Programa de Doutorado da Rede Nordeste de Biotecnologia

APLICAÇÃO DO BIOSSURFACTANTE DE *BACILLUS METHYLOTROPHICUS* UCP 1616 COMO COLETOR NO TRATAMENTO DE ÁGUAS OLEOSAS EM PROCESSOS DE FLOTAÇÃO

Marcos José Chaprão

Recife-PE

2020

MARCOS JOSÉ CHAPRÃO

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Ser feliz é reconhecer que vale a pena viver
Apesar de todos os desafios,
Incompreensões e períodos de crise.
Ser feliz é deixar de ser vítima dos problemas
E se tornar um autor da própria história.
É atravessar desertos fora de si,
Mas ser capaz de encontrar um oásis
No recôndito da sua alma.

É agradecer a Deus a cada manhã pelo milagre da vida.
Ser feliz é não ter medo dos próprios sentimentos.
É saber falar de si mesmo.
É ter coragem para ouvir um “não”.
É ter segurança para receber uma crítica,
Mesmo sendo injusta.

Pedras no caminho?
Guardo todas, um dia vou
Construir um castelo...

(Fernando Pessoa)

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RESUMO

As atividades ligadas ao setor petrolífero e de energia elétrica são as principais responsáveis pela produção de águas oleosas e seu descarte, demandando estratégias de tratamento e separação desses resíduos. A Flotação por Ar Dissolvido (FAD) é um dos processos de separação água-óleo mais utilizado. Essa tecnologia utiliza em sua maioria surfactantes químicos como coletores para potencializar a separação das partículas de óleo em suspensão. Como alternativa aos surfactantes sintéticos e tóxicos, o uso de biossurfactantes vem se destacando para o tratamento de efluentes industriais, por serem biodegradáveis e apresentarem baixa toxicidade. Diante dos desafios expostos e das necessidades de desenvolvimento e aperfeiçoamento das técnicas conhecidas, o presente trabalho apresenta uma solução eficiente utilizando um biossurfactante como coletor alternativo, produzido por *Bacillus methylotrophicus* UCP 1616 em um meio de cultivo econômico composto por resíduos industriais associado a tecnologias de flotação. Foram avaliadas a produção, estabilidade frente a uma grande variação de condições (pH, temperatura e salinidade), caracterização química (FTIR e ^{1}H RMN) do biotensoativo, toxicidade frente a sementes de vegetais e a aplicação do biotensoativo na remoção de óleo em solos e águas como agente de biorremediação. Após avaliação de suas propriedades, o biotensoativo foi formulado comercialmente, através da adição de um conservante (sorbato de potássio) e com tratamento térmico a 80 °C. Após a formulação, amostras do biossurfactante foram armazenadas à temperatura ambiente por 180 dias e suas propriedades tensioativas foram testadas. O biossurfactante de *B. methylotrophicus* foi aplicado em um sistema de FAD e em uma Câmara vertical de flotação por Pré-Saturação Induzida (CPSI) em escala de bancada, como coletor alternativo, sendo adicionado a um efluente oleoso sintético com 150 ppm de óleo lubrificante em água. Foi utilizado um Delineamento Composto Central Rotacional (DCCR) para avaliar a influência das variáveis independentes (vazão do efluente e vazão do biossurfactante formulado) na eficiência de remoção de óleo nos protótipos. Amostras do efluente tratado foram coletadas para avaliação do percentual de remoção de óleo por espectrofotometria. De acordo com os resultados obtidos, o biossurfactante desmonstrou redução da tensão superficial da água de 71 mN/m para 29 mN/m e boa estabilidade frente as condições estudadas. A concentração máxima de biossurfactante atingida foi de 10,0 g/l. A biomolécula foi considerada um lipopeptídeo baseado nos resultados da caracterização química, demonstrou ausência de toxicidade e exibiu potencial para a biorremediação de solo e de água contaminados por derivados de petróleo. O biossurfactante formulado demonstrou elevada estabilidade em ambos os métodos de conservação, com tolerância em ambientes com condições extremas. A eficiência da biomolécula formulada foi demonstrada pela taxa de remoção de óleo de 92% no protótipo FAD e de 99% na CPSI em escala de bancada. Os resultados demonstraram que o biossurfactante de *B. methylotrophicus* aumenta a eficiência do processo flotação, podendo auxiliar na mitigação e no gerenciamento de efluentes industriais e contribuindo para a redução da poluição ambiental causada por hidrocarbonetos derivados do petróleo.

Palavras-chave: Biossurfactante, *Bacillus methylotrophicus*, Formulação, FAD, microbolhas.

ABSTRACT

Activities related to the oil and electric energy sector are mainly responsible for the production of oily water and its disposal, requiring strategies for the treatment and separation of these residues. Dissolved Air Flotation (DAF) is one of the most used water-oil separation processes. This technology uses mostly chemical surfactants as collectors to enhance the separation of oil particles in suspension. As an alternative to synthetic and toxic surfactants, the use of biosurfactants has stood out for the treatment of industrial effluents, as they are biodegradable and have low toxicity. In view of the challenges exposed and the needs for development and improvement of known techniques, the present work presents an efficient solution using a biosurfactant as an alternative collector, produced by *Bacillus methylotrophicus* UCP 1616 in an economic culture medium composed of industrial waste associated with flotation technologies. Production was evaluated, stability in the face of a wide range of conditions (pH, temperature and salinity), chemical characterization (FTIR and ^{1}H NMR) of the biotensoactive, toxicity to vegetable seeds and the application of the biotensoactive in the removal of oil in soils and waters as a bioremediation agent. After evaluating its properties, the biotensoactive was formulated commercially, through the addition of a preservative (potassium sorbate) and with heat treatment at 80 ° C. After formulation, samples of the biosurfactant were stored at room temperature for 180 days and their surfactant properties were tested. The *B. methylotrophicus* biosurfactant was applied in a bench scale FAD system and in a vertical Chamber of flotation by Induced Pre-Saturation (CPSI) as an alternative collector, being added to a synthetic oily effluent with 150 ppm of lubricating oil in water. A Central Rotational Composite Design (CCRD) was used to evaluate the influence of independent variables (effluent flow and formulated biosurfactant flow) on the oil removal efficiency in prototypes. Samples of the treated effluent were collected to evaluate the percentage of oil removal by spectrophotometry. According to the results obtained, the biosurfactant showed a reduction in the surface tension of the water from 71 mN / m to 29 mN / m and high stability under the conditions studied. The maximum concentration of biosurfactant reached was 10.0 g / l. The biomolecule was considered a lipopeptide based on the results of chemical characterization, demonstrated absence of toxicity and exhibited potential for the bioremediation of soil and water contaminated by oil derivatives. The formulated biosurfactant demonstrated stability in both conservation methods, with tolerance in environments with extreme conditions. The efficiency of the formulated biomolecule was demonstrated by the oil removal rate of 92% in the DAF prototype and 99% in CIPS. The results showed that the biosurfactant of *B. methylotrophicus* increases the efficiency of the flotation process, being able to assist in the mitigation and management of industrial effluents and contributing to the reduction of environmental pollution caused by oil-derived hydrocarbons.

Keywords: Biosurfactant, *Bacillus methylotrophicus*, Formulation, FAD, microbubbles.

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A/O	Água em óleo
AIP	AgênciaInternacional de Petróleo
ANP	Agência Nacional do Petróleo
BETX	Bezenos, etilbezenos, toluenos e xilenos
CMC	Concentração Micelar Crítica
CO_2	Gás Carbônico
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
TSI	Torre de Saturação Induzida

SUMÁRIO

1. INTRODUÇÃO

O uso de combustível e de óleo pesado é inevitável no setor industrial e tem causado sérios problemas socioambientais. Em muitas partes deste setor como o transporte de combustíveis, a lubrificação de motores e máquinas, a lavagem de peças, pisos e máquinas impregnadas com resíduos de óleos e graxas, ocorrem vazamentos e a descarga de efluentes oleosos, resultando em impactos ambientais cumulativos (SOARES DA SILVA et al., 2018). O constante aumento dessas atividades industriais é o principal responsável pela produção de águas oleosas e pelo seu descarte. A eliminação de efluentes só é permitida após a remoção de óleo e sólidos suspensos para níveis aceitáveis pela legislação, exigindo sistemas para o tratamento e separação desses resíduos (RADZUAN et al., 2016; CHAPRÃO et al., 2018).

Neste contexto, uma das principais técnicas que tem se destacado com sucesso no tratamento de efluentes oleosos é o processo de flotação por ar dissolvido (FAD), que consiste na separação de água e óleo através da adesão de bolhas ar que as conduzem as partículas à superfície, onde são removidas. Desta forma, permitindo uma reutilização mais eficiente e econômica das fases envolvidas no processo (ALBUQUERQUE et al., 2012; ROCHA E SILVA et al., 2018).

A flotação geralmente envolve o uso de surfactantes químicos para aumentar a aderência às bolhas de ar. No entanto, novas diretrizes para a recuperação de água restringiram o uso desses produtos químicos (ROCHA E SILVA et al., 2015).

Leis ambientais mais rigorosas levaram à busca por tecnologias sustentáveis para auxiliar na mitigação e gestão de efluentes industriais, envolvendo o uso de compostos biodegradáveis para o tratamento de locais contaminados por hidrocarbonetos (ALMEIDA et al. 2016).

A biorremediação está entre as abordagens biológicas mais amplamente estudada no tratamento de ambientes contaminados com hidrocarbonetos. A baixa solubilidade desses compostos dificulta o acesso de microrganismos e a consequente biodegradação do poluente. Uma das soluções possíveis para a baixa disponibilidade de poluentes hidrofóbicos consiste no uso de biossurfactantes, que são uma opção atraente em comparação aos seus homólogos químicos (SILVA et al., 2014b; GEETHA et al., 2018). Estes tensoativos são naturalmente adquiridos de organismos vivos, tais como saponinas derivadas de plantas, sais biliares de

animais e lipopéptidos e glicolípidos produzidos por micróbios (ROCHA E SILVA et al., 2019).

Biossurfactantes ou surfactantes microbianos são metabolitos produzidos principalmente por bactérias e leveduras. Estes compostos são formados por estruturas moleculares com uma porção hidrofílica e uma porção hidrofóbica que tendem a particionar nas interfaces entre as fases líquidas com diferentes graus de polaridade (óleo / água e água / óleo), promovendo uma redução nas tensões superficial e interfacial, que confere a capacidade de detergência, emulsificação, lubrificação, solubilização e dispersão de fases (SANTOS et al., 2016). Os biossurfactantes apresentam inúmeras vantagens sobre os surfactantes de origem química, como baixa toxicidade, biodegradabilidade, estabilidade em ampla faixa de pH e em altas temperaturas, bem como tolerância a altas concentrações salinas (ROCHA E SILVA et al., 2019; BEZERRA et al., 2018).

Bactérias das famílias Pseudomonaceae e Bacillaceae são capazes de produzir biossurfactantes que podem ser utilizados para a remoção de derivados de petróleo e derivados de petróleo. Em particular, o *Bacillus subtilis* tem sido amplamente estudado em termos de produção de biossurfactante e é bem conhecido pela eficiente produção de um lipopeptídeo com atividade superficial denominado surfactina (GUDIÑA et al., 2016).

No entanto, o alto custo de produção de biossurfactantes é um fator limitante. Estratégias que possibilitam a produção econômica e a aplicação de biossurfactantes em processos ambientais são de fundamental importância (SINGH et al., 2018). Para contornar esse problema, os pesquisadores investigam o uso de resíduos industriais com alto teor de carboidratos e / ou lipídios como substrato de baixo custo para a produção de biossurfactantes (SANTOS et al. 2016). Isso envolve a seleção de substratos, condições de cultivo ideais para um microrganismo produtor de biossurfactante, aprimoramento dos processos de purificação e conservação (MULLIGAN et al., 2014).

A estabilidade de um biossurfactante é um fator essencial para a viabilidade de armazenamento a longo prazo, especialmente para um produto biotecnológico que deve atender critérios rigorosos para sua produção e aplicação no ambiente industrial. A durabilidade precisa ser alta para manter o produto em estoque com suas propriedades iniciais, de modo que esteja prontamente disponível para uso imediato em casos de aplicação urgente na ocorrência de um derramamento de

óleo. É, portanto, de fundamental importância desenvolver estratégias que possibilitem a produção, formulação e aplicação de biossurfactantes em processos industriais (FREITAS et al., 2016; SOARES DA SILVA et al., 2018).

Diante das necessidades de desenvolvimento e aprimoramento das técnicas atualmente conhecidas, o presente trabalho dispõe soluções efetivas no tratamento e controle de resíduos oleosos na área industrial. Desta forma, avaliou-se no presente trabalho a produção, natureza química e formulação comercial de um biossurfactante produzido por *Bacillus methylotrophicus* CCT1616 cultivado em resíduos industriais. Utilizou-se este biotensoativo como coletor alternativo no tratamento de águas oleosas associado a tecnologia de flotação, constituindo assim uma alternativa promissora, evitando impactos ambientais negativos provocados pelas indústrias.

2. OBJETIVOS

2.1. OBJETIVO GERAL

Caracterizar quimicamente e formular comercialmente um biossurfactante produzido por *Bacillus methylotrophicus* para aplicar como coletor natural no tratamento de águas oleosas a partir de sistemas de flotação.

2.2. OBJETIVOS ESPECÍFICOS

ETAPA I – PRODUÇÃO DO BIOSSURFACTANTE

- Produzir um biossurfactante em meio de baixo custo pela bactéria *Bacillus methylotrophicus CCT1616*.
- Determinar as curvas de crescimento do micro-organismo e produção do biossurfactante.

ETAPA II – ESTUDO DAS PROPRIEDADES TENSOATIVAS DO BIOSSURFACTANTE

- Determinar a estabilidade do biossurfactante sob diferentes condições de temperatura, concentração de NaCl e pH, com base na tensão superficial e índice de emulsificação.
- Isolar o biossurfactante para determinar a Concentração Micelar Crítica (CMC).
- Caracterizar bioquimicamente e determinar a toxicidade do biossurfactante.
- Testar a capacidade de remoção de poluente hidrofóbico adsorvido em areia e solo pelo biossurfactante.
- Determinar a capacidade de remoção do poluente hidrofóbico adsorvido em superfície porosa.
- Avaliar o potencial do biossurfactante como agente de biorremediação de petroderivado em água do mar.

ETAPA III – APLICAÇÃO DO BIOSSURFACTANTE FORMULADO EM SISTEMA DE FLOTAÇÃO POR AR DISSOLVIDO (FAD)

- Formular o biossurfactante a fim de obter um produto estável para comercialização e avaliar sua ação a partir da tensão superficial, dispersão de manchas de derivado de petróleo e capacidade de emulsificação.
- Testar o biossurfactante formulado como coletor no tratamento de água oleosa no protótipo de FAD em escala de bancada.
- Utilizar um Delineamento Composto Central Rotacional (DCCR) como ferramenta para selecionar as condições otimizadas de separação do óleo no protótipo de FAD.

ETAPA IV – APLICAÇÃO DO BIOSSURFACTANTE EM UMA CÂMARA VERTICAL DE PRÉ-SATURAÇÃ INDUZIDA (CPSI) NO TRATAMENTO DE EFLUENTE OLEOSO

- Investigar o potencial do biossurfactante bruto, previamente formulado e na versão isolada como coletor alternativo em um novo sistema de bancada, configurado como uma câmara de flotação com pré-saturação induzida (CPSI) no tratamento de efluente oleoso.
- Comparar a eficiência do biossurfactante frente a outros coletores naturais e sintéticos no sistema com pré-saturação induzida.

3. REVISÃO DA LITERATURA

3.1. IMPACTOS AMBIENTAIS CAUSADOS POR ÁGUAS OLEOSAS

Diversas fontes de energia renovável foram desenvolvidas e propostas para reduzir a dependência de combustíveis fósseis (GEETHA et al., 2018). Nas últimas décadas, devido ao crescimento populacional e aumento das atividades industriais, problemas ambientais têm se tornado cada vez mais rotineiros, ocasionando a poluição das águas superficiais e subterrâneas (CAI et al., 2018).

Um grande número de atividades industriais, especialmente da indústria do petróleo e da produção de energia elétrica, são as principais responsáveis pela produção de águas oleosas, por exigirem grandes quantidades de óleo pesado para o seu funcionamento (SOARES DA SILVA et al., 2018).

Derramamentos envolvendo hidrocarbonetos à base de petróleo, como combustível e óleo pesado, são inevitáveis no setor industrial e têm causado sérios problemas socioambientais (KARLAPUDI et al., 2018). Em muitas partes deste sistema, como o processo de perfuração e extração do petróleo, transporte de combustível, a lubrificação de motores e máquinas, a lavagem de peças, pisos e máquinas impregnadas com resíduos de óleo, etc., ocorrem vazamentos e a descarga de efluentes oleosos, resultando em impactos ambientais cumulativos (SOARES DA SILVA et al., 2018).

Quanto à legislação ambiental, independente da forma como o óleo se apresenta, o descarte nos corpos hídricos ou até mesmo a reutilização no processo só é permitido após a remoção do óleo e sólidos suspensos em níveis aceitáveis (SILVA et al., 2014a; SILVA et al., 2018).

Os métodos adotados para o tratamento de efluentes industriais são adaptados de acordo o rítimo das atividades da indústria, o que influencia nos volumes de águas oleosas produzidos, nível de contaminação da água, limites da legislação ambiental vigente, entre outros (RAJASULOCHANA E PREETHY, 2016).

Os sistemas de tratamento de águas residuais tendem a incorporar processos naturais, biológicos, físicos e químicos. Todos os processos podem ser definidos em termos de físico-química, bioquímica (incluindo microbiologia) e a velocidade do processo. As diferenças entre as instalações baseiam-se no tipo de tecnologia

utilizada e sua intensidade, bem como sobre as possíveis combinações de tecnologias (SALGOT; FOLCH; UNIT, 2018).

3.2. BIORREMEDIAÇÃO DE AMBIENTES IMPACTADOS POR ÓLEO

Atualmente, o controle e a redução da poluição são questões críticas enfrentadas pelos cientistas ambientais devido à rápida industrialização. O impacto ambiental provocado pelo descarte das águas oleosas produzidas é geralmente avaliado pela toxicidade dos constituintes e pela quantidade de compostos orgânicos. Os contaminantes presentes nas águas produzidas podem causar diferentes efeitos sobre o meio ambiente (SILVA et al., 2014b). Muitos dos compostos encontrados na água produzida são solúveis em óleos e permanecem junto a este durante o tratamento da água. Já outros, por serem solúveis em água, são descartados juntamente com a mesma. Após o descarte, alguns contaminantes tenderão a sair enquanto outros permanecerão dissolvidos. Os pesquisadores acreditam que os compostos solúveis, após o descarte, são os mais nocivos ao meio ambiente (BARKER; JONES, 2013).

A indústria do petróleo é uma das principais responsáveis pela liberação de poluentes de hidrocarbonetos no meio ambiente. Os hidrocarbonetos policíclicos aromáticos (PAHs) são os principais poluentes liberados no meio ambiente pelas atividades de exploração das indústrias de petróleo (VARJANI et al., 2017). Eles podem acumular-se no fundo da água por longos períodos em regiões próximas a descarga de petróleo. Pouco biodegradáveis, os PAHs são praticamente inatacáveis biológicas ou quimicamente na camada anaeróbica do sedimento. Estes hidrocarbonetos, aderidos a sedimentos, têm importante papel na intoxicação crônica, produzindo efeitos irreversíveis, como mutagêneses e/ou carcinogênese nos seres que mantêm contato com eles (TORMOEHLEN et al., 2014).

A necessidade de remediar áreas contaminadas tem levado ao desenvolvimento de novas tecnologias que enfatizam a detoxificação dos contaminantes de uma forma não convencional, ou seja, sem a utilização de métodos puramente químicos ou físicos. Nesse contexto, a aplicação da microbiologia para resolver problemas de poluição por derivados de petróleo no solo e na água ganha enorme importância econômica e ambiental através da biorremediação (SILVA et al., 2014b).

A tecnologia de biorremediação tornou-se um importante método de restauração de ambientes contaminados por resíduos de petróleo, pois utiliza a capacidade dos microrganismos em biodegradar ou biotransformar as mais diversas substâncias (KARLAPUDI et al., 2018). A determinação da tecnologia de biorremediação a ser aplicada em sítio, entretanto, dependerá das características dos contaminantes e, principalmente, do local onde ocorreu a contaminação. Estas respostas podem ser obtidas através de testes em escala piloto e do estudo detalhado das características envolvidas no processo (AZUBUIKE et a., 2016).

A biorremediação de solos e águas, entretanto, encontra alguns obstáculos associados à biodegradação dos hidrocarbonetos do petróleo, uma vez que esses compostos hidrofóbicos se ligam às partículas do solo e apresentam pouca solubilidade em água, resultando em baixa biodisponibilidade para os microrganismos e consequente paralização do processo (BROWN et al., 2017). Nesse contexto, a utilização de biossurfactantes surge como a tecnologia mais investigada dos últimos anos para a resolução deste problema, permitindo a dessorção e consequente solubilização dos hidrocarbonetos, facilitando, assim, a assimilação desses compostos pelas células microbianas (SANTOS et al., 2016).

3.3. LEGISLAÇÃO AMBIENTAL

A preocupação com o meio ambiente e, em especial, com o uso dos recursos hídricos tem levado os órgãos de controle ambiental a revisar e estipular limites mais rígidos para o descarte de efluentes industriais nas legislações em vigor. No Brasil, alguns órgãos ambientais foram criados com o intuito de estabelecer e fiscalizar o descarte de efluentes, podendo ser citada a criação do Conselho Nacional do Meio Ambiente (CONAMA) em 1981, um órgão consultivo e deliberativo do Sistema Nacional do Meio Ambiente (SISNAMA). A constituição brasileira de 1988 trata em capítulo específico as questões ambientais, bem como foi estabelecido nas décadas de 80 e 90 a política nacional e o sistema de gerenciamento dos recursos hídricos (Lei 9.433/1997). A resolução CONAMA nº 357/2005 classifica os corpos de água e estabelece as condições e padrões de lançamento de efluentes. A resolução CONAMA nº 430/2011 – “Dispõe sobre as condições e padrões de lançamento de efluentes, complementa e altera a Resolução nº 357/2005, do Conselho Nacional do Meio Ambiente – CONAMA. Além disso, a descoberta de novos campos de petróleo no mundo requer o uso de

tecnologias que deem suporte ao tratamento e reutilização de efluentes industriais, visando atender à legislação sem causar impactos ambientais devido ao seu uso (ROCHA E SILVA et al., 2018). Contudo, tratar águas oleosas tornou-se uma necessidade, sejam industriais ou domésticas. Assim, pesquisas por novas e melhores alternativas de tratamento de efluentes têm sido realizadas (CONAMA, 2005).

3.4. ÁGUAS OLEOSAS INDUSTRIAIS

Considerando que a água é uma das grandes preocupações para o desenvolvimento sustentável, a resolução brasileira CONAMA nº 430 de 13 de maio de 2011, que dispõe sobre as condições e padrões de lançamento de efluentes, estabelece que o limite permitido para óleos e graxas em um efluente é de 20 mg/L (BRASIL, 2011).

Existem grandes quantidades de águas residuais oleosas produzidas pelos processos de produção industrial e de vida diária. Estas águas residuais oleosas são uma das questões ambientais mais difundidas. Estes efluentes contêm uma grande quantidade de misturas emulsificadas de óleo / água. As águas residuais oleosas não são apenas prejudiciais ao meio ambiente, mas também afetam a saúde humana (CAI et al., 2018).

A produção ou geração de águas oleosas industriais é um problema importante para muitas indústrias, uma vez que muitos tipos de efluentes tendem a formar emulsões óleo / água estabilizadas difíceis de serem tratadas, tornando-se um desafio global (ROCHA E SILVA et al., 2018).

A forma de apresentação é a principal característica que define o grau de dificuldade com respeito à separação do óleo. Muitos efluentes oleosos apresentam-se em uma emulsão. A quantidade de sólidos em suspensão, distribuição de partículas, pH, temperatura, presença de produtos químicos, densidade de óleo, a qual também exerce influência no processo de separação (ROCHA E SILVA et al., 2018).

O óleo livre representa as dispersões grosseiras constituídas por gotas com diâmetro superior a 150 μm. Nesta forma é facilmente separado da água por processos convencionais de separação gravitacional. O óleo disperso apresenta diâmetro de gotas entre 50 e 150 μm e também pode ser removido por processos gravitacionais. Entretanto, a eficiência de separação dependerá fundamentalmente

da distribuição de tamanhos de gotas e da presença de agentes estabilizantes (LIU et al., 2015). Na forma emulsificado ou suspenso o diâmetro de gotas está, frequentemente, abaixo de 50 mm. Sua separação por processos gravitacionais é difícil (o sistema é estável). Normalmente utilizam-se os processos de filtração (filtros coalescedores) e a flotação, auxiliados por agentes desestabilizantes (COUTINHO et al., 2013).

3.4.1. Emulsões óleo/água

Uma emulsão é um sistema de dispersão, no qual as gotículas de um líquido são dispersas através de outro líquido imiscível, causado pela diferença de densidade entre as gotículas de ambas as fases que as envolve (ONUKI et al., 2014; LIANG et al., 2017).

Dependendo do tipo de líquido que forma a fase contínua, as emulsões são principalmente classificadas como óleo-em-água (O/A) ou água-em-óleo (A/O). Emulsão A/O é amplamente encontrado em pintura, produtos farmacêuticos, cosméticos, alimentos e, especialmente, em indústrias petroquímicas. São necessárias grandes quantidades de desemulsificantes para separar a água destas emulsões de modo a reduzir o teor de água no óleo bruto. Os biodesemulsificantes são uma classe de agentes biológicos utilizados para separar emulsões. Em comparação com os desmulsificantes químicos, eles podem ser aplicados a uma ampla gama de emulsões de petróleo bruto complicadas, não causam poluição secundária e são resistentes a alguns reagentes químicos, alterações de pH, salinidade e alta temperatura (COUTINHO et al., 2013).

