating time. Lower temperatures and a shorter heating time also favored the reduction in surface tension.

With regard to the salt concentration, the biosurfactant maintained its surface tension reducing capacity in media with up to 8% NaCl, with a tendency toward a reduction in surface tension with the increase in salt concentration, whereas emulsification capacity decreased in the presence of salt. According to Helvac et al.

(2004), electrolytes have a direct effect on carboxylate groups in glycolipids. The solution/air interface is negatively charged due to the ionised carboxylic acid groups in an alkaline medium, with strong repulsive forces between the molecules of the glycolipid. This negative charge is shielded by Na^+ ions, leading to the formation of a close-packed monolayer and consequent reduction in surface tension.

The emulsification results are promising with regard to the application of the biosurfactant from *P. aeruginosa* in the treatment of oily waters, as lubricating oil is normally present in the composition of the industrial effluents to be treated.

3.9 Critical micelle concentration of biosurfactant

The CMC is defined the minimum concentration of biosurfactant required for the maximum reduction in surface tension and the formation of micelles. Thus, a low CMC indicates an efficient biosurfactant (CAMPOS et al., 2013). In the present study, an increase in biosurfactant concentration led to a reduction in the surface tension of water from 70 to 26 mN/m, which was achieved at a concentration of 600 mg/l, with no further reduction occurring thereafter. This CMC differs from that reported for other glycolipids produced by *P aeruginosa* (SANTOS et al., 2016b). Different CMC values may be due to differences in the purity and composition of the glycolipid as well as differences with regard to the bacterial strain, medium and cultivation conditions (Silva et al. 2014).

3.10 Characterisation of biosurfactant

Figure 2 displays the FTIR spectrum of the biosurfactant isolated from *P. aeruginosa* UCP 0992. The vibration extending from 3300 to 3500 cm^{-1} is characteristic of O–

H stretching. The peak at 3000 to 2800 cm^{-1} is characteristic of aliphatic chains. The C=O group is evidenced around 1710 cm^{-1}. The peaks at 1550 to 1400 cm^{-1} may be due to a C double bond and the peak at ~ 1260 cm^{-1} corresponds to the ketone group.

Insert Figure 2

Figure 3 displays the ^{1}H NMR spectrum. The signals of the biosurfactant from *P. aeruginosa* UCP 0992 between δ 0.60 and 1.6 ppm suggest aliphatic and methyl groups. The signals between δ 2.0 and 2.2 ppm indicate the aldehyde group. The signals at δ 3.5 ppm and between δ 4.6 and 4.8 ppm are attributed to hydroxyl groups and those between δ 5.0 and 5.4 ppm correspond to double bounds.

Insert Figure 3

The GC-MS analysis of the biosurfactant was compared to data from the library. The chromatogram (Figure 4) displays two evident peaks. The first (45.53%) indicates a structure related to the carbonyl group and the second (28.21%) indicates to a structure containing a hydroxyl group. The molar mass of the structure was between 150 and 200 (m/z).

Insert Figure 4

The literature describes the characterisation of biosurfactants produced by species of *Pseudomonas sp.* using ^{1}H NMR, FTIR spectroscopy and GC-MS analysis (Charles Oluwaseun et al. 2017; Varjani and Upasani 2017; Moussa et al. 2014). Soares da Silva et al. (2017) obtained similar results to those of the present study in the characterisation of a biosurfactant obtained from *P. cepacia*, with peaks in the ^{1}H NMR spectrum between δ0.75 and 2.5 ppm indicating aliphatic and methyl

groups and peaks between δ2.0 and 4.8 indicating carbonyl and hydroxyl groups, respectively. In the FTIR spectrum, the same authors report absorption bands between 2966 and 2863 cm^{-1} for aliphatic groups and a band at 1700 $cm^{-1}$for C=O groups. In the present study, the ^{1}H NMR, FTIR spectroscopy and GC-MS analyses of the biosurfactant produced by *P. aeruginosa* UCP 0992 indicate a glycolipid.

3.11 Use of biosurfactant in DAF system for treatment of oily water

Collectors/coagulants are normally used to enhance separation efficiency in flotation systems. These collectors may be surfactants. Tensioactive agents are added to the pressurised water in DAF to reduce the air/water surface tension in the saturator, which enhances the separation process (MENEZES et al., 2011a). Some microbial biosurfactants have been successfully used in the removal of heavy metals from an acidic mine effluent using flotation columns. Biosurfactants from species of *Candida* were able to remove more than 90% of metallic cations (Albuquerque et al. 2012; Menezes et al. 2011; Sarubbo et al. 2015). Another study demonstrated the potential of a biosurfactant produced from *Candida*sp in the removal of oil in a semi-industrial scale DAF prototype, which increased the separation efficiency from 80 to 98% (ROCHA E SILVA et al., 2015). Based on these promising results, the present study tested a new bacterial biosurfactant as a replacement for synthetic surfactants in a laboratory-scale DAF system using a factorial design.

Table 3 displays the removal rates achieved with the application of the CCRD matrix using the crude biosurfactant from *P. aeruginosa* UCP 0992 as a collector. Removal efficiency ranged from 82 to 95%. Multiple regression analysis using RSM was performed to adjust the response function to the experimental data and

investigate the simultaneous influence of the four variables studied. The following quadratic polynomial equation best fit the data:

$$Y\,(\%) = 89.6925 - 2.64X_1 + 1.65X_1^2 + 2.35X_2 - 2.86X_2^2 + 2.10X_3 - 0.65X_3^2 + 4.14X_4 - 1.27X_4^2 + 0.56X_1X_2 - 0.79X_1X_3 + 0.16X_1X_4 + 0.66X_2X_3 + 0.75X_2X_4 - 0.19X_3X_4 \quad (2)$$

in which Y is separation efficiency (%) and X_1,X_2,X_3 and X_4 are coded values for oily water flow rate (mL/min), water + microbubble flow rate (mL/min), biosurfactant solution flow rate (L/min) and biosurfactant concentration (g/L), respectively. The optimal values from the CCRD were obtained by solving the regression equation and analysing the response surface contour plots. An oily water flow rate of 5.00 L/min, water + microbubble flow rate of 6.50 L/min, biosurfactant solution flow rate of 2.00 L/min and biosurfactant concentration of 0.35 g/L were the most favourable for the oil removal process using the biosurfactant, achieving a 94.88% separation rate (Run 8) (Table 3).

ANOVA was performed to test the significance and acceptability of the quadratic model (Table 4). The p-values and F-values (with 95 % confidence interval) indicate that all terms were statistically significant ($p < 0.05$; $F > 4$) with the exception of the interactions between the variables oily water flow rate (X_1) and biosurfactant concentration (X_4) and between biosurfactant solution flow rate (X_3) and biosurfactant concentration (X_4). The low pure error (0.36) indicates good reproducibility of the experimental data.

Insert Table 3

Insert Table 4

The explained variance (R^2 = 0.99595) ensured adequate fit (R = 0.9916) and the predicted versus actual values for separation efficiency determined by the model were very close to the straight line (Figure 5), which indicates that the model is suitable for predicting separation efficiency under the experimental conditions.

Insert Figure5

The effects and statistical significance of the forecasting model variables were graphically illustrated using Pareto charts (Figure 6). The linear term of factor X_4 contributed significantly, i.e., an increase in biosurfactant concentration implies an increase in separation efficiency. The linear term of factor X_2 also contributed positively to separation efficiency. However, the quadratic term of X_2 led to a reduction in efficiency. The linear term of factor X_1 contributed negatively to separation efficiency, but the quadratic term of factor X_1 contributed positively to separation efficiency. Both linear and quadratic terms of X_3 contributed positively to the increase in separation efficiency.

Insert Figure 6

Figure 7 displays the fitted response surface plot for separation efficiency obtained by the model of Eq. (2). Graphic representation enables the visualisation of the relationship between the response and experimental levels of each variable and type of interactions between test variables in order to deduce the optimum conditions. Better separation efficiency was found when the oily water flow rate was maintained at its minimum level and interacted with the water + microbubble flow rate (Fig. 7A), biosurfactant solution flow rate (Fig. 7B), and biosurfactant

concentration (Fig. 7C). However, these interactions were weak and did not produce well-defined regions in the graphs. The elliptic curve of the graph in Figure 7D indicates a high degree of interaction between water + microbubble flow rate and biosurfactant flow rate, with greater separation efficiency at the level just above the central region of the response surface. The combination of greater microbubble flow and greater biosurfactant concentration led to maximum separation efficiency (Fig. 7E) and the parallelism found in the graph of the interaction demonstrates that it is possible to predict separation efficiency by varying only one of these variables. Figure 7F shows that high separation efficiency was also achieved when both biosurfactant solution flow and biosurfactant concentration were maintained at their maximum levels.

Insert Figure 7

3.12 Flotation experiments following optimisation of operational conditions

After the optimisation of the operational conditions, new experiments were conducted replacing the crude biosurfactant with the isolated and formulated forms to determine the influence of the extraction process and addition of the chemical conservative potassium sorbate on the efficiency of the biomolecule. The results demonstrate that the isolated and formulated biosurfactants were capable of removing 95.89% and 94.75% of the oil, respectively. Control experiments (without the biosurfactant) conducted with the optimised operational conditions resulted in an oil removal rate of about 75% using the microbubble process alone, demonstrating the importance of adding the biosurfactant from *P. aeruginosa* UCP 0992 as a collector in the separation process.

Considering the 94.88% removal rate with the crude biosurfactant, the

formulated product (addition of the conservative) could be advantageous, as this form of the biosurfactant maintains stable tensioactive properties when stored for a period of 120 days, which demonstrates its potential for commercial use. However, the isolated biomolecule represents an additional cost, as the final price of the biosurfactant will be higher due to the inclusion of an extraction step.

Figure 8 shows the difference in the quality of the emulsion formed with and without the biosurfactant, demonstrating that the emulsification and consequent removal of the oil is substantially improved in the presence of the biosurfactant (Fig. 8B).

Insert Figure 8

The efficiency of the flotation-biosurfactant system is also evidenced when a concentration of oil of 15 g/L is used, which is much higher than the maximum limit permitted by Brazilian law (20 ppm) (CONAMA, 2011). This suggests that the DAF-biosurfactant combination will ensure adequate treatment of effluents under actual conditions of the treatment of industrial oily effluents and enable the reuse of the clean water. In a study conducted by Watcharasing et al. (2009), a 60% oil removal rate was found in a flotation system using synthetic surfactants. Thus, the biosurfactant tested herein achieved greater separation efficiency.

It is important to stress that the biosurfactant from *P. aeruginosa* UCP 0992, besides being used in its crude form, was produced in a culture medium prepared only with industrial waste products, which further reduces the costs. As substrates used in the production of biosurfactants account for 20 to 30% of the production cost, the final price of the biosurfactant from *P. aeruginosa* was calculated based on the price of pure biosurfactants available on the market, with a final price estimated to be around 0.6 to 1.5 €/kg (Hazra et al., 2012).

The low cost of the proposed DAF process is evident by the small amount of biosurfactant (350 ppm) required to achieve maximum efficiency (run number 8 in Table 3). Moreover, the DAF system is highly efficient, simple to operate and requires a low investment, as the implantation cost is approximately tenfold lower that that required for other methods, such as separation by continuous centrifugation. As many industries generate huge amounts of oily waters that require adequate treatment before being discarded or reused, the benefits of treating oily waters with the system developed herein demonstrates its considerable market potential.

4 **Conclusion**

The results obtained in the present study indicate that the efficiency of the DAF process for the treatment of oily water can be enhanced with the use of the biosurfactant produced by *P. aeruginosa*, as demonstrated by the significant increase in the oil removal rate. Thus, the system presented herein is a favourable strategy for decontaminating oily industrial effluents. Besides increasing the separation efficiency of the DAF system, the use of biosurfactants is a sustainable practice from the environmental standpoint, as it enables the use of industrial waste products, which are sometimes available from a single industrial complex. Moreover, the bench-scale prototype enables an analysis of characteristics that are fundamental to the establishment of parameter levels (set points) and equipment control strategies.

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Table 1

Experimental range and levels of independent variables for separation efficiency in DAF system

Variables	Range and levels				
	-2.0	**-1.0**	**0.0**	**+1.0**	**+2.0**
Oily water flow rate (L/min), X_1	2.5	5.0	7.5	10.0	12.5
Water + microbubbles flow rate (L/min), X_2	5.0	5.5	6.0	6.5	7
Biosurfactant solution flow rate (L/min), X_3	0.5	1.0	1.5	2.0	2.5
Biosurfactant concentration (g/L), X_4	0.05	0.15	0.25	0.35	0.45

Table 2

Influence of salt concentration, temperature, pH and heating time at 90 °C on surface tension reducing activity and emulsifying activity of cell-free broth containing biosurfactant from *P. aeruginosa* UCP0992 (results expressed as mean ± standard deviation)

pH	**Surface tension (mN/m)**	**EI (%)[a]**	**EI (%)[b]**	**EI (%)[c]**
4	33.4±0.2	80.5±4.0	20.5±2.0	20.0±3.0
8	24.0±0.5	90.3±4.0	55.5±3.9	58.2±2.0
10	26.5±0.5	90.0±5.1	40.1±1.9	35.3±4.3
Temperature (°C)	**Surface tension (mN/m)**	**EI (%)[a]**	**EI (%)[b]**	**EI (%)[c]**
5	20.0±0.4	60.0±5.0	40.3±3.5	42.2±3.
70	28.0±0.3	55.2±3.0	40.0±2.3	40.4±3.9
120	29.0±0.5	50.1±4.0	38.1±4.0	45.2±2.4
Heating time (min)	**Surface tension (mN/m)**	**EI (%)[a]**	**EI (%)[b]**	**EI (%)[c]**
20	22.0±0.4	50.0±3.9	40.0±3.2	43.0±2.7
40	26.6±0.2	45.1±4.0	39.0±2.7	45.0±3.2
60	26.5±0.3	48.0±3.0	40.1±1.3	43.0±2.0
Salt (%)	**Surface tension (mN/m)**	**EI (%)[a]**	**EI (%)[b]**	**EI (%)[c]**
2.0	26.3±0.2	75.0±3.3	70.1±1.8	95.0±2.8
5.0	25.3±1.1	80.1±2.8	35.0±2.0	40.0±2.1
8.0	20.6±1.0	45.0±3.0	15.0±3.0	20.3±3.2

[a]Emulsification index of motor oil; [b] Emulsification index of corn oil; [c] Emulsification index of soybean oil

Table 3

Central composite design matrix and experimental values of factors according to separation efficiency in DAF system with use of biosurfactant

Runs	Oily water flow rate (L/min), X_1	Water + microbubbles flow rate (L/min), X_2	Biosurfactant solution flow rate (L/min), X_3	Biosurfactant concentration (g/L), X_4	Separation efficiency (%), Y
1	-1.0	-1.0	-1.0	-1.0	87.38
2	-1.0	-1.0	-1.0	1.0	88.34
3	-1.0	-1.0	1.0	-1.0	89.37
4	-1.0	-1.0	1.0	1.0	90.22
5	-1.0	1.0	-1.0	-1.0	87.45
6	-1.0	1.0	-1.0	1.0	92.05
7	-1.0	1.0	1.0	-1.0	91.32
8	-1.0	1.0	1.0	1.0	94.88
9	1.0	-1.0	-1.0	-1.0	83.24
10	1.0	-1.0	-1.0	1.0	85.92
11	1.0	-1.0	1.0	-1.0	84.32
12	1.0	-1.0	1.0	1.0	86.43
13	1.0	1.0	-1.0	-1.0	86.98
14	1.0	1.0	-1.0	1.0	89.42
15	1.0	1.0	1.0	-1.0	88.79
16	1.0	1.0	1.0	1.0	90.52
17	-2.0	0.0	0.0	0.0	93.56
18	2.0	0.0	0.0	0.0	90.12

