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**APLICAÇÃO DE BIOSSURFACTANTES NA
REMEDIAÇÃO DE AREIA CONTAMINADA COM
HIDROCARBONETOS**

**RECIFE
2015**

CHAPRÃO, M. J. **Aplicação de Biossurfactantes na Remediação de Areia Contaminada com Hidrocarbonetos**

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Aplicação de Biossurfactantes na Remediação de Areia Contaminada com Hidrocarbonetos

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Incompreensões e períodos de crise.
Ser feliz é deixar de ser vítima dos problemas
E se tornar um autor da própria história.
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Mas ser capaz de encontrar um oásis
No recôndito da sua alma.

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É ter segurança para receber uma crítica,
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Pedras no caminho?
Guardo todas, um dia vou
Construir um castelo...

(Fernando Pessoa)

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LISTA DE ABREVIATURAS

CETESB - Companhia Ambiental do Estado de São Paulo

CMC – Concentração Micelar Crítica

HLB – Balanço Hidrofílico/Lipofílico

HPA – Hidrocarbonetos Policíclicos Aromáticos

HTP - Hidrocarbonetos Totais de petróleo

MEOR - Microbial Oil Recovery Enhancement

US EPA - Agência de Proteção Ambiental dos E.U.A.

RESUMO

Os biossurfactantes, compostos tensoativos produzidos por micro-organismos, vem sendo utilizados com sucesso na remediação de solos em função da eficiência dessas biomoléculas no aumento da dispersão e da biodegradação de hidrocarbonetos e de suas propriedades como biodegradabilidade e toxicidade reduzida. Nesse sentido, esse trabalho teve como objetivo investigar a aplicação de dois biossurfactantes, sendo um obtido da levedura *Candida sphaerica* e outro da bactéria *Bacillus* sp., produzidos a partir de resíduos industriais, na remediação de hidrocarbonetos de óleo lubrificante de motor em solo arenoso contaminado em laboratório. Os biossurfactantes foram testados na remoção do óleo em ensaios cinéticos e estáticos. Estudos comparativos com os surfactantes sintéticos Tween 80 e Triton X-100 também foram conduzidos. Em seguida, os biossurfactantes foram aplicados na biorremediação do solo arenoso contaminado a fim de avaliar a capacidade dessas biomoléculas em facilitar a biodegradação microbiana do contaminante. Ambos os biossurfactantes brutos e isolados mostraram eficiência na remoção do óleo de motor da areia contaminada em condições cinéticas (70-90 %), enquanto que os surfactantes sintéticos removeram entre 55 e 80 % do óleo sob as mesmas condições. O aumento na concentração dos biossurfactantes e dos surfactantes sintéticos não aumentou o percentual de remoção do óleo, enquanto que um tempo de contato de 5-10 min, sob agitação, foi suficiente para a remoção do óleo por todos os agentes tensoativos testados. O biossurfactante bruto e isolado de *C. sphaerica* foi capaz de remover cerca de 90 % de óleo de motor nas colunas empacotadas quando comparado com o biotensoativo de *B. sp.*, o qual removeu cerca de 40 % do óleo. Para os experimentos de degradação conduzidos na areia contaminada com o óleo de motor e enriquecida com melaço de cana de açúcar; no entanto, a degradação do óleo atingiu aproximadamente 100 % após 90 dias na presença das células do isolado de *B. sp.*, enquanto que o

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percentual de degradação do óleo não ultrapassou os 50 % na presença da *C. sphaerica*. A presença dos biossurfactantes aumentou a taxa de degradação do óleo em 10-20 %, especialmente durante os primeiros 45 dias de experimento, indicando que os biossurfactantes aceleraram a taxa de degradação dos hidrocarbonetos. Os resultados obtidos demonstraram que os biossurfactantes testados são promissores como agentes de remediação, podendo agir não só na remoção de óleos como também no aumento da capacidade de biodegradação de poluentes hidrofóbicos em solos.

Palavras-chave: *Candida sphaerica*. *Bacillus* sp. Biorremediação. Petróleo. Colunas empacotadas.