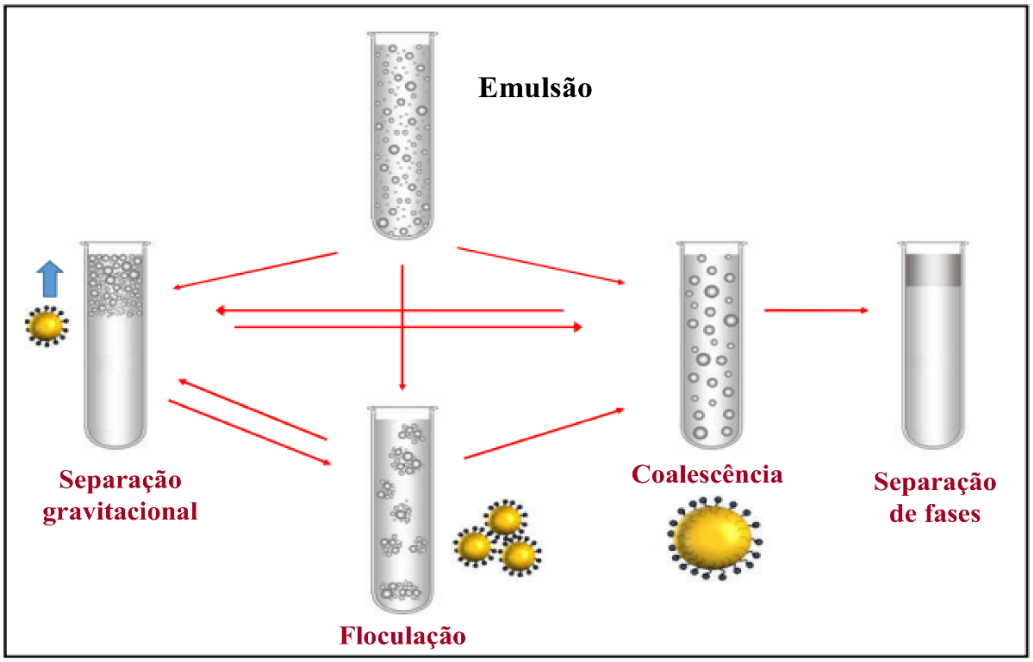
Durante o processo de produção de petróleo, a emulsificação do óleo pode se dar através do cisalhamento imposto por bombas, válvulas, constrições hidráulicas e outros equipamentos do processo. As partículas sólidas finamente divididas oriundas da própria formação produtora, assim como os produtos químicos residuais utilizados na desestabilização de emulsões água/óleo, e as moléculas surfactantes naturais do petróleo podem aumentar a proporção e a estabilidade do óleo emulsificado nas águas oleosas (RUBIO; SOUZA; SMITH, 2002).

3.4.2. Estabilidade de emulsões

A estabilidade de uma emulsão é a capacidade da mesma manter sua homogeneidade durante um certo período de tempo. Emulsões podem ser estabilizadas fisicamente ou quimicamente. Emulsões fisicamente estabilizadas são àquelas formadas sem a adição de surfactantes; a estabilidade é mantida por cargas elétricas inerentes ao sistema ou outras forças sob a influência de agentes estabilizadores (ZADYMOVA et al., 2016). Quando a água e o óleo são agitados mecanicamente, é possível produzir uma suspensão de gotículas de óleo na água - uma emulsão (WEN et al., 2016).

Uma vez formadas as gotas numa emulsão óleo-em-água durante a homogeneização, é importante mantê-las estáveis durante todo o tempo de vida esperado do produto (MCCLEMENTS, 2015). As emulsões podem tornar-se instáveis através de numerosos processos físico-químicos, que são frequentemente dependentes da natureza do emulsionante utilizado para estabilizar o sistema (Figura 1). Algumas das formas mais importantes pelas quais os emulsionantes podem influenciar a estabilidade da emulsão são delineadas abaixo, novamente com especial ênfase no comportamento dos emulsionantes naturais (MCCLEMENTS; GUMUS, 2016).

Figura 1. As emulsões óleo-em-água podem tornar-se fisicamente instáveis através de numerosos processos físico-químicos, incluindo separação por gravidade, floculação, coalescência e separação de fases



Fonte: Adaptado de MCCLEMENTS; JAFARI (2018)

3.4.3. Desestabilização de emulsões

A desestabilização de emulsões geralmente é necessária em alguns processos químicos e é particularmente importante para o tratamento de águas residuais emulsionadas. A quebra da emulsão (desemulsificação) ocorre através do rompimento das condições termodinâmicas na interface que leva ao rompimento das superfícies estáveis entre a massa e as fases internas. É, portanto, um processo importante antes do processamento de óleo a jusante, pois os agentes emulsionantes podem dificultar os processos de produção (ALMEIDA et al., 2016).

Emulsões de campo petrolífero representam um dos maiores problemas para a indústria de petróleo e são geradas em vários estágios de exploração, produção e recuperação de petróleo. Tais emulsões são frequentemente complexas e resultam da prevalência de moléculas anfifílicas no óleo, como a fração de resina contendo ácidos naftênicos e asfaltenos, além de sólidos finos, como argilas, escamas e cristais (REIS et al., 2013; ALMEIDA et al., 2016).

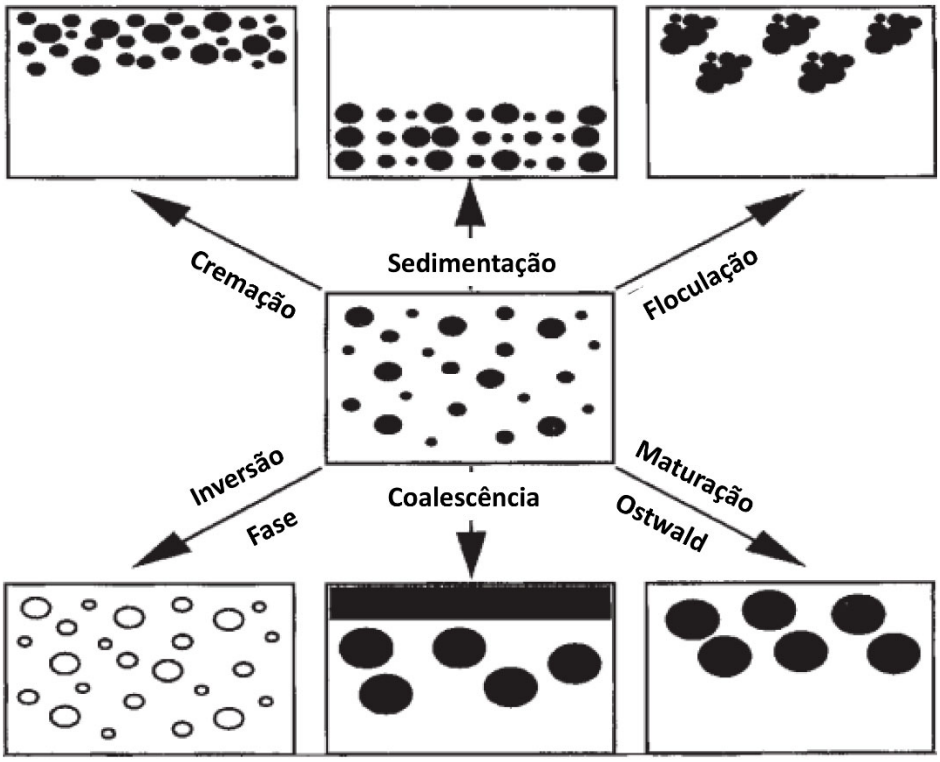
Segundo Tadros (2014), as emulsões podem ser classificadas de acordo com a natureza do emulsionante ou estrutura do sistema, onde vários processos relacionados à quebra de emulsões podem ocorrer no armazenamento, de acordo com:

- a distribuição do tamanho das partículas e a diferença de densidade entre as gotículas e a fase externa ou contínua;
- a diferença entre as forças de atração e repulsão determinam o fenômeno de floculação;
- a solubilidade das gotículas dispersas e a distribuição do tamanho das partículas, que pode ocasionar o envelhecimento de Ostwald;
- a estabilidade do líquido úmido entre as drenagens, que determina a coalescência;
- a inversão de fase, que é onde ocorre a troca entre a fase interna e a fase externa.

A desemulsificação é um processo desafiador que convencionalmente é realizado por métodos de tratamento físico, incluindo centrifugação, tratamento térmico, tratamento elétrico e / ou produtos químicos ou separação por membrana de ultrafiltração. Estes métodos são onerosos e constituem um problema de descarte, já que a maioria dos métodos químicos apresentam potencial para causar problemas ambientais (REIS et al., 2013; ZHU; GUO, 2016).

A desestabilização física de uma emulsão pode ser realizada usando quatro fenômenos diferentes: coagulação, floculação, sedimentação (creaming) e coalescência (ZHU E GUO, 2016). Estes fenômenos físicos envolvidos em cada processo de quebra não são simples, e requer uma análise das várias forças de superfície envolvidas. Além disso, os processos acima mencionados devem ser colocados simultaneamente em vez de consecutivamente, complica a análise (TADROS, 2009; 2014). Os vários processos de decomposição são ilustrados esquematicamente na Figura 2.

Figura 2. Representação esquemática dos vários processos de decomposição em emulsões



Fonte: Adaptado de TADROS (2009)

Um resumo de cada um dos processos de detalhamento acima é fornecido nas seguintes seções, juntamente com detalhes de cada processo e métodos para sua prevenção (TADROS, 2009):

- **Creaming e Sedimentação**

É um dos mecanismos de instabilidade mais comuns. Este processo resulta de forças externas, geralmente gravitacional ou centrífuga. Quando tais forças

excedem o movimento térmico do gotas (movimento browniano), um gradiente de concentração se acumula no sistema de tal forma que as gotas maiores se movem mais rapidamente para o topo (se a sua densidade for menor que do meio) ou para o fundo (se a sua densidade for maior que a do meio) do recipiente. Nos casos limites, as gotículas podem formar um (aleatório ou ordenado) na parte superior ou inferior do sistema, com o restante o volume ocupado pela fase líquida contínua.

- **Floculação**

Este processo refere-se à agregação das gotículas (sem qualquer alteração no tamanho da gota primária) em unidades maiores. É o resultado das atrações de van der Waals que são universais com todos os sistemas dispersos. A floculação ocorre quando não há repulsão suficiente para manter as gotas separadas a distâncias onde a atração de van der Waals é fraca. A floculação pode ser forte ou fraca, dependendo da magnitude da energia atrativa envolvida.

- **Envelhecimento de Ostwald**

Este efeito resulta da solubilidade definitiva das fases líquidas. Os líquidos que são referidos como imiscíveis têm frequentemente solubilidades mútuas que não são insignificantes. Com emulsões geralmente polidispersas, as gotículas menores terão maior solubilidade quando comparadas às gotículas maiores (devido a efeitos de curvatura). Com o tempo, as gotas menores desaparecem e suas moléculas se difundem para o volume e se depositam nas gotículas maiores. Com o tempo, a distribuição do tamanho das gotículas muda para valores maiores.

- **Coalescência**

Isso se refere ao processo de desbaste e ruptura do filme líquido entre as gotículas, com o resultado de que a fusão de duas ou mais gotículas ocorre para formar gotículas maiores. O caso limite para a coalescência é a separação completa da emulsão em duas fases líquidas distintas. A força motriz da coalescência é a superfície ou as flutuações do filme; isso resulta em uma abordagem próxima das gotículas, em que as forças de van der Waals são fortes e impedem sua separação.

- **Inversão de Fase**

Isso se refere ao processo pelo qual haverá uma troca entre a fase dispersa e o meio. Por exemplo, uma emulsão O / A pode, com o tempo ou mudança de condições, inverter para uma emulsão A / O. Em muitos casos, a inversão de fase passa por um estado de transição pelo qual múltiplas emulsões são produzidas.

3.4.4. Tratamento de efluentes oleosos

No Brasil, o aumento das atividades ligadas ao setor petrolífero e de energia, relacionado com a demanda energética do país e com as descobertas das reservas do Pré-Sal fez com que aumentasse também a quantidade dos efluentes oleosos gerados pelas refinarias e por usinas termoelétricas a óleo, demandando sistemas de tratamento e separação desse resíduo (ALMEIDA et al., 2016).

Frente às exigências cada vez mais rigorosas dos órgãos reguladores ambientais, o enquadramento da água oleosa gerada em diversas atividades industriais constitui-se, atualmente, em um dos maiores desafios à perfeita adequação ambiental de indústrias e prestadoras de serviços que atuam nos mais variados ramos de atividade. Para cumprimento da legislação, não basta simplesmente retirar o óleo que está presente na forma livre, sendo necessária também a remoção da quase totalidade do óleo que está presente na forma emulsionada (HENAUTH, 2015).

Em relação ao tratamento de efluentes, a busca por mecanismos que permitam reduzir a estabilidade de sistemas dispersos vem se intensificando. (YUAN; TONG; WU, 2011). As águas residuais contendo óleo podem ser tratadas através de diferentes métodos físicos, químicos e biológicos. As tecnologias de tratamento individuais disponíveis que foram relatadas incluem principalmente separação por gravidade, hidrociclone, sorção, precipitação química, flotação, filtração de membrana, oxidação química e biodegradação. Diferentes abordagens de tratamento são caracterizadas por diferentes requisitos de aplicação e padrões de tratamento (AN et al., 2017).

Existem vários separadores de óleo-água, que dependem principalmente de dois tipos de processo, separação por gravidade e separação por coalescência. O óleo e a água não são solúveis um no outro e a separação por gravidade utiliza a diferença de gravidade específica entre o óleo e a água. A separação tradicional por óleo e água geralmente depende do grande volume de água e do tamanho do

tanque. Para alcançar a alta eficiência de separação, espera-se que a separação óleo-água seja facilitada, tornando as gotículas de óleo maiores ou tornando o fluxo mais lento (AN et al., 2017).

O tratamento secundário realizado em refinarias corresponde ao tratamento biológico. Segundo Capps e Bradford (1993), o sistema de oxidação biológica é o principal processo do sistema de tratamento em efluentes contaminados com poluentes orgânicos. Os processos anteriores à oxidação biológica devem remover o óleo livre reduzindo a concentração de óleo no efluente de descarte até 50 mg/L. Outro processo crítico anterior a oxidação biológica é a equalização do fluxo que objetiva reduzir a mudança brusca das características do fluxo, como a temperatura, contaminantes, pH e salinidade (BROWN et al., 2017).

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).

3.5. FLOTAÇÃO

A flotação constitui um processo de separação sólido-líquido por gravidade, onde os sólidos presentes na suspensão são recuperados pela adesão dos mesmos a bolhas de gás (geralmente ar) como meio de transporte. Ao contrário do que ocorre na sedimentação gravitacional, o agregado (definido como agregado bolha-partícula) possui densidade menor que a densidade da suspensão. Este agregado ascende na fase aquosa permitindo, assim, a separação do óleo (DELIYANNI; KYZAS; MATIS, 2015; ROCHA E SILVA et al., 2018).

Sua utilização teve início no século passado e possui aplicação clássica no beneficiamento de minérios. Neste caso, a recuperação de espécies sólidas existentes em suspensões não homogêneas é baseada nas diferentes capacidades das partículas em suspensão de se aderirem às bolhas, permitindo uma separação seletiva. A técnica mostra-se particularmente vantajosa, em relação aos métodos tradicionais, quando a diferença entre as fases contínua e particulada é reduzida, como ocorre no tratamento de emulsões e suspensões floculentas (RUBIO et al., 2002).

Em efluentes oleosos, o princípio da flotação baseia-se principalmente na hidrofobicidade das partículas que causam a sua ligação com a bolha de ar através de força hidrofóbica. 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, sendo o soluto (matéria orgânica ou metal pesado, por exemplo), flotado para a superfície pela adição de um coletor, normalmente um surfactante apropriado, onde é recuperado no final do processo (MENEZES et al., 2011).

Sendo assim, existe uma série de produtos químicos que podem induzir ou melhorar a separação seletiva das fases. 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 alto peso molecular, sintéticas ou naturais.

A Flotação pode ser incorporada em regimes de tratamento de águas residuais, das seguintes maneiras (RUBIO; SOUZA; SMITH, 2002):

(1) Como uma unidade de processo para a remoção de contaminantes não separados por outros processos. Exemplos são encontrados na remoção de íons metálicos a partir de soluções diluídas de íons e na separação seletiva de íons valiosos.

(2) Como uma unidade de pré-tratamento antes de decantação primária, uma unidade-flash mais áspera.

(3) Como uma unidade de tratamento primário à frente de unidades de tratamento secundário, como lagoas de bio-oxidação.

(4) Como um processo de unidade para espessamento de lamas.

Os principais tipos de sistemas de flotação existentes são: eletro-flotação, flotação por ar disperso, flotação por ar dissolvido, flotação por aspersão (*nozzle*), flotação centrífuga, flotação rápida, flotação por cavitação e flotação em coluna (RUBIO *et al*., 2002).

Na eletro-flotação (EF), o princípio para a geração de microbolhas é a eletrólise de soluções aquosas com a produção de gás nos dois eletrodos. Tem aplicação em escala industrial na remoção de sistemas coloidais leves, tais como: emulsificação de óleo em água, íons, pigmentos, tintas e fibras. A vantagem desse processo é a clarificação da água e a desvantagem é a baixa quantidade de gás que flui por unidade de tempo, a emissão de gás hidrogênio, o custo do eletrodo e a manutenção e o volume de resíduo (sedimento) produzido.

Na flotação por ar disperso (FAD), as bolhas são formadas mecanicamente pela combinação de um agitador mecânico de alta velocidade e um sistema injetor de ar. Esta tecnologia faz uso da força centrífuga desenvolvida no processo. O gás (introduzido no topo) e o líquido se misturam completamente e, após passarem por um dispersor, múltiplas bolhas são formadas com tamanhos que variam de 700-1500μm de diâmetro. Este método, bastante conhecido no processo de flotação mineral, é também utilizado na indústria petroquímica para separação do sistema óleo-água.

Na flotação por ar dissolvido (FAD), as bolhas são formadas pela redução de pressão da água pré-saturada com ar a pressões mais altas que a atmosférica. A água supersaturada é forçada através de válvulas de agulha ou orifícios especiais, e nuvens de bolhas de 30-100μm de diâmetro são produzidas.

A flotação por aspersão (*nozzle*) utiliza um aspirador de gás (exaustor) para extrair ar da água reciclada, que em seguida é descarregada em um recipiente de

flotação (similar às máquinas convencionais de ar disperso) para desenvolver uma mistura de ar e água de duas fases. As bolhas formadas têm diâmetros que variam de 400-800μm. As vantagens deste processo incluem baixos custos iniciais e de consumo de energia (utiliza apenas uma bomba de ar), menor manutenção e maior tempo de vida do equipamento, porque a unidade não tem partes que se movem em alta velocidade.

Na flotação centrífuga é desenvolvido um campo centrífugo e a aeração ocorre tanto pela injeção de ar quanto pelos misturadores estáticos ou *nozzles*. O tamanho médio da bolha formada varia de 100-1000μm. O pulverizador de ar hidrociclone (ASH) pode ser classificado como a unidade de flotação centrífuga e consiste em um sistema de aeração onde o ar é pulverizado através de uma parede de um tubo poroso encamisado, havendo a formação de numerosas pequenas bolhas pelo rodamoinho de alta velocidade na fase aquosa.

Na flotação rápida, a célula de flotação apresenta um grande potencial para separação sólido/líquido e líquido/líquido no processo mineral. Sua principal vantagem é a grande quantidade de gás que flui por unidade de tempo, alta eficiência e custo moderado do equipamento. Além disso, sem partes móveis, a célula de flotação rápida tem baixo consumo de potência e baixo custo de manutenção. A célula consiste em uma zona de aeração/contato, uma zona de bolha-partícula e uma zona de limpeza ou formação de espuma. O tamanho médio da bolha formada varia de 100 a 600μm de diâmetro.

A flotação por cavitação (CAF) utiliza um aerador que extrai ar do ambiente e injeta microbolhas diretamente na água residuária. É utilizada em indústrias alimentícias, especialmente na indústria de laticínios, tintas e em curtumes, para remover sólidos suspensos, gorduras, óleos e graxas. Em relação aos diferentes equipamentos de flotação existentes para tratamento de efluentes líquidos, um dos principais é a coluna de flotação.

3.5.1. Aplicações da Flotação

A flotação é cada vez mais utilizada no tratamento de resíduos, através da introdução de novos dispositivos de flutuação superiores, levando a novas e melhores aplicações para a remediação de minerais, águas e sólidos contaminados da indústria, etc. A aplicação cruzada da flotação nas diversas áreas de interesse deve levar a procedimentos novos e aprimorados na indústria mineral e

metalúrgica, nas indústrias químicas e petrolíferas e no tratamento de águas residuais industriais e domésticas (RUBIO et al., 2007; ALBUQUERQUE et al., 2012). A Tabela 1 resume as algumas aplicações ambientais da flotação em áreas distintas.

Tabela 1. Algumas aplicações ambientais da flotação

AMBIENTE (SÓLIDO / LÍQUIDO, SÓLIDO / LÍQUIDO / LÍQUIDO OU LÍQUIDO / SEPARAÇÃO LÍQUIDA):
• Tratamento de compostos orgânicos (plantas de extração por solventes), óleos, gorduras e corantes (ágatas);
• Tratamento de efluente com metais pesados (As^{+3}, Cr^{+3} / Cr^{+6}, Cd^{+2}, Pb^{+2}, $Mn+^{2}$, Ni^{+2}, Cu^{+2}, Zn^{+2}, Se^{+2}) e ânions (CrO_4, S^{-2}, AsO_4, PO_4, MoO_4);
• Reciclagem de água (filtros): Ânions e remoção de íons de cálcio;
• Treatment of AMD – Acid Mine Drainage and water reuse.
PROCESSO INDUSTRIAL
• Separação de proteínas;
• Remoção de impurezas na indústria de cana-de-açúcar;
• Separação de óleos, gorduras, surfactantes (sabonetes), remoção de odores e resíduos sólidos na indústria de alimentos;
• Reciclagem de plásticos, pigmentos, corantes e fibras;
• Separação de tinta de papel, borracha, resinas, pigmentos de toner de impressora;
• Remoção de óleo emulsionado na indústria química e petroquímica;
• Espessamento de lodo ativado;
• Reutilização (reciclagem) de águas industriais (PET, lavagem de veículos, aviões).
OUTROS
• Remoção-separação de micro-organismos (algas, fungos, bactérias);
• Separação de metais para química analítica;
• Tratamento de solos: remoção de pesticidas, óleos e elementos radioativos;
• Tratamento de águas industriais no controle de corrosão, remoção de sabões, detergentes;
• Tratamento de águas para uso industrial e doméstico;
• Tratamento de esgoto (remoção de flocos biológicos, sólidos suspensos).

FONTE: RUBIO et al. (2007)

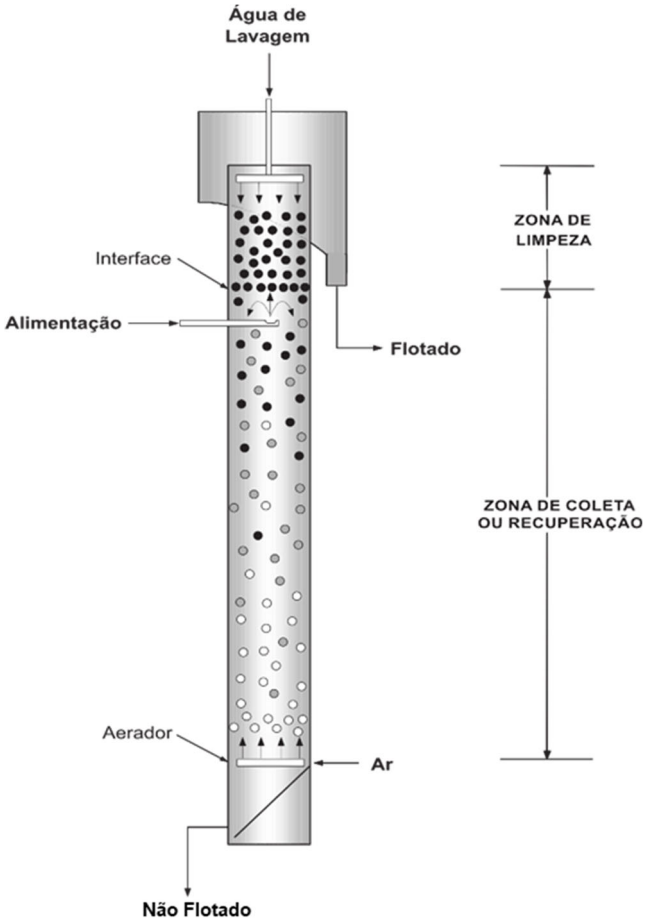
3.5.2. Flotação em coluna

As colunas de flotação surgiram da necessidade de resolver os problemas de grandes perdas de partículas de baixos teores e granulometrias na corrente de descarte de circuitos de flotação convencional. O principal motivo para essas perdas é que os equipamentos de flotação convencional geram bolhas com diâmetros de 600-3000 micrômetros, consideradas grandes para separação de partículas finas. As ineficiências do processo de flotação foram amenizadas com a criação das colunas (ALVES et al., 2017).

A coluna pode ser dividida em duas regiões principais: a de coleta e a de limpeza. A região de coleta representa a região compreendida entre o ponto de injeção de ar e o ponto de alimentação da suspensão. Nesta região ocorre a colisão entre as partículas dispersas e as bolhas, pois as partículas descendentes na suspensão entram em contato direto com as bolhas ascendentes. Se o tempo de contato for suficiente para que ocorra a adesão das partículas hidrofóbicas à superfície da bolha, tem-se a formação do agregado bolha-partícula, responsável pela separação das espécies. A região de limpeza está compreendida entre o ponto de alimentação e a adição da água de lavagem. Nesta região, as partículas não flotáveis que foram arrastadas pelas bolhas são forçadas a retornar à região de coleta, sob a ação da água de lavagem. O fluxo da água de lavagem também força a suspensão alimentada a se mover descendentemente, evitando a contaminação do produto concentrado no topo da coluna (AQUINO et al., 2010; DA LUZ et al., 2018).

O sistema de injeção de ar deve assegurar a distribuição homogênea das bolhas no interior da coluna e um tamanho de bolha uniforme, de forma a garantir as condições de estabilidade requeridas no processo. Usualmente, são utilizados dispersores internos, onde o ar atravessa um meio poroso. A Figura 3, ilustra uma coluna típica de flotação.

Figura 3. Representação esquemática de uma coluna de flotação



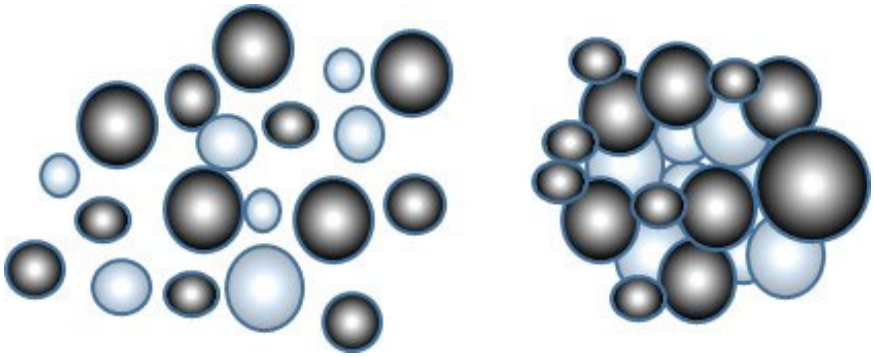
Fonte: Aquino et al., 2010

3.5.3. Flotação por Ar Dissolvido

O processo de flotação por ar dissolvido (FAD) surgiu no século passado, na primeira metade da década de vinte. A patente original do processo foi editada em 1924 na Escandinávia para Niels Peterson; Carl Sven, tendo como objetivo a recuperação de fibras da indústria do papel. Houve uma grande evolução nesse método e atualmente ele é amplamente empregado, sendo que os principais setores que utilizam este processo são as indústrias petroquímicas, de papel e de processamento de alimentos, unidades de tratamento de água potável e sistemas espessadores de lodos industriais e municipais (RUBIO *et al*., 2002).