19	0.0	-2.0	0.0	0.0	82.34
20	0.0	2.0	0.0	0.0	84.31
21	0.0	0.0	-2.0	0.0	84.76
22	0.0	0.0	2.0	0.0	90.11
23	0.0	0.0	0.0	-2.0	78.79
24	0.0	0.0	0.0	2.0	94.23
25	0.0	0.0	0.0	0.0	88.35
26	0.0	0.0	0.0	0.0	89.44
27	0.0	0.0	0.0	0.0	89.93
28	0.0	0.0	0.0	0.0	90. 05

Table 4

Analysis of variance (ANOVA) for separation efficiency in pilot scale DAF system with use of biosurfactant

Factor	Sum of squares	Degrees of freedom	Mean square	F-ratio	p-value[b]
X_1 (L) [c]	41.6593	1	41.6593	342.1478	0.000344
X_1 (Q) [d]	16.2855	1	16.2855	133.7529	0.001388
X_2 (L)	33.2291	1	33.2291	272.91	0.000482
X_2 (Q)	49.0776	1	49.0776	403.0738	0.000270
X_3 (L)	26.5020	1	26.5020	217.6608	0.000675
X_3 (Q)	2.5741	1	2.5741	21.14146	0.019335
X_4 (L)	102.7548	1	102.7548	843.9243	8.957E-05
X_4 (Q)	9.6393	1	9.6393	79.16778	0.002993
X_1 (L) x X_2 (L)	1.2656	1	1.2656	10.39456	0.048434
X_1 (L) x X_3 (L)	2.4964	1	2.4964	20.50290	0.020151
X_1 (L) x X_4 (L)	0.0992	1	0.0992	0.814934	0.433205
X_2 (L) x X_3 (L)	1.7161	1	1.7161	14.09431	0.033020
X_2 (L) x X_4 (L)	2.2650	1	2.2650	18.60262	0.022953
X_3 (L) x X_4 (L)	0.1369	1	0.1369	1.124358	0.366789
Lack of Fit	0.8710	10	0.0871	0.715372	0.700035
Pure Error	0.3653	3	0.1218		
Total square sum	305.5641	27			

[a] R^2 = 0.99595; adjusted R^2 = 0.9916.
[b] $p \leq 0.05$ – significant at 5% level.
[c] (L) = linear effect.
[d] (Q) = quadratic effect.

Figure captions

Fig.1. Bench DAF Prototype used for treating oily aqueous phase.

Fig.2. FTIR spectrum of biosurfactant extract produced by *P.aeruginosa* UCP0992 cultivated in mineral medium supplemented with 4% soybean frying oil and 0.5% corn steep liquor.

Fig.3. ^{1}H NMR spectrum (CD_3OD, 300 MHz) of biosurfactant isolated from *P.aeruginosa* UCP0992 cultivated in mineral medium supplemented with 4% soybean frying oil and 0.5% corn steep liquor.

Fig.4. Chromatogram of biosurfactant produced by *P.aeruginosa* UCP0992 showing peaks for structures containing carbonyl and hydroxyl groups.

Fig. 5. Plot of predicted vs. actual separation efficiency achieved using biosurfactant produced by *P. aeruginosa* UCP 0992.

Fig. 6. Pareto chart showing effects of observed factors and combined impact on separation efficiency in DAF system with use of biosurfactant.

Fig. 7. Response surface plots and contour plots for maximum separation efficiency generated using data in Table 5; Inputs = 28 experimental runs carried out under conditions established by CCRD; separation efficiency as function of (A) oily water

flow rate and microbubble water flow rate; (B) biosurfactant solution flow rate and oily water flow rate; (C) biosurfactant concentration and oily water flow rate; (D) microbubble water flow rate and biosurfactant solution flow rate; (E) biosurfactant concentration and microbubble water flow rate; (F) biosurfactant concentration and biosurfactant solution flow rate.

Fig. 8. Separation of oily water. A) DAF system functioning without biosurfactant; B) DAF system functioning with biosurfactant from *P. aeruginosa* UCP 0992. Note formation of floated complex resulting action of microbubbles combined with biosurfactant studied (right).

Figure 1

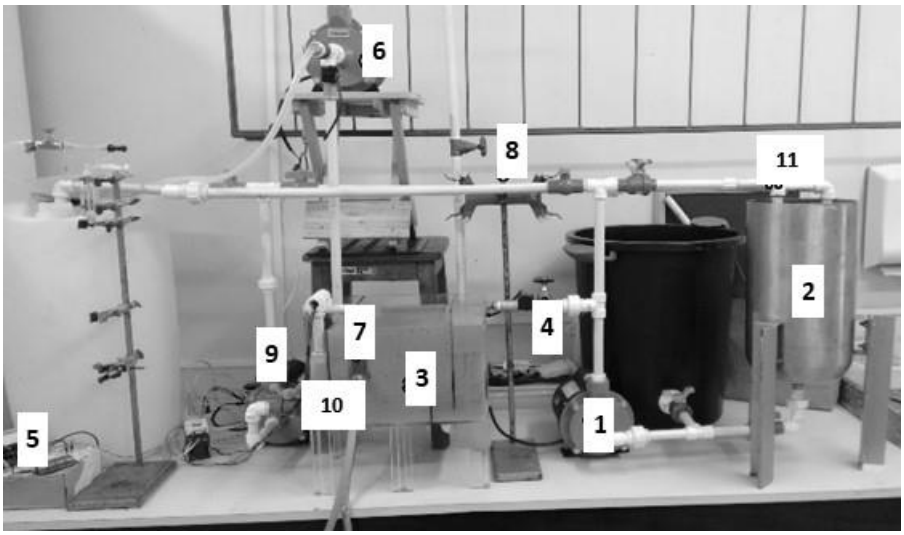
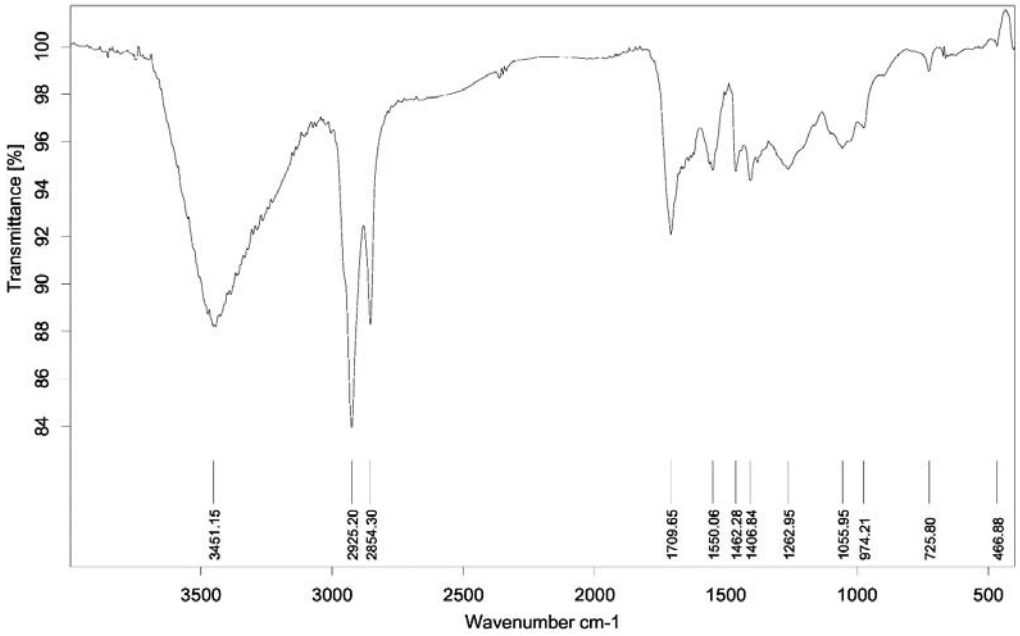


Figure 2


Transmittance [%]
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98
96
94
92
90
88
86
84
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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 3

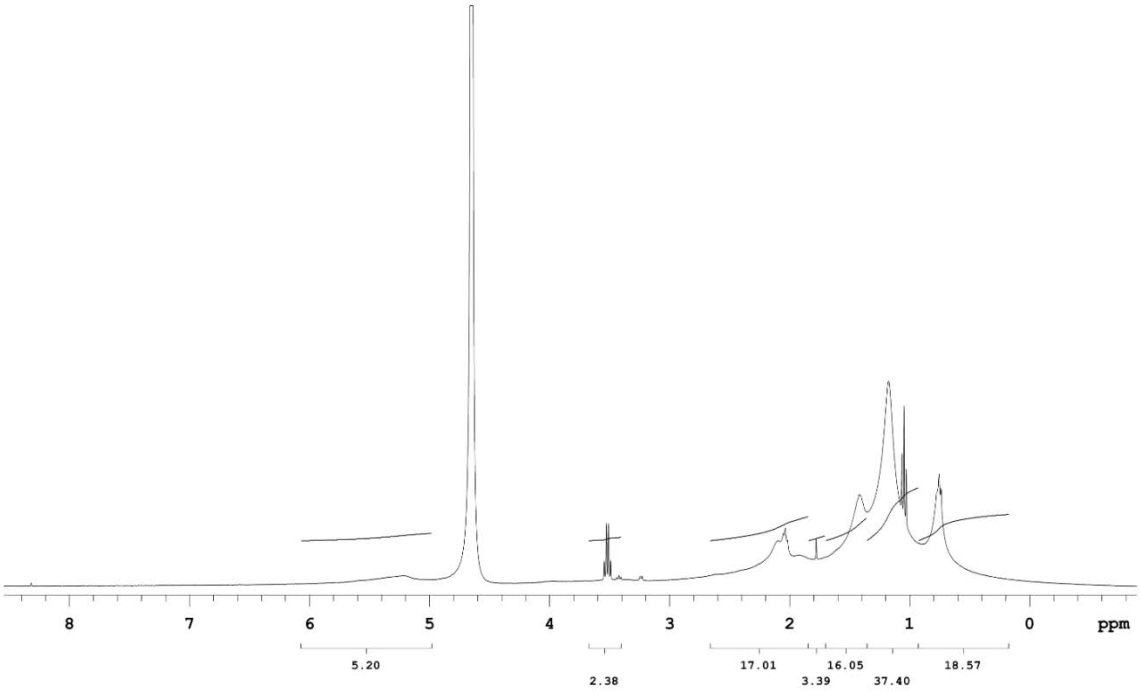
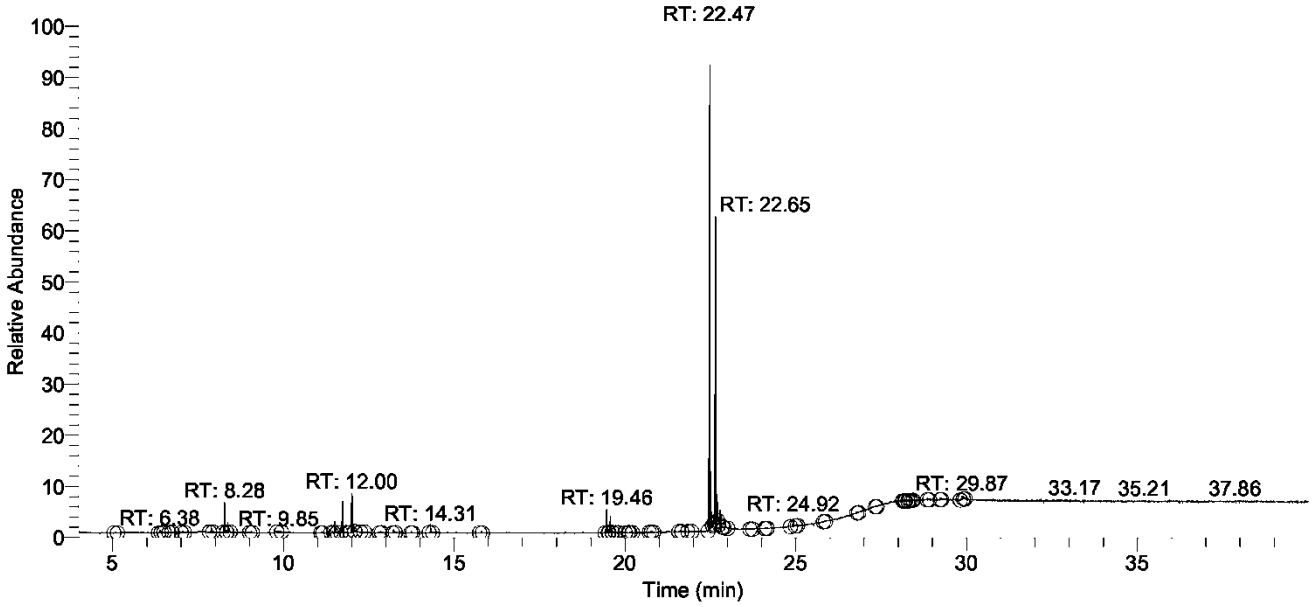


Figure 4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RT: 9.85
RT: 12.00
RT: 14.31
RT: 19.46
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RT: 29.87
33.17
35.21
37.86
Relative Abundance
Time (min)
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70
60
50
40
30
20
10
0
5
10
15
20
25
30
35