ABSTRACT

The biosurfactants, tension active compounds produced by microorganisms, have been successfully used in bioremediation processes and soil washing since the efficiency of these biomolecules in increasing the dispersion and the biodegradation of hydrocarbons. Their properties such as low toxicity and biodegradability are also very attractive for environmental applications. Thus, this study investigated potential application of two biosurfactants for enhanced removal capability and biodegradation of motor oil contaminated sand under laboratory conditions. The biosurfactants were produced by the yeast *Candida sphaerica* and by the bacterium *Bacillus* sp. cultivated in low-cost substrates. The ability of removing motor oil from soil by the two biosurfactants was identified and compared with that of the synthetic surfactants Tween 80 and Triton X-100. Both crude and isolated biosurfactants showed excellent effectiveness on motor oil removal from contaminated sand under kinetic conditions (70-90%), while the synthetic surfactants removed between 55 and 80% of the oil under the same conditions. The increase in biosurfactants and synthetic surfactants concentration did not enhance the removal of oil. A contact time of 5-10 min under agitation seemed to be enough for oil removal with the biosurfactants and synthetic surfactants tested. The crude and the isolated biosurfactant produced by *C. sphaerica* were able to remove high percentages of motor oil from packeded columns (around 90%) when compared to the biosurfactant from *Bacillus* sp. (40%). For the degradation experiments conducted in motor oil contaminated sand enriched with sugar cane molasses, however, oil degradation was approximately 100% after 90 days in the presence of *B. sp.* cells, while the percentage of oil degradation did not exceed 50 % in the presence of *C. sphaerica*. The presence of the biosurfactants increased the degradation rate in 10-20%, especially during the first 45 days of the experiments, indicating that biosurfactants acted as efficient enhancers for hydrocarbon biodegradation. The results indicated the

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biosurfactants enhancing capability on both removal and rate of motor oil biodegradation in soil systems.

Keywords: *Candida sphaerica*. *Bacillus* sp. Bioremediation. Petroleum. Sand-packed column.

CAPÍTULO 1

1 INTRODUÇÃO

Com o avanço da tecnologia, o meio-ambiente tem sido, cada vez mais, atingido pela geração de poluentes pelas indústrias. As refinarias de petróleo, por exemplo, são fontes potenciais de poluição ambiental, uma vez que seus hidrocarbonetos são aplicados como combustíveis derivados de petróleo e como precursores de compostos sintéticos em grandes volumes. Quantidades consideráveis de produtos petrolíferos contaminam o solo e as águas subterrâneas como consequência de vazamentos e derramamentos de processos de refino e transporte de petróleo e tanques de armazenamento (SILVA et al., 2014).

A cada ano, cerca de 1.680.000 gal (~ 40 mil barris) de petróleo são derramados por insuficiência de gasodutos e mais de 200 mil de tanques de armazenamento subterrâneo nos EUA, causando grandes riscos ambientais (HUESEMAN, 2004). O derramamento de petróleo é responsável por aproximadamente 15 % de todos os incidentes de poluição na Inglaterra, com cerca de nove ocorrências por dia, resultando em um milhão de toneladas de derramamento de óleo em ecossistemas terrestres a cada ano (STROUD et al., 2007).

No Brasil, mais especificamente no Estado de São Paulo, os valores orientadores para solos são descritos para os hidrocarbonetos policíclicos aromáticos (HPA), o que corresponde à soma das concentrações de dez compostos prioritários selecionados pela CETESB. Vale ressaltar que no Brasil não existe uma legislação específica para HPA total, bem como para hidrocarbonetos totais de petróleo (HTP) (ANDRADE et al., 2010).

A Agência de Proteção Ambiental dos E.U.A. (US EPA) tem proposto várias tecnologias para o tratamento dos solos contaminados por hidrocarbonetos derivados do petróleo, incluindo métodos químicos, físicos e biológicos (U.S. EPA, 2001; BRITISH PETROLEUM, 2010).

Um dos métodos mais investigado é a biorremediação, que utiliza a habilidade de degradação natural de plantas ou microrganismos, usualmente fungos ou bactérias, para converter parcialmente os contaminantes em compostos menos tóxicos ou totalmente em gás carbônico e água.

No Brasil, até o momento, apenas alguns estudos foram realizados abordando a temática de biorremediação de solos. Ao contrário, em outros países, como nos Estados Unidos, essa questão é estudada com frequência, tanto no meio acadêmico como no industrial. Por isso, nos últimos anos, a biorremediação de solos tem se tornado tema recorrente de muitos estudos acadêmicos, gerando literatura técnico-científica abundante (ANDRADE et al., 2010).

Embora a biorremediação seja um método efetivo e ambientalmente compatível, o tempo e os custos necessários para tratamento nesse processo não são viáveis para o tratamento de uma grande quantidade de resíduos. Contudo, alguns métodos tais como a lavagem de solos, utilizados para separação dos contaminantes sem provocar danos químicos ao solo, podem aumentar acentuadamente a velocidade da taxa de biodegradação (FRACCHIA et al., 2012; BANAT, 2010). O método de lavagem do solo é rápido e eficiente, apresentando potencial de aplicação na remoção de uma grande quantidade de poluentes (URUM et al., 2004).