Os princípios básicos de funcionamento da 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 (Figura 4) (SILVA et al., 2014a).

Figura 4. Incorporação das bolhas de gás nas gotas de óleo e incorporação ao floco no processo de flotação

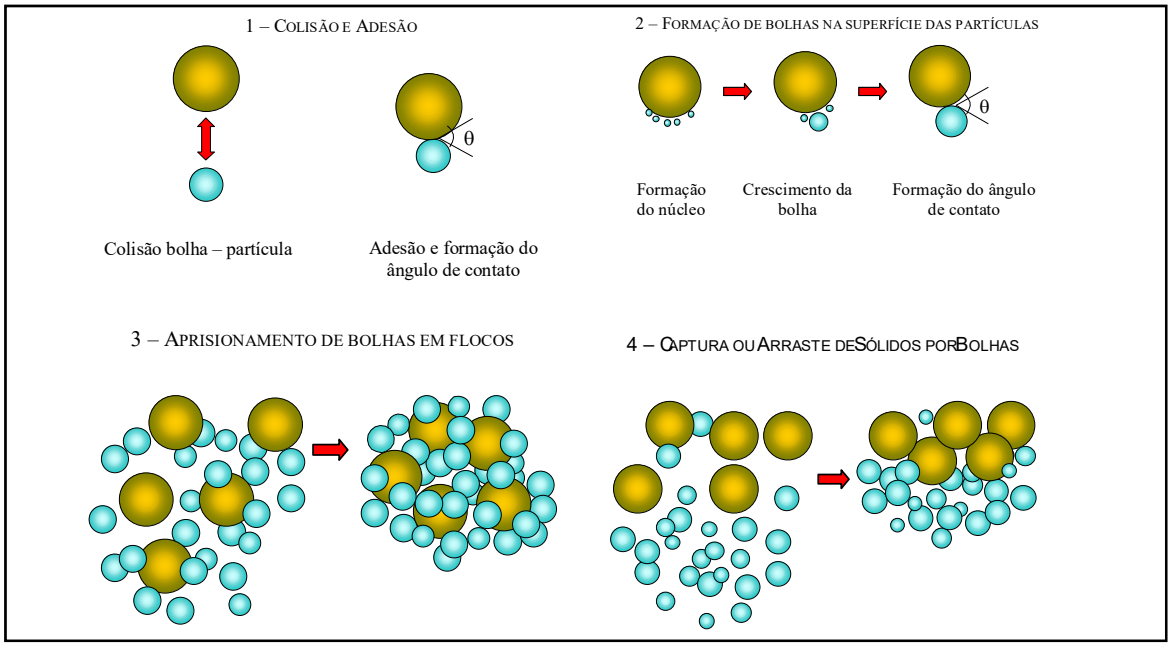


Os mecanismos da FAD resultam em elevada eficiência de coleta, visto que microbolhas são mais reativas devido a alcançar maiores áreas superficiais, cerca de quarenta vezes maior do que na flotação convencional (diâmetro de bolha 200 – 6500 μm) (RUBIO, SOUZA, SMITH, 2002; FAUSTINO et al., 2017). De acordo com Edzwald (2010), as microbolhas apresentam diâmetros entre 10 - 100 μm.

Pesquisas recentes revelaram a presença de nanobolhas geradas conjuntamente com microbolhas no processo FAD, assim como seu desempenho e contribuição na captura de poluentes da água. A flotação assistida por microbolhas e nanobolhas revela-se uma técnica promissora para o tratamento de água potável e águas residuais, bem como para a processamento de contaminantes na indústria (ETCHEPARE et al., 2016; AZEVEDO et al., 2016; CALGAROTO et al., 2016).

Na FAD com microbolhas, 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 (Figura 5) (RUBIO, 2002).

Figura 5. Fenômenos de colisão, adesão, nucleação, aprisionamento e captura de partículas e agregados por microbolhas



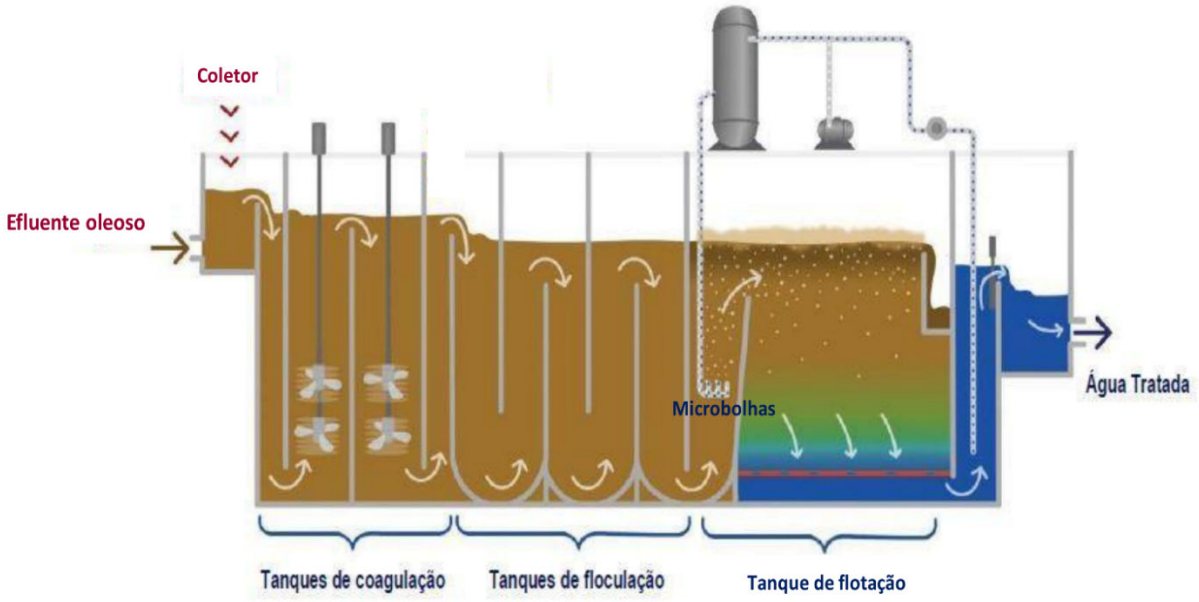
Fonte: Rubio, (2003)

Este processo de flotação é um dos mais econômicos e efetivos métodos de recuperação-remoção de sólidos, óleos emulsionados, microorganismos, 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 FAD consiste na formação e aplicação de microbolhas geradas pela despressurização e passagem forçada de um volume de água saturada com ar à pressão elevada (3 a 5 kgf/cm²), através de uma válvula de constrição de fluxo do tipo Venturi, placa de orifício ou válvula agulha (RUBIO et. al., 2002; AZEVEDO et al., 2017). As unidades (agregados) formadas por microbolhas e partículas

dissolvidas em água apresentam uma densidade aparente menor do que o meio aquoso e, dessa forma, “flutuam” ou "flotam" até a superfície de um reator (célula de flotação) ou interface liquido/ar, onde são removidos (Figura 6), (PENG et al., 2009; MENEZES et al., 2011).

Figura 6. Esquema simplificado do funcionamento da FAD



Fonte: adaptado de https://www.tratamentodeagua.com.br

Contudo é necessário observar alguns pontos essenciais para o êxito do processo, dentre os quais, os mais importantes são: a resistência do ar, o arraste e a distribuição do tamanho das bolhas, o grau de agitação, o tempo de residência das bolhas na polpa, o teor de sólidos, tamanho das partículas, a gravidade, a forma das partículas e os reagentes de flotação (ROCHA E SILVA et al., 2015). O tempo de retenção, taxa de reciclo e a tensão superficial são parâmetros fundamentais para o aumento da eficiência do processo de separação.

3.5.3.1. Tempo de Retenção

O processo de flotação dispõe de duas etapas: o tempo de acondicionamento, onde os reagentes se adsorvem sobre as superfícies dos compostos hidrofóbicos, somente sob agitação. Durante o acondicionamento ocorrem as transformações físico-químicas necessárias à adsorção dos componentes. Este parâmetro varia muito para cada sistema, podendo ser de 3 a 30 minutos para sistemas industriais.

A segunda etapa é a própria flotação, iniciada pela injeção de microbolhas na emulsão, havendo a interação do óleo com as bolhas de ar (SILVA, 2008; ARRUDA et al., 2010).

O regime hidrodinâmico, a cinética de flotação (influenciando no tempo de retenção) e a razão gás/líquido são características a serem consideradas no processo flotação. Normalmente há uma relação entre a velocidade de ascensão da bolha com o seu diâmetro, à medida que o diâmetro da bolha aumenta, maior é a velocidade de ascensão e menor seu tempo de retenção no interior do sistema de flotação, causando assim uma redução no tempo de interação com as partículas hidrofílicas em suspensão. Este parâmetro influencia diretamente no desempenho do processo de flotação (FINCH et al., 2014).

3.5.3.2. Taxa de Reciclo

A taxa de recirculação pode ser considerada um parâmetro fundamental para o bom desempenho do processo de flotação, pois está diretamente relacionada com o nível de alimentação de água bruta ou efluente no sistema. A taxa de reciclo depende intimamente do fluxo de água a ser tratada e da concentração de sólidos presentes nesta (EDZWALD, 2010).

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., 2011). Ao avaliar os parâmetros de maior influência no processo de FAD, Faustino et al. (2017), observaram que, a elevação da taxa de reciclo contribui para menores valores de turbidez residual. Porém, visto que valores excessivos de reciclo resultam no aumento do gasto energético, do consumo de água e da umidade do flotado.

3.5.3.3. Tensão Superficial

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, exercendo uma força de

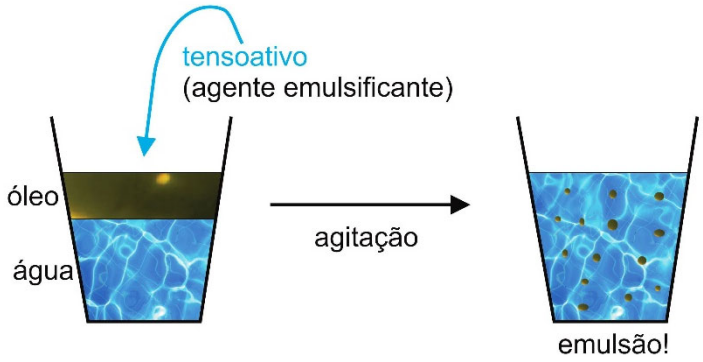
atração entre as moléculas dos líquidos. Isto acontece, porque no interior do material, átomos e moléculas estão rodeados pelo mesmo material. Já na superfície, átomos e moléculas possuem as mesmas características apenas de um lado, sendo que no outro lado possuem moléculas diferentes ou nenhuma molécula (BALL, 2006; PACWA-PŁOCINICZAK et al., 2011).

A variação da tensão superficial é um dos fatores que influem diretamente no tamanho de bolha. A utilização de coletores no processo de flotação tem como principais funções, controlar o tamanho de bolha, produzindo bolhas menores, aumentando assim a probabilidade de colisão bolha-partícula, e promover a estabilidade da espuma (SOBHY e TAO, 2013). Devido a bipolaridade o tensoativo adsorve na superfície da bolha de maneira a ficar com a parte hidrofílica voltada para a água e a hidrofóbica para a bolha. Esse processo diminui o esforço inicial do sistema para a formação de espuma, já que o mesmo tem o efeito de diminuir a tensão superficial da solução (FINCH et al., 2014). A dosagem dos coletores, geralmente com características coagulantes, está relacionada com o grau de clarificação do efluente final. Normalmente, os coletores possuem características específicas que visam facilitar a separação dos poluentes (MENEZES *et al*., 2011; SILVA et al., 2014a).

3.5.3.4. Surfactantes

Surfactantes são compostos compostos de moléculas anfipáticas com uma porção hidrofílica e porção hidrofóbica que particionam na interface óleo-água ou ar-água (SILVA et al., 2014b). A porção apolar é frequentemente uma cadeia hidro carbonada, 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 (nas quais óleos podem ser solubilizados em água ou vice – versa), formação de espuma, dispersão e detergência, o que as tornam mais versáteis em processos químicos e industriais (MAO et al., 2015; SANTOS et al., 2016; BEZERRA et al., 2018), como mostra a Figura 7.

Figura 7. Formação de microemulsões a partir da adição de um surfactante



Fonte: http://mundopoo.com.br/2017/11/11/entendendo-os-surfactantes.com.br

Surfactantes podem reduzir a tensão superficial da água. A tensão superficial consiste na força de atração existente entre as moléculas dos líquidos na sua superfície. Quanto maior a atração entre as moléculas de um líquido, maior a tensão. Dessa forma, um agente tensoativo ou surfactante tem a capacidade de quebrar essa força de atração, reduzindo assim o valor da tensão superficial das moléculas em questão. Assim, quanto menor a tensão superficial do surfactante, maior a sua efetividade, uma vez que será mais fácil a ocorrência de interação entre moléculas imiscíveis (SANTOS et al., 2016; ROCHA E SILVA et al., 2018).

A Concentração Micelar Crítica (CMC), é a concentração de surfactante na qual conjuntos de moleculas organizadas, conhecidas como micelas, são formadas e correspondem ao ponto em que o agente tensoativo atinge a menor tensão superficial estável (BEZERRA et al., 2018).

Os surfactantes são uma das importantes classes de produtos químicos, não só devido ao seu uso comum no dia-a-dia, mas também porque eles têm uma grande variedade de aplicações. A utilização dos surfactantes se concentra nas indústrias de produtos de limpeza (sabões e detergentes), petróleo, cosméticos e produtos de higiene, agricultura e saúde (VARJANI, 2017; ZHAO et al., 2018).

A produção mundial de surfactantes excede três milhões de toneladas por ano, sendo a maioria utilizada como matéria-prima para fabricação de detergentes para uso doméstico. Alguns exemplos de surfactantes iônicos utilizados comercialmente incluem ésteres sulfatados ou sulfatos de ácidos graxos (aniônicos) e sais de amônio quaternário (catiônico) (BARROS et al., 2007).

A maioria dos surfactantes disponíveis comercialmente são produzidas quimicamente a partir de derivados de petróleo. Entretanto, os problemas causados por tais agentes (geralmente tóxicos e difíceis de quebrar por meio da ação microbiana) e as novas legislações de controle do meio ambiente, têm motivado os consumidores a buscar por surfactantes naturais como alternativa aos produtos existentes (VIJAYAKUMAR; SARAVANAN, 2015; SANTOS et al., 2016). O desenvolvimento e a utilização de surfactantes totalmente biodegradáveis podem aliviar a crescente preocupação com o meio ambiente e aumentar a aceitação desta tecnologia de separação. Neste contexto, surge a utilização de surfactantes biológicos como alternativa para o aumento da eficiência de flotação.

3.6. BIOSSURFACTANTES

Os biossurfactantes (conhecidos cientificamente como biossurfactantes ou comercialmente como biodetergentes), são moléculas superficialmente ativas produzidas por organismos vivos, especialmente plantas e micro-organismos. Durante várias décadas, eles têm atraído o interesse como alternativas promissoras aos atuais surfactantes à base de petróleo (OTZEN, 2017; ROCHA E SILVA et al., 2019).

Estudos recentes mostram que os surfactantes microbianos, têm habilidade para solubilizar e mobilizar efetivamente compostos orgânicos e inorgânicos adsorvidos em solos e em águas contaminadas. Estas biomoléculas apresentam excelentes vantagens eu seu uso, como toxidade reduzida, alta solubilidade na presença de substâncias orgânicas e inorgânicas, biodegradabilidade, resistência a altas temperaturas, salinidade e pH. Essas características permitem seu uso na indústria de cosméticos, farmacêutica e de alimentos (ROCHA E SILVA et al., 2019).

A capacidade de um emulsionante para reduzir a tensão interfacial é, portanto, altamente dependente de suas características moleculares, tais como seu peso molecular e o número e localização de grupos hidrofílicos e hidrofóbicos (MCCLEMENTS; JAFARI, 2018). Estes compostos compreendem uma grande variedade de estruturas químicas, como glicolípidios, lipopéptídios, proteína-polissacarídeo complexos, fosfolipídios e ácidos graxos produzidos por micro-organismos cultivados em substratos insolúveis (óleos, resíduos e hidrocarbonetos) e substratos solúveis (hidratos de carbono) (SOARES DA SILVA et al. 2017). As

classificações dessas estruturas e os rendimentos em biossurfactante são determinados a partir da escolha do(s) substrato(s) utilizado(s) (FREITAS et al., 2016; GUDIÑA et al., 2016). Durval et al. (2019), realizaram um estudo para selecionar as melhores fontes de carbono e nitrogênio utilizadas na produção de um biossurfactante microbiano com capacidade para potencializar a degradação de óleo em água do mar.

Novos biossurfactantes com propriedades atrativas para a remoção de óleos e de metais têm sido produzidos e isolados a partir de resíduos industriais, os quais têm despertado grande interesse como alternativa de baixo custo para a produção, uma vez que a escolha do substrato possa representar uma redução de até 40% do custo total do processo (SARUBBO et al., 2015). Resíduos industriais, tais como milhocina (ROCHA E SILVA et al., 2014), glicerol (SILVA et al., 2010), vinhaça (OLIVEIRA et al., 2013), óleo soja residual (LUNA et al., 2011; RUFINO et al., 2014), óleo de canola residual (SILVA et al., 2013) gordura animal (SANTOS et al., 2013) e melaço (ALMEIDA et al., 2016), entre outros, têm sido descritos como substratos para a produção de biossurfactantes (SOARES DA SILVA et al., 2017).

Uma grande variedade de micro-organismos produz biossurfactantes, alimentando-se de substâncias que são imiscíveis em água. Muitas dessas biomoléculas são produzidas por leveduras e vêm sendo estudados com mais ênfase na última década. As leveduras *Candida lipolytica, C. sphaerica* e *C. bombicola* são as mais comumente utilizadas para a produção de biossurfactantes (LUNA et al., 2015; SILVA et al., 2014b).

As bactérias do gênero *Pseudomonas* são conhecidas por sua capacidade de degradar hidrocarbonetos e de metabolizar vários compostos orgânicos complexos. Este gênero produz grandes quantidades de raminolipídeos classificados como glicolipídeos e apresentam diversas aplicações biotecnológicas, em especial na área ambiental e na indústria de petróleo. Dentre as bactérias, *Bacillus subtilis* é outro micro-organismo bem conhecido por sua eficiência na produção de um lipopeptídeo com ótima atividade de superfície denominado surfactina (SARUBBO et al., 2015; ROCHA E SILVA et al., 2019). 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 já conhecidos (LIU; LIN; CHANG, 2015).

3.6.1. Propriedades tensoativas dos biossurfactantes

As propriedades físicas e químicas dos biossurfactantes, como redução da tensão superficial, capacidade espumante, capacidade emulsificante e estabilizante, Concentração Micelar Crítica baixa, solubilidade e poder detergente são muito importantes na avaliação de seu desempenho e na seleção de matérias-primas com potencial de produção destes agentes (SANTOS et al., 2016). Apesar da diversidade de composição química e das propriedades, algumas características são comuns à maioria dos biossurfactantes. Muitas dessas características representam uma série de vantagens sobre os surfactantes químicos convencionais (BEZERRA et al., 2018), tais como:

- Biodegradabilidade: os biossurfactantes são mais facilmente degradados na água e no solo, o que os torna adequados para aplicações na biorremediação e tratamento de resíduos (SILVA et al., 2014b).
- Compatibilidade com o ambiente e toxicidade reduzida: Os biossurfactantes oferecem mais segurança à população, sem os efeitos alérgicos apresentados pelos produtos artificiais, o que permite seus usos na indústria de cosméticos, farmacêutica e de alimentos (CAMPOS et al., 2013).
- Elevada seletividade: A presença de grupos funcionais específicos, permite especificidade nas aplicações, como a desintoxicação de poluentes específicos (KAPADIA; YAGNIK, 2013).
- Estabilidade: Demonstram atividade estável em condições extremas de temperaturas, pH e salinidade (CAMPOS et al., 2013).
- Atividade de superfície: A propriedade de maior importância para avaliar as atividades dos biossurfactantes é a medida de alterações nas tensões superficial e interfacial, bem como da estabilização ou desestabilização de emulsões e do balanço hidrofílico/lipofílico. As tensões existentes entre as fases ar/água e óleo/água são conhecidas como tensão superficial e tensão interfacial, respectivamente (SANTOS et al., 2016). A tensão superficial diminui quando a concentração de biossurfactante 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. A concentração dessas micelas forma a Concentração Micelar Crítica (CMC). Esta concentração corresponde à mínima concentração de surfactante necessária para que a

tensão superficial seja reduzida ao máximo. Quando a CMC é atingida, várias micelas são formadas. 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 (PACWA-PLOCINICZAK et al., 2011).

Outra vantagem dos biossurfactantes reside no fato de não serem compostos derivados do petróleo, fator importante à medida que os preços do petróleo aumentam. Além disso, a estrutura química e as propriedades físicas dos biossurfactantes podem ser modificadas através de manipulações genéticas, biológicas ou químicas, permitindo o desenvolvimento de produtos para necessidades específicas (NITSCHKE et al., 2011).

Todas essas características contribuem para a aplicabilidade de biossurfactantes em diferentes indústrias (ŁAWNICZAK et al., 2013; SILVA et al., 2014b).

3.6.2. Aplicação de biossurfactantes na indústria de petróleo

A indústria do petróleo e correlatas, tais como as refinarias, termelétricas a óleo e outros processos industriais em grande escala, é o maior campo de utilização dos biossurfactantes, os quais podem ser aplicados de forma eficaz em toda a cadeia de processamento do petróleo (extração, transporte e armazenagem), além de poderem ser utilizados como agentes inibidores da corrosão de equipamentos, oleodutos e tanques de estocagem, bem como coadjuvantes na formulação de combustíveis, oferecendo uma série de vantagens sobre os seus homólogos sintéticos (ALMEIDA et al., 2016).

Pesquisas e relatórios técnicos atuais já demonstraram a viabilidade da utilização de biossurfactantes no processo de recuperação microbiana melhorada de petróleo (MEOR), o qual consiste na recuperação de petróleo residual aprisionado nos capilares a partir de um reservatório empobrecido. No MEOR, os biossurfactantes diminuem as forças de capilaridade que impedem a circulação de óleo através dos poros rochosos, quebrando as películas de óleo das rochas, prolongando, assim, a vida do reservatório. O MEOR, portanto, se torna menos caro em comparação com a recuperação química, visto que os biossurfactantes utilizados no processo são mais eficientes e podem ser obtidos de micro-organismos a partir de substratos de baixo custo (SARAFZADEH et al., 2014).

O petróleo bruto precisa, eventualmente, ser transportado por longas distâncias dos campos de extração até as refinarias. Esse transporte quase sempre acarreta dificuldades operacionais que limitam a sua viabilidade econômica. Os principais problemas são baixa fluidez devido ao elevado grau de viscosidade e deposição de asfaltenos ou parafinas, que pode causar problemas com entupimentos no gasoduto (CERÓN-CAMACHO et al., 2013, ALMEIDA et al., 2016), Biossurfactantes alto peso molecular (bioemulsificantes) têm sido extensivamente estudados, neste contexto, devido à sua excelente capacidade para estabilizar emulsões óleo-em-água, os quais se ligam fortemente às gotículas de óleo, formando uma barreira eficaz que evita a coalescência da gota, ajudando na redução da viscosidade durante o transporte em gasoduto. Dentre os bioemulsificantes mais potentes para esta finalidade, o Emulsan tem se destacado na literatura como a opção mais promissora (PERFUMO et al., 2010).

Grandes quantidades de petróleo e óleos combustíveis também são armazenadas diariamente em tanques de armazenamento. A manutenção destes tanques requer lavagem periódica. No entanto, os resíduos e as frações pesadas do petróleo que se acumulam no fundo e nas paredes dos tanques de armazenamento são altamente viscosos e se tornam depósitos sólidos que não podem ser removidos com bombeamento convencional (ALMEIDA et al., 2016). A remoção deste material requer lavagem com solventes e limpeza manual, que requer uma mão de obra intensiva, procedimento caros, perigosos e demorados (ROCHA E SILVA et al., 2019). O uso de biossurfactantes microbianos neste processo tem se mostrado um procedimento alternativo de limpeza bastante promissor para diminuir a viscosidade desses depósitos, por meio da formação de emulsões de óleo-em-água, facilitando o bombeamento dos resíduos e permitindo a recuperação posterior do óleo quando a emulsão é quebrada, destacando-se, para esta finalidade, os biossurfactantes produzidos pelas linhagens *Gordonia* sp., *P. aeruginosa* SH 29 e *P. cepacia* CCT6659, dentre outras (MATSUI et al., 2012; ROCHA e SILVA et al., 2014).

3.6.3. Biossurfactantes como coletores na flotação

No Brasil, o aumento das atividades ligadas ao setor petrolífero e de energia, relacionado com a demanda energética do país e com as descobertas das reservas do Pré-Sal fez com que aumentasse também a quantidade dos efluentes oleosos

gerados pelas refinarias e por usinas termoelétricas a óleo, demandando sistemas de tratamento e separação desse resíduo (ALMEIDA et al., 2016). Frente às exigências cada vez mais rigorosas dos órgãos reguladores ambientais, o enquadramento da água oleosa gerada em diversas atividades industriais constitui-se, atualmente, em um dos maiores desafios à perfeita adequação ambiental de indústrias e prestadoras de serviços que atuam nos mais variados ramos de atividade. Para cumprimento da legislação, não basta simplesmente retirar o óleo que está presente na forma livre, sendo necessária também a remoção da quase totalidade do óleo que está presente na forma emulsionada (HENAUTH, 2015).

Nesse contexto, uma das principais técnicas de separação água-óleo que vem se destacado com sucesso no país é o processo de flotação por ar dissolvido (FAD), que é comumente utilizado no tratamento de águas oleosas de processos industriais (ALBUQUERQUE et al., 2012).

Esses efluentes podem ter óleo em sua forma livre ou na forma de uma emulsão (ROCHA E SILVA et al., 2018). O composto solubilizado em água é difícil de remover e requer processos químicos especiais, como a extração com solventes e / ou uso de tratamento biológico (JAMALY; GIWA; HASAN, 2015). O óleo emulsionado requer o uso de processos mais sofisticados. Nesses casos, a flotação deve ser combinada com métodos auxiliares, como a adição de compostos tensoativos (ZADYMOVA et al., 2016).

Processos de flotação é geralmente auxiliado pela adição de coletores, utilizando surfactantes sintéticos apropriados capazes de possibilitar maior interação entre fluidos incompatíveis (SARUBBO et al., 2015). Estes coletores possuem características específicas que visam promover a adesão à fase dispersa e facilitar a separação dos poluentes (MENEZES et al., 2011). Os surfactantes são capazes de quebrar as forças de atração entre as moléculas de água, reduzindo a tensão superficial entre as duas fases e permitindo, assim, uma maior interação entre líquidos incompatíveis (SARUBBO et al., 2015). Entretanto, o uso de surfactantes sintéticos nesses processos de separação, embora tenha como principal objetivo o controle da poluição, tem sido questionado devido à toxicidade desses coletores químicos (ALMEIDA et al., 2016).