Figure 5

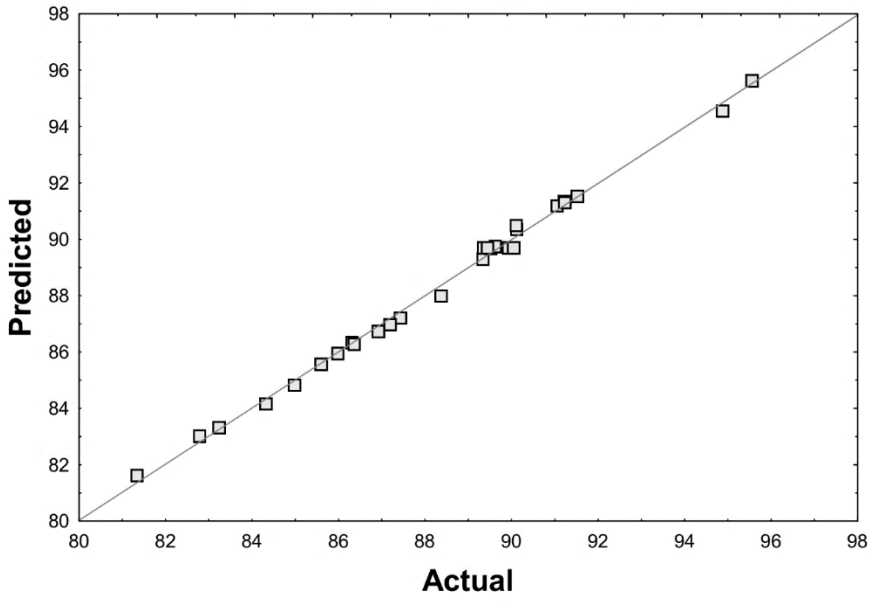

Predicted
Actual
98
96
94
92
90
88
86
84
82
80
80
82
84
86
88
90
92
94
96
98

Figure 6

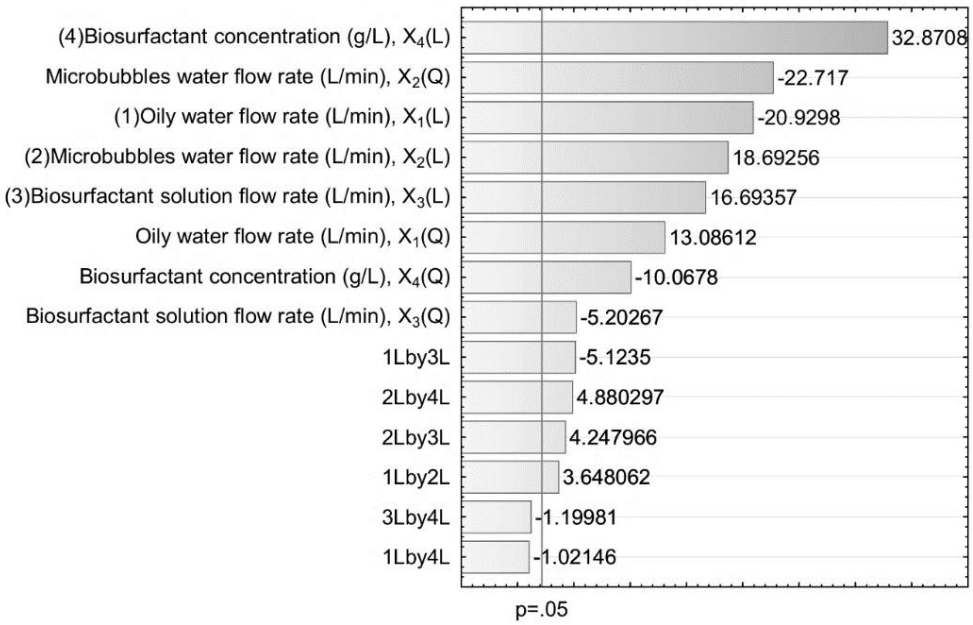

(4)Biosurfactant concentration (g/L), X4(L)
32.8708
Microbubbles water flow rate (L/min), X2(Q)
-22.717
(1)Oily water flow rate (L/min), X1(L)
-20.9298
(2)Microbubbles water flow rate (L/min), X2(L)
18.69256
(3)Biosurfactant solution flow rate (L/min), X3(L)
16.69357
Oily water flow rate (L/min), X1(Q)
13.08612
Biosurfactant concentration (g/L), X4(Q)
-10.0678
Biosurfactant solution flow rate (L/min), X3(Q)
-5.20267
1Lby3L
-5.1235
2Lby4L
4.880297
2Lby3L
4.247966
1Lby2L
3.648062
3Lby4L
-1.19981
1Lby4L
-1.02146
p=.05

Figure 7

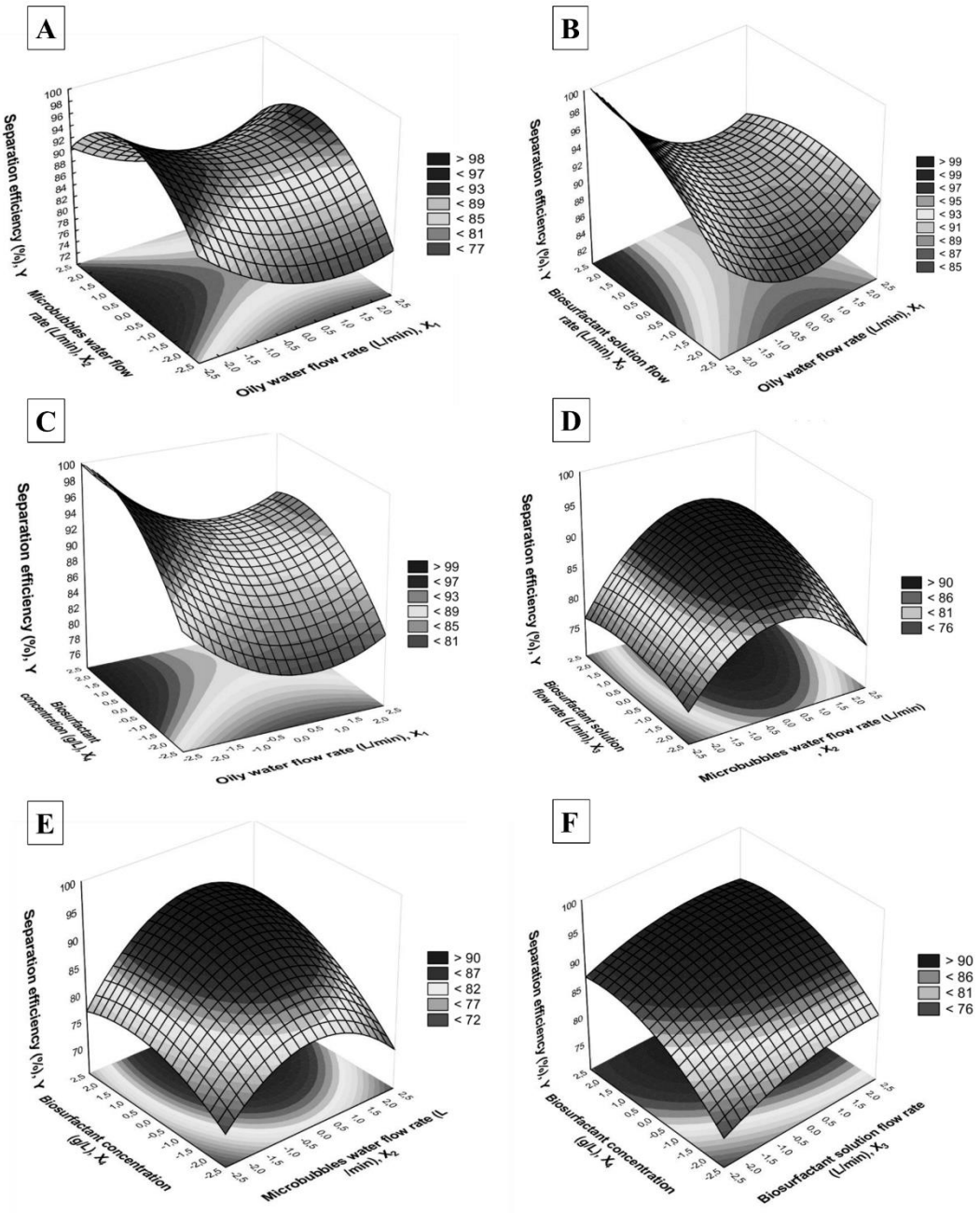
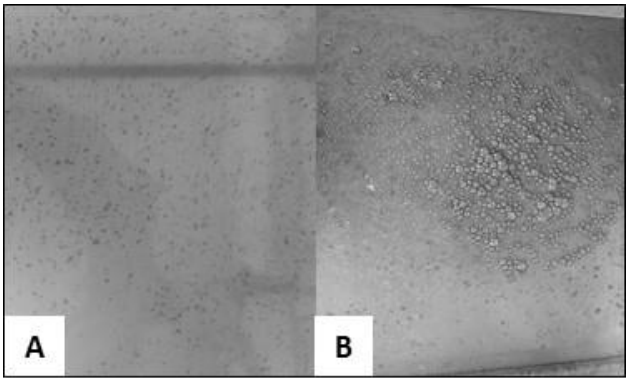

A
Separation efficiency (%), Y
Microbubbles water flow rate (L/min), X2
Oily water flow rate (L/min), X1
> 98
< 97
< 93
< 89
< 85
< 81
< 77
B
Separation efficiency (%), Y
Biosurfactant solution flow rate (L/min), X2
Oily water flow rate (L/min), X1
> 99
< 99
< 97
< 95
< 93
< 91
< 89
< 87
< 85
C
Separation efficiency (%), Y
Biosurfactant concentration (g/L), X3
Oily water flow rate (L/min), X1
> 99
< 97
< 93
< 89
< 85
< 81
D
Separation efficiency (%), Y
Biosurfactant solution flow rate (L/min), X3
Microbubbles water flow rate (L/min), X2
> 90
< 86
< 81
< 76
E
Separation efficiency (%), Y
Biosurfactant concentration (g/L), X3
Microbubbles water flow rate (L/min), X2
> 90
< 87
< 82
< 77
< 72
F
Separation efficiency (%), Y
Biosurfactant concentration (g/L), X3
Biosurfactant solution flow rate (L/min), X2
> 90
< 86
< 81
< 76

Figure 8



5.3 CAPÍTULO 3 - Use of bacterial biosurfactants as natural collectors in the dissolved air flotation process for the treatment of oily industrial effluent

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(F.I. = 1,87)

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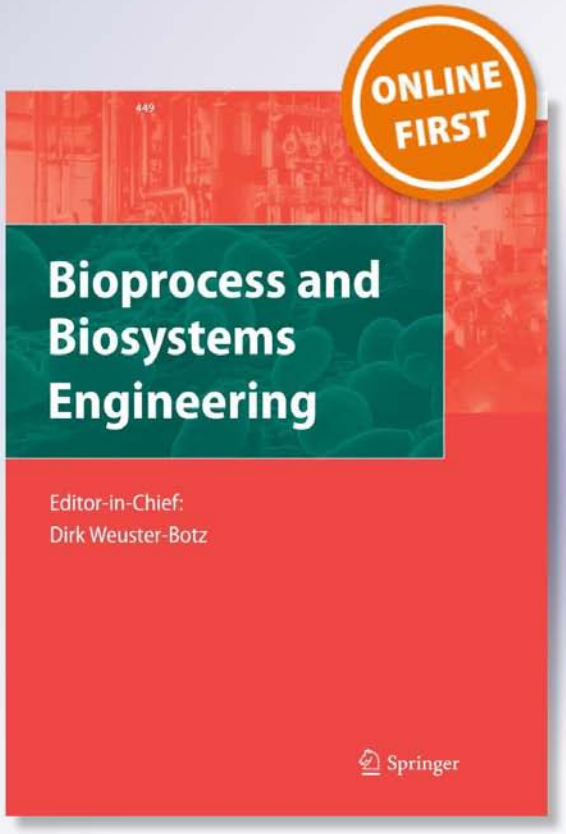

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RESEARCH PAPER

Use of bacterial biosurfactants as natural collectors in the dissolved air flotation process for the treatment of oily industrial effluent

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Abstract
The aim of the present study was to investigate the separation of oil from water using a bench-scale DAF prototype with the addition of biosurfactants isolated from *Pseudomonas cepacia* CCT6659 and *Bacillus cereus* UCP1615. The best operating conditions for the DAF prototype were determined using a central composite rotatable design. The results demonstrated that the biosurfactants from *P. cepacia* and *B. cereus* increased the oil separation efficiency from 53.74% (using only microbubbles) to 94.11 and 80.01%, respectively. The prediction models for both DAF-biosurfactant systems were validated, showing an increase in the efficiency of the DAF process from 53.74% to 98.55 and 70.87% using the formulated biosurfactants from *P. cepacia* and *B. cereus*, respectively. The biosurfactant from *P. cepacia* was selected as the more promising product and used for the treatment of oily effluent from a thermoelectric plant, achieving removal rates ranging between 75.74 (isolated biosurfactant) and 95.70% (formulated biosurfactant), respectively.

Keywords Biosurfactant · *Bacillus cereus* · *Pseudomonas cepacia* · Dissolved air flotation · Oily water · CCRD

Introduction

The continual expansion of the processing industry and the extensive use of petroleum-related products in the majority of industries (automobile industry, aircraft factories, chemical industries, machinery, etc.) constitutes a source of pollution due to the increase in the frequency of oil spills during the transportation, storage and distribution of crude oil and petroleum-based products, which causes serious environmental harm [1, 2]. Moreover, the mixture of oil and water occurs in the production, transportation and refining stage of the petroleum industry as well as during the use of petroleum-based products [3]. The shearing caused by pumps, valves and other equipment leads to the mixture of phases and the formation of stable emulsions. In the production stage, oily water is formed by the water used to extract the petroleum [4]. This water is often discarded into the environment without due treatment stipulated by environmental laws.

The reuse of effluents from industrial processes has become increasingly more common due to the environmental and economic appeal of this practice, as there are incentives to reduce production costs and aggregate value to the company in terms of sustainability [5]. The release of effluents into the environment is regulated by environmental and public health agencies. Regardless of the final destination, such effluents must undergo some type of treatment and meet the requirements for proper disposal [6]. According to environmental legislation, the disposal or the reuse of oily water is only permitted after the removal of the oil and suspended solids [7]. According to Brazilian resolution nº 430 from May 13th, 2011, of the Conselho Nacional do Meio Ambiente (CONAMA [National Environment Council]), which stipulates the conditions for the release of effluents, 20 mg/L is the permitted limit for an oily effluent [6]. Regarding the

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disposal of oily water generated by the exploration/production of petroleum, CONAMA resolution no. 393/2007 stipulates that these waters must be limited to a monthly mean concentration of oil and grease of up to 29 mg/L, with a maximum daily concentration of 42 mg/L [8].

One of the main methods for the treatment of oily water involves the use of gravitational separators, such as a sedimentation tank, centrifuge, and hydrocyclone. [4, 5]. Treatment leads to the removal of only approximately 200 mg/L of oil, due mainly to the presence of oil-in-water emulsions, which are difficult to remove through simple flotation and require auxiliary methods, such as the use of coagulants and surfactants [2]. The addition of these agents makes the process much more efficient, enabling the removal of a larger amount of oil in comparison to other methods [10].

Flotation is a particle separation process involving adherence to bubbles. The oil particle–bubble union has less density than the aqueous medium and floats to the surface of the flotation cell, facilitating the removal of the oil [9]. The basic operating principles of dissolved air flotation (DAF) are quite simple (contact between solid particles and air bubbles and subsequent floating to the surface for removal), but some essential variables are required for the successful use of this method [2]. Moreover, the flotation separation method as a part of pollution control or for the treatment of water is often criticised due to the probable toxicity of the chemical surfactants employed as collectors in this process, such as sodium dodecyl sulfate and sodium oleate [5–7, 11].

Surfactants are compounds composed of amphipathic molecules with a hydrophilic portion and hydrophobic portion that partition at the oil–water or air–water interface. The apolar potion is often a hydrocarbon chain, whereas the polar portion may be ionic (cationic or anionic), nonionic or amphoteric. These characteristics enable surfactants to reduce surface and interfacial tensions as well as form microemulsions in which hydrocarbons can be solubilised in water or water can be solubilised in hydrocarbons [1–12].

Some alternatives have emerged to make this process environmentally more sustainable, such as the use of biosurfactants (biological molecules) in DAF and the use of natural adsorbents, which have a number of advantages over synthetic surfactants and adsorbents, such as better environmental compatibility, biodegradability, low toxicity (posing no risk to workers) and specificity as well as resistance to high temperatures, salinity and a broad pH range [5].

Central composite rotational design (CCRD) has been effectively employed recently to select optimum variables to enhance the oil separation efficiency of DAF systems [9, 13]. CCRD constitutes a statistical tool for examining the relationship between one or more response variables and quantitative set of experimental factors. As the correlation between the response and independent variables is generally unknown at the onset of a process, the first step in CCRD is to approximate the function (variable response) by analyzing the independent variables [14, 15].