A biorremediação de solos e águas também encontra outros 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, o que reduz biodisponibilidade para os microrganismos e limita, conseqüentemente, a transferência de massa para a biodegradação. Segundo Kuyukina et al. (2005), a penetração do óleo através do solo é um processo extremamente complexo incluindo fatores físicos, químicos e biológicos.

A chave do processo para o aumento da biodisponibilidade dos óleos contaminantes é o transporte da carga poluente para a fase aquosa. Nesse contexto, a utilização de compostos surfactantes surge como alternativa para o

aumento da solubilidade dos óleos, permitindo a dessorção e consequente solubilização dos hidrocarbonetos, facilitando, assim, a assimilação desses compostos pelas células microbianas (BURGHOFF, 2012).

Estudos recentes mostram que os surfactantes microbianos, metabólitos produzidos por bactérias e leveduras, têm habilidade para solubilizar e mobilizar efetivamente compostos orgânicos adsorvidos no solo (WANG et al., 2008). Alguns surfactantes sintéticos, como o Triton X – 100 e o Tween 80 também apresentam habilidade de aumentar a concentração dos compostos não polares na fase aquosa. Contudo, o uso de surfactantes sintéticos está associado a efeitos tóxicos e à resistência à biodegradação desses compostos (PACWA-PLOCINICZAK et al., 2011).

Comparados com os surfactantes sintéticos, os biossurfactantes, em geral, exibem forte compatibilidade ambiental, maior atividade superficial, toxicidade reduzida e alta biodegradabilidade (MULLIGAN, 2009). Por essa razão, os biossurfactantes são fortes candidatos à utilização na biorremediação de solos contaminados e ambientes aquáticos (MARCHANT; BANAT, 2012), além de serem produzidos por fontes renováveis como a fermentação microbiana, apresentando, assim, vantagem química frente aos similares sintéticos.

De acordo com a literatura, do ponto de vista ambiental, a substituição de surfactantes químicos por biossurfactantes reduz o ciclo de vida das emissões de CO₂ em 8 %. Nesta base, estima-se que 1,5 milhões de toneladas de emissões de CO₂ sejam evitadas (RAHMAN; GAKPE, 2008). Os biossurfactantes ocupam cerca de 10 % da produção total mundial de tensoativos (cerca de dez milhões de toneladas por ano), sendo fornecidos para vários setores industriais além do petrolífero, como o alimentício (produção de emulsificantes em alimentos), o farmacêutico (formulação de hidratantes, cremes, medicamentos), o médico (produção de agentes antimicrobianos), o agrícola (produção de fertilizantes pra solos) e o civil (tratamento de resíduos, esgotos) entre outros (VAN BOGAERT et al., 2011).

A aplicação de biossurfactantes para remover os contaminantes oleosos de solos é uma tecnologia onde a eficiência de remoção é impulsionada principalmente pelas propriedades físico-químicas dos biossurfactantes. Ao reduzir a tensão superficial e interfacial, os biossurfactantes aumentam as áreas de superfície de compostos insolúveis que conduzem a uma maior mobilidade e biodisponibilidade dos óleos. Em consequência, os biossurfactantes aumentam a biodegradação e a remoção dos compostos insolúveis (HAZRA et al., 2012).

2 OBJETIVOS

2.1 Objetivo Geral

Aplicar dois biossurfactantes na descontaminação ambiental de derivado de petróleo em solo arenoso.

2.2 Objetivos Específicos

- Produzir dois biossurfactantes, um a partir do isolado da bactéria *Bacillus* sp. e outro a partir da levedura *Candida sphaerica*, em meios e condições previamente estabelecidos.
- Isolar os biossurfactantes produzidos.
- Conferir as propriedades surfactantes e tensoativas dos biossurfactantes isolados em condições previamente estabelecidas.
- Conferir as propriedades surfactantes e tensoativas de dois surfactantes sintéticos.
- Testar a capacidade de remoção de derivado de petróleo pelos biossurfactantes e surfactantes sintéticos em areia utilizando frascos através de ensaios cinéticos.
- Avaliar a concentração e o tempo de contato dos biossurfactantes e dos surfactantes sintéticos na eficiência de remoção do óleo nos ensaios cinéticos.
- Testar a capacidade de remoção de derivado de petróleo pelos biossurfactantes e surfactantes sintéticos em areia utilizando colunas empacotadas através de ensaios estáticos.
- Avaliar a concentração dos biossurfactantes e dos surfactantes sintéticos na eficiência de remoção do óleo nos ensaios em colunas empacotadas.
- Testar a capacidade de degradação de derivado de petróleo pelos microrganismos e seus respectivos agentes surfactantes em consórcio na areia contaminada.