Assim, os biossurfactantes surgem como uma excelente opção sustentável aos coletores sintéticos na flotação por ar dissolvido, uma vez que esses compostos naturais aumentam a eficiência do processo e reduzem significativamente os

impactos na saúde humana e no meio ambiente, dando uma maior credibilidade à flotação como método de separação (ROCHA E SILVA et al., 2019). A Tabela 2 apresenta algumas aplicações industriais de biossurfactantes em sistemas de FAD.

Tabela 2. Emprego industrial de biossurfactantes em sistemas de FAD

Micro-organismo/Tipo do biossurfactante	**Aplicação**	**Referências**
Pseudomonas cepacia e CCT 6659 e Bacillus cereus UCP 1615	separação das fases óleo/água	SILVA et al., 2018
Candida lipolytica UCP0988 e *Candida sphaerica* UCP0995/ Sophorolipídeos	Remoção de metais pesados Fe e Mn	MENESES et al., 2011
Candida sphaerica UCP0995/Sophorolipídeos	Separação água/óleo	ROCHA E SILVA et al., 2015
Pseudomonas aeruginosa/ Rhaminolipídeos	Remoção de metais pesados Cr e Fe	ABYANEH; FAZAELIPOOR, 2016
Bacillus e Pseudomonas / Lipopeptídeos e glicolipídeos	separação das fases óleo/água	COSTA, 2016
Saponina e Raminolipídeo	Melhoramento do desempenho hidrodinâmico da flotação	WANG, 2013
Lactobacillus pentosus/ glicolipídio	Agente espumante na flotação	VECINO et al., 2013
Pseudomonas aeruginosa ZJU211 / Mistura de Mono e di raminolipídeos	Tratamento do resíduo oleoso após a flotação	LONG et al., 2013

Fonte: Autor da tese (2020)

Rocha e Silva et al. (2015), utilizaram um biossurfactante obtido a partir do micro-organismo *Candida sphaerica* e demonstraram que o biotensoativo potencializou a eficiência de separação de material oleoso de um sistema FAD em escala piloto, de 80,0% para 95,0%, tornando a técnica de flotação uma tecnologia

mais limpa e eficaz na separação de óleo-água. Recentemente, Silva et al. (2018) investigaram a separação do óleo em água usando um sistema de FAD, com e sem o uso de biossurfactantes produzidos por *Pseudomonas cepacia* e *Bacillus cereus* nas mesmas condições. Os biossurfactantes aumentaram a eficiência de separação água/óleo de 53,74% (utilizando apenas microbolhas) para 94,11 e 80,01%, respectivamente. Chaprão et al. (2018), utilizando um biossurfactante microbiano em um protótipo horizontal (DAF), alcançou uma taxa de 92% de remoção de óleo em um efluente sintético.

Nesse sentido, demonstra-se o potencial de utilização desses agentes biotecnológicos para aumentar a eficiência dos processos de separação de poluentes orgânicos e inorgânicos gerados nas indústrias.

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4. ARTIGOS DERIVADOS DA TESE

4.1. CAPÍTULO 1 - Production of a biosurfactant from *Bacillus methylotrophicus* UCP1616 for use in the bioremediation of oil-contaminated environments

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Production of a biosurfactant from *Bacillus methylotrophicus* UCP1616 for use in the bioremediation of oil-contaminated environments

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Abstract

The aim of the present study was to produce a microbial biosurfactant for use in the bioremediation of environments contaminated with petroleum products. *Bacillus methylotrophicus* was isolated from seawater taken from a port area and cultivated using industrial waste as substrate (corn steep liquor and sugarcane molasses [both at 3%]). Surface tension measurements and motor oil emulsification capacity were used for the evaluation of the production of the biosurfactant, which demonstrated stability in a broad range of pH and temperature as well as a high concentration of saline, with the reduction of the surface tension of water to 29 mN/m. The maximum concentration of biosurfactant (10.0 g/l) was reached after 144 hours of cultivation. The biosurfactant was considered to be a lipopeptide based on the results of proton nuclear magnetic resonance and Fourier transformed infrared spectroscopy. The tests demonstrated that the biosurfactant is innocuous and has potential for the bioremediation of soil and water contaminated by petroleum products. Thus, the biosurfactant described herein has a low production cost and can be used in environmental processes.

Keywords: surfactant; *Bacillus*; bioavailability; bioremediation; industrial waste.

Introduction

Spills often occur during oil exploration and transport and cause serious environmental problems. Mechanical recovery with the use of sorbents is a promising oil removal method and involves the transference of oil from a contaminated area to a temporary storage facility. However, the majority of sorbents used in this process end up in landfills or incinerators and are therefore an additional source of pollution, the treatment of which translates to an increase in the cost of the oil recovery method (Almeida et al. 2016).

Stricter environmental laws have led to the search for sustainable technologies involving the use of biodegradable compounds for the treatment of hydrocarbon-contaminated sites. For such, surfactants acquired from living organisms have been tested, such as plant-derived saponins, bile salts from animals and microbial-produced lipopeptides and glycolipids. These natural compounds with surfactant properties are denominated biosurfactants (Campos et al. 2013).

Biosurfactants have hydrophilic and hydrophobic moieties that act between fluids with different polarities (such as oil and water), enabling access to hydrophobic substrates through an increase in the area of contact of insoluble compounds as well as enhanced mobility and bioavailability, leading to the biodegradation of these substrates. These features enable biosurfactants to lower both surface and interfacial tension as well as form microemulsions by which hydrocarbons can be solubilised in water or vice versa. Therefore, biosurfactants have applications in industries due to their properties of detergency, lubrication, emulsification, solubilisation, foaming capacity and phase dispersion (Almeida et al. 2016).

Studies report the potential for the use of biosurfactants, such as a lipoprotein produced from *Bacillus subtilis* known as surfactin and a group of glycolipids produced by the bacterium *Pseudomonas aeruginosa* known as rhamnolipids. Although extremely efficient, these two types of biosurfactant are expensive due to the substrates employed in their production and the level of purity required for applications in the pharmaceutical and medical fields (Santos et al. 2016b).

The advantages biosurfactants have over synthetic surfactants include low toxicity and stability in the presence of high temperatures, a broad pH range and high concentrations of saline. These characteristics contribute to the applicability of these biomolecules in different steps of the petroleum production chain (Almeida et

al. 2017a). Thus, natural surfactant compounds constitute a sustainable option for enabling the dispersion and solubilisation of hydrocarbons and facilitating the assimilation of these compounds by microbial cells (Silva et al. 2014).

However, the high production cost of biosurfactants is a limiting factor. To circumvent this problem, researchers have investigated the use of industrial waste with high carbohydrate and/or lipid content as low-cost substrate for the production of biosurfactants (Santos et al. 2016b). Among the industrial waste used for this purpose, the literature describes corn steep liquor (Rocha e Silva et al. 2014), glycerol (Silva et al. 2010), residual soybean oil (Luna et al. 2016), animal fat (Santos et al. 2016a; 2017), vegetable fat (Gusmão et al. 2010) and molasses (Almeida et al. 2017a).

Strategies that enable the low-cost production and application of biosurfactants in environmental processes are of fundamental importance. This involves the selection of optimum substrates and cultivation conditions for a biosurfactant-producing microorganism and the improvement of purification processes (Santos et al. 2016b). Bacteria of the genus *Bacillus* are some of the main working tools for biotechnological applications. These bacteria produce a variety of products, such as extracellular enzymes, biopolymers, biopesticides and biosurfactant, from renewable resources (Joshi et al. 2013). Moreover, according to the US Food and Drug Administration, the products from these bacteria as “Generally Regarded as Safe”.

Thus, the aim of the present study was to describe the production kinetics, characterisation and stability of a biosurfactant produced by *Bacillus methylotrophicus* cultivated with industrial waste and adequate culture conditions. The biosurfactant in question has potential application as an adjuvant in

bioremediation processes of hydrophobic pollutants generated by the petroleum industry.

Materials and methods

Identification of microorganism

The microorganism was isolated from a port area contaminated with complex hydrocarbons stemming from nautical activities in the city of Recife, Brazil (08° 03 '14 "S, 34° 52' 52" W). The continental waters of the region have a surface temperature of 27 to 28 °C. Salinity is close to 3% due to the influence of coastal rivers (estuarine area). Seawater samples were collected from the surface layer and placed in sterile plastic containers for transportation to the laboratory. Sea water samples were subjected to a serial dilution process. Aliquots of dilutions were placed in Petri dishes containing solid medium formulated with (per liter): 15.0 g Agar, 2.5 g yeast extract, 1.0 g glucose and tryptone 5.0 g, pH 7.0. The dishes were incubated at 28 °C for 24 hours. Cultures were stored at 4 °C to maintain viability and preserved in glycerol at -80 °C.

The isolate was submitted to genomic deoxyribonucleic acid (DNA) extraction with the DNeasy Blood and Tissue Kit (Qiagen) using the technical procedures recommended by the manufacturer. The polymerase chain reaction (PCR) method was applied to the genetic material from the microorganism using universal oligonucleotides (forward 5’-AGAGTTTGATCATGGCTCAG-3’; reverse 5’-GGTTACCTTGTTACGACTT-3’) (Skwor et al. 2014), which amplify a fragment (approximately 1500 bp) of the 16s ribosomal RNA (rRNA) coding region (Srinivasan et al. 2015).

The reactions were performed with the aid of the PCR Master Mix [1x] (Promega) using 100 ηg of the target DNA and 20 pmol of each oligonucleotide in a final volume of 25 µl. The GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) was used for the amplification reactions, which consisted of initial denaturation at 95°C for 5 minutes. This was followed by 30 cycles of denaturation for 45 seconds at 94°C, annealing of the oligonucleotides for 45 seconds at 52°C and extension for 1 minute at 72°C (each cycle). Final extension was performed for 6 minutes at 72°C.

The amplicons were submitted to agarose gel electrophoresis, purified with the aid of the ExoSAP-IT® PCR Product Cleanup (Affymetrix) and sequenced in the ABI Prism 3100 (Applied Biosystems). The Basic Local Alignment Search Tool (BLAST) was used to compare the sequences to those found in the GenBank database.

Culture media

Nutrient agar was used for the maintenance of the bacteria and had the following composition (per litre): meat extract 5.0 g, peptone 10.0 g, NaCl 5.0 g, Agar 5.0 g, pH 7.0. Nutritive broth was used for the growth of the inoculum and had the following composition: meat extract 5.0 g, peptone 15.0 g, NaCl 5.0 g, K_2HPO_4 5.0 g, pH 7.0.

The mineral medium described by Bushnell & Haas (1941) (1.0 g of KH_2PO_4, 1.0g of K_2HPO_4, 0.2 g of $MgSO_4.7H_2O$, 0.2 g of $CaCl_2.H_2O$ and 0.05 g of $FeCl_3.6H_2O$) was used for biosurfactant production. The carbon source was molasses and the nitrogen source was corn steep liquor, both at a concentration of 3% (Chaprão et al. 2015). The constituents were solubilised and sterilised in an autoclave at 121ºC for 20 minutes. The pH of the medium was adjusted to 7.0. Surface tension was 56 mN/m prior to inoculation.

Corn steep liquor (20 to 26% lactic acid, 21 to 45% protein, 8% ash, 3% sugar and 0.9 to 1.2% fat) was obtained from Corn Products do Brasil (Cabo de Santo Agostinho, PE, Brazil). Sugarcane molasses (75% dry matter, 9 to 12% non-sugar organic matter, 2.5% protein, 1.5 to 5.0% potassium and 1% phosphorus, calcium and magnesium [Santos et al. 2016b]) was obtained from a processing plant in Vitória de Santo Antão, Brazil.

Preparation of inoculum

After 24 hours in the nutrient agar medium, fresh cultures were transferred to Erlenmeyer flasks containing 50 ml of nutrient broth. Orbital stirring was performed at 150 rpm and 28°C for 10 to 14 hours until reaching an optical density of 0.7 (corresponding to 10^7 colony-forming units/ml) at 600 nm. This reading was used with the inoculum at a concentration of 3% (v/v).

Biosurfactant production

One-litre Erlenmeyer flasks containing 500 ml of the production medium were incubated with the inoculum (3%) to obtain the biosurfactant through fermentation. The flasks were kept under orbital stirring at 200 rpm for 144 hours at a temperature of 28ºC. Throughout the culture process, aliquots were withdrawn for the determination of biomass, surface tension, pH and biosurfactant yield. The stability of the biomolecule was determined after 144 hours of cultivation by varying temperature, pH and the concentration of NaCl, as described in the following sections.

Determination of biomass and pH

For the dry weight determination of biomass, 100 ml of culture were centrifuged at 4500 x g for 20 minutes at 10ºC and the supernatant was discarded. The cell pellet was washed twice with cold distilled water to remove residual culture medium and centrifuged again. The biomass was then dried at 50°C for 24 hours. Dry weight was determined by gravimetry on an analytical scale and pH was determined using a potentiometer.

Determination of surface tension

An automatic surface tensiometer (Sigma 700, KSV Instruments LTD, Finland) with a Du Nuoy ring was used to determine surface tension and the critical micelle concentration (CMC), achieving values of 29 mN/m and 0.5% (w/v), respectively, as described by Chaprão et al. (2015).

Emulsification index

For the determination of the emulsification index (EI) (Cooper and Goldenberg, (1987), 2 ml of motor oil were added to 2 ml of the cell-free broth and vortexed for 2 min at high speed. The EI was calculated after 24 h [(height of emulsion layer/total height of mixture) x 100] at different temperatures (0, 5, 70, 100 and 120ºC), pH values (2, 4, 6, 8, 10 and 12) and concentrations of NaCl (2.0, 4.0, 6.0, 8.0 and 10.0%).

Biosurfactant stability

The cell-free broth was submitted to different temperatures, pH values and concentrations of NaCl to determine the stability of the surfactant activity. For such, surface tension and emulsification activity were measured as described above (Silva

et al. 2017).

Isolation of biosurfactant

The method described by Silva et al. (2010) was used to isolate the biosurfactant from the cell-free broth. The pH of the supernatant was adjusted to 2.0, followed by the addition of an equal volume of $CHCl_3/CH_3OH$ (2:1). After shaking 15 min, the mixture was left to stand until the separation of the phases. The procedures were repeated twice. A rotary evaporator was used to concentrate the product from the pooled organic.

Chemical composition of biosurfactant

The Labtest kit (Labtest Diagnóstica, SA, Brasil) was used for the determination of the protein concentration in the isolated biosurfactant. The phenol-sulfuric acid method was employed for the determination of carbohydrates, using D-glucose as the standard. Lipid extraction was performed with $CHCl_3/CH_3OH$ (1:1 and 2:1, v/v). Following evaporation of the organic extracts under vacuum, the lipid content was determined using the gravimetric method (Manocha et al. 1980).

Determination of ionic charge of biosurfactant

The double diffusion method was used to determine the ionic charge of the biosurfactant. Wells were made in uniformly spaced rows in 1% agar. The biosurfactant was placed in some wells. Sodium dodecyl sulphate (20 Mm) as the anionic substance and barium chloride (50 mM) as the cationic substance were placed in others. Precipitation lines indicating the ionic nature of the biosurfactant were monitored for a period of 48 hours (Silva et al. 2010).

Nuclear magnetic resonance spectroscopy

The extracted biosurfactant was dissolved in deuterated chloroform (CDCl3) for the determination of the respective ^{1}H NMR spectra. (The spectrometer (Agilent 300Mz) operated at 300.13 MHz) and readings were performed at 25°C. Chemical displacement (δ) in comparison to tetramethylsilane was determined on a ppm scale.

Infrared spectroscopy

Fourier transformed infrared (FTIR) spectroscopy (FTIR 400, Perkin Elmer) was used for the characterisation of the extract of the biosurfactant recovered from the supernatant of the *B. methylotrophicus* UCP 1616 isolate (resolution: 4 cm^{-1} in the wave number region from 400 to 4000 cm^{-1}.

Application of biosurfactant for removal of hydrophobic pollutant adsorbed to soil and sand in flasks

The removal of motor oil from contaminated soil was evaluated by saturating 50 g of *in natura* sand and sandy soil (50% sand, 48% clay and 2% silt) with a 10% motor oil solution (Rufino et al. 2013) in the laboratory. The soil was placed in 250-ml Erlenmeyer flasks with 50 ml of the cell-free broth (crude biosurfactant). Distilled water (50 ml) was used as the control. After shaking at 200 rpm and 28ºC for 24 hours, the supernatant was removed and the broth was discarded. Hexane was used to extract the residual oil from the soil, which was then weighed (Luna et al. 2011).

Removal of hydrophobic compound adsorbed to porous surface

Marine rocks (coral reef fragments) were collected from Suape Beach in the city of Ipojuca, Brazil (mean pore size: 230 μm to 520 μm; approximate porosity: 72%). The material was soaked in residual motor oil and the volume required for complete coverage was recorded. Each rock was then washed with the cell-free broth in a 100-ml beaker. Following extraction with hexane, gravimetry was used to determine the amount of residual oil on the rocks for the calculation of the removal rate (%).

Bioremediation test

The method described in the Standard Methods for the Examination of Water and Wastewater (APHA, 2005) was used for the bioremediation tests. One hundred ml of fresh seawater obtained from the Suape Petrochemical Complex in the state of Pernambuco, Brazil, were placed in 250-ml Erlenmeyer flasks with 1.0% motor oil and isolated biosurfactant solutions at concentrations of 0.25% (½ CMC) and 0.5% (CMC). Incubation was performed in an orbital shaker at 150 rpm and 28°C. After one, seven, 14, 21 and 28 days, samples were analysed and the most probable number method was used to estimate the quantity of microorganisms.

Phytotoxicity test

Following the method as described by Tiquia et al. (1996), seed germination and root growth tests were performed with the cabbage *Brassica oleracea* (var. *capitata*) to determine the phytotoxicity of the biosurfactant. Isolated biosurfactant solutions at concentrations of 0.25% (½ CMC) and 0.5% (CMC) in distilled water were prepared. Pure distilled water was used as the control. After incubation in the absence of light, seed germination, root growth (≥ 5 mm) and the germination index (GI) were calculated.

Statistical analyses

Surface tension, stability and emulsification 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 ($p < 0.05$). The Statistica® program, version 12.0 was used for the statistical analyses.

Results and discussion

Identification of microorganism

The reduction in biosurfactant production costs depends on the identification of low-cost substrates and microorganisms with adequate genotypic and phenotypic characteristics for the production of active biomolecules (Banat et al. 2014). In the present study, the isolate was identified based on 16S rRNA sequencing, which revealed that it a member of the genus *Bacillus*, with maximum similarity to the 16S rRNA sequence for *Bacillus methylotrophicus* (99% similarity of the partial sequence). After molecular identification, the microorganism isolated from a port region was deposited in the Culture Bank of the *Núcleo de Pesquisas em Ciências Ambientais* (NPCIAMB) [Environmental Science Research Centre] at the *Universidade Católica de Pernambuco* [Catholic University of Pernambuco] and registered in the World Federation Culture for Collection (WFCC), catalogued as UCP 1616.

According to Jennings and Tanner (2000), biosurfactant-producing microorganisms are found in hydrocarbon-contaminated soil and bacteria that produce biosurfactants account for up to 35% of aerobic heterotrophs. A large number of reports are found in the literature on the isolation of *B. licheniformis* from oil reservoirs (Joshi et al. 2013). Petroleum-degrading bacteria are found in similar

habitats and produce biosurfactants, due to the considerable hydrocarbon content in the surrounding environment. Indeed, hydrocarbon-contaminated environments constitute a favourable habitat for biosurfactant-producing microorganisms, many of which belong to the genus *Bacillus*.

Microorganism growth curves and biosurfactant production

During the cultivation of *B. methylotrophicus* in the mineral medium with 3.0% sugarcane molasses and 3% corn steep liquor for 144 hours, the surface tension was reduced from 56 mN/m to approximately 29 mN/m during the exponential growth phase, with peak biosurfactant production (approximately 5.5 g/l) occurring after 24 h of growth, followed immediately by the stationary growth phase. The maximum concentration of biosurfactant was 10.0 g/l, which was achieved in the exponential growth phase of the microorganism, followed by a reduction in the stationary growth phase (beginning at 40 hours), which may be related to the consumption of the biosurfactant due to the scarcity of nutrients in this phase of the growth curve. The stability of the surface tension, on the other hand, can be attributed to the saturation of the medium surface by surfactant molecules. Therefore, biosurfactant production by *B. methylotrophicus* is associated with growth, as a nearly parallel relationship was found among biosurfactant production, cell growth and the reduction in surface tension.

These results are in agreement with data described by Rocha e Silva et al. (2014) using industrial waste for the production of 5.2 g/l of a bacterial biosurfactant, which achieved a surface tension of 27.57 mN/m after 144 hours of cultivation at 250 rpm using corn steep liquor and waste frying soybean oil. Evaluating a biosurfactant produced by *Bacillus subtilis* in a medium supplemented with corn steep liquor, Gudiña et al. (2015) report the best surface tension results (29.70 to

31.00 mN/m) after 48 and 72 hours of cultivation, indicating that production of the biomolecule occurred in both the exponential growth phase and stationary phase. Soares da Silva et al. (2016) report similar results in the production of a bacterial biosurfactant using 3% corn steep liquor and 2% waste frying canola oil, with the onset of biosurfactant production in the exponential growth phase and a minimum surface tension value of 27.00 mN/m in the stationary phase after 48 hours of cultivation, when maximum biosurfactant production occurred. Thus, different substrates affect the outcome, which underscores the importance of the choice of substrate to the efficient production of a biosurfactant.

The pH of the culture medium was monitored throughout the 144 hours of cultivation. When the greatest reduction in surface tension occurred, pH was 6.6 and increased to 8.7 by the end of cultivation due to the continued production of metabolites by the microorganism. The formation of metabolic products causes changes in both pH and surface tension (Abdel-Mawgoud et al. 2008). Alvarez et al. (2015) found that changes in pH also influenced the growth of *Bacillus amyloliquefaciens* and biosurfactant production. Moreover, the acidity of the medium is correlated with efficiency in the synthesis of biosurfactants by microorganisms.

Stability of biosurfactant under different conditions of temperature, NaCl concentration and pH determined based on surface tension and emulsification index

Although biosurfactants exhibit diversity in terms of chemical composition and properties, some common characteristics are found in the majority of these biomolecules, many of which offer advantages over synthetic surfactants, such as tolerance to high temperatures and a broad pH range (Campos et al. 2013). It is

therefore important to study the influence of such variables when considering the application of these metabolites in bioremediation processes.

Table 1 displays the stability of the biosurfactant under extreme conditions of salinity, temperature and pH through the determination of surface tension and the emulsification index. No significant changes in surface tension occurred when the biosurfactant was subjected to high concentrations of NaCl. These results are promising, since concentrations of NaCl of 2% are enough to inhibit the action of synthetic surfactants (Santos et al. 2016b).

The biosurfactant produced in the present study proved to be stable at temperatures of 0, 5, 70, 100 and 120ºC, as demonstrated by the lack of substantial changes in surface tension throughout this temperature range. Similar results have been described for a biosurfactant produced by *Bacillus subtilis* in a medium supplemented with glycerol (Bezza and Chirwa, 2015). Santos et al. (2018) found a slight increase in surface tension following exposure of a biosurfactant to temperatures between 100 and 120ºC, with values ranging from 29.06 mN/m to 30.19 mN/m.

No significant changes in surface tension occurred when the biosurfactant was submitted to pH 2, 4, 6, 8 and 10, but a discrete increase in surface tension was found at pH 12. Soares da Silva et al. (2017) found that the surface tension of the biosurfactant remained relatively stable between pH 6.0 and 12.0 (28 to 29 mN/m), whereas surface tension increased slightly below pH 6.0, reaching 32 mN/m at pH 2.0. The denaturation of protein components or the increase in the ionisation of the medium can cause a change in surface temperature at extreme pH values (Santos et al. 2018).

In addition to surface tension, the stability of oil/water emulsions is widely used as an indicator of surface activity. Although the ability of a molecule to form a stable

emulsion is not always associated with a reduction in surface tension, the combination of two immiscible liquids (oils) results in the formation of an emulsion (Tadros 2013). The identification and, above all, the understanding and control of factors that affect the stability of emulsions have been the object of studies since the 1980s. The oil fraction, type and concentration of tensoactive agents and stabilisers and the difference in density between the phases are some of the variables that exert an influence on the stability of an emulsion. Emulsions remain stable in the presence of surfactants through reductions in interfacial tension and the degree of coalescence. Therefore, the stability of an emulsion is related to the balance among the oil, water and surfactant established by the action of the latter (Mohamed et al. 2017).

The emulsification activity of the biosurfactant produced by *B. methylotrophicus* was determined for several immiscible substrates in water. Table 1shows the influence of different temperatures, NaCl concentrations and pH values on the emulsification activity of the biosurfactant. A gradual reduction in the emulsification index (EI) of motor oil occurred when the concentration of NaCl was increased in the biosurfactant solution. The same occurred with the increase in temperature, with maximum EIs at the lowest temperatures investigated, which are considered extreme from the environmental standpoint. The emulsification of the motor oil by the biosurfactant was reduced with the increase in pH, especially between pH 10.0 and 12.0, whereas the EI was 100% at extremely acid pH.

For the biosurfactant from *B. methylotrophicus*, it is likely that the increase in the concentration of NaCl led to a weaker formation of the oil-water-biosurfactant emulsion complex due to the affinity of NaCl with water molecules, causing a reduction in the action of the biosurfactant and an imbalance in this complex. With regard to the thermal variation, it is possible that the increase in temperature

reduced the viscosity of the motor oil, thereby diminishing the interaction between the biosurfactant and oil. For pH, it is possible that this variable promoted some change in the biosurfactant, enabling more or less interaction with the motor oil as a function of its composition and structure.

The increases in temperature and the concentration of NaCl led to the enhancement of the tensoactive property, but not the emulsification capacity of the biosurfactant. Indeed, a good surfactant is not always a good emulsifier (Campos et al. 2013).

Biosurfactant characterisation

Microbial biosurfactants are classified as lipids, glycolipid, lipopeptides and polysaccharide-protein complexes. The biochemical composition of a biosurfactant is related to genetics of the microorganism as well as the substrates used in the production medium (Gudiña et al. 2017; Rufino et al. 2013). The biochemical analysis revealed that the biosurfactant isolated in the present study is composed of 83.76% proteins and 16.24% lipids, demonstrating that it is a lipopeptide. The agar double diffusion tests revealed that the biosurfactant produced by *B. methylotrophicus* has an anionic nature. Similar results are reported for a lipopeptide produced by *B. mojavensis* I4 submitted to the same test (Ghazala et al. 2017). The properties of lipopeptide surfactants produced by the genus *Bacillus* demonstrate the considerable potential of these natural compounds in biotechnological applications, such as the bioremediation of environments polluted with hydrocarbons (Parthipan et al. 2017).