The aim of the present study was to evaluate the efficiency of a DAF prototype involving biosurfactants produced from the bacteria *Bacillus cereus* and *Pseudomonas cepacia* as collectors for the removal of oil from water.

Materials and methods

Materials

All reagents were of the highest purity available. A synthetic effluent formulated with motor oil was used in the optimization experiments. The motor oil is commercially available for use in flex engines (gasoline, VNG and alcohol), type SAE 20W-50, with a synthetic guard (PETROBRAS, Brazil) [paraffin-based lubricating oil (complex mixture of hydrocarbons) with performance-enhancing additives].

Microorganisms

The following bacterial strains were used for the production of biosurfactants: *Bacillus cereus* UCP 1615 maintained at the culture bank of the Centre for Environmental Science of the Catholic University of Pernambuco and *Pseudomonas cepacia* CCT 6659, which was obtained from the culture bank of the André Tosello Research and Technology Foundation located in the city of Campinas (state of São Paulo, Brazil).

Inoculum maintenance and growth media

A nutrient agar medium was used for the maintenance of the bacteria. The medium was composed of peptone (1.0%), meat extract (0.5%), NaCl (0.5%) and agar (1.5%) at pH 7.0. The constituents were solubilised and sterilised in an autoclave at 121 °C for 20 min. A nutritive broth was used for the growth of the inoculum and was composed of peptone (1.5%), meat extract (0.5%), NaCl (0.5%) and K_2HPO_4 (0.5%) at pH 7.0. Young cultures obtained after 24 h in the nutrient agar medium were transferred to Erlenmeyer flasks containing 50 mL of nutritive broth, which were maintained under orbital agitation at 200 rpm for 10–14 h at 28 °C until reaching an optical density of 0.7 (corresponding to an inoculum of 10^7 colony-forming units/mL) at 600 nm. This reading was used with the inoculum at concentrations of 1.5–5.0% (v/v).

Media and biosurfactant production

Two biosurfactants were produced in media containing industrial waste using previously established methods. The

mineral medium used for the cultivation of *Bacillus cereus* contained K_2HPO_4 (0.87 g/L), $MgSO_4.7•H_2O$ (0.6 g/L), NaCl (0.1 g/L), KCl (0.2 g/L), Tris–(hydroxymethyl)aminomethane (6.5 g/L) and yeast extract (0.05 g/L) and was supplemented with 2% waste soy frying oil and 0.12% peptone [16]. The mineral medium used for the cultivation of *Pseudomonas cepacia* contained 0.05% KH_2PO_4, 0.1% K_2HPO_4, 0.05% $MgSO_4.7•H_2O$, 0.01% KCl and 0.001% $FeSO_4.7H_2O$ and was supplemented with 2% residual canola oil and 3% corn steep liquor [17].

For the production of the biosurfactants, fermentation was performed in 500-mL Erlenmeyer flasks containing 100 mL of the production medium and incubated with the inoculum obtained after growth in the nutritive broth. The flasks were maintained under orbital agitation at 200 rpm for 48 h for *B. cereus* and 60 h for *P. cepacia* at a temperature of 28 °C.

Determination of surface tension

Surface tension was measured in the cell-free broth using a KSV Sigma 700 tensiometer (Finland) with a Du Noüy ring.

Isolation of biosurfactants

Different methods were used to isolate the biosurfactants produced by the bacteria. For the biosurfactant from *B. Cereus* UCP 1615, the pH of the cell-free broth was adjusted to 2.0 with a 6 M HCl solution. Next, the same volume of chloroform/methanol (2:1, v/v) was added. The mixture was shaken vigorously for 15 min and left to rest for the separation of the phases. The organic phase was removed and the procedure was repeated two more times. The product was concentrated from the organic phases collected using a rotary evaporator, then dissolved in methanol, concentrated a second time by evaporation of the solvent at 45 °C and weighed [18]. The biosurfactant from *P. aeruginosa* CCT 6659 was extracted from the cell-free broth after centrifugation at 5000x*g* for 30 min. The pH of the supernatant was adjusted to 2.0 using 6.0 M NaCl solution and an equal volume of $CHCl_3/CH_3OH$ (2:1) was added. The mixture was shaken vigorously for 15 min and left to rest for the separation of the phases. The organic phase was removed and the procedure was repeated. The product was concentrated from the organic phases collected using a rotary evaporator, then dissolved in methanol and concentrated a second time by evaporation of the solvent at 45 °C.

Preparation of biosurfactant formulations

Potassium sorbate (0.2%), which is a low-cost conservative that inhibits the growth of mold widely used in the production and conservation of foods, was added to the cell-free broth (crude biosurfactant) of each strain. The concentration of the preservative was chosen according to the results obtained previously in our laboratories by Freitas et al. [19].

Experimental design

A central composite rotatable design (CCRD) with four variables and 28 experiments was used to evaluate the influence of the following independent variables on the efficiency of the oil separation process: oily water flow rate, microbubble flow rate, biosurfactant solution flow rate and biosurfactant concentration. The values of the independent variables are listed in Table 1.

Application of biosurfactants in oil removal process using dissolved air flotation

The tests were performed using a laboratory-scale DAF unit (Fig. 1). Ten litres of water was mixed with 50 g of motor oil with the aid of a pump (1) in the storage tank (2) for 1 h to obtain good oil/water saturation. The aquarium (3) was filled with 15 L of drinking water. After recirculation in the storage tank, the synthetic oily effluent was fed into the aquarium with the same pump, entering through a valve (4). The flow rate was monitored using an *Arduino Uno* board, diluting the oily water, which was then bombarded with microbubbles generated through cavitation using a centrifugal pump (5) for the separation of the oily phase in the supernatant. The interaction between the microbubbles and oil droplets at the base of the aquarium gave rise to flocs comprised of oil, biosurfactant and air, which floated due to the much lower density in comparison to the water, forming a layer of oily foam, which was collected in the collection portion of the DAF device (6). To enhance the efficiency of the process, quantities of biosurfactant were introduced into the biosurfactant tank (7). A return pump (8) connected

Table 1 Experimental range and levels of independent variables for separation efficiency in DAF system

Variables	Range and levels				
	−2.0	−1.0	0.0	+1.0	+2.0
Oily water flow rate (L/min), X_1	2.5	5.0	7.5	10.0	12.5
Microbubble flow rate (L/min), X_2	5.0	5.5	6.0	6.5	7
Biosurfactant solution flow rate (L/min), X_3	0.5	1.0	1.5	2.0	2.5
Biosurfactant concentration (g/L), X_4	0.05	0.15	0.25	0.35	0.45

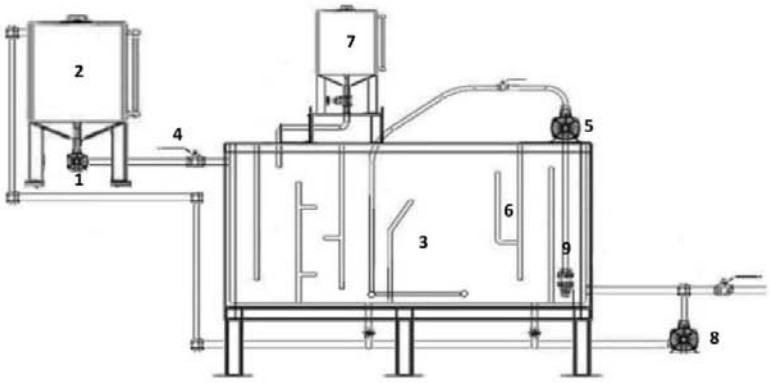

Fig. 1 Bench-scale DAF prototype for the treatment of oily water

to the treated water section (9) was used for recirculation, returning the treated effluent to the storage tank (2) for collection and subsequent analysis without coming into contact with the initial oily effluent. Several experiments were conducted with different flow rates and concentrations of the biosurfactant in its isolated and formulated forms. The DAF prototype was also run without the biosurfactant. Each assay lasted 5 min.

Removal of hydrophobic contaminant by flotation

The residual oil was extracted from the samples of synthetic oily water with the same volume of hexane (1:1, v/v). The mixture was shaken for 15 min and left to rest for the separation of the phases. The organic phase was removed and the procedure was repeated five times. The oil contained in this phase was submitted for analysis in a UV–Vis spectrophotometer (SP-22-BIOSPECTRO) at 330 nm. For the determination of the calibration curve, a standard solution of motor oil (5000 mg/L) was diluted in *n*-hexane at concentrations ranging from 1 to 1000 mg/L. *n*-Hexane was used as the blank to calibrate the device [20].

Separation of oily water from thermoelectric plant effluent

After the optimization experiments, an oily effluent collected from the Candeias thermoelectric plant (Candeias Energy Company) in the city of Candeias, in Bahia State, Brazil, was tested in the DAF prototype. This effluent is largely composed of heavy oil, motor oil and diesel oil. The effluent from the plant needed to undergo an initial separation of the oily water prior to the flotation process due to the high degree of saturation of the heavy oil in the supernatant layer. The crude oily water from this effluent was subsequently investigated with regard to its physicochemical characteristics. The biosurfactant selected for the prior step was applied to the flotation process in its isolated and formulated forms to investigate the oil removal capacity (as described above) and increase the efficiency of the process.

Determination of the effluent turbidity

The turbidity of the effluent was monitored following the CONAMA Resolution 430/2011 [6]. The turbidity meter (TB 1000, MS Tecno PON) was calibrated prior to the analysis.

Statistical analysis

ANOVA, the determination of regression coefficients and the construction of graphs were performed with the aid of the Statistica® program, version 12.0. All determinations were performed at least three times.

Results and discussion

The petroleum industry produces large volumes of oily water during drilling and extraction processes, which poses a considerable environmental problem. Dissolved air flotation (DAF) is a process that has been successfully employed to separate hydrocarbons from water. Biosurfactants are produced by microorganisms and constitute a promising, sustainable technology for increasing DAF efficiency due to the fact that these natural products are biodegradable and nontoxic. Recent studies have shown that microbial surfactants have the ability to solubilise and mobilise adsorbed organic compounds in contaminated water [5].

Table 2 displays the CCRD matrix with the oil removal efficiency results using the biosurfactant isolated from *P.cepacia* CCT 6659 as an alternative collector. Removal rates varied significantly, ranging from 6 to 95%. Multiple regression analysis was employed to adjust the response function to the experimental data and investigated the

Table 2 Central composite design matrix and experimental values of observed factors on separation efficiency in DAF system using biosurfactant isolated from *P. cepacia* CCT 6659 as alternative collector

Runs	Oily water flow rate (L/min), X_1	Microbubble flow rate (L/min), X_2	Biosurfactant solution flow rate (L/min), X_3	Biosurfactant concentration (g/L), X_4	Separation efficiency (%), Y
1	−1.0	−1.0	−1.0	−1.0	63.67
2	−1.0	−1.0	−1.0	1.0	68.87
3	−1.0	−1.0	1.0	−1.0	48.72
4	−1.0	−1.0	1.0	1.0	47.29
5	−1.0	1.0	−1.0	−1.0	38.53
6	−1.0	1.0	−1.0	1.0	65.37
7	−1.0	1.0	1.0	−1.0	46.27
8	−1.0	1.0	1.0	1.0	94.11
9	1.0	−1.0	−1.0	−1.0	60.36
10	1.0	−1.0	−1.0	1.0	55.15
11	1.0	−1.0	1.0	−1.0	17.44
12	1.0	−1.0	1.0	1.0	27.16
13	1.0	1.0	−1.0	−1.0	10.46
14	1.0	1.0	−1.0	1.0	13.93
15	1.0	1.0	1.0	−1.0	8.29
16	1.0	1.0	1.0	1.0	25.83
17	−2.0	0.0	0.0	0.0	76.27
18	2.0	0.0	0.0	0.0	6.08
19	0.0	−2.0	0.0	0.0	65.56
20	0.0	2.0	0.0	0.0	43.50
21	0.0	0.0	−2.0	0.0	32.14
22	0.0	0.0	2.0	0.0	80.00
23	0.0	0.0	0.0	−2.0	15.40
24	0.0	0.0	0.0	2.0	68.66
25	0.0	0.0	0.0	0.0	26.70
26	0.0	0.0	0.0	0.0	30.87
27	0.0	0.0	0.0	0.0	31.28
28	0.0	0.0	0.0	0.0	29.87

simultaneous influence of the four variables studied. The following quadratic polynomial equation best fits the data:

$$Y_1(\%) = 29.6800 - 16.44X_1 + 2.00X_1^2 + 5.42X_2 + 5.34X_2^2 + 1.44X_3 + 5.72X_3^2 + 8.77X_4 + 2.21X_4^2 - 7.33X_1X_2 - 3,82X_1X_3 - 3.31X_1X_4 + 9.60X_2X_3 + 5.46X_2X_4 + 2,71X_3X_4, \quad (1)$$

in which Y_1 (%) is the oil separation efficiency and X_1, X_2, X_3 and X_4 are the coded values for oily water flow rate (mL/min), microbubble flow rate (mL/min), biosurfactant solution flow rate (L/min) and biosurfactant concentration (g/L), respectively. The best result regarding oil separation efficiency was obtained in Run 8 (Table 2), with an oily water flow rate of 5.00 L/min, microbubble flow rate of 6.50 L/min, biosurfactant solution flow rate of 2.00 L/min and biosurfactant concentration of 0.35 g/L as the most favourable levels of the variables for the oil removal process, reaching a maximum separation rate of 94.11%.

Analysis of variance (ANOVA) was performed to investigate the significance of the proposed quadratic model (Table 3). The p value < 0.05 and $F > 4$ (with a 96% confidence interval) indicate that all terms were statistically significant. The low pure error value (4.29) indicates the good reproducibility of the experimental data. The coefficient of determination ($R^2 = 0.8691$) ensured the goodness-of-fit of the model ($R = 0.72812$), as the predicted values for separation efficiency determined by the multiple polynomial equation were very close to the slope (Fig. 2), which indicates that the proposed model is adequate for the prediction of separation efficiency under the experimental conditions tested.