3 REVISÃO DE LITERATURA

3.1 Surfactantes

Os surfactantes constituem uma classe de compostos químicos utilizados em diversos setores industriais. Esses compostos são formados por estruturas moleculares contendo porções hidrofílicas e hidrofóbicas que tendem a se distribuir nas interfaces entre fases fluidas com diferentes graus de polaridade (óleo/água) (MUTHUSAMI et al., 2008), promovendo a redução da tensão superficial e interfacial, conferindo a capacidade de detergência, emulsificação, lubrificação, solubilização e dispersão de fases (DELEU; PAQUOT, 2004; GAUTAM; TYAGI, 2006; NITSCHKE et al., 2007).

A importância comercial dos surfactantes torna-se evidente a partir da tendência do mercado em aumentar a produção desses compostos em decorrência da diversidade de utilizações industriais (CALVO et al., 2009). As aplicações industriais são classificadas de acordo com seus usos: 54 % como detergentes, 13 % nas indústrias têxteis, de couro e de papel, 10 % em processos químicos, outros 10 % nas indústrias farmacêuticas e de cosméticos, 3 % na indústria de alimentos, 2 % na agricultura e os 2 % restantes em outras aplicações (MUTHUSAMY et al., 2008).

A grande maioria dos surfactantes disponíveis comercialmente é sintetizada a partir de derivados de petróleo. Entretanto, a preocupação ambiental entre os consumidores, combinada a novas legislações de controle do meio ambiente têm levado à procura por surfactantes naturais como alternativa aos produtos existentes (NITSCHKE; PASTORE, 2002).

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

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

Os compostos de origem microbiana que exibem propriedades surfactantes, isto é, diminuem a tensão superficial e possuem alta capacidade emulsificante, são denominados biossurfactantes e consistem em subprodutos metabólicos de bactérias, leveduras e fungos filamentosos (SINGH et al., 2007).

3.2 Estimativa da Atividade Surfactante

As atividades surfactantes podem ser determinadas por medidas de alterações nas tensões superficial e interfacial, estabilização ou desestabilização de emulsões e balanço hidrofílico/lipofílico (HLB).

A propriedade de maior importância para os agentes tensoativos é a tensão superficial, que é a força de atração existente entre as moléculas dos líquidos (PACWA-PŁOCINICZAK et al., 2011).

Define-se como superfície o limite entre um líquido e o ar e como interface o limite entre dois líquidos. Dessa forma, as tensões existentes entre as fases ar/água e óleo/água são conhecidas como tensão superficial e tensão interfacial, respectivamente (BANAT et al., 2010).

A tensão superficial é facilmente medida quantitativamente por um tensiômetro. Esta medição é a base da maior parte das avaliações iniciais para identificar a presença de um surfactante no meio. A tensão superficial ar/água para a água destilada é de aproximadamente 72 mN/m (ou dynes/cm) e a tensão interfacial para a água destilada contra n-hexadecano é de aproximadamente 40mN/m. Tipicamente, surfactantes podem diminuir esses valores para cerca de 30-40 mN/m e 1 mN/m, respectivamente (PARKINSON, 1985).

A tensão superficial diminui quando a concentração de surfactante no meio aquoso aumenta, ocorrendo a formação de micelas, que são moléculas

anfipáticas agregadas com as porções hidrofílicas posicionadas para a parte externa da molécula e as porções hidrofóbicas para a parte interna. 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 (CORTIS; GHEZZEHEI, 2007; VAN et al., 2006).

A eficiência e a efetividade são características básicas essenciais que determinam um bom surfactante. A eficiência é medida através da CMC, enquanto que a efetividade está relacionada com as tensões superficiais e interfaciais (BARROS et al., 2007).

Uma emulsão é formada quando uma fase líquida é dispersa como gotículas microscópicas em outra fase líquida contínua. Dois tipos de emulsões podem ser formadas: água-em-óleo (a/o) (surfactante mais solúvel em óleo) e óleo-em-água (o/a) (surfactante mais solúvel em água). A estabilidade de uma emulsão depende de muitos fatores, incluindo o tamanho das gotículas dispersas, que é favorecida através da redução da tensão interfacial. A presença de emulsificantes e de desemulsificantes estabiliza ou desestabiliza as emulsões, respectivamente. A capacidade emulsificante é analisada pela habilidade do surfactante em gerar turbidez devido à suspensão de hidrocarbonetos, como n-hexadecano, em um sistema aquoso em análise, enquanto que a capacidade de desemulsificação é geralmente avaliada pelo efeito do agente de-emulsionante sobre emulsões normais preparadas com agentes tensioativos sintéticos (PARKINSON, 1985).