Figure 2 shows the results of the ^{1}H NMR analysis of the biosurfactant, which suggests the presence of a methyl functional group in the region situated between 0 and 1 ppm. The signals between 1 and 1.8 ppm correspond to hydrogens linked

to the aliphatic carbon chain. In the region situated between 1.8 and 2.2 ppm, signals of possible hydrogens linked to double bonds are found. The signal between 2.2 and 2.4 ppm may indicate the presence of hydrogens linked to carbon adjacent to carbonyl. The signal in the region between 4 and 4.2 ppm is strong evidence of the presence of hydroxyls in the molecule. The signals between 5.2 and 5.6 ppm are derived from hydrogens found in carbons that have a double bond.

Figure 3 displays the results of the ^{13}CNMR analysis, showing signals of aliphatic carbons in the region between 0 to 40 ppm, carbons linked to hydroxyls in the signal at 60 ppm, double bonds between 120 and 140 ppm, and carboxyl acids in the signal near 180 ppm.

As shown in Figure 4, the FITR spectrum of the isolated and purified biosurfactant from *B. methylotrophicus* exhibited signal amplification at 3466 cm^{-1}, which is characteristic of hydroxyls in carboxylic acids. Carbonyl was detected at 1681 cm^{-1} and possible deformations caused by the double bonds were detected at 1463 cm^{-1}, confirming the results obtained by NMR.

In a study conducted by Bezza and Chirwa (2015), a purified and isolated product of *B. subtilis* CN2 was strongly adsorbent in the bands indicating groups of peptides and the presence of an aliphatic chain. Hazra et al. (2015) found peaks indicating an aliphatic and carbonyl chains across the FTIR spectrum of the isolate. These results are similar to previous reports on lipopeptide biosurfactants, most of which have a fatty acid nature.

According to Araujo et al. (2013), the fatty acid composition of lipopeptides is controlled by the abundance of fatty acid precursor coenzymes in the cell; moreover, the composition and size of the fatty acid chains vary depending on the medium used, which could result in greater specific surfactant activity. However, Youssef et al. (2011) found that a variation in the percentage of fatty acids produced by *B.*

subtilis led to a change in the surface activity of the biosurfactant. In contrast to the present results, the percentage of fatty acids found in the cited study was likely correlated with the efficiency of the tensoactive properties of the biosurfactant evaluated.

Jha et al. (2016) report a partially purified biosurfactant with bands characteristic of aliphatic and peptide chains, demonstrating similarity with cyclic lipopeptides produced by bacilli. Several strains of *Bacillus* are able to produce surfactin, which is a bacterial cyclic lipopeptide that is considered to be one of the most effective biosurfactants, with a wide range of applications, such as use in environmental bioremediation and antibacterial treatments (Ghazala et al. 2017).

Based on the findings, the chemical profile of the biosurfactant described herein has different polarities, with a chemical structure basically composed of hydroxylated fatty acids. Although it presents amphiphilic characteristics, a detailed analysis is needed to define its definitive chemical structure.

Application of biosurfactant in removal of hydrophobic pollutant adsorbed to soil and sand in flasks

The treatment of contaminated soils requires biodegradable washing products with low toxicity that pose no risks to the environment. Biosurfactants remove oil and heavy metals through desorption, solubilisation and dispersion of the contaminants in soil, enabling the recovery and even the reuse of the contaminating substance. Petroleum-based hydrocarbons adsorbed to soil particles are difficult to remove and degrade. Biosurfactants are capable of emulsifying hydrocarbons by increasing their solubility in water and reducing the surface tension, which facilitates the detachment of these oily substances from soil particles (Rufino et al. 2013).

Microbial Enhanced Oil Recovery (MEOR) can be evaluated using a bench-scale design, which is an economical model that simulates oil recovery operations (Silva et al. 2014). In the tests with the crude biosurfactant from *B. methylotrophicus*, the tensioactive agent was capable of removing 63% of the oil from the sand samples and 25% from the sandy soil (Table 2). According to Adrion et al. (2017), contaminant removal rates are affected by the biosurfactant type and concentration, its affinity with the contaminant, interactions with acidic or alkaline additives and the characteristics of the soil. Using a biosurfactant, Rufino et al. (2013) report a 30% removal rate of oil from clay soil by the cell-free broth and removals rates of 33.1 to 37.3% from sand using the isolated biosurfactant. In some experiments, the required biosurfactant concentration is related to its sorption or bond to the soil particles. When removing oil from soil, the efficiency of a biosurfactant depends on its physicochemical characteristics (hydrophobicity and ionic charge) and the characteristics of the soil, making it difficult to predict its effects (Santos et al. 2017).

Washing of hydrophobic compound adsorbed to a porous surface

The literature offers few reports on the removal of oil from porous surfaces and few methods have been adequate for cleaning contaminants from delicate coral reefs, which are difficult to access. Although widely employed, physical removal methods and chemical dispersants are inadequate due to the damage caused to the corals and the further contamination of the environment. Therefore, the use of biosurfactants is an attractive option when an ecosystem is exposed to an oil spill. Sobrinho et al. (2013) report a 60% motor oil removal rate from a porous surface using a crude biosurfactant, demonstrating the dispersant potential of the biosurfactant. Luna et al. (2016) report a 70% removal rate of motor oil from a porous surface using a biosurfactant produced by *Candida bombicola*. In the present

investigation, the motor oil removal rate was around 70% after manual shaking for five minutes using the biosurfactant isolated from *B. methylotrophicus* (Figure5), which demonstrates the viability of applying this biosurfactant as a biological dispersant to remove hydrophobic pollutants from sensitive ecosystems, such as coral reefs.

Application of biosurfactant as bioremediation agent

The potential use of the biosurfactant as a bioremediation agent for seawater contaminated with a petroleum product was investigated. The activity of autochthonous marine bacteria and fungi in the biodegradation process was monitored for 28 days (Figure 6). The results achieved with the addition of the biosurfactant at both ½ the CMC and the CMC were better than those achieved in the control (absence of biosurfactant), as an accentuated increase in the amount of autochthonous microorganisms was found throughout the incubation time (Figure 6). Microbial growth remained constant in the absence of motor oil (Figures 6A and 6C). In contrast, maximum bacterial growth peaks in the presence of petroleum products were found on Day 7 and Day 14 with the biosurfactant at ½ the CMC and the CMC, respectively (Figure 6B) and peaks of fungal growth were found on the 7th and 21st days at ½ the CMC and the CMC, respectively (Figure 6D), with a subsequent reduction in growth.

Likewise, Santos et al. (2016a) found that a biosurfactant from *Candida lipolytica* at concentrations of ½ the CMC, the CMC and twice the CMC favored the growth of autochthonous microorganisms during 30 days of cultivation. Rocha e Silva et al. (2014) report the same effect on the growth of autochthonous marine bacteria and fungi in the region of the Suape Port; a biosurfactant isolated from *Pseudomonas cepacia* accelerated the growth of the microorganisms during the 30-

day cultivation period and served as a solubilising agent for the motor oil, thereby facilitating its biodegradation.

The solubilisation oil depends on the ability of a biosurfactant to increase the interactions between the hydrophobic constituents and the aqueous phase (Dadrasnia and Ismail 2015). A biosurfactant promotes emulsification which increases the surface area of the oil column and facilitates the interaction between the hydrophobic substrate and surface of the microbial cells, leading to greater bioavailability of the contaminant and, consequently, increasing the degradation rate of the hydrophobic compound (Patowary et al. 2017). However, the increase in bioavailability can release toxic by-products resulting from the microbial metabolism of the oil constituents, which, depending on the hydrophobic pollutant, increases the toxicity of the medium (Almeida et al. 2017b). The toxic effect of the motor oil was clearly seen during the development of the microorganisms from the Suape Port (Figs. 6B and 6D), which demonstrated an accentuated decline in growth in the presence of the oil that was not seen on the growth curve in the absence of the contaminant (Figs. 6A and 6C). The bacteria were more sensitive to the toxicity and exhibited no further growth by the 28th day of cultivation (Fig. 6B). In contrast, fungi were more resistant (Fig. 6D).

Phytotoxicity test

For use in environmental applications, a biosurfactant must have no toxicity. Ecotoxicity bioassays are analytical methods that enable characterising the toxicity of chemical substances (Soares da Silva et al. 2017). In the present study, toxicity of the biosurfactant produced by *B. methylotrophicus* to seeds of the cabbage *Brassica oleracea var. captata* was evaluated using the germination index, which is a combination of relative seed germination and relative root growth. Table 3 displays

the phytotoxicity results. The solutions of the isolated biosurfactant at ½ the CMC (0.25%) and the CMC (0.5%) had no inhibitory effect on seed germination or the elongation of the roots of the cabbage plant, as the germination index was 73 and 66%, respectively. Moreover, secondary root growth and the emergence of leaves occurred with all solutions tested, which is in agreement with data reported in the literature. Santos et al. (2017) found the occurrence of germination in three vegetable species even in the presence of high concentrations of a biosurfactant produced by *C. lipolytica*. Soares da Silva et al. (2017) found that a biosurfactant from *P. cepacia* had no inhibitory effects on the germination or root elongation of cabbage seeds.

Conclusions

B. methylotrophicus UCP 1616 cultivated with industrial waste has potential as a biosurfactant producer. The biosurfactant demonstrated attractive tensioactive properties, with potent surface activity, high emulsification activity and efficiency when submitted to a range of temperatures, pH values and salt concentrations. The chemical characterisation of the new biosurfactant revealed a likely lipopeptide nature. The biomolecule exhibited low toxicity to cabbage seeds and stimulated the growth of autochthonous microorganisms during the biodegradation of motor oil, which enables its safe use in environmental applications. The results of bench scale tests simulating environments impacted by the petroleum industry demonstrate that the tensioactive agent has considerable capacity for the removal of hydrophobic contaminants. The new biosurfactant described in this paper is promising for application in bioremediation processes in both marine and terrestrial environments contaminated with petroleum products.

Compliance with Ethical Standards

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Conflict of Interest: Marco José Chaprão, Rita de Cássia F. Soares da Silva, Raquel D. Rufino, Juliana M. Luna, Valdemir A. Santos and Leonie A. Sarubbo declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Table 1 Influence of salt concentration, temperature and pH on reduction in surface tension and emulsification activity in motor old by cell-free broth containing biosurfactant from *B. methylotrophicus* cultivated in mineral medium supplemented with 3.0% corn steep liquor and 3.0% molasses for 48 h at 200 rpm and 28 ºC (data expressed as mean ± standard deviation)

NaCl (%)	**Surface tension (mN/m)**	**Emulsification Index (%)**
0	29.00 ± 0.25	100.00 ± 3.12
2.0	28.90 ± 0.25	100.00 ± 3.12
4.0	28.00 ± 0.15	87.00 ± 3.45
6.0	27.80 ± 0.10	43.50 ± 4.11
8.0	27.05 ± 0.50	45.00 ± 4.10
10.0	27.00 ± 0.30	10.50 ± 3.35
12.0	27.00 ± 0.25	35.00 ± 4.30
Temperature (°C)	**Surface tension (mN/m)**	**Emulsification Index (%)**
0	29.90 ± 0.14	100.00 ± 2.19
5	29.00 ± 0.34	98.00 ± 4.12
70	28.00 ± 0.22	85.00 ± 5.02
100	27.00 ± 0.25	45.00 ± 4.03
120	27.50 ± 0.21	55.50 ± 3.10
pH	**Surface tension (mN/m)**	**Emulsification Index (%)**
2	29.00 ± 0.11	100.00 ± 2.97
4	31.00 ± 0.20	82.50 ± 3.08
6	28.00 ± 0.31	75.50 ± 4.09
8	28.50 ± 0.31	59.00 ± 4.03
10	29.40 ± 0.12	15.00 ± 2.21
12	34.00 ± 0.15	10.00 ± 3.05

Table 2 Removal rates of motor oil adsorbed to *in natura* sand and sandy soil by cell-free broth containing biosurfactant from *B. methylotrophicus* cultivated in mineral medium supplemented with 3% corn steep liquor and 3% molasses (data expressed as mean ± standard deviation)

	Removal (%)	
Type of sand	**Crude biosurfactant**	**Distilled water**
***In natura* sand**	63.0 ± 0.5	15.0 ± 0.3
Sandy soil	25.0 ± 0.7	10.0 ± 0.4

Table 3 Phytotoxicity of biosurfactant isolated from *B. methylotrophicus* cultivated in mineral medium supplemented with 3% corn steep liquor and 3% molasses on *Brassica oleracea* seeds

Cabbage seeds	Phytotoxicity parameters (%)	Biosurfactant concentration	
		½ x CMC (0.25 %)	CMC (0.5 %)
Brassica oleracea	**Germination index**	73.00 ± 0.39	100.00 ± 0.31
	Root growth	76.00 ± 0.21	66.00 ± 0.15
	Seeds germinated	96.00 ± 0.11	66.00 ± 0.22

FIGURE CAPTIONS

Fig. 1 Growth, pH, surface tension and biosurfactant concentration curves during cultivation of *B. methylotrophicus* in mineral medium supplemented with 3% corn steep liquor and 3% molasses

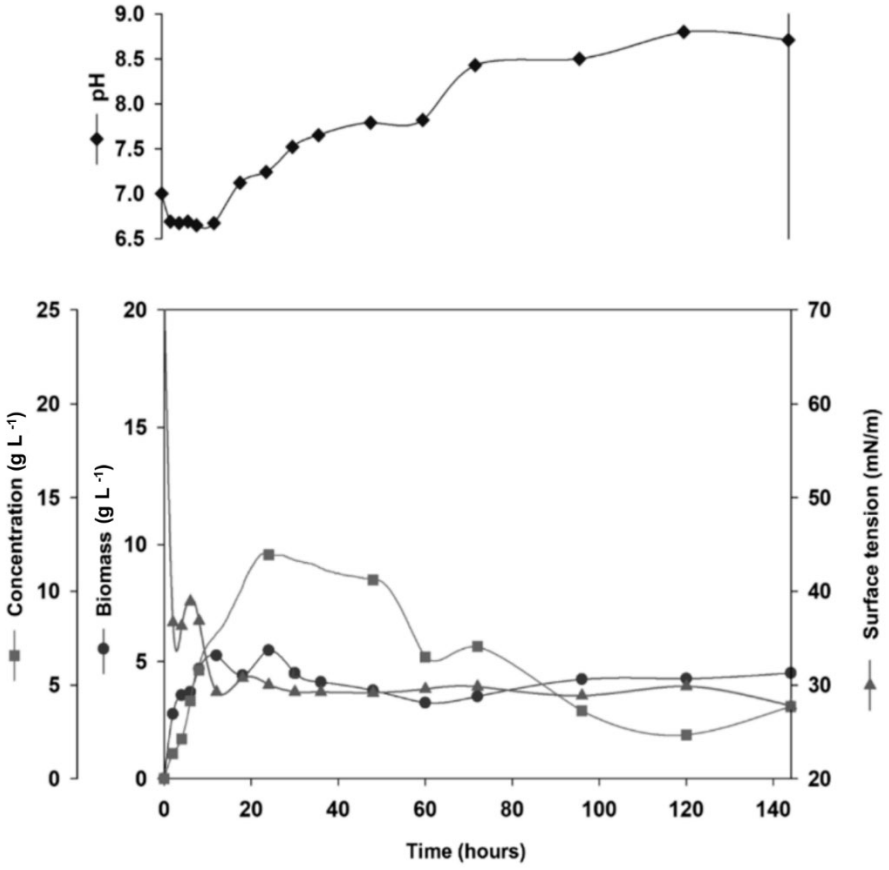
Fig. 2 ^{1}H NMR spectrum (CD_3OD, 300 MHz) of biosurfactant isolated from *B. methylotrophicus* cultivated in mineral medium supplemented with 3% corn steep liquor and 3% molasses

Fig. 3 ^{13}C NMR spectrum (CD_3OD, 300 MHz) of biosurfactant isolated from *B. methylotrophicus* cultivated in mineral medium supplemented with 3% corn steep liquor and 3% molasses

Fig. 4 Infrared (FTIR) spectrum for biosurfactant isolated from *B. methylotrophicus* cultivated in mineral medium supplemented with 3% corn steep liquor and 3% molasses

Fig. 5 Removal of motor oil adsorbed to marine rocks by biosurfactant from *B. methylotrophicus* cultivated in mineral medium supplemented with 3% corn steep liquor and 3% molasses. (A) Rock completely covered with oil prior to removal. (B) Rock after removal process

Fig. 6 Influence of isolated biosurfactant (½ x CMC and CMC) from *B. methylotrophicus* on growth of autochthonous microorganisms. A: bacteria in seawater; B: bacteria in seawater in presence of motor oil; C: fungi in seawater; D: fungi in seawater in presence of motor oil


pH
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8.5
8.0
7.5
7.0
6.5
Concentration (g L -1)
Biomass (g L -1)
Surface tension (mN/m)
Time (hours)
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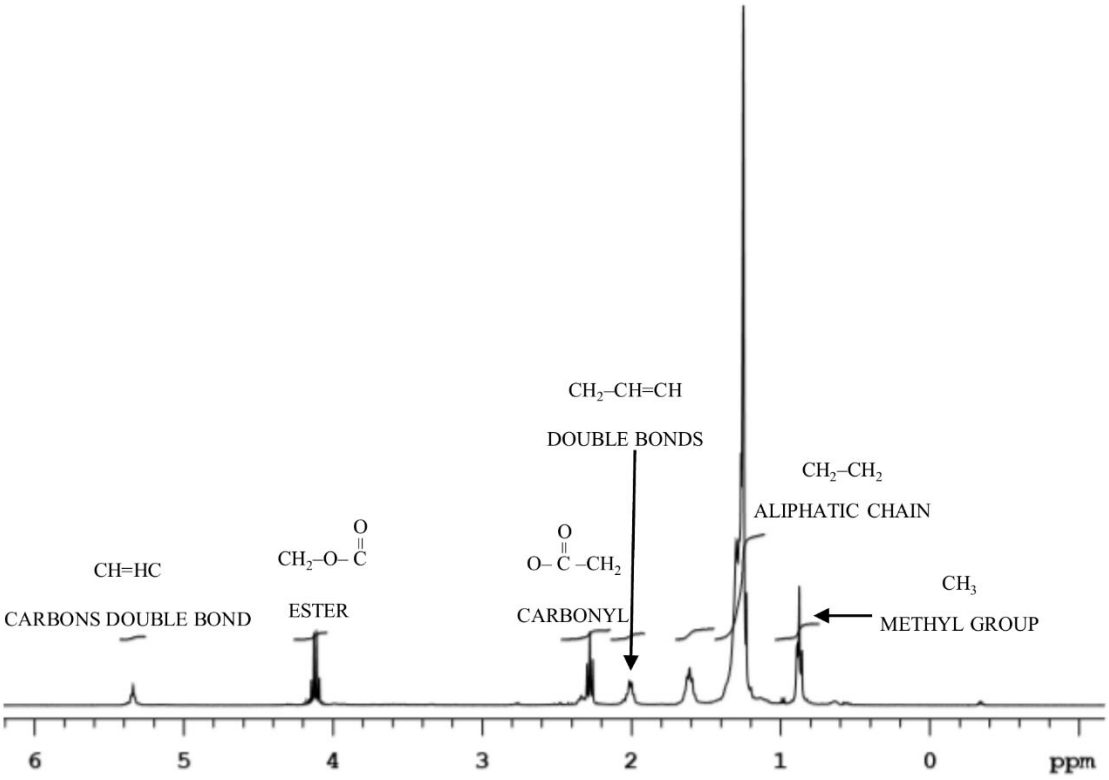
20
40
60
80
100
120
140
5
10
15
25
30
50
70

Fig. 1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DOUBLE BONDS
CH2–CH2
ALIPHATIC CHAIN
CH=HC
CARBONS DOUBLE BOND
CH2–O–C
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ESTER
O–C–CH2
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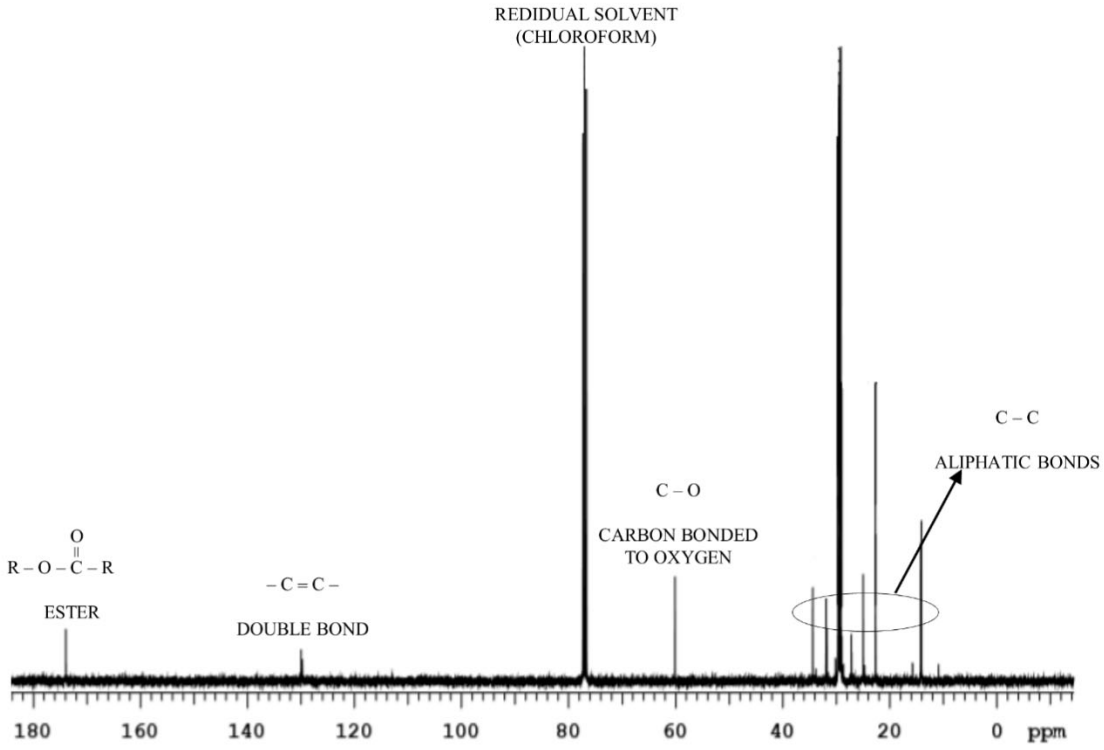
Fig. 2


REDIDUAL SOLVENT
(CHLOROFORM)
C – C
ALIPHATIC BONDS
C – O
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ESTER
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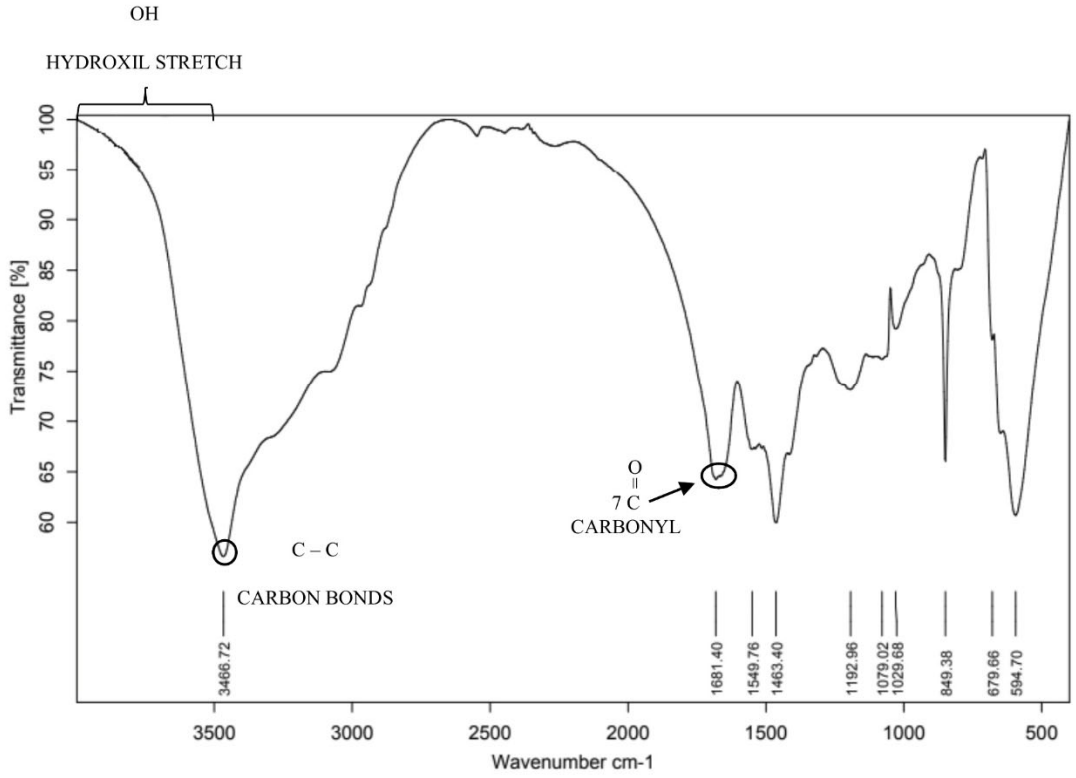
Fig. 3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HYDROXIL STRETCH
Transmittance [%]
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95
90
85
80
75
70
65
60
C – C
CARBON BONDS
O
7 C
CARBONYL
3466.72
1681.40
1549.76
1463.40
1192.96
1079.02
1029.68
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679.66
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3000
2500
2000
1500
1000
500
Wavenumber cm-1