Table 3 Analysis of variance (ANOVA) for separation efficiency in bench-scale DAF system with using biosurfactant isolated from *P. cepacia* CCT 6659 as alternative collector

Factor	Sum of squares	Degrees of freedom	Mean square	*F* ratio	*p* value[a]
X_1 (L)	6487.55284	1	6487.55284	1509.59919	0.0000375096969
X_1 (Q)	96.1100315	1	96.1100315	22.3639991	0.0179187652
X_2 (L)	704.058337	1	704.058337	163.828476	0.00102902234
X_2 (Q)	684.3477	1	684.3477	159.241976	0.0010731302
X_3 (L)	49.5650042	1	49.5650042	11.5333612	0.0425838757
X_3 (Q)	786.586375	1	786.586375	183.032059	0.00087338013
X_4 (L)	1846.085	1	1846.085	429.568513	0.000245637353
X_4 (Q)	117.738325	1	117.738325	27.3967218	0.0135708778
X_1 (L)×X_2 (L)	860.395556	1	860.395556	200.206837	0.000764711815
X_1 (L)×X_3 (L)	233.554806	1	233,554806	54,3462466	0,00516025119
X_1 (L)×X_4 (L)	175.099056	1	175,099056	40,7440833	0,00778525648
X_2 (L)×X_3 (L)	1475.52016	1	1475,52016	343,341178	0,000343041684
X_2 (L)×X_4 (L)	477.531756	1	477,531756	111,117639	0,00182348392
X_3 (L)×X_4 (L)	117.559806	1	117,559806	27,3551819	0,013599341
Lack of fit	2036.44523	10	203,644523	47.3863742	0.00443506412
Pure error	12.8926	3	4.29753333		
Total square sum	15655.1905	27			

$R^2 = 0.8691$; adjusted $R^2 = 0.72812$

L linear effect, *Q* quadratic effect

[a] $p \leq 0.05$ significant at 5% level

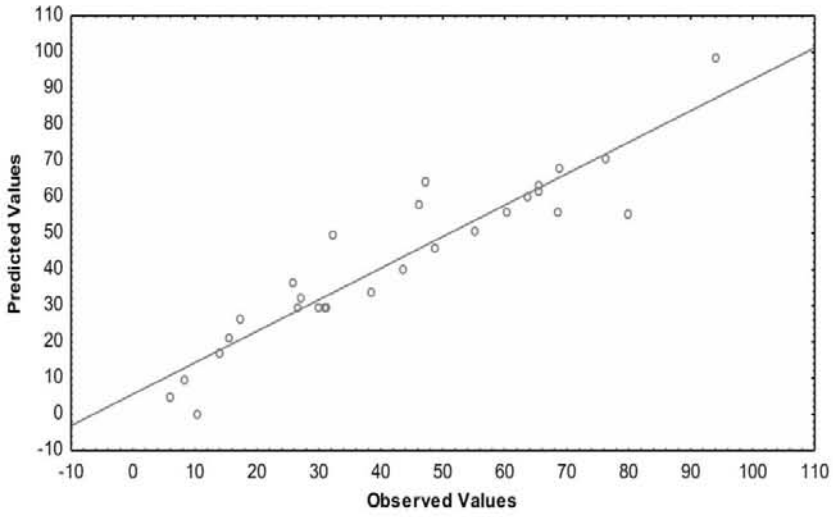

Fig. 2 Plot of predicted vs. actual separation efficiency achieved using biosurfactant isolated from *P. cepacia* CCT 6659

The effects and statistical significance of the variables in the prediction model are shown graphically in the Pareto chart (Fig. 3). The linear term of X_1 contributed significantly with a negative sign, i.e., a lower oily water flow rate enabled better oil removal efficiency. The linear term X_2 led to an increase in separation efficiency at lower levels of this variable, i.e., lower microbubble flow rate. The linear and quadratic terms X_3 and X_4 contributed positively to the increase in separation efficiency.

Table 4 displays the CCRD matrix with the oil removal efficiency results using the biosurfactant isolated from *B. Cereus* UCP 1615. The best oil separation efficiency occurred in Run 7, using an oily water flow rate of 5.00 L/min, microbubble flow rate of 6.50 L/min, biosurfactant

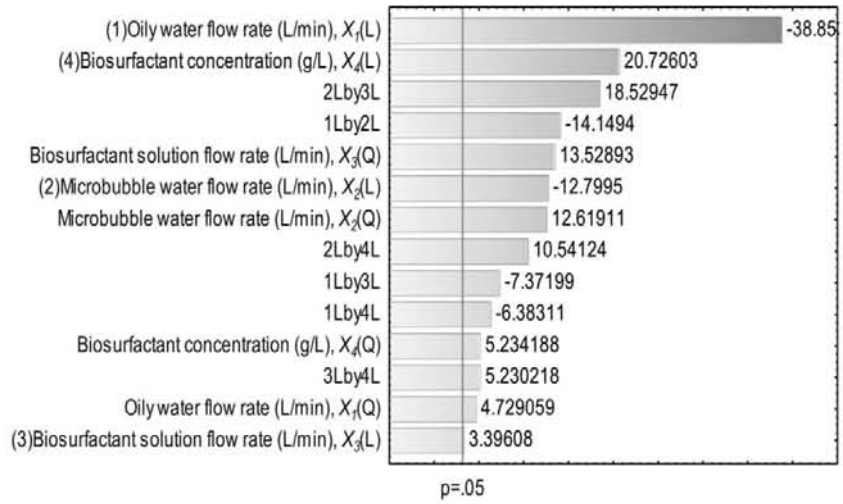

Fig. 3 Pareto chart showing effects of observed factors and combined impact on separation efficiency in DAF system using biosurfactant isolated from *P. cepacia* CCT 6659

Table 4 Central composite design matrix and experimental values of observed factors on separation efficiency in DAF system using biosurfactant isolated from *B. cereus* UCP 1615 as alternative collector

Runs	Oily water flow rate (L/min), X_1	Microbubble flow rate (L/min), X_2	Biosurfactant solution flow rate (L/min), X_3	Biosurfactant concentration (g/L), X_4	Separation efficiency (%), Y
1	−1.0	−1.0	−1.0	−1.0	45.77
2	−1.0	−1.0	−1.0	1.0	65.09
3	−1.0	−1.0	1.0	−1.0	40.92
4	−1.0	−1.0	1.0	1.0	50.03
5	−1.0	1.0	−1.0	−1.0	46.34
6	−1.0	1.0	−1.0	1.0	40.99
7	−1.0	1.0	1.0	−1.0	80.01
8	−1.0	1.0	1.0	1.0	47.61
9	1.0	−1.0	−1.0	−1.0	44.42
10	1.0	−1.0	−1.0	1.0	43.46
11	1.0	−1.0	1.0	−1.0	27.7
12	1.0	−1.0	1.0	1.0	28.00
13	1.0	1.0	−1.0	−1.0	39.53
14	1.0	1.0	−1.0	1.0	12.00
15	1.0	1.0	1.0	−1.0	34.57
16	1.0	1.0	1.0	1.0	19.05
17	−2.0	0.0	0.0	0.0	52.65
18	2.0	0.0	0.0	0.0	28.69
19	0.0	−2.0	0.0	0.0	37.98
20	0.0	2.0	0.0	0.0	29.15
21	0.0	0.0	−2.0	0.0	34.25
22	0.0	0.0	2.0	0.0	33.26
23	0.0	0.0	0.0	−2.0	36.14
24	0.0	0.0	0.0	2.0	30.85
25	0.0	0.0	0.0	0.0	44.35
26	0.0	0.0	0.0	0.0	44.21
27	0.0	0.0	0.0	0.0	44.24
28	0.0	0.0	0.0	0.0	44.55

solution flow rate of 2.00 L/min and biosurfactant concentration of 0.15 g/L, with a maximum separation rate of 80.01%. An empirical model was applied to adjust the oil separation efficiency and the variables of the process using the biosurfactant isolated from *B. Cereus* UCP 1615 as an alternative collector. The following quadratic polynomial equation best fits the data:

$$Y_2(\%) = 44.3375 - 9.00X_1 + 0.12X_1^2 - 1.79X_2 - 1.66X_2^2 - 0.49X_3 - 1.61X_3^2 - 2.65\,X_4 - 1.67X_4^2 - 3.22X_1X_2 - 3.15X_1X_3 - 2.15X_1X_4 + 5.90X_2X_3 - 6.79X_2X_4 - 1.50X_3X_4, \quad (2)$$

in which Y_2 (%) is the oil separation efficiency and X_1, X_2, X_3 and X_4 are the coded values for oily water flow rate (mL/min), microbubble flow rate (mL/min), biosurfactant solution flow rate (L/min) and biosurfactant concentration (g/L), respectively. The evaluation of the empirical model was performed using ANOVA (Table 5). The p value < 0.05 and $F > 4$ (with a 96% confidence interval) indicate that all terms were statistically significant. The good reproducibility of the experimental data was demonstrated by low pure error value (0.024). The oily water flow rate was the most important factor to the increase in separation efficiency, followed by biosurfactant concentration. The coefficient of determination ($R^2 = 0.86695$) ensured the goodness-of-fit of the model ($R = 0.72367$), as the predicted X observed values were distributed close to the slope (Fig. 4), thereby validating the prediction model.

The Pareto chart (Fig. 5) shows the statistical significance of the variables in the prediction model. The most significant to the oil removal process were X_1, X_4 and X_2, which contributed a negative sign, i.e., lower oil water and microbubble flow rates and a lower concentration of the biosurfactant from *B. cereus* UCP 1615 contributed to the increase in separation efficiency.

The findings demonstrate that the most significant variables for the biosurfactants of both strains used as alternative collectors were exactly the same (Figs. 3, 5) and the levels selected for the best results were quite similar (Tables 2, 4). Comparing the performance of the two biosurfactants, the concentration of the tensioactive agent from *B. Cereus* UCP 1615 was half that of the tensioactive agent from *P.cepacia* CCT 6659 under the best conditions selected. The biosurfactant from *P.cepacia* CCT 6659 also demonstrated good removal efficiency on Run 22 (80% oil removal rate) (Table 2), with a higher oily effluent flow rate (enabling a greater volume of treated water) and a lower biosurfactant concentration in comparison to the condition with the best result (Run 8), which is of greater interest in terms of the cost of the process.

Table 5 Analysis of variance (ANOVA) for separation efficiency in bench-scale DAF system using biosurfactant isolated from *B. cereus* UCP 1615 as alternative collector

Factor	Sum of squares	Degrees of freedom	Mean square	F ratio	p value[a]
X_1 (L)	1943.1001	1	1943.1001	82016.1845	0.0000000938863253
X_1 (Q)	0.34620026	1	0.34620026	14.612744	0.0315164775
X_2 (L)	76.8626042	1	76.8626042	3244.2886	0.0000119209214
X_2 (Q)	65.8276565	1	65.8276565	2778.51522	0.0000150379683
X_3 (L)	5.69400417	1	5.69400417	240.337847	0.000583136478
X_3 (Q)	62.105794	1	62.105794	2621.41937	0.0000164085382
X_4 (L)	168.593004	1	168.593004	7116.13102	0.0000036718521
X_4 (Q)	67.226169	1	67.226169	2837.54495	0.0000145715552
X_1 (L)×X_2 (L)	166.216556	1	166.216556	7015.82369	0.00000375085213
X_1 (L)×X_3 (L)	159.201306	1	159.201306	6719.71746	0.00000400139668
X_1 (L)×X_4 (L)	73.9170062	1	73.9170062	3119.95805	0.0000126399898
X_2 (L)×X_3 (L)	557.786306	1	557.786306	23543.5655	0.000000610373951
X_2 (L)×X_4 (L)	736.715306	1	736.715306	31095.9679	0.000000402128034
X_3 (L)×X_4 (L)	35.9700062	1	35.9700062	1518.25563	0.0000371898632
Lackof fit	625.847442	10	62.5847442	2641.63535	0.0000109157168
PureError	0.071075	3	0.0236916667		
Total square sum	4704.51732	27			

$R^2 = 0.86695$; adjusted $R^2 = 0.72367$

L linear effect, *Q* quadratic effect

[a] $p \leq 0.05$ significant at 5% level

Table 6 displays the comparative data for the production variables as well as the performance of the biosurfactants from *P. cepacia* CCT 6659 and *B. cereus* UCP 1615 regarding the separation efficiency of the DAF process. The tensioactive agent from *P. cepacia* CCT 6659 proved to be more promising for application in the DAF-biosurfactant

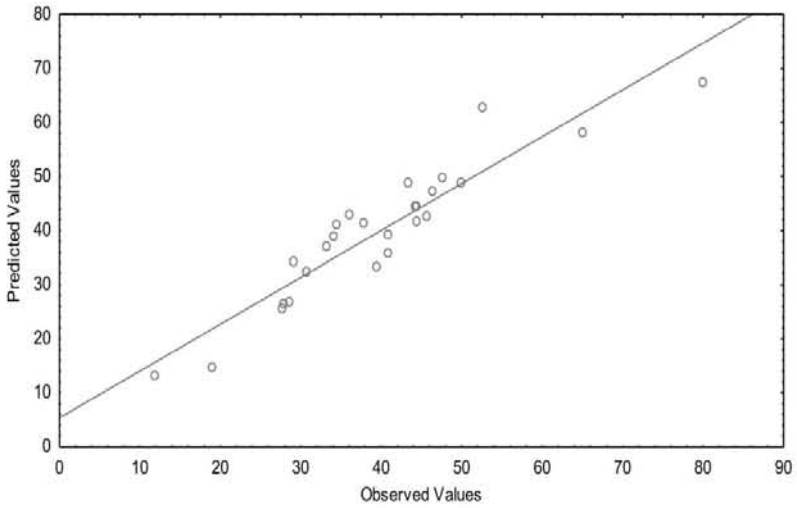

Fig. 4 Plot of predicted vs. actual separation efficiency achieved using biosurfactant isolated from *B. cereus* UCP 1615

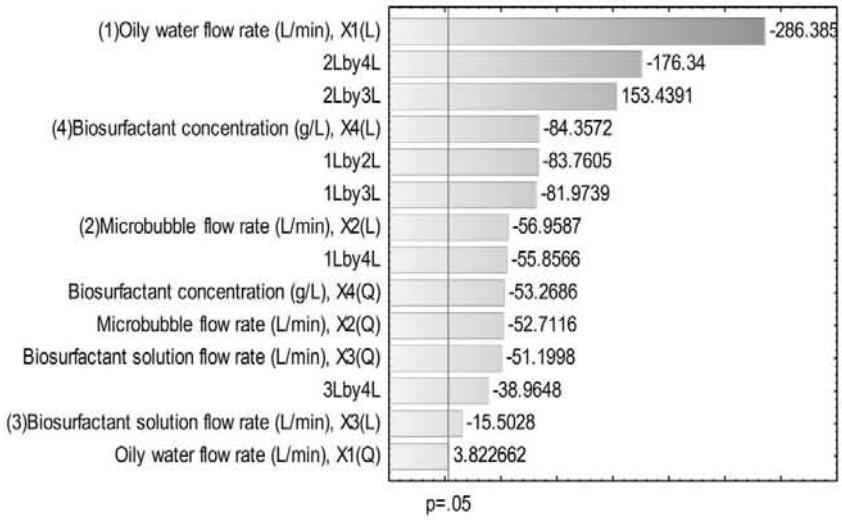

Fig. 5 Pareto chart showing effects of observed factors and combined impact on separation efficiency in DAF system using biosurfactant isolated from *B. cereus* UCP 1615

Table 6 Comparative performance of isolated biosurfactants from *P. cepacia* CCT 6659 and *B. cereus* UCP 1615 regarding separation efficiency in DAF process

Biosurfactants	Surface tension (mN/m)[a,b]	CMC (mg/L)[a,b]	Yield (g/L)[a,b]	Maximum separation efficiency (%)	Model determination coefficient (R^2)
Isolated from *P. cepacia* CCT 6659	27.01	600	8.01	94.11	0.8691
Isolated from *B. cereus* UCP 1615	27.50	500	4.60	80.01	0.8669

[a]Data obtained from Silva et al. [14]

[b]Data obtained from Silva et al. [16]

system, as this strain produced twice the yield in terms of the concentration of the biomolecule in comparison to the biosurfactant from *B. cereus* UCP 1615. Moreover, the biosurfactant from *P. cepacia* CCT 6659 achieved a better removal efficiency result. The biosurfactants from both strains demonstrated equivalent characteristics with regard to the other variables.