O valor do HLB indica se o surfactante irá promover emulsões água-em-óleo ou óleo-em-água através da comparação do seu valor com valores de HLB de surfactantes conhecidos. A escala de HLB pode ser construída escolhendo-se o valor de 1 para o ácido oléico e o valor de 20 para o oleato de sódio e usando uma faixa de mistura destes dois componentes em diferentes proporções para obter os valores intermediários. Emulsificantes com HLB

menores que seis favorecem a estabilização de emulsões água-em-óleo, enquanto emulsificantes com HLB entre 10 e 18 possuem um efeito oposto, favorecendo emulsões óleo-em-água (PARKINSSON, 1985).

3.3 Biossurfactantes

A maioria dos biossurfactantes conhecidos é produzida em substratos insolúveis em água como hidrocarbonetos sólidos e líquidos, óleos e gorduras, embora muitos tenham sido obtidos a partir de substratos solúveis, ou pela combinação destes (VAN et al., 2006).

A possibilidade de produção dos biossurfactantes a partir de substratos renováveis e de diferentes espécies microbianas, além da possibilidade de variação de inúmeros parâmetros culturais como tempo de cultivo, velocidade de agitação, pH do meio e nutrientes adicionados, permite a obtenção de compostos com características estruturais e propriedades físicas distintas, o que os tornam comparáveis ou superiores aos surfactantes sintéticos em termos de eficiência, embora os custos de produção ainda não permitam uma maior competitividade com seus similares petroquímicos (CANET et al., 2002). A CMC dos biossurfactantes (medida de sua eficiência) varia entre 1-2000 mg/L, enquanto que a tensão interfacial (óleo/água) e superficial ficam em torno de 1 e 30 mN/m respectivamente.

Dados sobre a CMC são escassos e, mais uma vez, difíceis de interpretar ou correlacionar. A comparação entre os valores de CMC de biossurfactantes e de seus equivalentes químicos está apresentada na Tabela 1 e mostra valores de CMC muito mais baixos no caso dos biossurfactantes. Em princípio, quanto menor a CMC, mais eficaz o surfactante e mais favorável, do ponto de vista econômico, a sua utilização em processos industriais (BOGNOLO, 1999).

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

Agente surfactante	CMC (mg/L)
Fosfatidil etanolaminas	30
Ácidos fosfatídicos	70
Raminolípídeo	20
Surfactina	11
Alquil benzeno sulfonato	590
Lauril sulfato de sódio	2 000 – 2 900

Fonte: Bognolo (1999).

3.4 Classificação dos Biossurfactantes

Os surfactantes sintéticos são classificados de acordo com a carga iônica que reside na parte polar da molécula. Em função da presença ou ausência de cargas elétricas, podem ser aniônicos, catiônicos, não-iônicos ou anfotéricos (MANEERAT, 2005; RON; ROSENBERG, 2001).

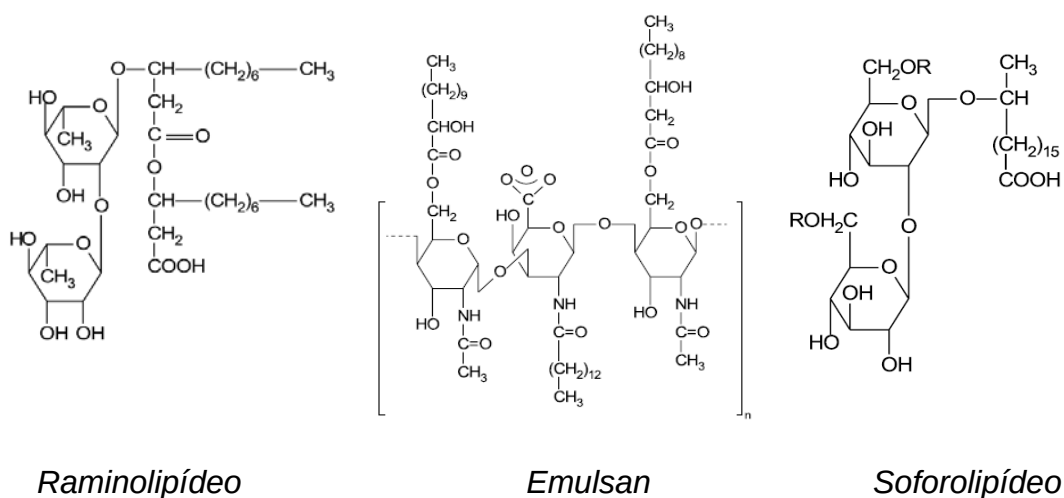
A maioria dos biossurfactantes é aniônica ou neutra. Apenas alguns são catiônicos, como os que contêm grupamentos amina. A parte hidrofóbica é caracterizada por ácidos graxos de cadeia longa, enquanto que a porção hidrofílica pode ser um carboidrato, um aminoácido, um peptídeo cíclico, fosfato, um ácido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