Fig. 4

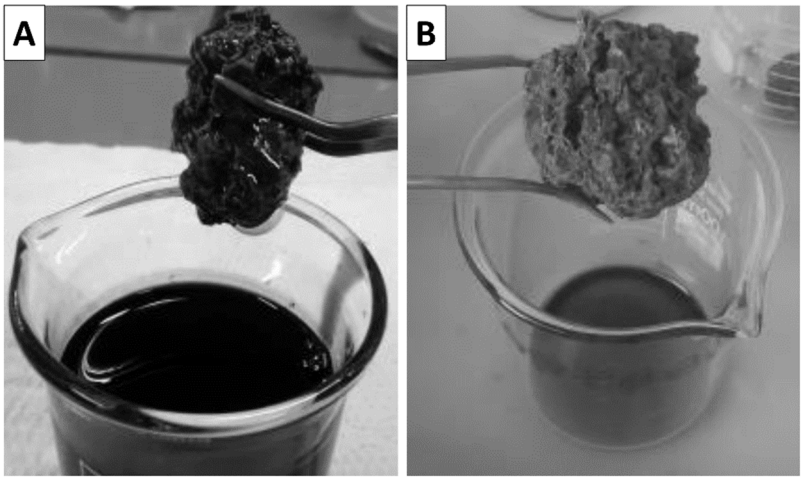

Fig. 5

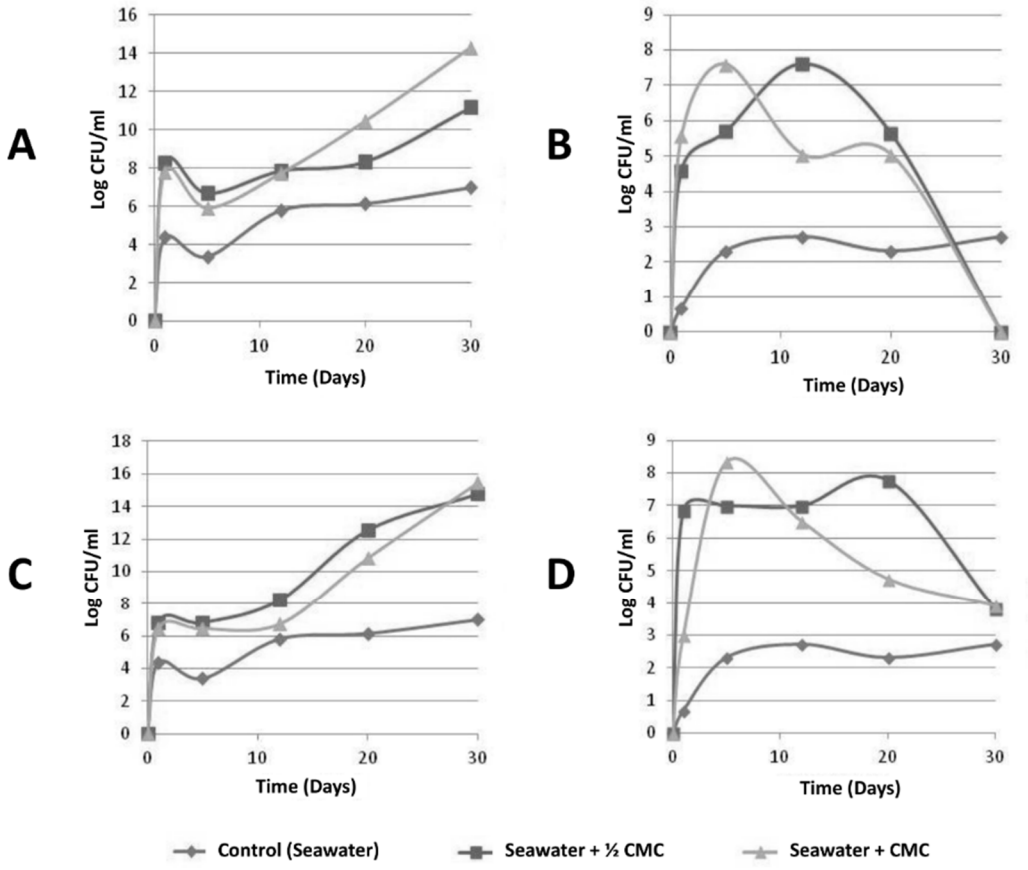
A
B
C
D
Log CFU/ml
Time (Days)
Control (Seawater)
Seawater + ½ CMC
Seawater + CMC

Fig. 6

4.2. CAPÍTULO 2 - Formulation and application of a biosurfactant from *Bacillus methylotrophicus* as collector in the flotation of oily water in industrial environment

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Formulation and application of a biosurfactant from *Bacillus methylotrophicus* as collector in the flotation of oily water in industrial environment

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Abstract

The present study describes the formulation of a biosurfactant produced by *Bacillus methylotrophicus* UCP1616 and investigates its long-term stability for application as a collector in a bench-scale dissolved air flotation (DAF) prototype. For formulation, the conservative potassium sorbate was added to the biosurfactant with or without prior heat treatment at 80 °C for 30 min. After formulation, the biosurfactant samples were stored at room temperature for 180 days and the tensioactive properties of the biomolecule were determined with different pH values, temperatures and concentrations of salt. Then, a central composite rotatable design was used to evaluate the influence of the independent variables (effluent flow rate and formulated biosurfactant flow rate) on the oil removal efficiency in the DAF prototype. The formulated biosurfactant demonstrated good stability in both conservation methods, with tolerance to a wide pH range, salinity and high temperatures, enabling its use in environments with extreme conditions. The efficiency of the formulated biomolecule through heating and addition of sorbate was demonstrated by the 92% oil removal rate in the DAF prototype. The findings demonstrate that the biosurfactant from *Bacillus methylotrophicus* enhances the efficiency of the DAF process, making this technology cleaner. This biosurfactant can assist in the mitigation and management of industrial effluents, contributing toward a reduction in environmental pollution caused by petroleum-based hydrocarbons.

Keywords: biosurfactant; formulation; *Bacillus methylotrophicus*; DAF; oily effluent.

1. Introduction

Several sources of renewable energy have been developed and proposed to reduce humanity's dependence on fossil fuels (Geetha et al., 2018). However, a large number of industrial activities, especially those in the petroleum industry and the production of electrical energy, require heavy oil to function. Spills involving

petroleum-based hydrocarbons, such as fuel and heavy oil, are an inevitable part of the industrial sector and have caused serious social-environmental problems. In many parts of this system, such as the transportation of fuel, the lubrication of motors and machinery, the washing of parts, floors and machines impregnated with oil residue, etc., leaks and the discharge of oily effluents occur, resulting in cumulative environmental impacts (Soares da Silva et al., 2018).

The disposal of effluents is only permitted after the removal of oil and suspended solids to acceptable levels (Radzuan et al., 2016). One of the main methods for successfully separating oil from water during the treatment of effluents is dissolved air flotation, in which oil droplets adhere to air bubbles and rise to the surface, where they are removed. This process enables the reuse of the phases in an efficient, economical manner. Flotation often involves the use of chemical surfactants to enhance adherence to the air bubbles (Rocha e Silva et al., 2015). However, new guidelines for water recovery have restricted the use of these chemical products. In this context, the aim of petroleum biotechnology is to employ biological processes in the exploration, production, transformation and refinement of petroleum as well as assist in the mitigation and management of industrial effluents, thereby contributing to a reduction in pollution (Almeida et al., 2016).

Bioremediation is among the most widely studied biological approaches to the treatment of environments contaminated with hydrocarbons. The low solubility of hydrocarbons hinders the access of microorganisms and the consequent biodegradation of the pollutant. One of the possible solutions to the low availability of hydrophobic pollutants consists of the use of biosurfactants, which are an attractive option in comparison to their chemical counterparts (Silva et al., 2014; Geetha et al., 2018). Biosurfactants or microbial surfactants are metabolites produced mainly by bacteria and yeasts. These compounds are formed by

molecular structures with a hydrophilic portion and a hydrophobic portion that tend to partition at the interfaces between liquid phases with different degrees of polarity (oil/water and water/oil), promoting a reduction in surface and interfacial tensions, which confers the capacity of detergency, emulsification, lubrication, solubilisation and the dispersion of phases (Santos et al., 2016). Biosurfactants have numerous advantages over surfactants of a chemical origin, such as low toxicity, biodegradability, stability in a wide pH range and at high temperatures as well as tolerance to high saline concentrations (Rocha e Silva et al., 2018). Bacteria from the families Bacillaceae and Pseudomonaceae are capable of producing biosurfactants that can be used for the removal of petroleum and petroleum-based products. In particular, *Bacillus subtilis* has been widely studied in terms of biosurfactant production and is well known for its efficient production of a lipopeptide with surface activity denominated surfactin (Gudiña et al., 2016).

The stability of a biosurfactant is an essential factor to the viability of long-term storage, especially for a biotechnological product that must meet rigorous criteria for its production and application in the industrial environment. Durability needs to be high in order to maintain the product in stock with its initial properties so that it is readily available for immediate use in cases of urgent application in the occurrence of an oil spill. It is therefore of fundamental importance to develop strategies that enable the production, formulation and application of biosurfactants in industrial processes (Soares da Silva et al., 2018).

Thus, the aim of the present study was to formulate a biosurfactant produced by the bacterium *Bacillus methylotrophicus* CCT1616 for the commercial application of the biomolecule as a collector in the treatment of oily water using a bench-scale DAF prototype and evaluate this bioprocess for the treatment of oily effluent stemming from industrial activities.

2. Materials and Methods

2.1. Microorganism

The bacterium *Bacillus methylotrophicus* UCP1616 isolated from the port area in the city of Recife, state of Pernambuco, Brazil and deposited in the culture bank of the Centre for Environmental Sciences of the Catholic University of Pernambuco was used at the biosurfactant producer.

2.2. Inoculum growth medium

Young cultures of the bacterium obtained after 24 hours of cultivation in a nutrient agar medium were transferred to Erlenmeyer flasks containing 50 mL of nutritive broth with the following composition: meat extract (5.0 g/L), peptone (15.0 g/L), NaCl (5.0g/L), K_2HPO_4 (5.0g/L) and distilled water (1.0 L) at pH 7.0. The mixture was maintained under orbital agitation at 150 rpm for 10 to 14 hours 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 a concentration of 3% (v/v).

2.3. Production medium

Biosurfactant production was performed in the mineral medium described by Bushnell and Hass (1941) composed of KH_2PO_4 (1.0 g/L), K_2HPO_4 (1 g/L), $MgSO_4.7H_2O$ (0.2 g/L), $CaCl_2.H_2O$ (0.2 g/L) and $FeCl_3.6H_2O$ (0.05g/L). The mineral medium was supplemented with 3% sugarcane molasses and 3% corn steep liquor (Chaprão et al., 2015). Fermentation for the production of the biosurfactant was performed in Erlenmeyer flasks containing 500 mL of the medium for 48 hours at 28ºC and at 200 rpm. The broth containing the biosurfactant was centrifuged at 5000

rpm for 30 min to separate the microbial biomass and obtain the crude biosurfactant. Corn steep liquor was acquired from Corn Products do Brasil in the city of Cabo de Santo Agostinho, state of Pernambuco, Brazil. Sugarcane molasses was acquired from a local sugar processing plant in the city of Vitória de Santo Antão, state of Pernambuco, Brazil.

2.4. Stabilisation of broth with surfactant property

The broth containing the biosurfactant (crude biosurfactant) was submitted to two conservation methods: a) the addition of 0.2% potassium sorbate, which is a food conservative considered safe and non-toxic that is capable of inhibiting microbial growth; and b) heating at 80 °C for 30 min followed by the addition of 0.2% potassium sorbate. After the conservation treatments, the crude biosurfactant was stored at room temperature (28 to 30 °C) for up to 180 days. Samples were withdrawn at 15, 30, 70, 110 and 180 days to study long-term stability. During each evaluation, the biosurfactant samples were submitted to changes in pH (5.0, 7.0 and 9.0), addition of NaCl (1, 3 and 5% p/v) and heating to 40 °C and 50 °C. The following properties were analysed to select the better conservation method: surface tension, emulsification activity and dispersing capacity of motor oil in seawater (Soares da Silva et al., 2018).

2.5. 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. The platinum ring was immersed into the broth and the force required to pull it through the liquid-air interface was recorded.

2.6. Determination of emulsification activity

For the determination of emulsification activity, samples of the cell-free broth were analysed based on the method described by Cooper and Goldenberg (1987), using 2.0 mL of a hydrophobic compound (motor oil) to which 2.0 mL of the biosurfactant was added in a test tube. The mixture was vortexed for two minutes. After 24 h, the emulsion percentage was calculated by dividing the height (in centimetres) of the emulsified phase by the total height of the mixture.

2.7. Determination of dispersion capacity

The dispersion capacity of an oil slick was simulated in the laboratory by contaminating samples of water with motor oil in a Petri dish. The formulated biosurfactant at a concentration of 1.0% was added at biosurfactant-to-oil proportions of 1:2, 1:8 and 1:25 (v/v). The mean diameter of the clear zones of triplicate experiments was measured and calculated as the rate of the Petri dish diameter (dispersion index), as described in Rocha e Silva et al. (2014).

2.8. Synthetic oily solution

A commercially available lubricating oil (SAE 20W-50) with a synthetic protector (Petrobras, Brazil) for use on “Flex” engines (gasoline, natural gas and alcohol) was used for the synthetic oily solution. This oil is composed of complex blend of hydrocarbons and additives to enhance its performance. The oil was weighed on an analytical scale and transferred to a recipient with de-ionised water. The mixture was submitted to a homogeniser at 2000 rpm for 30 minutes. The concentration of oil was 5 g/L, which is above the concentration of an effluent composed of oily water (emulsified oil) at a thermoelectric plant (evaluated at 10 ppm, discarding the free oil content, as free oil can be separated using simpler physical methods, such as continuous sedimentation).

2.9. Experiments with bench-scale DAF prototype

The DAF prototype was constructed in acrylic to enable the visualisation of the formation of the microbubbles and separation of the contaminant from the water. The prototype had a capacity of 15 L. The flotation chamber was 0.262 m in height, 0.240 m in length and 0.240 m in width (Figure 1). The flotation system was loaded with 50 g of motor oil mixed into 10 L of water and homogenised by a pump (1) in the storage tank (2) for one hour for complete oil/water dispersion. The flotation tank (3) was filled with 15 L of distilled water. After circulating in the storage tank, the oily effluent was fed into the flotation chamber with the aid of the same pump (1), entering through a valve (4). The flow rate was verified using an *Arduino*® UNO sensor (5). The contaminated water entered into contact with the microbubbles formed by the injection of a controlled quantity of air in the aspiration line of another pump (6). The interaction between the oil droplets dispersed at the base of the DAF device and microbubbles led to the formation of flocs composed of oil and water that floated to the surface due to the lower density in comparison to the water, forming a layer of oily foam, which was collected in the separation chamber (7). The quantities of crude and formulated biosurfactant were dosed using a burette (8) to enhance the efficiency of the process. The return pump (9) connected to the treated water tank (10) re-circulated the treated effluent to the storage tank without coming into contact with the initial oily effluent, from where it could be collected (11) for subsequent analysis. Each experiment in the DAF system lasted approximately five minutes (Silva et al., 2018).

Insert Figure 1

2.10. Experimental planning for the evaluation of oil separation efficiency using the formulated biosurfactant

A central composite rotatable design (CCRD) was used for the analysis of the effect of the addition of the biosurfactant on the oil separation efficiency in the DAF system. Two two-factor CCRDs were used to evaluate the influence of the independent variables (effluent flow rate [X_1] and formulated biosurfactant flow rate [X_2]) on the response variable (removal of the contaminant). The values of the independent variables are specified in Table 1 and the coded planning matrix is displayed in Table 2.

Insert Table 1, Insert Table 2

2.11. Dissolved air flotation assays

Each assay was conducted with previous agitation performed in the 30-L tank for one hour to favour the mixture of the effluent. Prior to the end of this period, the microbubble flow was initiated to enable the clear visualisation of the continuous movement of the bubbles. The effluent and biosurfactant were then released into the 15-L flotation tank in accordance with the flow rates specified in the factorial planning.

Before and after a fixed time of five minutes for each assay, aliquots were extracted with n-hexane (1:1, v/v) to measure the initial concentration (C_I) and final concentration (C_F) of oil in the water through spectrophotometric analysis at a wavelength of 330 nm. The results of this analysis enabled the calculation of the removal rate after five minutes of flotation, using Equation 1:

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

in which C_I is the concentration of oil (mg/L) fed into the system and C_F is the concentration of oil (mg/L) at the output of the system.

2.12. Statistical Analysis

All determinations regarding the stability tests were performed at least three times. Means and standard errors were calculated using the Microsoft Office Excel 2016.

Analysis of variance (ANOVA) with 95% confidence intervals was used to determine the significance of the effects. ANOVA, the determination of regression coefficients and the construction of graphs were performed with the aid of the *Statistica* program, version 12.0 (Statsoft Inc, USA).

3. Results and Discussion

3.1. Stability of the formulated biosurfactant

Investing in productive efficiency, that is, the need to maximize production factors in order to obtain higher levels of productivity and profitability, is a challenge that must be evaluated. It is necessary to assess a new product to identify the main bottlenecks in the production system, since gains in efficiency are only transformed into financial gains if the biotechnological processes are effective.

Long-term stability is one of the requirements for developing a new biotechnological product and putting it on the market. The properties of a stable commercial product should not change drastically with the fluctuations in pH, temperature and salinity encountered in the industrial environment (Soares da Silva et al., 2018). To ensure a commercial bioproduct, the crude biosurfactant produced by *B. methylotrophicus* was submitted to two conservation methods and its

tensioactive properties were analyzed for a period of 180 days of storage to determine the shelf life of the product being offered on the market (Freitas et al., 2016; Soares da Silva et al., 2018). The behaviour of the biosurfactant after its formulation was evaluated under specific environmental conditions of pH, temperature and the presence of salt. The tensioactive properties (*i.e.*, surface tension, emulsification activity and dispersion capacity) were evaluated to select the more adequate conservation method for future applications.

Figure 2 displays surface tension results of the biosurfactant submitted to the conservation processes (addition of 0.2% potassium sorbate [A] and heat treatment with addition of 0.2% potassium sorbate [B]) after storage for different periods of time followed by variations in pH (5, 7 and 9), temperature (40 and 50°C) and NaCl concentrations (1, 3 and 5%). With both conservation methods, the biosurfactant demonstrated stability when exposed to the different pH values tested throughout the entire storage time. Surface tension was around 26.6 mN/m at pH 5, 28 mN/m at pH 7 and 29.5 mN/m at pH 9. In the samples submitted to different concentrations of NaCl, a discrete increase in surface tension was found (around 29 and 30 mN/m) in comparison to the control, followed by a reduction throughout the storage time to around 27 and 28 mN/m.

Insert Figure 2

Emulsification activity consists of the capacity to blend immiscible liquids in a stable manner (Santos et al., 2016; Soares da Silva et al., 2017). Figure 3 displays emulsification activity results of biosurfactant submitted to the conservation processes (addition of 0.2% potassium sorbate [A] and heat treatment with addition of 0.2% potassium sorbate [B]) after storage for different periods of time followed by exposure to variations in pH (5, 7 and 9), temperature (40 and 50°C) and NaCl concentrations (1, 3 and 5%). The biosurfactant remained stable under all conditions

tested, reaching approximately 100% emulsification of the motor oil with both conservation methods throughout the 180 days of storage, especially after having been submitted to the heat treatment and addition of potassium sorbate (Fig. 3B). Moreover, the biosurfactant demonstrated better results with the lower concentrations of salt (1 and 3%), achieving 95% emulsification in the first 70 days of the experiment, especially after having been submitted to the heat treatment. A discrete reduction in emulsification activity occurred in the presence of salt at concentrations of 3 and 5% at the day 15 of evaluation after the addition of the potassium sorbate, which did not invalidate the efficiency of the biomolecule when considering the other results throughout the storage time (Fig. 3A). The biosurfactant submitted to heat treatment, however, achieved 95% emulsification during 30 days of storage when submitted to a 5% concentration of salt (Fig. 3B). The results regarding the biosurfactant submitted to variations in temperature (40 and 50°C) were better in the first 30 days of storage, achieving 96% emulsification with both conservation methods (Fig. 3A-B). After 70 days of storage, the emulsification index did not surpass 50% for the biosurfactant conserved with potassium sorbate alone (Fig. 3A).

Insert Figure 3

Biosurfactants are emerging as a promising alternative to chemical dispersants, accelerating the natural dispersion and degradation of hydrocarbons released into the environments through the solubilisation of oily compounds (Freitas et al., 2016). Figure 4 displays the motor oil dispersion capacity of the biosurfactant produced by *B. methylotrophicus* submitted to the conservation process with the addition of 0.2% potassium sorbate after storage for different periods of time followed by exposure to variations in pH (5, 7 and 9), temperature (40 and 50 °C) and concentrations of NaCl (1, 3 and 5%) at biosurfactant-to-oil proportions of (A)

1:2, (B) 1:8 and (C) 1:25 (v/v). The three proportions of the biosurfactant demonstrated similar behaviour under all conditions evaluated. At Time 0, the dispersion capacity of the biosurfactant did not surpass 15% at any proportion tested. The best results were found at proportions of 1:8 and 1:25 (v/v) after 70 days of storage, reaching approximately 100% oil dispersion.

Insert Figure 4

Figure 5 displays the motor oil dispersion capacity of the biosurfactant produced by *B. methylotrophicus* submitted to the conservation process with heat treatment and the addition of 0.2% potassium sorbate after storage for different periods of time followed by exposure to variations in pH (5, 7 and 9), temperature (40 and 50 °C) and concentrations of NaCl (1, 3 and 5%) at biosurfactant-to-oil proportions of (A) 1:2, (B) 1:8 and (C) 1:25 (v/v). The formulated biosurfactant demonstrated the best dispersant capacity after 70 days of storage. On the first day of the experiment, the dispersant capacity did not surpass 10% under any of the conditions tested. The best performance was achieved at biosurfactant-to-oil proportions of 1:8 and 1:25 (v/v), reaching 90% dispersion.

Insert Figure 5

Freitas et al. (2016) submitted a biosurfactant from *Candida bombicola* to conservation procedures and found that the addition of potassium sorbate and heat treatment were the most promising. Soares da Silva et al. (2018) studied a biosurfactant produced by the bacterium *Pseudomonas cepacia* and found that the biotensioactive agent was stable under all conditions investigated, especially after being submitted to fractionated tyndallization and the addition of potassium sorbate.

The stability evaluations in the present study revealed that the tensioactive properties of the biosurfactant produced by *B. methylotrophicus* remained practically constant throughout the 180-day storage time, demonstrating the long-

term stability of the biosurfactant. When treating industrial environments contaminated by spilled petroleum-based products, the time and costs involved make the treatment of large amounts of contaminants unviable. Therefore, any product that assists in the clean up should be maintained in stock so that it is available for immediate use in the occurrence of an unexpected accident.

3.2. Evaluation of formulated biosurfactant as a collector in the treatment of oily effluent in a bench-scale DAF prototype

The flotation phenomenon is normally assisted by the addition of a collector, which is generally an appropriate surfactant (Albuquerque et al., 2012). Environmentally sustainable alternatives are being explored, such as the use of biosurfactants as collectors in DAF systems, in an effort to reduce the environmental impact of this type of activity and lend greater credibility to flotation as a separation method (Menezes et al., 2011; Vecino et al., 2013). Surfactants are capable of breaking the forces of attraction between molecules and immiscible substances, leading to a reduction in surface tension between two phases and enabling greater interaction between incompatible liquids (Sarubbo et al., 2015). Thus, biological surfactants can be employed as an environmentally friendly option to enhance the efficiency of the DAF process.

From the results obtained in the stability tests, the biosurfactant formulated through heating and addition of sorbate was selected for application as a collector in the treatment of the oily effluent in the DAF prototype.

A CCRD was applied with two factors (effluent flow rate [X_1] in L/min and formulated biosurfactant flow rate [X_2] in L/min). The planning matrix shown in Table 2 displays the coded and real factors. The response variable for the definition of the optimized conditions of the experiments was oil removal rate from the synthetic

effluent tested. A total of twelve experiments ($2^k + 2k + 4$) were run. The preliminary tests revealed that the highest removal rates occurred when the middle ranges of the factors were employed (Table 3). The repetition on the central point is responsible for the statistical validity of the other experiments, which were situated in the centre of the star formed by the distribution of the experiments (Greenland et al., 2016).

Insert Table 3

Table 4 displays the coefficients of the factors and their interactions as well as the p-values. None of the p-values has higher larger 0.05, indicating no need to discard the respective coefficients, as all were within the 95% confidence interval. The p-values confirm the high F values calculated, contributing to the significance of the model (Westland, 2015). Lack of fit, which was used as one of the test parameters of the adequateness of the model, was considerable. Therefore, one must consider this together with other criteria for the adoption of the statistical prediction and optimization model. An experimental error less than 1% demonstrates an excellent performance on the part of the researcher in the execution of the experiments, contributing positively to the significance of the model. Moreover, the R^2 value demonstrates that 91.5% of the variability in the oil removal rate is explained by the model.

Insert Table 4

Equation 2 represents the model comprised of the coefficients estimated for the prediction model obtained from the CCRD after the analysis of variance (ANOVA) was applied to the data. The equation also represents the response surface of the oil removal rate as a function of the effluent flow rate (X_1) and formulated biosurfactant flow rate (X_2) illustrated in Figure 6. Regarding the ranges of the effluent and formulated biosurfactant flow rates, an increase in the removal

rate was found. This was followed by a drop in the removal rate, indicating the optimal flow rates for this phenomenon under these working conditions.

$$Y(\%) = -157.5 + 84.9 \cdot X_1 - 9.4 \cdot {X_1}^2 - 388.1 \cdot X_2 - 188.9 \cdot {X_2}^2 - 28.3 \cdot X_1 \cdot X_2 \quad (2)$$

Insert Figure 6

Figure 7 displays a graph with level curves of the corresponding increases in oil removal illustrated in Figure 6. Considerable interaction was found between the effluent and biosurfactant flow rates, represented by the parallelism between the level curves. Maximum oil removal was achieved when the effluent flow rate was 3.7 L/min and the biosurfactant flow rate was 0.55 L/min. This experimental condition was replicated four times to confirm the value predicted by the CCRD.

Insert Figure 7

This linearity of the results is also shown in Figure 8, which compares the observed values to those predicted by the model of Equation 3, with a linear regression coefficient of 92.8%.

Insert Figure 8

The results demonstrate that the formulated biosurfactant was capable of removing 92.00% of the oil. Control experiments without the addition of the biosurfactant were performed with the same DAF operating conditions, in which oil removal with only the action of the microbubbles was around 60.00%. This result demonstrates the importance of the addition of the biosurfactant from *B. methylotrophicus* as the collector in the oil removal process.

The flotation system plus the action of the biosurfactant proved to be efficient when one considers the high concentration of oil tested (5 g/L), which is higher than the maximum concentration permitted by environmental legislation in Brazil (20 ppm) (CONAMA, 2011). This result indicates the potential of the DAF-biosurfactant

process for the treatment of oily effluents and the possibility of reusing the treated water.

In an experiment with a DAF system using synthetic and biological surfactants, Albuquerque et al. (2012) demonstrated that the biosurfactant produced by *C. lipolytica* performed better than the chemical surfactant sodium oleate in the removal of heavy metals. In another experiment, a biosurfactant potentiated the separation efficiency of oily material in a pilot-scale DAF system from 80.0% to 95.0%, demonstrating that the process was effective with the use of the biotensioactive agent, making flotation a cleaner technology and effective at separating oil from water (Rocha e Silva et al., 2015).

The potential use of the biosurfactant formulated in the present study, with its tensioactive properties preserved over a period of 180 days, is promising from the economic standpoint, as the biomolecule was produced from industrial waste products at low cost and applied without the need for downstream steps, which correspond to approximately 60% of the final cost of obtaining a biosurfactant currently on the market (Santos et al., 2016). Therefore, this study demonstrates the potential for the use of this biotechnological agent to enhance the efficiency of treatment processes for oily effluents generated by industrial activities.

4. Conclusion

The results of the present study indicate that the biosurfactant produced by *B. methylotrophicus* did not undergo any significant changes in terms of tensioactive properties during 180 days of storage following two conservation processes, demonstrating stability and resistance to extreme conditions of pH, salinity and temperature. The heat-treated conservation method achieved better results. The dispersion capacity, which is a very important factor for a tensioactive agent, was

better in the highest proportions of oil tested, demonstrating the potential of this biosurfactant for application in the containment of large oil spills. The formulated biosurfactant from *B. methylotrophicus* added to the bench-scale DAF system led to a significant increase in the oil removal rate, making the oil separation process more complete for the treatment of effluents from an industrial environment. Besides enhancing the separation efficiency of DAF systems, the use of biosurfactants constitutes a sustainable practice that enables the use of industrial waste products. Therefore, the biosurfactant described herein is a promising product with applications in different steps of oily effluent treatment in the industrial environment.