For the validation of both prediction models (Eqs. 1 and 2), the biosurfactants from *P.cepacia* CCT 6659 and *B. Cereus* UCP 1615 were evaluated in their formulated forms using the best process conditions (Runs 8 and 7, respectively). The isolated biosurfactant was used during the previous variable optimisation process to avoid interference in the statistical error of the models due to the occurrence of other biomolecules in the cell-free broth. Table 7 displays the results of the application of the models. The formulated biosurfactant from *P. cepacia* CCT 6659 demonstrated an excellent performance in removing the oily fraction compared to the 53.74% oil removal rate without the use of the biosurfactant as the alternative collector (using only microbubbles). In contrast, the oil removal efficiency of the formulated biosurfactant from *B. cereus* UCP 1615 decreased around 10% in comparison to this biosurfactant in its isolated form. Therefore, the formulated biosurfactant from *P. cepacia* CCT 6659 was selected for the subsequent evaluations.

Separation of oily water from effluent from thermoelectric plant

Table 8 displays the physicochemical characteristics of an actual oily effluent from the Candeias thermoelectric plant (Candeias Energia S/A). The effluent contained a significant quantity of oily content and was highly turbid, likely due to the high content of suspended solids. A considerable amount of sulphide was also detected. Using the Brazilian environmental legislation (CONAMA Resolution 430/2011) [6] as reference, the oil, grease and turbidity of the effluent were above acceptable limits and adequate treatment was required.

Table 7 Oil fraction removal percentage using biosurfactants from *P. cepacia* CCT 6659 and *B. cereus* UCP 1615 and microbubbles in flotation process

Conditions	Oily fraction removal (%)
Oily effluent (control)	–
Microbubbles	53.74 ± 0.01
Formulated biosurfactant from *P. cepacia* CCT 6659	98.25 ± 0.02
Formulated biosurfactant from *B. cereus* UCP 1615	70.87 ± 0.01

Results are expressed as mean ± standard deviation

Table 8 Physicochemical characterisation and chemical properties of oily effluent from thermoelectric plant (Candeias Energia S/A)

Parameter	Value/unit	CONAMA 430/2011 specifications
Oil and grease[a]	103 mg/L	20 mg/L
Sulphide[a]	0.45 mg/L	1 mg/L
pH	7.2	5–9
Turbidity	120.22 NTU	40 NTU
Conductivity[a]	310 mS/m	–
BOD[a]	98 mg/L	–
COD[a]	500 mg/L	–
Suspended solids[a]	95.70	–

[a]Data obtained from Candeias thermoelectric plant (Candeias Energia S/A)

The efficiency of the treatment process was evaluated based on the removal of the oily fraction and turbidity of the effluent in the flotation system. Table 9 displays the results of the tests performed with the bench-scale DAF prototype and the isolated and formulated forms of the biosurfactant from *P. cepacia* CCT 6659 applied to the effluent from the Candeias thermoelectric plant. The data indicate significant oil removal with both forms of the biosurfactant when compared to the process with the microbubbles alone. In particular, the formulated biosurfactant achieved an oil removal rate that left the effluent below the limit established by legislation (≤ 20 mg/L). The formulated biosurfactant from *P. cepacia* CCT 6659 achieved a reasonable reduction in turbidity of the effluent (around 18.93%), whereas the isolated form achieved a reduction in turbidity of around

Table 9 Oil removal rates using flotation process and total turbidity of oily effluent from thermoelectric plant (Candeias Energia S/A) using the biosurfactant from *P.cepacia* CCT 6659 in formulated and isolated forms as alternative collector

Conditions	Removal of oily fraction (%)	Turbidity (NTU)	Removal (mg/L)
Oily effluent (control)	–	120.22 ± 0.03	–
Microbubbles alone	63.65 ± 0.02	106.52 ± 0.02	65.56 ± 0.03
Isolated biosurfactant from *P. cepacia* CCT 6659	75.74 ± 0.01	51.39 ± 0.01	78.01 ± 0.01
Formulated biosurfactant from *P. cepacia* CCT 6659	95.70 ± 0.02	97.46 ± 0.01	98.57 ± 0.02

Results are expressed as mean ± standard deviation

57.25% (to 51.39 NTU), which is near the limit specified by legislation (≤40 NTU).

These data demonstrated that, under actual conditions of the routine of the thermoelectric plant, the treatment of the oily effluent by combining DAF with a bacterial biosurfactant would ensure adequate effluent treatment on the industrial scale and enable the reuse of the clean water for different activities within the plant. The DAF system alone was not sufficient to bring the oily effluent to within the maximum limits established by Brazilian legislation [6]. The formulated form of the biosurfactant from *P.cepacia* CCT 6659 proved to be more adequate as an alternative collector for reducing the oily content of the effluent. In turn, the isolated biosurfactant from *P.cepacia* CCT 6659 proved to be more indicated for application in a subsequent step of the process, assisting in the reduction in the turbidity of the effluent associated with a continuous water recirculation system in the DAF process.

Few studies report the use of biological surfactants as collectors/coagulants in dissolved air flotation processes. One of the first studies conducted in Brazil on the development of DAF-biosurfactant systems for the treatment of oily water involved the use of a crude biosurfactant obtained from the yeast *Candida sphaerica* UCP 0995 cultivated in industrial waste during 6 days of fermentation. The authors reported an increase in the efficiency of separating oil from a synthetic effluent from 80.0% (using microbubbles alone) to 98.0% (in the presence of the biosurfactant) in a pilot-scale DAF system [9]. In a study conducted by Lins et al. [21], a crude biosurfactant obtained from *C. lipolytica* UCP 0988 (cultivated for 3 days using industrial waste as the substrate) led to an improvement in oil removal from an actual effluent comprised of waste frying oil in a pilot DAF system, achieving an oil separation rate of 95.5%. In a more recent study, Luna et al. [13] applied a crude biosurfactant from *C. guilliermondii* UCP 0992 in the treatment of a synthetic oily effluent using a bench-scale DAF system, achieving an increase in the removal efficiency from 40% (microbubbles alone) to 92% with the crude biosurfactant.

The better performance of DAF systems with the use of a biosurfactant as an adjuvant in the process is undeniable. In the present study, biosurfactants obtained in a rapid-growth fermentative medium (2–2.5 days) were used rather than long-growth media for yeasts (3–5 days), the biosurfactants of which have previously been employed more in innovative DAF-biosurfactant systems for the treatment of oily water. Moreover, the biosurfactants were evaluated in their isolated and formulated forms, which ensures greater stability and durability of the end product and offers an advantage over the use of the cell-free broth (crude biosurfactant) [19]. The formulated form of the biosurfactant from *P. cepacia* CCT 6659, which was selected for use in the DAF system in the present study, has previously been characterised with regard to its economic viability for use on an industrial scale [22], which ensures the technical–economic viability of the DAF–biosurfactant system presented herein.

Conclusion

The present study demonstrated the considerable potential of biosurfactants as alternative collectors in a DAF system. The central composite rotatable design proved to be an excellent method for the identification of optimum variables to enhance the oil separation efficiency of a DAF system. The biosurfactant produced by *P. cepacia* CCT 6659 enabled a significant increase in the oil removal rate. Moreover, the use of industrial waste products in the acquisition of the biosurfactant is in line with current concerns for sustainable practices. The treatment of industrial effluents and oily water using the DAF–biosurfactant system developed herein can contribute to a reduction in environmental degradation and the improvement in the quality of water resources. Therefore, the present study describes a favourable, efficient strategy for the decontamination of oily industrial effluents.

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Compliance with ethical standards

Conflict of interest This work is free from any conflict of interest.

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6. CONCLUSÕES

Os resultados obtidos nessa pesquisa permitem as seguintes conclusões

- A utilização de resíduos industriais como substratos de baixo custo foi favorável ao crescimento da bactéria *P. aeruginosa* UCP 0992 e à produção do biossurfactante.
- A maximização da produção do biossurfactante de *P. aeruginosa* UCP 0992em biorreator foi alacancada com a utilização do planejamento fatorial, possibilitando futura aplicações dustriais do tensoativo na redução da contaminação provocada por petróleo e derivados em ambientes industriais a um custo reduzido.
- O biossurfactante produzido reduziu consideravelmente a tensão superficial da água, demonstrando elevada capacidade emulsionante e dispersante de compostos hidrofóbicos.
- Obiossurfactante demonstrou estabilidade em condições ambientais extremas de salinidade, temperatura e variações de pH.
- O biossurfactante obtido foi caracterizado como um tensoativo de natureza glicolipídica.
- O biossurfactante produzido apresentou potencial de remoção de derivado de petróleo adsorvido em areia sob condições estáticas e cinéticas.
- A bactéria e seu biossurfactanteapresentaram grande potencial de aplicação em processos de biorremediação de óleo em água do mar e em areia.
- Apresença do biossurfactante potencializou a capacidade de degradação do óleo pela bactéria em água do mar e em areia.
- A aplicação dobiossurfactantecomo coletor natural aumentou a eficiência de separação de óleo no protótipo de FAD de bancada.
- O protótipo de FAD pode ser aplicado no tratamento de águas oleosas, a depender do tipo de efluente e dos níveis de remoção de óleo exigidos pela legislação amboental, na ausência do biossurfactante como coletor.
- A maximização da separação do óleo foi alacancada com a utilização do planejamento fatorial.
- O biossurfactante bruto, isolado ou formulado pode ser aplicado com eficiência no protótipo da FAD, sendo a seleção da forma de aplicação dependente das condições disponíveis a nível industrial.

- A viabilidade do sistema FAD-Biossurfactante foi comprovada pela eficiência de remoção de óleo por dois biossurfactantes produzidos por *Psedomonas cepacia* CCT6659 e outro por *Bacillus cereus* UCP1615, previamente caracterizados.
- O biossurfactante de *Pseudomonas cepacia* CCT6659 na sua forma formulada, se mostrou efetivo como coletor alternativo na eficiência do processo FAD para a remoção de óleo e na redução da turbidez do efluente de usina termoelétrica.

APÊNDICE

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Biosurfactant Application as Alternative Collectors in Dissolved Air Flotation System

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The effluent production of oily water type has generated many environmental problems for several industries. The use of flotation as a separation process of oily waters has been described, although it has been sometimes criticized due to the toxicity of collectors. The development and use of biodegradable surfactants may enhance the further acceptance of this separation technology. In this sense, the dissolved air flotation (DAF) process continues to be widely used in industries, both for water and wastewater supplies. The use of collectors is essential to improve the efficiency of the process, due to its specific characteristics that facilitate the adhesion of the particles and, consequently, the separation of the pollutants. These surface-active molecules of biological origin also have several advantages over synthetic surfactants such as higher biodegradability, higher foaming, less toxicity, better environmental compatibility, more tolerant to pH, salt, and temperature variation, and higher selectivity for metals and organic compounds and can be synthesized from renewable feedstocks. The aim of this study was to investigate a water-oil separation by DAF, with and without the addition of biosurfactant produced by *Pseudomonas cepacia* CCT 669 in mineral medium and formulated with 2.0% of corn steep and 3.0% of canola waste frying oil at 28°C for 60 h under 200 rpm. The experiments used to compare the effects of the addition of biosurfactant followed an experimental planning CCRD, where the response variable was the separation efficiency. Results indicated the biosurfactant added a considerable value to the process, increasing from 41.0% to 98.0% the separation efficiency, presenting potential of application as a collector of oily contaminants in the DAF process form.

1. Introduction

Petroleum industry and related industries 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. Effluent production of oily water type has generated many environmental problems for several industries (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). In this context, the flotation process has proven to be quite efficient, with the capability of removing a larger amount of oil in comparison to other methods (Albuquerque et al. 2012).

Flotation is a bubbles adhesion based particle separation process. The oil particle-bubble union has less density than the aqueous medium and floats to the surface of the flotation chamber, where the oil particles are removed (Bahadori et al. 2013). Flotation was first used in mineral processing and has long been employed in solid/liquid separation processes that involve the use of stable foams to recover mineral particles (Peng et al. 2009).

With the industrial sector development, the flotation process application was improved, leading to the emergence of dissolved air flotation (DAF), which involves solute removal through adsorption, co-precipitation

or occlusion in a floc transporter and subsequent release by the addition of an adequate tensioactive agent (Beneventi et al. 2009). With DAF, the water is saturated with pressurised air through a nozzle, forming bubbles that reach the flotation chamber, which is at atmospheric pressure. The air becomes supersaturated and precipitates from the solution in the form of small bubbles (Babaahmadi, 2010; Rocha e Silva et al. 2015).
The use of flotation as a separation process of oily waters have been widely employed wastewater treatments, of oil industries (Bahadori et al., 2013; Rocha e Silva et al. 2015). The dissolved air flotation may be considered as a clean technology since it uses small quantities of coagulant and air to promote separation. The size, speed, and bubbles, along with the velocity gradient are important parameters to control the efficiency of the process and operating costs (Babaahmadi, 2010). On the other hand, this technique has been sometimes criticized due to the toxicity of the synthetic surfactants used as collectors in this process (Menezes et al., 2011). Surfactants are compounds composed of amphipathic molecules with a hydrophilic portion and a hydrophobic portion that partition at the oil/water or air/water interface. The apolar portion is often a hydrocarbon chain, whereas the polar portion may be ionic (cationic or anionic), non-ionic or amphoteric. These characteristics enable surfactants to reduce surface and interfacial tension and form microemulsions, in which hydrocarbons can be solubilised in water or vice versa (Almeida et al. 2016).
Currently, the development and use of biodegradable surfactants (biosurfactants) has helped to increase acceptance of this separation technology (Rocha e Silva et al., 2015). Biosurfactants are amphipathic molecules that reduce the surface and interfacial tensions of liquids. Such compounds have a predilection for interfaces of dissimilar polarities (liquid–oil) and are soluble in both organic (non-polar) and aqueous (polar) solvents (Silva et al., 2014). These surface-active molecules of biological origin also have several advantages over synthetic surfactants such as higher biodegradability, higher foaming, less toxicity, better environmental compatibility, more tolerant to pH, salt, and temperature variation, and higher selectivity for metals and organic compounds and can be synthesized from renewable feedstocks (Menezes et al., 2011).
The aim of this study was to investigate a water-oil separation by DAF, with addition of biosurfactant, in a pilot-scale DAF system. The experiments used to evaluate the effects of biosurfactant addition followed an experimental planning CCRD, where the response variable was the oil removal efficiency.

2. Materials and Methods

2.1 Materials

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

2.2 Bacterial strain and inoculum preparation

A strain of *P. cepacia* CCT6659 was provided from the culture collection of the Fundação André Tosello de Pesquisa e Tecnologia, Campinas city, São Paulo, Brazil. The microorganism was maintained in nutrient agar slants at 4°C. For pre-culture, the strain from a 24-h culture on nutrient agar was transferred into 50 ml nutrient broth to prepare the seed culture. The cultivation condition for the seed culture was 28°C, 200 rpm, and 24h of incubation time.

2.3 Biosurfactant production

The fermentation for the biosurfactant production was carried out in distilled water containing 2% of canola oil residual, 3% of corn steep liquor, 0.2% $NaNO_3$, 0.05% KH_2PO_4, 0.1% K_2HPO_4, 0.05% $MgSO_4$.$7H_2O$, 0.01% KCl and 0.001% $FeSO_4$.$7H_2O$. After media preparation, the pH was adjusted to 7.0 and these were autoclaved at 121°C for 20 minutes. The culture was incubated in a rotary Marconi MA832 shaker (Marconi Laboratory equipment, SP, Brazil) for 60 h at 200 rpm (Silva et al., 2013).