Os biossurfactantes são comumente classificados de acordo com a natureza bioquímica ou com a espécie microbiana produtora. Quanto à estrutura, podem ser classificados em cinco grandes grupos (RAHMAN; GAKPE, 2008):

- Glicolipídeos, cujo grau de polaridade depende dos hidrocarbonetos utilizados como substratos. São exemplos os raminolipídeos produzidos por *Pseudomonas aeruginosa*, e os soforolipídeos produzidos por espécies de *Candida*.
- Lipossacarídeos, os quais normalmente possuem massa molar elevada e são solúveis em água, como o conhecido Emulsan, emulsificante extracelular produzido por hidrocarbonetos a partir da bactéria *Acinetobacter calcoaceticus*.
- Lipopeptídeos, como a surfactina, produzida por *Bacillus subtilis*, um dos biossurfactantes mais poderosos já relatados na literatura.
- Fosfolipídeos, estruturas comuns a muitos micro-organismos, como o biossurfactante de *Corynebacterium lepus*.
- Ácidos graxos e lipídeos neutros (alguns classificados como glicolipídeos) e proteínas hidrofóbicas.

A Figura 1 ilustra algumas estruturas de biossurfactantes, cujos exemplos apresentam o lipossacarídeo emulsan e os glicolipídeos raminolipídeo e soforolipídeo

Figura 1 - Estruturas típicas de biossurfactantes glicolipídicos



Fonte: Mukherjee et al.(2006).

3.5 Micro-organismos Produtores de Biossurfactantes

Uma grande variedade de micro-organismos, incluindo bactérias, leveduras e fungos filamentosos é capaz de produzir biossurfactantes com diferentes estruturas moleculares (DELEU; PAQUOT, 2004).

As bactérias dos gêneros *Pseudomonas* e *Bacillus* são descritas na literatura como grandes produtoras de biossurfactantes.

Os raminolipídeos produzidos por *Pseudomonas aeruginosa* têm sido extensivamente estudados (ABDEL-MAWGOUD et al., 2010; CHRZANOWSKI et al., 2012; HENKEL et al., 2012; NITSCHKE et al., 2011). A composição e os rendimentos dependem do tipo do fermentador, do pH, da composição dos nutrientes, dos substratos e das temperaturas utilizadas (MULLIGAN, 2005).

Os *Bacillus subtilis* são produtores de lipopeptídeos, como a chamada surfactina, a qual contém sete aminoácidos ligados aos grupos carboxila e hidroxila do ácido C14 (BARROS et al., 2007; LU et al., 2007). Concentrações de surfactina menores que 0,005 % reduzem a tensão superficial para 27 mN/m, tornando o surfactina um dos mais poderosos biossurfactantes. A solubilidade e a capacidade surfactante da surfactina, por outro lado, depende do tipo de resíduo utilizado como substrato (HUE et al., 2001).

Entre as leveduras, espécies de *Candida* têm sido largamente empregadas com sucesso na fermentação de hidrocarbonetos e, conseqüentemente, para produção de biossurfactantes (GUSMÃO et al., 2010; LUNA et al., 2011a,b; 2013; RUFINO et al., 2007; 2008; SARUBBO et al., 1999; 2001; 2006; 2007; SARUBBO; CAMPOS-TAKAKI, 2011).

A Tabela 2 resume as principais classes de biossurfactantes e os respectivos micro-organismos produtores descritos na literatura.