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Table 1

Values of independent variables at -1.41, -1.00, 0.00, +1.00 and +1.41 levels for evaluation of removal of motor oil from synthetic effluent

	Coded levels of variable				
Variable	**-1.41**	**-1.00**	**0.00**	**+1.00**	**+1.41**
Effluent flow rate (L/min), X_1	2.39	3.00	4.50	6.00	6.62
Formulated biosurfactant flow rate (L/min), X_2	0.08	0.20	0.50	0.80	0.98

Table 2

CCRD planning matrix with two variables for evaluation of removal of motor oil from synthetic effluent by action of formulated biosurfactant

Assay	Effluent flow rate (L/min), X_1	Formulated biosurfactant flow rate (L/min), X_2
1	3.00	0.20
2	6.00	0.20
3	3.00	0.80
4	6.00	0.80
5	2.39	0.50
6	6.62	0.50
7	4.50	0.08
8	4.50	0.98
9	4.50	0.50
10	4.50	0.50
11	4.50	0.50

Table 3

CCRD planning matrix and results: Removal of hydrophobic contaminant by formulated biosurfactant from *B. methylotrophicus* as collector in bench-scale DAF prototype

Assay	Effluent flow rate (L/min), X_1	Formulated biosurfactant flow rate (L/min), X_2	Oil removal (%) Y
1	3.00	0.20	62
2	6.00	0.20	81
3	3.00	0.80	48
4	6.00	0.80	16
5	2.39	0.50	83
6	6.62	0.50	18
7	4.50	0.08	36
8	4.50	0.98	82
9	4.50	0.50	92
10	4.50	0.50	91
11	5.50	0.50	91
12	4.50	0.50	92

Table 4

Values of parameters obtained in variance of analysis (ANOVA) of quadratic polynomial model

Factors	Quadratic sum	Degrees of freedom	Quadratic mean	Calculated F	p-value
(1) QE (L)	3651.872	1	3651.872	10955.61	0.000002
QE (Q)	2890.000	1	2890.000	8670.00	0.000003
(2) QB (L)	338.700	1	338.700	1016.10	0.000068
QB (Q)	1849.600	1	1849.600	5548.80	0.000005
1L by 2L	650.250	1	650.250	1950.75	0.000026
Lack of Fit	800.428	3	266.809	800.43	0.000075
Pure Error	1.000	3	0.333		
Total SS	9416.000	11			

R^2 = 91.50%

Figure Captions

Fig. 1. Bench-scale DAF prototype for treatment of synthetic oily effluent.

Fig. 2. Surface tension of biosurfactant produced by *B. methylotrophicus* over 180 days of storage submitted to (A) addition of 0.2% potassium sorbate or (B) heat treatment and addition of 0.2% potassium sorbate.

Fig. 3. Emulsification activity of biosurfactant produced by *B. methylotrophicus* over 180 days of storage submitted to (A) addition of 0.2% potassium sorbate or (B) heat treatment and addition of 0.2% potassium sorbate.

Fig. 4. Dispersion capacity of motor oil by biosurfactant produced by *B. methylotrophicus* over 180 days of storage submitted to conservation method with addition of 0.2% potassium sorbate at biosurfactant-to-oil proportions of (A) 1:2, (B) 1:8 and (C)1:25 (v/v).

Fig. 5. Dispersion capacity of motor oil by biosurfactant produced by *B. methylotrophicus* over 180 days of storage submitted to conservation method with heat treatment and addition of 0.2% potassium sorbate at biosurfactant-to-oil proportions of (A) 1:2, (B) 1:8 and (C)1:25 (v/v).

Fig. 6. Response surface graph of effects of effluent flow rate and biosurfactant flow rate on oil removal rate from synthetic effluent in DAF system.

Fig. 7. Graph with level curves corresponding to removal of oil from synthetic effluent by formulated biosurfactant in DAF system.

Fig. 8. Observed and predicted results of CCRD for flotation with formulated biosurfactant.

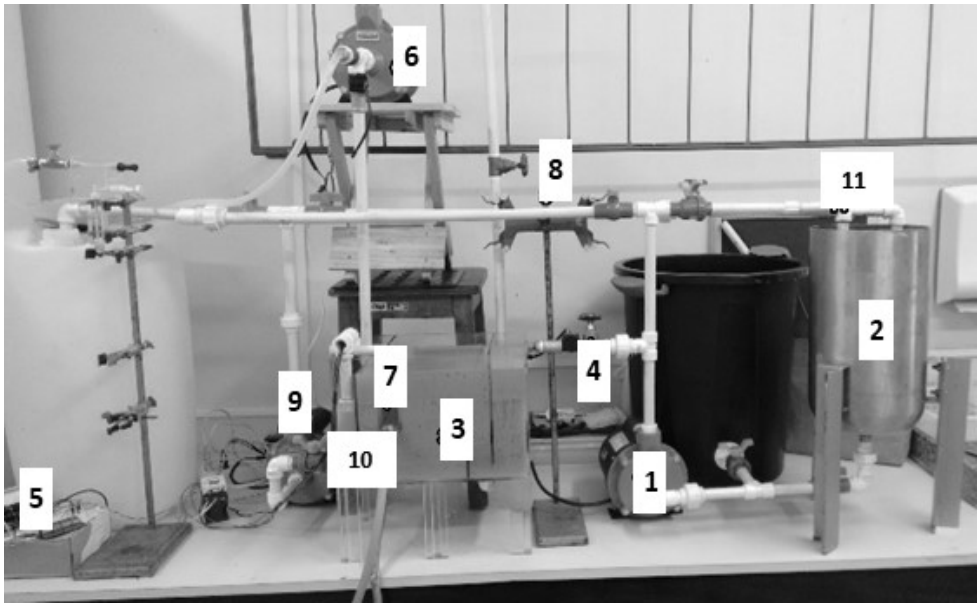

Fig. 1.

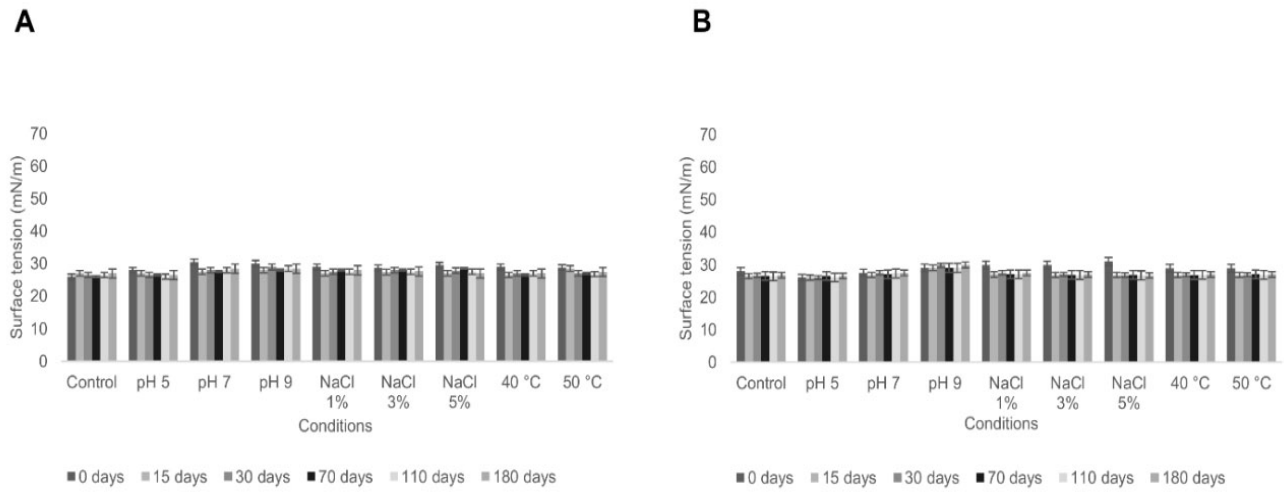
A
B
Surface tension (mN/m)
70
60
50
40
30
20
10
0
Control
pH 5
pH 7
pH 9
NaCl 1%
NaCl 3%
NaCl 5%
40 °C
50 °C
Conditions
0 days
15 days
30 days
70 days
110 days
180 days

Fig. 2

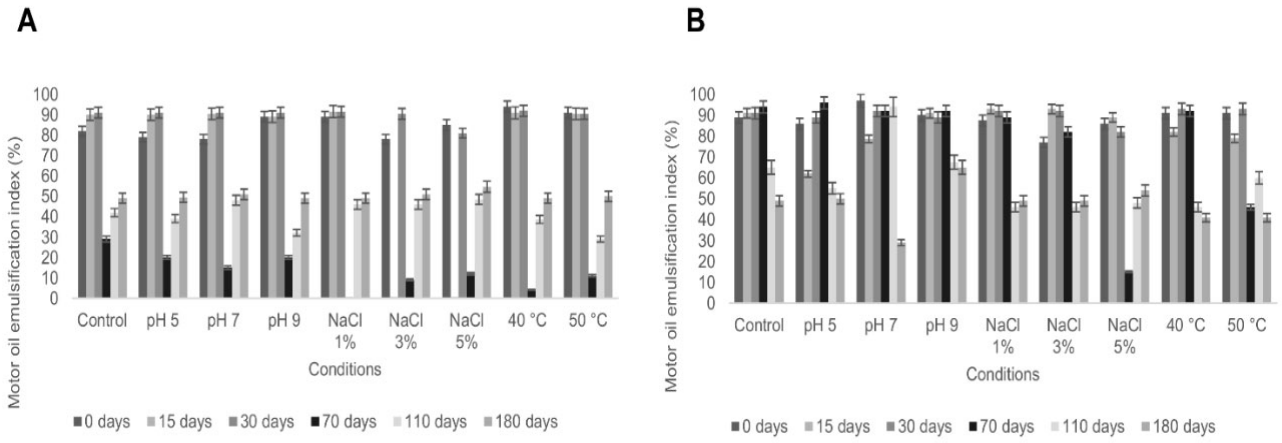
A
B
Motor oil emulsification index (%)
100
90
80
70
60
50
40
30
20
10
0
Control
pH 5
pH 7
pH 9
NaCl 1%
NaCl 3%
NaCl 5%
40 °C
50 °C
Conditions
0 days
15 days
30 days
70 days
110 days
180 days

Fig. 3.

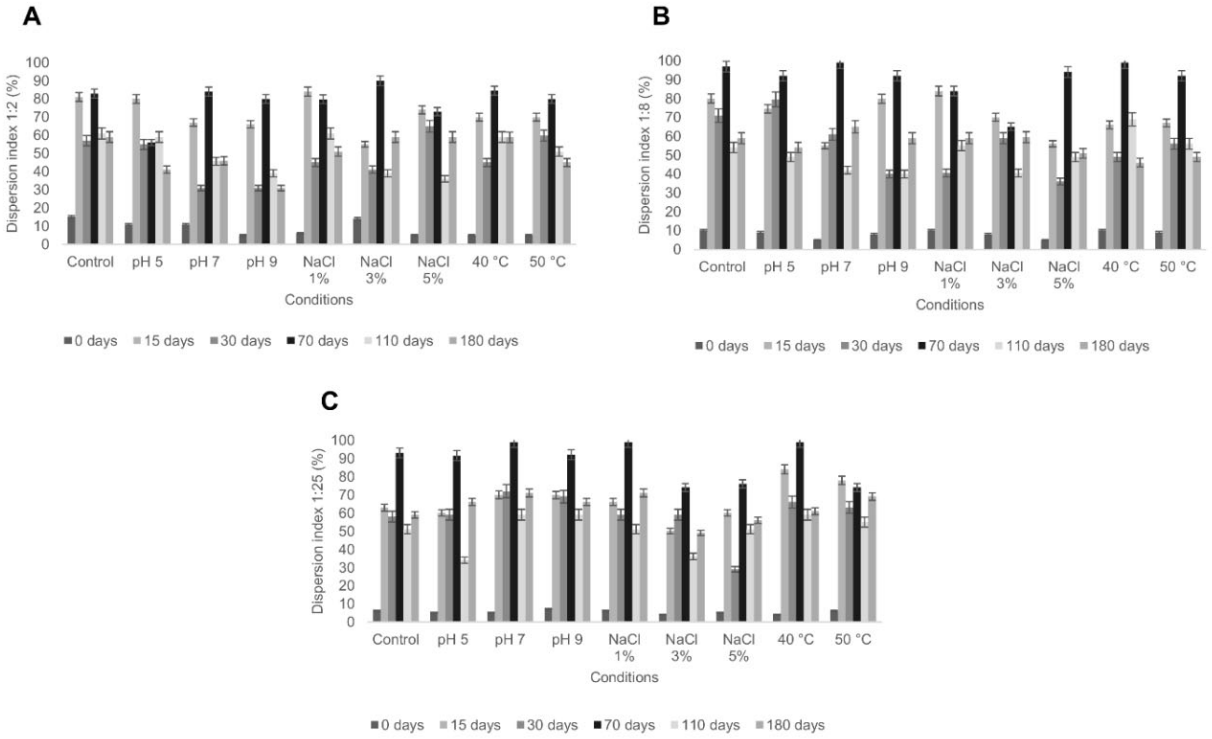
A
Dispersion index 1:2 (%)
100
90
80
70
60
50
40
30
20
10
0
Control
pH 5
pH 7
pH 9
NaCl 1%
NaCl 3%
NaCl 5%
40 °C
50 °C
Conditions
0 days
15 days
30 days
70 days
110 days
180 days
B
Dispersion index 1:8 (%)
Conditions
C
Dispersion index 1:25 (%)
Conditions

Fig. 4.

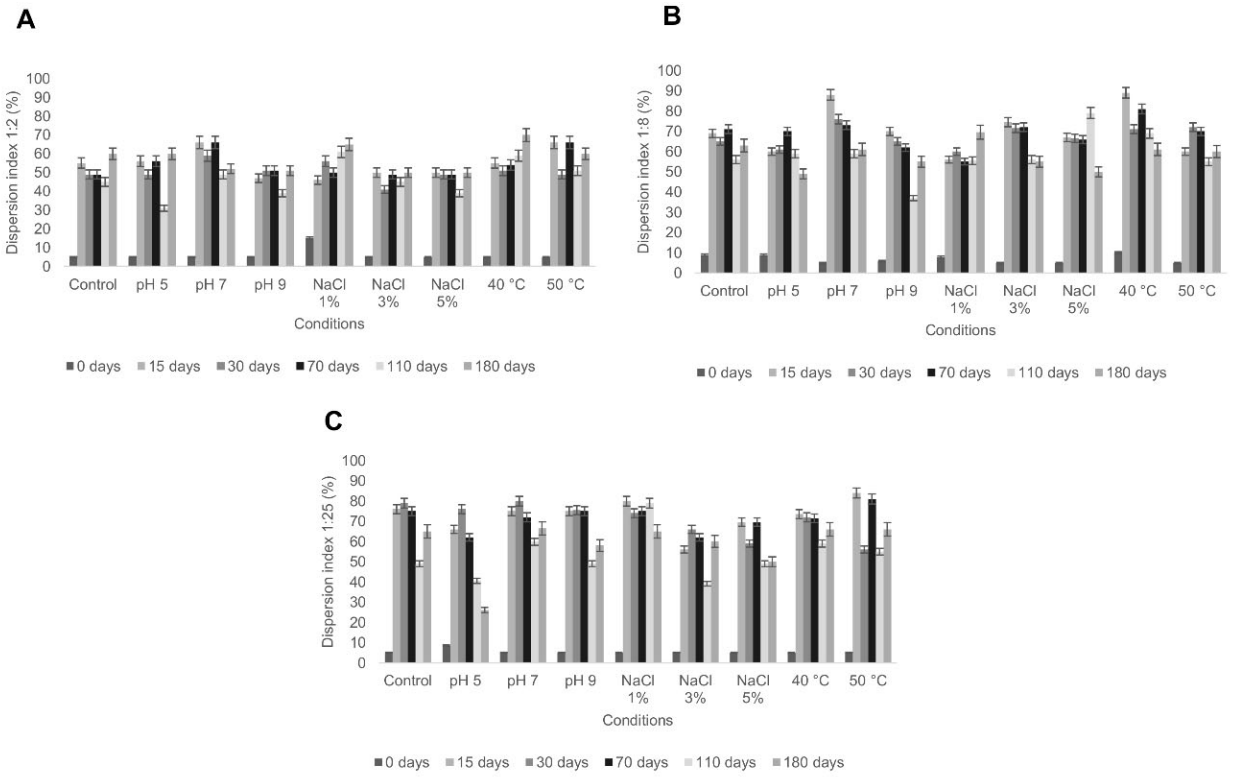
A
Dispersion index 1:2 (%)
100
90
80
70
60
50
40
30
20
10
0
Control
pH 5
pH 7
pH 9
NaCl 1%
NaCl 3%
NaCl 5%
40 °C
50 °C
Conditions
0 days
15 days
30 days
70 days
110 days
180 days
B
Dispersion index 1:8 (%)
Conditions
C
Dispersion index 1:25 (%)
Conditions

Fig. 5.

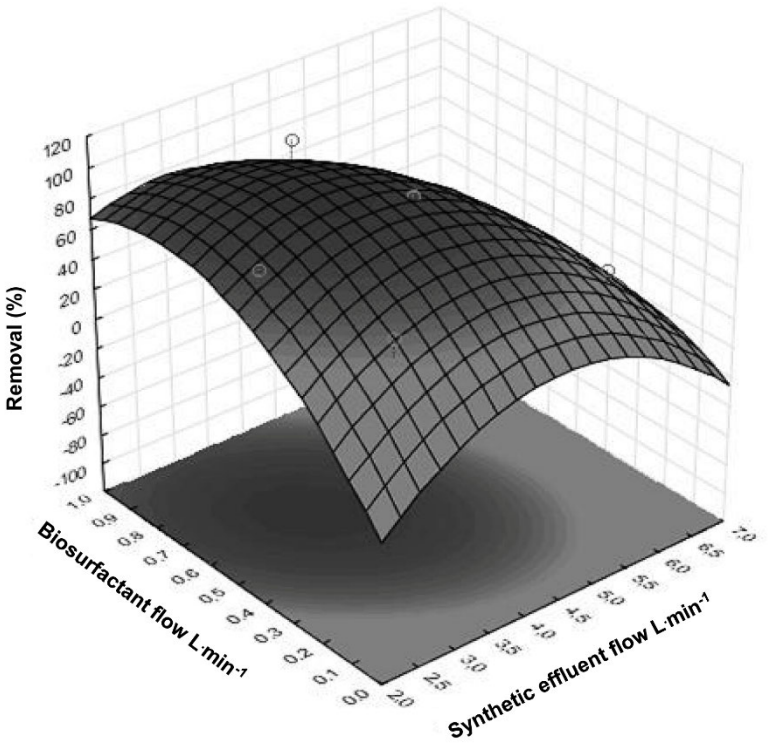
Removal (%)
120
100
80
60
40
20
0
-20
-40
-60
-80
-100
Biosurfactant flow L·min-1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Synthetic effluent flow L·min-1
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0

Fig. 6.

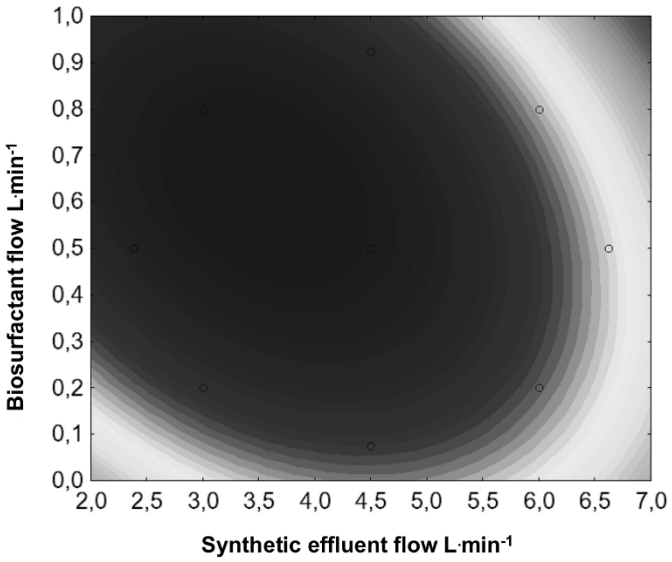
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
Biosurfactant flow L·min-1
2,0
2,5
3,0
3,5
4,0
4,5
5,0
5,5
6,0
6,5
7,0
Synthetic effluent flow L·min-1

Fig. 7.

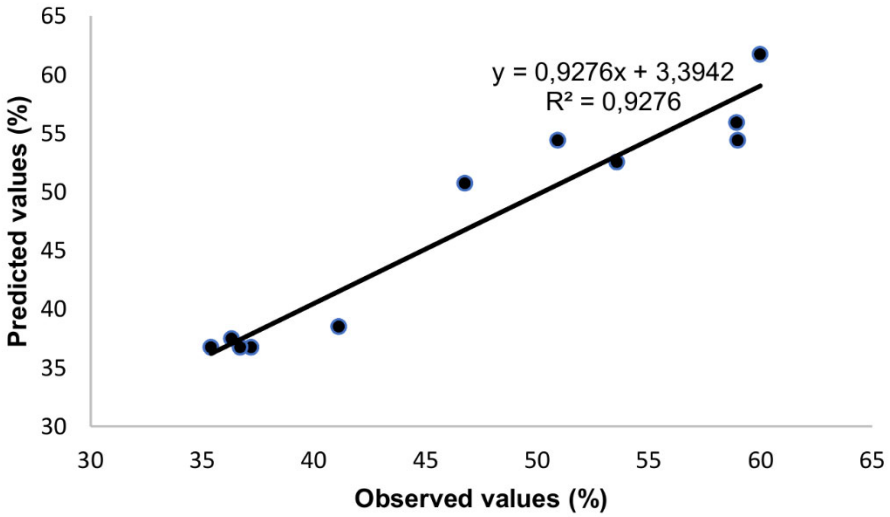
y = 0,9276x + 3,3942
R² = 0,9276
Predicted values (%)
Observed values (%)
65
60
55
50
45
40
35
30
30
35
40
45
50
55
60
65

Fig. 8.

4.3. CAPÍTULO 3 – Application of a biosurfactant from *Bacillus methylotrophicus* as a collector in an oily water flotation system with an induced air pre-saturation chamber

ARTIGO SUBMETIDO PARA PUBLICAÇÃO NO PERIÓDICO INTERNACIONAL JOURNAL OF PETROLEUM SCIENCE AND ENGINEERING

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Application of a biosurfactant from *Bacillus methylotrophicus* as a collector in an oily water flotation system with an induced air pre-saturation chamber

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Abstract

The treatment of oily water generated during industrial activities is a priority in the current scenario. To remediate this problem, numerous separation technologies have been applied, such as flotation employing the saturation of the effluent with microbubbles of air. The market has driven the development and use of nontoxic, biodegradable surfactants, which can serve as alternative collectors in flotation procedures and further increase the acceptance of this separation technology. In the present study, we performed a comparative analysis using a biosurfactant produced by *Bacillus methylotrophicus* CCT1616 and commercial surfactants as collectors. The collectors were applied interacting with the action of microbubbles in a bench-scale induced-air pre-saturation chamber (IAPSC) for the treatment of oily effluent. The results demonstrated that the IAPSC system achieved the removal of 80.16% of the oil with the action of the microbubbles alone. The water/oil separation capacity of the biosurfactant from *B. methylotrophicus* CCT1616 in comparison to the commercial collectors was demonstrated by the 99.00% removal rate of the oil from the effluent. The use of this biomolecule enhanced the separation efficiency of the prototype. This result is quite promising as a process that can be used to meet industrial demands. In conclusion, the use of biosurfactants as collectors is a promising alternative for the treatment of oily effluents in flotation processes.

Keywords: Biosurfactant; *Bacillus methylotrophicus*; oily water; induced pre-saturation; collector.

1. Introduction

Due to the growth of human populations and the increase in industrial activities, environmental problems have become increasingly commonplace in recent decades, causing the pollution of surface water and groundwater (Cai et al., 2018). Contamination due to petroleum products is one of the most widespread concerns and numerous cleaning technologies have been developed to remediate this problem (Chaprão et al., 2018; Mnif et al., 2017; Rocha e Silva et al., 2019).

Treatment methods for industrial wastewater vary depending several factors, such as the volume involved, the composition of the effluent and the limits of environmental legislation in each country. Examples of treatment processes for oily water are electroflotation, dissolved air flotation, induced air flotation, column flotation, hydrocyclones and decanters (Prakash et al., 2018). The purpose of these treatments is the reduction in the concentration of the oil dispersed in the water so that it can either be discarded after reaching the limit permitted by environmental law or reused in the industrial process (Rajasulochana and Preethy, 2016).

Flotation is one of the most indicated technologies for the treatment of oily water generated during industrial activities. Its high degree of efficiency and the ability to control physical variables, such as microbubble size, hydraulic retention time, etc., make it stand out among current oil-water separation methods (Rocha e Silva et al., 2018b).

The application of the flotation process was perfected with the development of the industrial sector, with the emergence of dissolved air flotation (DAF), which is a process involving the removal of a solute by adsorption through the action of microbubbles or nanobubbles by coprecipitation or by an *in situ* occlusion of the

transporting floc, which is then propelled by the addition of an adequate tensioactive agent (Albuquerque et al., 2012).

The use of flotation as a separation process has been criticised due to the probable toxicity of the collectors used in this process, which are chemical surfactants (Menezes et al., 2011). Thus, oil is not the only determinant factor of toxicity in the environment. Evidence shows the considerable presence of polycyclic aromatic hydrocarbons in oils dispersed by chemical tensioactive agents, which cause greater toxicity to aquatic organisms (Silva et al., 2014). Thus, alternatives have been evaluated to replace toxic synthetic surfactants in flotation processes, such as microbial biosurfactants, which are biodegradable biomolecules with low toxicity (Almeida et al., 2016). Biosurfactants are considered the biotechnological compounds of the 21st Century and can be applied in the most diverse industrial fields as well as treatment processes for oily effluents (Singh et al., 2019).

The efficiency of flotation systems can be enhanced by adjusting the operational parameters, such as the pre-saturation of the effluent and the use of biodegradable tensioactive agents, which enhance the adhesion of the microbubbles to the oil droplets (Rocha e Silva et al., 2018b). The pre-saturation of the effluent reduces one of the steps in the DAF chamber, which is the contact step, leaving only the flotation step and separation of the oily foam from the liquid phase (Rubio et al., 2002).

The aim of the present study was to evaluate the use of a biosurfactant produced by *Bacillus methylotrophicus* CCT1616 as a collector combined with induced air pre-saturation for the treatment of oily effluent. The experiments were conducted in a laboratory-scale induced air pre-saturation chamber.