2.4 Surface tension measurement

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., 2014).

2.5 CCRD Experimental factorial design

In order to study biosurfactant potential as an alternative collector on the oil-water separation in a DAF pilot scale system prototype (Figure 1), central composite rotational design (CCRD) application was performed. The independent variables were coded at five levels (-2.00, -1.00, 0.00, +1.00, +2.00) and the complete design consisted of 28 experimental points including 4 replications of the central points. The coded levels of

the independent variables used in the experimental design are listed in Table 1. The response variable was the oil removal efficiency of the pilot prototype. All determinations were performed at least three times. ANOVA, regression coefficients and the construction of graphs were performed using the Statistica® program, version 10.0 (Statsoft Inc, USA).

Table 1: Experimental range and levels of independent variables for oil removal efficiency in the pilot scale DAF system with use of the biosurfactant

Levels	Oily Water Flow (X_1)*	Microbubble Flow (X_2)*	Biosurfactant Flow (X_3)*	Biosurfactant Concentration (X_4)**
-2.00	2.50	5.00	0.50	0.05
-1.00	5.00	5.50	1.00	0.15
0.00	7.50	6.00	1.50	0.25
1.00	10.00	6.50	2.00	0.35
2.00	12.50	7.00	2.50	0.45

** (L/min); ** (g/L)*

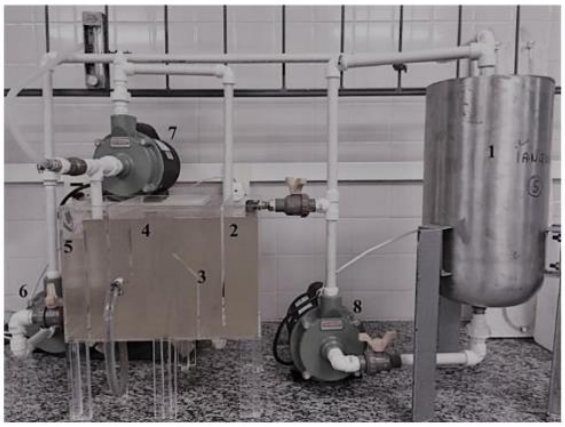

Figure 1: Three dimensional scheme of pilot scale DAF system. Oily water storage tank (1); Flotation chamber (2); Second section where separation between treated water and oily foam formed (3); Oily foam collectors (4); Chamber of treated water collection (5); Return pump for treated water (6); Microbubble production pump (7); and oily water production pump and DAF chamber feed (8)

3. Results and Discussion

3.1 Biosurfactant production

The properties and integrity of the biosurfactant produced were verified prior to the tests for the study of its potential as an alternative collector. The biosurfactant produced was able to reduce the surface tension of the culture medium from 55.0 mN/m to 28.0 mN/m corroborating with the results obtained by Soares da Silva et al (2017) under the same conditions evaluated.

3.2 Evaluation of water-oil separation efficiency using biosurfactant in the DAF system

The CCRD matrix and corresponding results are given in Table 2.
As a result, the effluent flow rate of 5.00 L/min, microbubble water flow of 5.50 L/min, biosurfactant flow rate of 1.00 L/min and biosurfactant concentration of 0.35 g/L were the more favorable parameters for the oil removal process using this type of biosurfactant, reaching a percentage of removal of 98.25% (Run 2), compared with 41.20% of oil removal without the use of the biosurfactant as an alternative collector. The results were not favorable for biosurfactant action of the in the DAF process occurred in the tests 10 and 11 (11.08 and 12.09%, respectively), which was probably due to the considerable increase of the effluent flow (oily water) and low concentration of the biosurfactant which must have influenced negatively in the micelles formation and compromised the collision between the microbubbles and the particles of the oil.

Table 2: Experimental design results matrix and values of observed factors on separation efficiency in the pilot scale DAF system with use of biosurfactant

Runs	Oily Water Flow (X_1)	Microbubble Flow (X_2)	Biosurfactant Flow (X_3)	Biosurfactant Concentration(X_4)	Removal efficiency (%)(Y)
1	5.00	5.50	1.00	0.15	63.20
2	5.00	5.50	1.00	0.35	98.25
3	5.00	5.50	2.00	0.15	85.18
4	5.00	5.50	2.00	0.35	73.83
5	5.00	6.50	1.00	0.15	65.38
6	5.00	6.50	1.00	0.35	49.76
7	5.00	6.50	2.00	0.15	37.82
8	5.00	6.50	2.00	0.35	80.86
9	10.00	5.50	1.00	0.15	80.20
10	10.00	5.50	1.00	0.35	11.08
11	10.00	5.50	2.00	0.15	12.09
12	10.00	5.50	2.00	0.35	71.74
13	10.00	6.50	1.00	0.15	63.95
14	10.00	6.50	1.00	0.35	36.36
15	10.00	6.50	2.00	0.15	29.59
16	10.00	6.50	2.00	0.35	30.39
17	2.50	6.00	1.50	0.25	51.38
18	12.50	6.00	1.50	0.25	19.08
19	7.50	5.16	1.50	0.25	34.13
20	7.50	6.84	1.50	0.25	42.42
21	7.50	6.00	0.50	0.25	40.00
22	7.50	6.00	2.50	0.25	51.37
23	7.50	6.00	1.50	0.05	38.89
24	7.50	6.00	1.50	0.45	73.44
25	7.50	6.00	1.50	0.25	51.88
26	7.50	6.00	1.50	0.25	46.61
27	7.50	6.00	1.50	0.25	47.29
28	7.50	6.00	1.50	0.25	50.54

The Pareto diagram shown in Figure 2 shows the statistical significances of the studied variables at p-values (< 0.05). As can be observed, the Oily Water Flow (X_1) was the most significant variable for the process and its increase causes a decrease in the efficiency of oil removal by the system. The Biosurfactant Flow did not present statistical significance (X_3), however, its concentration (Biosurfactant Concentration) was positively correlated with the oil removal efficiency.

Figure 3 displays the fitted response surface plots for oil removal efficiency shown by Pareto diagram of the statistically significant interactions. The combination of greater oily water flow and greater microbubble flow led to maximum oil removal efficiency (Fig. 3A) and the elliptic curve found in the graph indicates a high degree of interaction of these variables. Figure 3B shows that high oil removal efficiency was also achieved when both oily water flow and biosurfactant concentration were maintained at their maximum levels. Better oil removal efficiency was found when the biosurfactant flow and biosurfactant concentration was maintained at its maximum level (Fig. 3C), however, these interactions were weak and did not produce well-defined regions in the graphs.

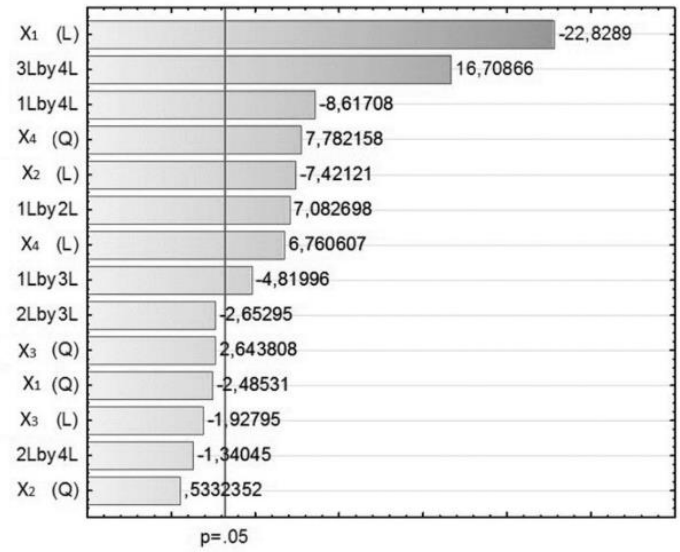

Figure 2: Pareto's Chart of the Central Composed Rotate Design.

In another study conducted by Rocha e Silva et al (2015), a biosurfactant obtained from *Candida sphaerica* UCP 0995 also promotes an improvement of the oil removal in the DAF system. The authors reported increasing separation efficiency from 80.0 to 98.0% in the presence of biosurfactants. The results demonstrate a better performance of the bench scale system using the biosurfactant as a coadjuvant in the DAF process, compared to the action of the microbubbles only without the use of this alternative collector. This confirms the potential of these microbial biomolecules as an aid in the removal of hydrophobic compounds in Dissolved Air Flotation. In addition, it is important to highlight that the biosurfactant from *P. cepacia* CCT 669 was produced in a culture medium prepared only with industrial waste products, which further reduces the process costs, since substrates used in the production of biosurfactants account for 20 to 30% of the production cost (Hazra et al., 2012; Santos et al., 2016). The low cost of the proposed DAF process is evident by the small amount of biosurfactant (350 ppm) required to achieve maximum efficiency (run number 2 in Table 2). As many industries generate huge amounts of oily waters that require adequate treatment before being discarded or reused, the benefits of treating oily waters with the system developed herein demonstrates its considerable market potential. Therefore, biosurfactants are promising coagulants and/or dispersants capable of increasing the efficiency of this technique.

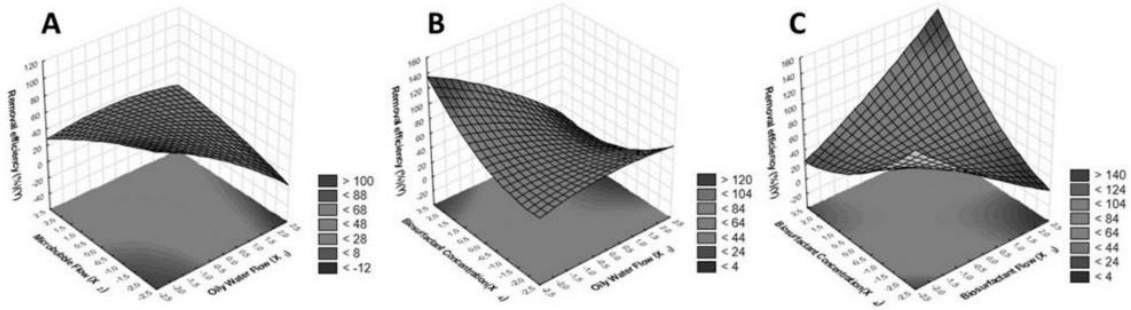

Figure 3: Response surface plots and contour plots for maximum removal efficiency for the 28 experimental runs carried out under conditions established by CCRD; removal efficiency as function of (A) oily water flow and microbubble flow; (B) oily water flow and biosurfactant concentration; (C) biosurfactant flow and biosurfactant concentration.

4. Conclusions

The present study demonstrated the effectiveness of using a central composite rotational design to identify the optimum parameters for increase oil removal efficiency in DAF system. The above results confirm the great potential of the biosurfactant to be used as an alternative collector, since these microbial surfactants act as

true "molecular glues", interacting with the oil and the air bubbles, facilitating the oil transportation during the process flotation, as evidenced by the results presented.

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

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ABSTRACT

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

1. Introduction

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

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

The most important advantage of biosurfactants over chemical

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

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

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

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

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

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

2. Materials and methods

2.1. Materials

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

2.2. Bacterial strain and preparation of seed culture

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

2.3. Fermentation media

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

2.4. Biomass determination

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

2.5. Emulsifying activity with different hydrophobic compounds

Emulsification index (EI) was measured using the method described by Cooper and Goldenberg (1987), whereby 2 ml of a liquid hydrophobic compound (motor oil, lubricating oil, diesel, kerosene, n-hexadecane and vegetables oils) was added to 2 ml of the culture broth free of cells in a graduated screwcap test tube, and vortexed at high speed for 2 min. The emulsion stability was determined after 24 h and the emulsification index was calculated by dividing the measured height of the emulsion layer by the mixture's total height and multiplying by 100.

2.6. Surface tension and CMC determination

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

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

was obtained from the plot of surface tension against surfactant concentration.

2.7. Effect of environmental factors on biosurfactant activity

The effect of addition of different concentrations of NaCl on the activity of the biosurfactant was investigated in the cell-free broth. A specific concentration of NaCl (2–12%, w/v) was added and surface tension and emulsification activity were determined as previously stated. The cell-free broth was also maintained at a constant temperature (0, 5, 28, 70, 100 and 120 °C) for 60 min and used for surface tension and emulsification measurements. The effect of pH on surface tension and emulsification was evaluated after adjustment of the broth pH to 2, 4, 6, 8, 10 and 12 with 6.0 M NaOH or HCl.

2.8. Biosurfactant isolation

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

2.9. Biosurfactant characterization by thin-layer chromatography

After isolating the biosurfactant, a sample of 0.1 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., R_f values. Chromatograms were developed with chloroform: methanol: acetic acid (65:15:2, v/v) and the detection was done by the following methods: (1) exposure to iodine vapours for lipid stains, (2) exposure to the Molish reagent for sugar detection and (3) exposure to 1% ninhydrin solution for free amino groups. The reagents were sprayed, and the plates were heated for 30–40 min at 110 °C until the appearance of the respective colour (Deshpande and Daniels, 1995; Santos et al., 2002).

2.10. Determination of biosurfactant ionic character

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

2.11. Nuclear magnetic resonance spectroscopy

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

2.12. Fourier transform infrared spectroscopy

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

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

The fatty acids sample (hydrophobic moiety) of the biosurfactant was analysed on gas chromatograph-mass spectrometer system (Thermo Scientific Trace 1300 - ISQ Single Quadrupole) equipped with a TGMS-5 column (30 m × 0.25 mm. 0.25 µm 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 1 µl sample was injected. Helium was used as carrier gas. The injector and detector temperatures were maintained at 300 and 280 °C, respectively.

2.14. Toxicity against Artemia saline as indicator

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

2.15. Phytotoxicity assay

The phytotoxicity of the biosurfactant was evaluated in static test by seed germination and root elongation of two cabbages species (*Brassica oleracea* var. *botrytis* L. *and Brassica oleracea* var. *capitata*) according to Tiquia et al. (1996). Solutions of the isolated biosurfactant were prepared with distilled water in concentrations at ½ × CMC (300 mg/L), at the full CMC (600 mg/L) and twice the CMC (1200 mg/L). The toxicity was determined in sterilized Petri dishes (1× 10 cm) containing Whatman N° 1 filter paper. The seeds were pre-treated with Sodium hypochlorite and 10 seeds were inoculated in each Petri dish which was inoculated with 5 ml of the test solution at 27 °C. After five days of incubation in the dark, the seed germination, root elongation (≥ 5 mm) and germination index (GI, a factor of relative seed germination and relative root elongation) were determined as follows:

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

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

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

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

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

Biosurfactant suitability for enhanced oil recovery was carried out using artificially contaminated sand with 10% of motor oil as described by Luna et al. (2011). Samples of 50 g of 40/50 mesh (0.3–0.42 mm)

and 20/30 mesh (0.6–0.85 mm) fractions of the contaminated Brazilian standard sand NBR 7214 (1982) were transferred to 250-ml Erlenmeyer flasks, which were submitted to the following treatments: addition of 50 ml distilled water (control) or 50 ml of the cell-free broth or 50 ml of a solution of the isolated biosurfactant at ½ × CMC (300 mg/L), at the full CMC (600 mg/L) and twice the CMC (1200 mg/L). The samples were incubated on a rotary shaker (150 rpm) for 24 h at 27 °C and then were centrifuged at 5 000*g* for 20 min for separation of the laundering solution and the sand. The pH of the samples was also measured before and after the treatment. The amount of oil residing in the sand after the impact of biosurfactant was gravimetrically determined as the amount of material extracted from the sand by hexane.