Tabela 2 - Principais classes de biossurfactantes e micro-organismos produtores

Classes	Micro-organismos
Glicolipídeos	
Raminolipídeos	<i>Pseudomonas aeruginosa</i>
Soforolipídeos	<i>Torulopsis bombicola</i> , <i>T. apicola</i>
Trealolipídeos	<i>Rhodococcus erythropolis</i> , <i>Mycobacterium</i> sp.
Lipopeptídeos e lipoproteínas	
Peptídeo-lipídeo	<i>Bacillus licheniformis</i>
Viscosina	<i>Pseudomonas fluorescens</i>
Serrawetina	<i>Serratia marcescens</i>
Surfactina	<i>Bacillus subtilis</i>
Subtilisina	<i>Bacillus subtilis</i>
Gramicidina	<i>Bacillus brevis</i>
Polimixina	<i>Bacillus polymyxa</i>
Ácidos graxos, lipídeos neutros e fosfolipídeos	
Ácidos graxos	<i>Corynebacterium lepus</i>
Lipídeos neutros	<i>Nocardia erythropolis</i>
Fosfolipídeos	<i>Thiobacillus thiooxidans</i>
Surfactantes poliméricos	
Emulsan	<i>Acinetobacter calcoaceticus</i>
Biodispersan	<i>Acinetobacter calcoaceticus</i>
Liposan	<i>Candida lipolytica</i>
Carboidrato-lipídeo-proteína	<i>Pseudomonas fluorescens</i>
Manana-lipídeo-proteína	<i>Candida tropicalis</i>
Surfactantes particulados	
Vesículas	<i>Acinetobacter calcoaceticus</i>
Células	Várias bactérias

Fontes: Muthusamy et al.(2008); Pacwa-Plociniczak et al.(2011).

3.6 Propriedades dos Biossurfactantes

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

Apesar da diversidade de composição química e de propriedades, algumas características são comuns à maioria dos biossurfactantes. Muitas dessas características representam vantagens sobre os surfactantes convencionais (NITSCHKE et al., 2007):

- atividade superficial e interfacial: os biossurfactantes são mais eficientes e mais efetivos do que os surfactantes convencionais, pois produzem menor tensão superficial a menores concentrações;
- tolerância à temperatura, pH e força iônica: muitos biossurfactantes podem ser utilizados sob condições extremas. O lipopeptídeo de *Bacillus licheniformis* JF-2, por exemplo, é estável a temperaturas em torno de 75 °C, por até 140 horas e pH entre 5 e 12. Os biossurfactantes suportam concentrações de 10 % de sal, enquanto que 2 % de NaCl são suficientes para inativar surfactantes convencionais;
- biodegradabilidade: os biossurfactantes são facilmente degradados por bactérias e outros micro-organismos microscópicos na água e no solo, o que os torna adequados para aplicações na biorremediação e tratamento de resíduos;
- baixa toxicidade: os biossurfactantes têm recebido maior atenção devido à crescente preocupação da população com os efeitos alérgicos dos produtos artificiais; além disso, sua baixa toxicidade permite o uso em alimentos, em cosméticos e em produtos farmacêuticos;
- disponibilidade: biossurfactantes podem ser produzidos a partir de matérias-primas largamente disponíveis, além da possibilidade de serem produzidos a partir de resíduos industriais;

- especificidade: biossurfactantes, sendo moléculas orgânicas complexas com grupos funcionais específicos também serão específicos em suas ações. Essa propriedade pode ser de grande interesse da detoxificação de poluentes específicos ou em determinadas aplicações nas indústrias farmacêutica, cosmética ou alimentícia;
- biocompatibilidade e digestibilidade, o que garante a aplicação dessas biomoléculas nas indústrias farmacêutica, cosmética e alimentícia.

A despeito das vantagens, alguns pontos desfavoráveis devem ser citados, como (RAHMAN; GAKPE, 2008):

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

3.7 Fisiologia dos Biossurfactantes

Os biossurfactantes são produzidos por uma grande variedade de micro-organismos, excretados ou atacados às células, principalmente quando cultivados em substratos insolúveis em água (TAN, 2000).

A função do surfactante em uma célula microbiana, no entanto, não está completamente elucidada. Contudo, existem especulações sobre o envolvimento destes na emulsificação de substratos insolúveis. O contato direto da célula com as gotas de hidrocarbonetos e sua interação com as gotas emulsificadas têm sido descritos.

O principal papel fisiológico dos biossurfactantes é permitir aos micro-organismos crescerem em substratos insolúveis em água, através da redução da tensão superficial entre as fases, tornando o substrato mais disponível para ingestão e metabolismo. Os mecanismos moleculares do processo de ingestão destes substratos (ex. alcanos) não estão completamente elucidados. A ingestão direta dos hidrocarbonetos dissolvidos na fase aquosa, o contato direto das células com grandes gotas de hidrocarbonetos assim como a interação com gotas emulsificadas (emulsão) têm sido descritos (TAN, 2000). Além da emulsificação da fonte de carbono, os biossurfactantes também estão envolvidos na adesão das células microbianas ao hidrocarboneto. A adsorção celular dos micro-organismos aos substratos insolúveis e a excreção de compostos surfactantes juntos permitem o crescimento em tais fontes de carbono.