2. Materials and Methods

2.1. Materials

The anionic surfactant sodium dodecyl sulfate (SDS), furnished by VETEC LTD., Brazil, with a critical micelle concentration (CMC) of 0.0085 mol/L, and a commercial anionic biosurfactant classified as a rhamnolipid (CMC of 300 mg/L), furnished by SIGMA-ALDRICH BRASIL, a subsidiary of MERCK, were used in the experiments. The biosurfactant from *B. methylotrophicus* CCT1616 was previously characterised by Chaprão et al. (2018a) and has a CMC of 600 mg/L. The substances used as substrates for the production of the biosurfactant were sugarcane molasses as the carbon source, obtained from a local sugar processing plant in the municipality of Vitória de Santo Antão, and corn steep liquor as the nitrogen source, obtained from Ingredion Brazil in the municipality of Cabo de Santo Agostinho, both located in the state of Pernambuco, Brazil.

A synthetic oily effluent was used, consisting of a concentration 50 ppm of motor oil in water produced through the combined flow of water and oil into the flotation system to obtain the homogenisation of the effluent. The waste motor oil was obtained from an automotive maintenance establishment in the city of Recife, Pernambuco, Brazil. According to current Brazilian legislation, 20 ppm is the maximum concentration of oil permitted in industrial effluents (Brasil, 2011).

2.2. Micro-organism

The bacteria *Bacillus methylotrophicus* CCT1616 isolated from the port area of the city of Recife, PE, Brazil, and deposited in the Culture Bank of the Environment Science Research Centre of *Universidade Católica de Pernambuco* was used as the biosurfactant producer (Chaprão et al., 2018a).

2.3. Growth medium for inoculum

Young cultures of the bacteria obtained after 24 hours of cultivation in AN medium were transferred to Erlenmeyer flasks containing 50 mL of nutritive broth with the following composition: meat extract (5.0 g/L), peptone (15.0 g/L), NaCl (5.0 g/L), K_2HPO4 (5.0 g/L) and distilled water (1.0 L), pH 7.0. The solution was kept under orbital stirring at 150 rpm for 10 to 14 hours at 28°C to obtain 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 a concentration of 3% (v/v) (Chaprão et al., 2018b).

2.4. Production of biosurfactant

The biosurfactant was produced in the mineral medium described by Bushnell and Hass (1941) composed of KH_2PO_4 (1.0 g/L), K_2HPO_4 (1 g/L), $MgSO_4.7H_2O$ (0.2 g/L), $CaCl_2.H_2O$ (0.2 g/L) and $FeCl_3.6H_2O$ (0.05 g/L). The medium was supplemented with 3% sugarcane molasses and 3% corn steep liquor. Fermentations for the production of biosurfactant were performed in Erlenmeyer flasks containing 500 mL of the medium for 48 hours at 28ºC and 200 rpm. The broth containing the biosurfactant was centrifuged at 5000 rpm for 30 min to separate the microbial biomass and obtain the crude biosurfactant (Chaprão et al., 2018b).

2.5. Measurement of surface tension

Surface tension was measured in the cell-free broth (crude biosurfactant) using a tensiometer (KSV Sigma 700, Finland) with a du Noüy ring. The platinum ring was immersed into the broth and the force required to pull it through the liquid-air interface was recorded (Chaprão et al., 2018a).

2.6. Formulation of biosurfactant

Potassium sorbate (0.2%) was added to the cell-free broth containing the crude biosurfactant as a conservative and the solution was stored under sterile conditions in a hermitically sealed recipient at room temperature (Soares da Silva et al., 2019).

2.7. Extraction of biosurfactant

A solution of HCl (6.0 M) was used to adjust the pH of the cell-free broth to 2.0, followed by the addition of an equal volume of $CHCl_3$/CH_3OH (2:1, v/v). After vigorous shaking for 15 min, the mixture was set to rest until the separation of the phases. The organic phase was removed and the procedure was repeated twice more. A rotary evaporator was used to concentrate the product from the organic phases, obtaining a viscous, yellowish product, which was dissolved in methanol and concentrated by evaporating the solvent at 45°C. The isolated biosurfactant was weighed and the concentration was expressed as g/L (Ibrahim et al., 2013; Durval et al., 2018).

2.8. Experimental flotation design

The trials were performed in a bench-scale induced air pre-saturation chamber (IAPSC), as shown in the schematic representation (Fig. 1). One hundred litres of oily effluent were used with each collector tested: crude biosurfactant from *B. methylotrophicus* (1.5 L), formulated biosurfactant from *B. methylotrophicus* (1.5 L), isolated biosurfactant from *B. methylotrophicus* at ½ x CMC (300 mg/L), commercial rhamnolipid biosurfactant at ½ x CMC (150 mg/L) and SDS at ½ x CMC (1.2 g/L). Each mixture was homogenised for approximately 30 minutes to obtain a uniform

water/oil/collector distribution. The oily effluent without the addition of collector was used as the control.

Procedure: Oily water is pumped from the feed tank (1) by a centrifugal pump (2) adapted to previously saturate the effluent to be treated with atmospheric air. The air enters the system quantified and regulated with the aid of a rotameter (3) and needle valve (4). The saturation process occurs within the pump and its feed line under a pressure of 5.5 bar maintained with the aid of a control valve (5). The affluent of the process (pre-saturated with microbubbles of air) enters the flotation chamber (6) where the oil-water separation occurs. It is essential to maintain the level of the effluent at the height of the lid of the chamber (7) so that the oily foam can be pushed to the tube connected to the highest part of the lid (8) for discharge. The treated water exits through the base of the flotation chamber by a tube that forms a hydraulic seal (9) for the maintenance of the level of the saturated effluent in the chamber. A valve is installed at the base of the flotation chamber for the withdrawal of samples of the treated water (10). A portion of tube is connected to the top of the tube that forms the seal; this portion of tube is open to the atmosphere (11) to break the vacuum during maintenance or cleaning of the system. A gate valve is maintained closed during the operation of the system to produce the vacuum (Venturi effect) (12) during the de-obstruction of the oily foam discharge tube.

Insert Figure 1

The experimental flotation system (Fig. 1) has a chamber with an effective volume of 3.4 L and operates with a flow of 2.0 $L \cdot min^{-1}$. Under these conditions, the hydraulic retention time is 2:50 min. The air flow for the saturation of the synthetic effluent is 2.0 $L \cdot min^{-1}$. Samples of treated effluent were collected after 4, 8, 12, 16 and 20 minutes of the process for the determination of the percentage of oil removal.

2.9. Quantification of oil removed during process

Oil was extracted from samples of the treated synthetic oily effluent with the same volume of hexane (1:1, v/v). The mixture was shaken vigorously for 15 min and set to rest for the separation of the phases. The organic phase was removed. After the extractions, the results were obtained using a UV-Vis spectrophotometer (SP-22-BIOSPECTRO) and readings were performed at a wavelength of 330 nm in relation to the calibration curve prepared with a standard solution of the oil at 5000 mg/L in a 100-mL volumetric flask. The solutions were diluted in n-hexane at concentrations ranging from 1 to 1000 mg/L beginning with the initial sample. N-hexane was used at the blank to calibrate the device. The solvent was analytical grade and adequate for the spectrophotometric equipment (Emmandi et al., 2014). All experiments were performed in triplicate at room temperature (27°C) and mean values are reported.

3. Results and Discussion

3.1. Evaluation of collector efficiency in flotation system with induced air pre-saturation

Although use is still quite limited, biosurfactants have demonstrated promising results when employed as collectors in flotation processes. Innovations in this field will determine the sustainability and economic viability of this technology. However, it is not easy to implement clean technologies in traditional industrial processes (Rocha e Silva et al., 2018b).

The three collectors tested in the present study (the biosurfactant under study, a commercial biosurfactant and a synthetic surfactant) exhibited satisfactory removal rates (Fig. 2). The flotation trials performed with the biosurfactant from

Bacillus methylotrophicus UCP1616 in its crude, formulated and isolated forms achieved ascending removal rates with the increase in the residence time of the collector in relation to the effluent. The crude biosurfactant removed 98.0%, the formulated biosurfactant removed 98.39% and the isolated biosurfactant removed 99.0% of the oil after 20 minutes. Besides being efficient, the crude biosurfactant has the advantage of being obtained as a lower cost due to the non-need for extraction and/or purification steps and can be easily obtained in large volumes with the use of industrial bioreactors. The use of a crude biosurfactant results in a reduction on the order of 70% of the total cost of the process (Santos et al., 2016). The commercial rhamnolipid biosurfactant achieved a 90.52% oil removal rate and the synthetic surfactant (SDS) achieved an 86.74% removal rate. It should be pointed out that the flotation trial without the addition a collector (action of microbubbles alone) also had ascending removal rates with the increase in time, achieving 80.16%.

Recent studies have demonstrated the successful use of microbial biosurfactants as collectors in flotation processes. Chaprão et al. (2018a) also used the biosurfactant from *Bacillus methylotrophicus* UCP1616 in a horizontal DAF prototype, achieving a 92% oil removal rate from a synthetic effluent. Rocha e Silva et al. (2015) investigated the removal of petroleum products emulsified in water in a pilot-scale DAF with and without the use of a biosurfactant. The biosurfactant aggregated considerable value to the process, increasing the separation efficiency from 80.0% to 98.0%. Silva et al. (2018b) investigated the separation of oil from water using a pilot-scale horizontal DAF prototype with and without the use of a microbial biosurfactant and obtained an increase in water/oil separation efficiency from 41.0% to 98.0%. Other types of biosurfactants produced by species of the genus *Candida* have also been successfully employed in the flotation of heavy

metals, with cation removal rates higher than 90% in DAF columns (Albuquerque et al., 2012; Menezes et al., 2011).

The present results reveal the advantages of using an induced air pre-saturation chamber (IAPSC) over the horizontal DAF system cited by Chaprão et al. (2018a). The IAPSC system led to a 7% increase in oil removal efficiency in comparison to the horizontal DAF using the same biosurfactant produced by *B. methylotrophicus*. The greater efficiency of the IAPSC is due to the injection of air in favour of the effluent flow (concurrent flow). In contrast, the air flow is orthogonal in relation to the horizontal flow of the effluent in a traditional DAF system. Figs. 2 and 3 show an important increase in efficiency in the trials performed with a 20-minute operating time, which was due to the greater effluent residence time, enabling greater contact with the injected air.

Insert Figure 2

One-way analysis of variance (ANOVA) was performed to determine statistically significant differences among the means of the responses induced by variations in the independent variables. The differences were statistically significant ($p < 0.0001$). Using ANOVA, a diagnostic model was created, in which the values approached the slope, demonstrating the supposition of normality. The boxplot enables visualizing the distribution of the experimental data (mean, maximum and minimum values) (Fig. 3).

Insert Figure 3

Conclusion

The biosurfactant produced by *Bacillus methylotrophicus* demonstrated promising results in comparison to commercial collectors in the comparative analysis. The present findings reveal the considerable potential of this biosurfactant for use as a collector in the treatment of oily effluents in a bench-scale flotation system with an induced air pre-saturation chamber. The biosurfactant produced by *B. methylotrophicus* led to a significant increase in the oil removal rate, making the oil effluent treatment process more adequate to an industrial environment.

Acknowledgments

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Figure captions

Fig. 1. Vertical bench-scale flotation system with induced air pre-saturation of effluent. Feed tank (1), centrifugal pump (2), rotameter (3), needle valve (4), control valve (5), flotation chamber (6), lid of the chamber (7), discharge (8), hydraulic seal tube (9), valve for the withdrawal of samples of the treated water (10), tube open to the atmosphere (11), gate valve (12).

Fig. 2. Oil removal efficiency at different times in flotation process with induced air pre-saturation of oily effluent in absence and presence of different collectors.

Fig. 3. Boxplot of flotation trials.

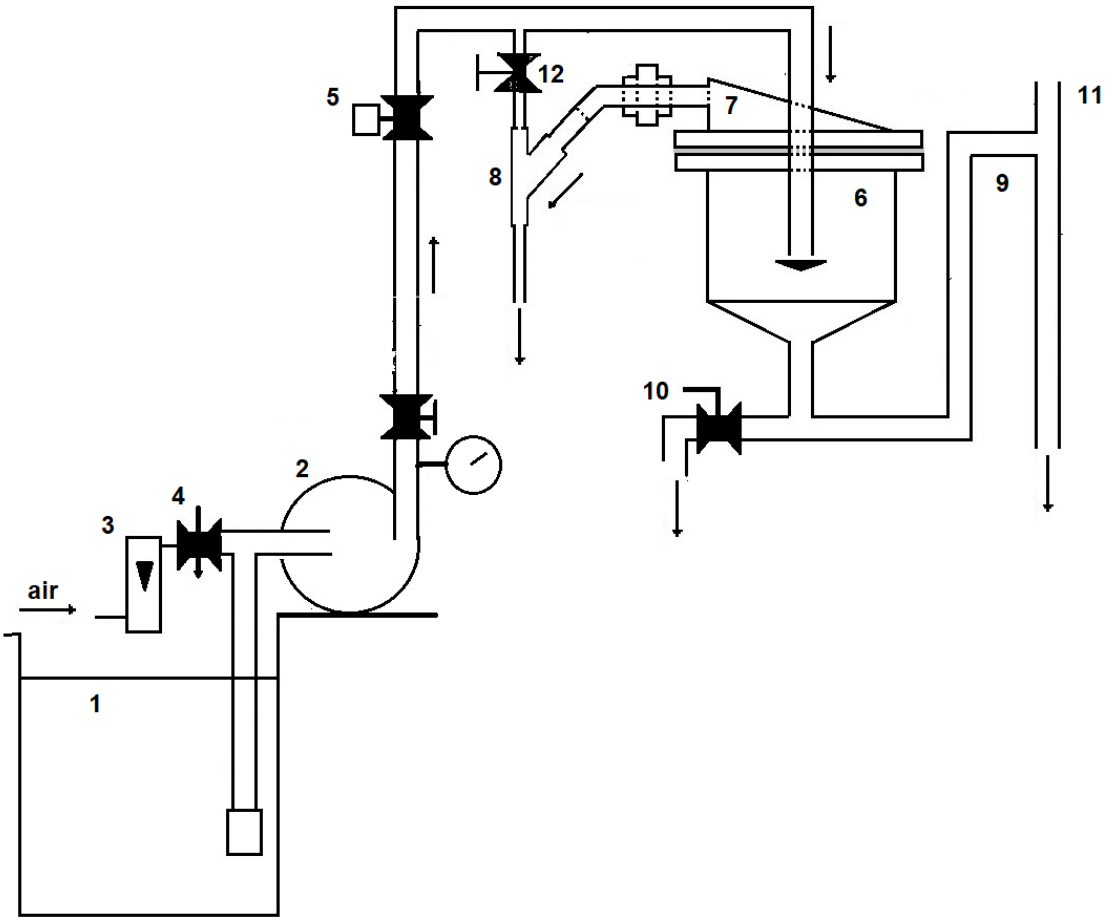
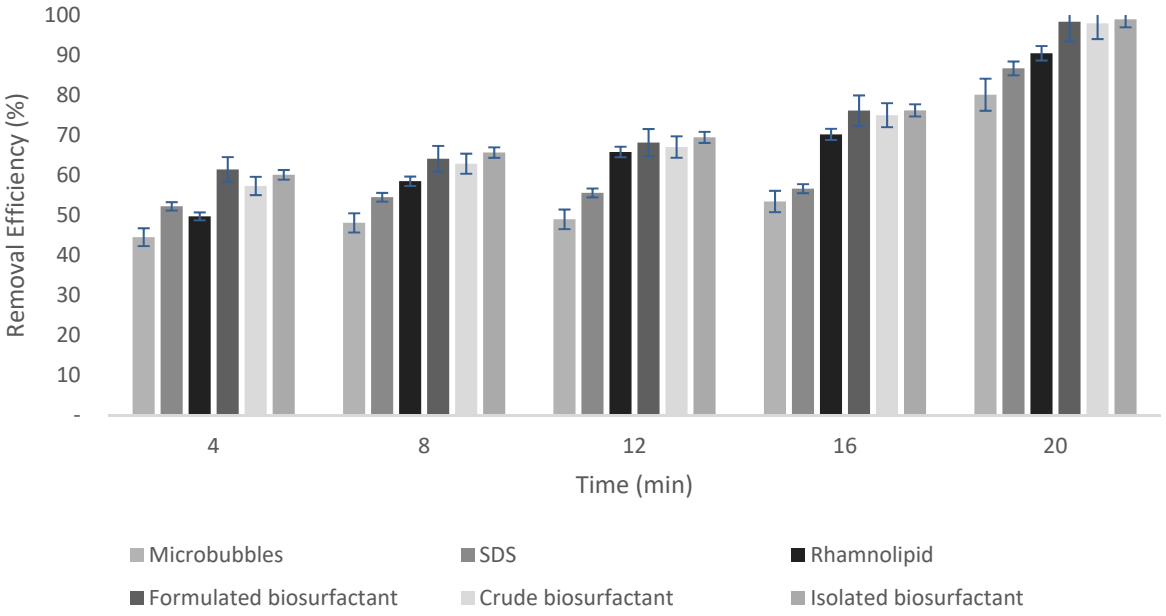


Fig. 1.


Removal Efficiency (%)
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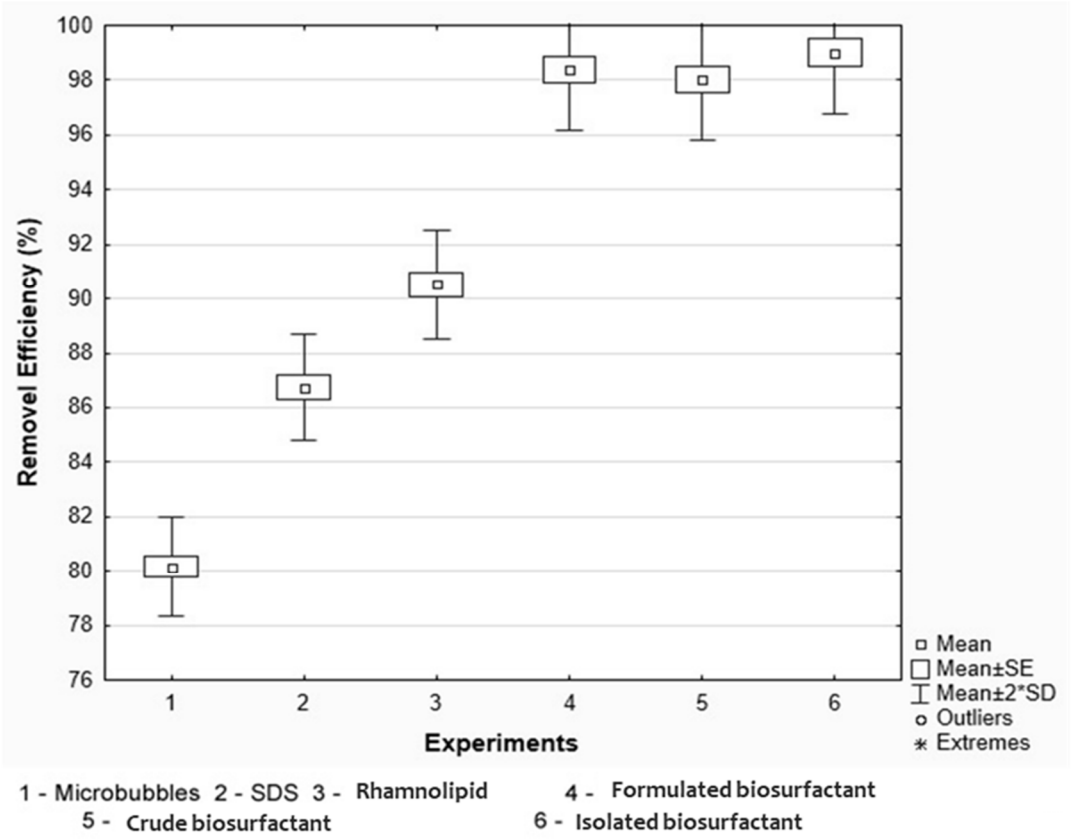
Time (min)
Microbubbles
SDS
Rhamnolipid
Formulated biosurfactant
Crude biosurfactant
Isolated biosurfactant

Fig. 2.


Removel Efficiency (%)
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Experiments
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1 - Microbubbles 2 - SDS 3 - Rhamnolipid 4 - Formulated biosurfactant
5 - Crude biosurfactant 6 - Isolated biosurfactant

Fig. 3.

5. CONCLUSÕES

Com os resultados obtidos foi possível alcançar as seguintes conclusões:

- A produção de biossurfactante por *Bacillus methylotrophicus* CCT1616 utilizando resíduos industriais foi satisfatória, possibilitando uma redução dos custos de obtenção do biotensoativo.
- O biossurfactante demonstrou estabilidade reduzindo consideravelmente a tensão superficial e apresentou capacidade de emulsificação elevada frente a condições ambientais extremas de salinidade, temperatura e variações de pH.
- Baseado nos resultados de caracterização química, o biossurfactante obtido foi considerado um lipopeptídeo.
- O biossurfactante produzido por *B. methylotrophicus* apresentou baixa toxicidade as sementes de *Brassica oleracea*, demonstrando possibilidade de atuação em solo.
- O biossurfactante produzido apresentou potencial de remoção de derivado de petróleo adsorvido em areia e solo arenoso sob condições cinéticas.
- O biotensoativo demontrou viabilidade de aplicação como dispersante biológico para remoção de poluentes hidrofóbicos em superfícies porosas, como recifes de coral.
- O biossurfactante demonstrou inocuidade e grande potencial de aplicação em processos de biorremediação de petroderivado em água do mar.
- O biossurfactante formulado manteve as suas propriedades tensoativas estáveis durante um longo período de armazenamento, permitindo sua produção industrial associada a uma logística de estoque para aplicação imediata.
- A eficiência do bioproduto formulado e aplicado como coletor alternativo foi demonstrada pela alta taxa de remoção de óleo no protótipo FAD de bancada.
- O biossurfactante de *B. methylotrophicus* contribuiu positivamente para elevar a eficiência de separação do óleo na Câmera de flotação com pré-saturação induzida (CPSI) de bancada.
- Os resultados demonstram que o biossurfactante de *B. methylotrophicus* aumenta a eficiência do processo FAD, tornando esta tecnologia mais limpa.

APÊNDICES

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Production of a biosurfactant from *Bacillus methylotrophicus* UCP1616 for use in the bioremediation of oil-contaminated environments

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Abstract

The aim of the present study was to produce a microbial biosurfactant for use in the bioremediation of environments contaminated with petroleum products. *Bacillus methylotrophicus* was isolated from seawater taken from a port area and cultivated using industrial waste as substrate (corn steep liquor and sugarcane molasses [both at 3%]). Surface tension measurements and motor oil emulsification capacity were used for the evaluation of the production of the biosurfactant, which demonstrated stability in a broad range of pH and temperature as well as a high concentration of saline, with the reduction of the surface tension of water to 29 mN/m. The maximum concentration of biosurfactant (10.0 g/l) was reached after 144 h of cultivation. The biosurfactant was considered to be a lipopeptide based on the results of proton nuclear magnetic resonance and Fourier transformed infrared spectroscopy. The tests demonstrated that the biosurfactant is innocuous and has potential for the bioremediation of soil and water contaminated by petroleum products. Thus, the biosurfactant described herein has a low production cost and can be used in environmental processes.

Keywords Surfactant · *Bacillus* · Bioavailability · Bioremediation · Industrial waste

Introduction

Spills often occur during oil exploration and transport and cause serious environmental problems. Mechanical recovery with the use of sorbents is a promising oil removal method and involves the transference of oil from a contaminated area to a temporary storage facility. However, the majority of sorbents used in this process end up in landfills or incinerators and are therefore an additional source of pollution, the treatment of which translates to an increase in the cost of the oil recovery method (Almeida et al. 2016).

Stricter environmental laws have led to the search for sustainable technologies involving the use of biodegradable compounds for the treatment of hydrocarbon-contaminated sites. For such, surfactants acquired from living organisms have been tested, such as plant-derived saponins, bile salts from animals and microbial-produced lipopeptides and glycolipids. These natural compounds with surfactant properties are denominated biosurfactants (Campos et al. 2013).

Biosurfactants have hydrophilic and hydrophobic moieties that act between fluids with different polarities (such as oil and water), enabling access to hydrophobic substrates through an increase in the area of contact of insoluble compounds as well as enhanced mobility and bioavailability, leading to the biodegradation of these substrates. These features enable biosurfactants to lower both surface and interfacial tension as well as form microemulsions by which hydrocarbons can be solubilised in water or vice versa. Therefore, biosurfactants have applications in industries due to their properties of detergency, lubrication,

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Formulation and application of a biosurfactant from *Bacillus methylotrophicus* as collector in the flotation of oily water in industrial environment

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ABSTRACT

The present study describes the formulation of a biosurfactant produced by *Bacillus methylotrophicus* UCP1616 and investigates its long-term stability for application as a collector in a bench-scale dissolved air flotation (DAF) prototype. For formulation, the conservative potassium sorbate was added to the biosurfactant with or without prior heat treatment at 80 °C for 30 min. After formulation, the biosurfactant samples were stored at room temperature for 180 days and the tensioactive properties of the biomolecule were determined with different pH values, temperatures and concentrations of salt. Then, a central composite rotatable design was used to evaluate the influence of the independent variables (effluent flow rate and formulated biosurfactant flow rate) on the oil removal efficiency in the DAF prototype. The formulated biosurfactant demonstrated good stability in both conservation methods, with tolerance to a wide pH range, salinity and high temperatures, enabling its use in environments with extreme conditions. The efficiency of the formulated biomolecule through heating and addition of sorbate was demonstrated by the 92% oil removal rate in the DAF prototype. The findings demonstrate that the biosurfactant from *Bacillus methylotrophicus* enhances the efficiency of the DAF process, making this technology cleaner. This biosurfactant can assist in the mitigation and management of industrial effluents, contributing toward a reduction in environmental pollution caused by petroleum-based hydrocarbons.

1. Introduction

Several sources of renewable energy have been developed and proposed to reduce humanity's dependence on fossil fuels (Geetha et al., 2018). However, a large number of industrial activities, especially those in the petroleum industry and the production of electrical energy, require heavy oil to function. Spills involving petroleum-based hydrocarbons, such as fuel and heavy oil, are an inevitable part of the industrial sector and have caused serious social-environmental problems. In many parts of this system, such as the transportation of fuel, the lubrication of motors and machinery, the washing of parts, floors and machines impregnated with oil residue, *etc.*, leaks and the discharge of oily effluents occur, resulting in cumulative environmental impacts (Soares da Silva et al., 2018).

The disposal of effluents is only permitted after the removal of oil and suspended solids to acceptable levels (Radzuan et al., 2016). One of the main methods for successfully separating oil from water during the treatment of effluents is dissolved air flotation, in which oil droplets adhere to air bubbles and rise to the surface, where they are removed. This process enables the reuse of the phases in an efficient, economical manner. Flotation often involves the use of chemical surfactants to enhance adherence to the air bubbles (Rocha e Silva et al., 2015). However, new guidelines for water recovery have restricted the use of these chemical products. In this context, the aim of petroleum biotechnology is to employ biological processes in the exploration, production, transformation and refinement of petroleum as well as assist in the mitigation and management of industrial effluents, thereby contributing to a reduction in pollution (Almeida et al., 2016).

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