2.17. Application of the biosurfactant in hydrophobic contaminant spreading

The oil displacement test was carried out slowly by dropping of 15 µl of motor oil onto the surface of 40 ml of distilled water layer contained in a Petri dish (15 cm in diameter) that spread all over the water surface area. This was followed with the addition of 10 µl of the cell-free broth or aqueous solutions containing the isolated surfactant at ½ × CMC (300 mg/L), at the full CMC (600 mg/L) and twice the CMC (1200 mg/L) onto the surface of the oil layer. The average value of the diameters of the clear zones of triplicate experiments was measured and recorded then calculated as percentage of the Petri dish diameter (Ohno et al., 1996).

2.18. Application of the biosurfactant in hydrophobic contaminant cleaning test

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

2.19. Statistical analysis

All surface tensions, biosurfactant concentrations and emulsification activities determinations were performed at least three times. Means and standard errors were calculated using the Microsoft Office Excel 2003 (Version 7).

3. Results and discussion

3.1. Biosurfactant production and growth kinetics

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

Fig. 1 shows the pattern of biosurfactant formation and cell growth of *P. cepacia* CCT6659 in the mineral previously optimized medium (Silva et al., 2013a) containing 2% waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$ at 28 °C during 60 h under agitation speed of 250 rpm. *P. cepacia* started to produce biosurfactant soon after inoculation along with cell growth. Surface tension measurements were used as an indirect measure of surfactant production and to evaluate the efficiency of the produced biosurfactant. The culture broth surface tension reached the minimum value of 27 mN/m after 12 h, while the accumulation of biosurfactant was gradually increasing. The maximum biosurfactant production (8 g/l) occurred during the stationary phase of the culture (48–60 h). At this point, it was observed not only the maximum biosurfactant accumulation, but also the highest biomass

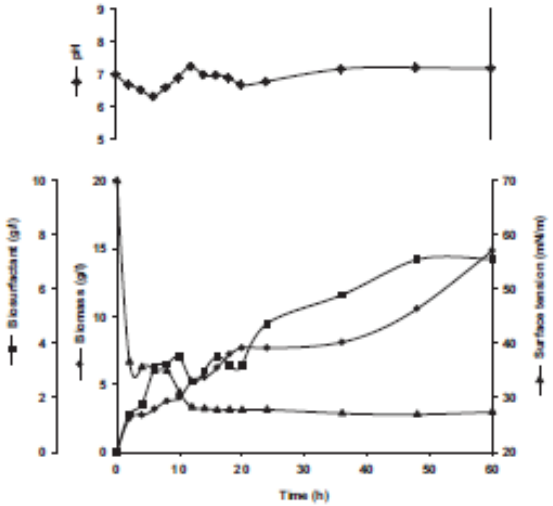

Fig. 1. Growth, pH, surface tension and biosurfactant concentration profiles of *P. cepacia* CCT6659 grown in mineral medium supplemented with 2% waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$ during 60 h at 250 rpm and 28 °C.

concentration (about 15.0 g/l dry weight). The rapid increase of biomass during the stationary phase is related to the diauxic growth phenomenon, which typically occurs when less complex nutrients sources present in the medium are depleted, forcing the microorganism to the consumption of the complex sources. The surface tension, on the other hand, remained constant because the CMC had been reached. Biosurfactant production by *P. cepacia* was growth-associated since there was an almost parallel relationship between biosurfactant production, cell growth and surface tension reduction.

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

Different kinetic profiles for biosurfactant production can be observed as described in the literature. Cha et al. (2008), for example, observed that the production of the biosurfactant from *P. aeruginosa* cultivated in an optimized medium containing 2% acidified soybean oil was found to be a function of cell growth. Biosurfactant was produced at a concentration of 5.0 g/l, with a cell concentration of 25 g/l. George and Jayachandran (2009), on the other hand, observed that the production of 9.2 g/l rhamnolipid biosurfactants using orange fruit peelings from *P aeruginosa* MTCC2297 was growth independent. A surface tension reduction up to 31.1 mN/m was obtained. The rhamnolipid production by *Pseudomonas aeruginosa* cultivated in minimal media provided with n-heptadecane as sole carbon source under shake-flask conditions started at 48 h of incubation and lasted till the end of incubation period (7 days). The surface tension of distilled water was reduced from 72.0 to 30.0 mN/m (Raza et al., 2009).

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

Wu et al. (2008) described the production of 3.7 and 2.6 g/l of biosurfactant for olive oil and soybean oil, respectively, from *P. aeruginosa* EM1, while Sousa et al. (2011) obtained 1.2 g/l biosurfactant from *P. aeruginosa* MSIC02 grown in hydrolyzed glycerin for a surface tension value of 29.3 mN/m. Oliveira et al. (2009) used experimental design tools to study the effects of process conditions on surfactant

Table 1
Emulsification index (EI) of hydrophobic substrates by the cell-free broth containing the biosurfactant from *P. cepacia* CCT6659 cultivated in mineral medium supplemented with 2% waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$ during 60 h at 250 rpm and 28 °C.

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

production during batch tests conducted using a strain of *P. alcaligenes* growing on palm oil. The authors obtained 2.3 g/l biosurfactant after 48 h of bioprocess, with a surface tension of 31 mN/m. The use of sequencing batch reactors for biosurfactant production from *P. aeruginosa* SP4 growing in palm oil and glucose during 48 h showed a surface tension reduction to 28–30 mN/m (Pansiripat et al., 2010).

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

3.2. Biosurfactant emulsification capacity

A practical measurement of a surface-active compound utility is its ability to turn immiscible liquids into stable emulsions. Table 1 presents the hydrophobic substrates tested for emulsification by the cell-free broth containing the biosurfactant from *P. cepacia* CCT6659. Motor oil of car engine was the best substrate while n-hexadecane and soybean vegetable oil were the poorest. The cell-free broth containing the biosurfactant obtained after 60 h of cultivation was able to emulsify 90% motor oil. The water-oil emulsions showed to be compact and remained stable for more than six months at room temperature, suggesting that the addition of such biosurfactant into a remediation process may enhance the availability of the recalcitrant hydrocarbon. The vegetable oils were particularly not good substrates for emulsification by the biosurfactant from *P. cepacia* (data not shown).

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

3.3. Biosurfactant stability related to surface tension and emulsification

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

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

As described in material and methods section, various amounts of NaCl were added to the cell-free broth and mixed completely and then surface tension was measured. As seen, the biosurfactant maintained

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

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

[a] Emulsification index of motor oil.
[b] Emulsification index of lubricating oil.

the capacity of reducing the surface tension up to 12% NaCl, while 80–90% of the original emulsifying activity of both hydrocarbons was retained at concentrations up to 12%. These results could be interpreted as a good salt resistance of the biosurfactant produced by *P. cepacia* under conditions of this work. Since the sea salinity in the world is around 3%, the biosurfactant from *P. cepacia* CCT6659 could be applied in saline environments.

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

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

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

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

Considering that the purification accounts for up to 60% of the total production cost of biosurfactants and the economic considerations in the oil industry, most biosurfactants would require either whole-cell

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

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

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

3.5. Biosurfactant characterization

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

The agar double diffusion method showed the anionic nature of the biosurfactant. A similar result had been observed for the biosurfactant from *P. aeruginosa* UCP0992 (Silva et al., 2010) and *P. fluorescens* 495 (Meylheuc et al., 2001), both submitted to the same test. The biosurfactant isolated from *P. cepacia* was characterized by TLC. The TLC analysis revealed the *Rf* (retention factor) value of 0.75 (Fig. 2c). The spot showed positive reactions for sugars with Molish reagents and for lipids with iodine vapours, but negative reactions for amino groups with ninhydrin, suggesting its glycolipid nature.

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

3.6. ^{1}H NMR spectroscopy

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

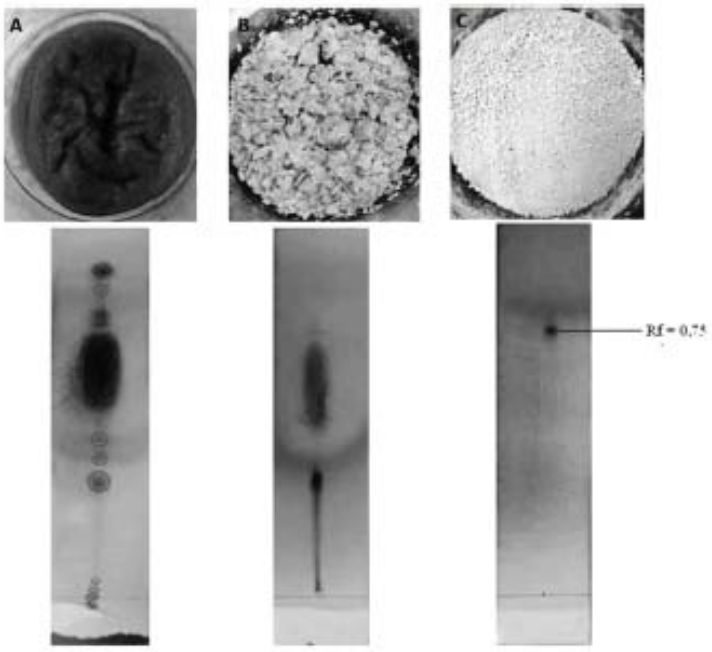

Fig. 2. TLC of the biosurfactant from *P. cepacia* CCT6659 grown in mineral medium supplemented with 2% waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$ during 60 h at 250 rpm and 28 °C. (A) Crude extract forms a viscous sticky oily residue with brown colour. (B) Crude extract after partial purification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

Table 4
Phytotoxicity of the biosurfactant from *P. cepacia* CCT6659 cultivated in mineral medium supplemented with 2% waste frying oil, 3% corn steep liquor and 0.2% on two cabbages species.

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

Table 5
Removal of motor oil adsorbed in standard sand samples by the biosurfactant produced by *P. cepacia* CCT6659 cultivated in mineral medium supplemented with 2% waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$ and by distilled water (as the control).

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

biosurfactant from *Serratia rubidaea* SNAU02 demonstrating 86% germination at a concentration of 500 mg/ml when compared with the control with regard to the vegetal species.

3.10. Application of the biosurfactant in hydrophobic contaminant removal

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

Removals in excess of 70% were observed for all solutions tested, with a maximum removal of 96% at 2 × CMC. This result can be considered good when compared to the results obtained by Rocha e Silva et al. (2014) also using a biosurfactant from *P. cepacia* (93%). Thus, the biosurfactant produced was efficient in the removal of hydrophobic compounds under kinetic conditions of agitation. It was also observed that the particle size of the sands did not exercise great influence on the percentage removal of the pollutant, neither the biosurfactant concentration, suggesting the ability of the biosurfactant to be applied in many kinds of soils and in its crude form. The possibility of using the cell-free broth can contribute to increase the use of biosurfactants in applications such as enhanced oil recovery or bioremediation, as their purification constitutes a relevant portion of the overall production costs.

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

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

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

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

Table 6
Application of the biosurfactant from *P. cepacia* CCT6659 in hydrophobic contaminant spreading and hydrophobic contaminant recovering from the walls of the beakers.

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

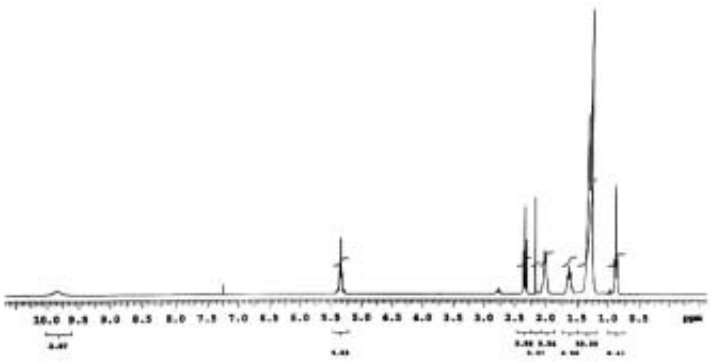

Fig. 3. ^{1}H NMR spectrum (CD_3OD, 300 MHz) of the isolated biosurfactant from *P. cepacia* CCT6659 cultivated in mineral medium supplemented with 2% waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$.

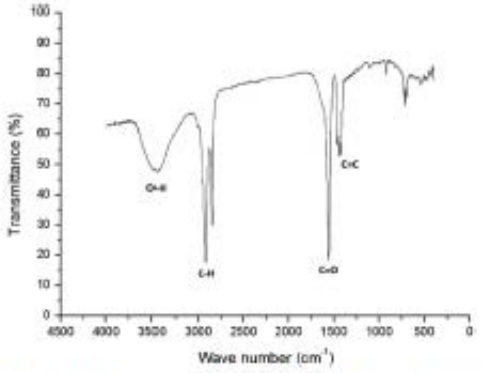

Fig. 4. FTIR spectrum for biosurfactant extract produced by *P. cepacia* CCT6659 cultivated in mineral medium supplemented with 2% waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$.

3.7. Fourier transform infrared spectroscopy

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

3.8. GC-MS analysis of fatty acids

The fatty acid composition of the biosurfactant was analysed by GC-MS and compared with the library data. It was found that the biosurfactant is mainly comprised of long chain fatty acids, mainly C-18 long fatty acids (Fig. 5). The major fatty acid found was C-18 Octadecanoic acid (89.49%). Octadecanoic acid was also previously found as the main fatty acid in various studies of purified glycolipids. Saravanakumari and Mani (2010) have isolated a biosurfactant from

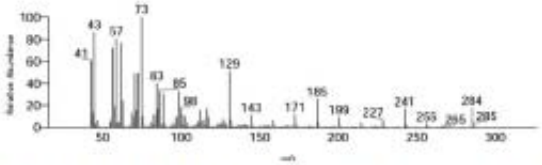

Fig. 5. GC-MS separation of the biosurfactant produced by the *P. cepacia* CCT6659 showing peaks for the octadecanoic acid.

Lactococcus lactis which also contains octadecanoic acid as a fatty acid chain linked to the sugar moiety. Octadecanoic acid was also found as the major fatty acid type in the cell bound biosurfactant produced by *Lactobacillus pentosus* (Vecino et al., 2015). Based on the results obtained by ^{1}H NMR, FTIR spectroscopy, TLC and GC-MS analysis, it is possible to say that the biosurfactant studied shows a glycolipidic nature.

3.9. Biosurfactant toxicity

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

The biosurfactant from *P. cepacia* was tested for its toxicity in a short term bioassay using brine shrimp, as shown in Table 3. The isolated biosurfactant did not show toxicity to larvae with increasing biosurfactant concentration and not even the cell-free broth after 24 hs. The acute toxicity tests of the surfactant JE1058BS produced by the bacterium *Gordonia* sp. against two species of marine larvae, *Mysidopsis bahia* (shrimp) and *Menidia beryllina* (fish), also showed the low toxicity of this biosurfactant (Saeki et al., 2009). Based on the *Artemia salina* toxicity test, *P. cepacia* biosurfactant proved to be innocuous (Table 3), as expected for a biologically derived surface-active agent (Camacho-Chab et al., 2013).

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

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

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

4. Conclusions

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

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