3.8 Biossíntese e Regulação dos Biossurfactantes

As vias metabólicas primárias, nomeadamente hidrocarbonetos e carboidratos, estão envolvidas na síntese das porções hidrofóbica e hidrofílicas, respectivamente (DESAI; BANAT, 1997). As vias metabólicas da síntese deste dois grupos de precursores são diversas e utilizam enzimas

específicas. Em muitos casos, as primeiras enzimas para a síntese destes precursores são regulatórias; conseqüentemente, a despeito da diversidade, existem alguns princípios comuns sobre sua síntese e regulação. As vias metabólicas das porções hidrofílicas e hidrofóbicas têm sido extensivamente estudadas e estão documentadas na literatura. De acordo com Syldatik e Wagner (1987), existem as seguintes possibilidades para a síntese e relação entre as duas porções de um biossurfactante: a) as porções hidrofílica e hidrofóbica são sintetizadas pela síntese de novo por dois caminhos diferentes; b) a porção hidrofílica seria sintetizada pela síntese de novo e a porção hidrofóbica seria induzida pelo substrato; c) a porção hidrofóbica seria sintetizada pela síntese de novo e a porção hidrofílica seria dependente do substrato e d) a síntese de ambas as porções seria dependente do substrato.

Em geral, três mecanismos, nomeadamente indução, repressão e nitrogênio e íons multivalentes operam na regulação da produção de um biossurfactante. A indução da síntese de lipídeos pela adição de ácidos graxos de cadeia longa, hidrocarbonetos ou glicerídeos ao meio de crescimento de *Torulopsis magnoliae* ou a síntese de trealolipídeos por *Rhodococcus erythropolis* pela adição de hidrocarbonetos e a produção de um glicolípido pela adição de alcanos em *Pseudomonas aeruginosa* têm sido reportados.

A regulação por nitrogênio ou íons metálicos também possui um papel importante na síntese de surfactantes. A síntese de raminolipídeos em *Pseudomonas aeruginosa* após a exaustão do nitrogênio, na fase estacionária de crescimento, foi observada por vários pesquisadores. Contrariamente, a adição de uma fonte de nitrogênio provocou uma inibição na síntese de raminolipídeos por células na fase estacionária de *P. aeruginosa*.

A limitação de cátions multivalentes também causa a produção de biossurfactantes. Guerra-Santos et al. (1984) demonstraram que, pela limitação da concentração de sais de magnésio, cálcio, potássio, sódio e elementos traços, um alto rendimento de raminolipídeos pode ser alcançado em *Pseudomonas aeruginosa*. A limitação de ferro, por exemplo, estimula a

produção de agentes surfactantes em *P. fluorescens* e *P. aeruginosa*, enquanto que a adição de sais de ferro e manganês estimulam a produção de biossurfactantes em *B. subtilis* e *Rhodococcus* sp.

3.9 Economia na Produção de Biossurfactantes

Nos processos biotecnológicos a economia é sempre um fator importante, especialmente nos casos de produção de biossurfactantes. O sucesso da produção de biossurfactantes depende do desenvolvimento de processos de baixo custo e da seleção de substratos pouco custosos, uma vez que esses representam de 10 a 30 % do custo total de produção. Os biossurfactantes têm que competir com os surfactantes petroquímicos considerando três aspectos: custo, funcionalidade e capacidade de produção junto à necessidade de aplicação pretendida (BANAT et al., 2010; CAMEOTRA; MAKKAR, 1998; COIMBRA et al., 2009; MUTHUSAMY et al., 2008; RUFINO et al., 2008).

O alto custo de produção dos biossurfactantes pode ser absorvido, se usados em pouca quantidade, na produção de cosméticos, de medicamentos e de alimentos; por outro lado, para aplicações mais abrangentes, como a recuperação de óleos, a qual requer altos volumes de surfactantes a um baixo custo, o alto custo de produção ainda dificulta a utilização dessas biomoléculas (CAMEOTRA; MAKKAR, 1998).

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

A produção de biossurfactante pode ser espontânea ou induzida pela presença de compostos lipofílicos, por variações de pH, temperatura, aeração e velocidade de agitação, ou ainda, quando o crescimento celular é mantido

sob condições de estresse, como baixas concentrações da fonte de nitrogênio (DESAI; BANAT, 1997).

A produção de biossurfactantes a um baixo custo é dificultada quando se faz necessário uma refinação extensiva. Para o desenvolvimento de processos deve-se usar biossurfactantes capazes de serem recuperados por técnicas simples e baratas. O processo mais comum de recuperação dos biossurfactantes é a extração com solventes (clorofórmio-metanol, dicloremetano-metanol, butanol, acetato de etila, pentano, hexano e ácido acético, entre outros). Todavia, têm sido reportadas técnicas de precipitação dos produtos como a precipitação por sulfato de amônio, centrifugação por cristalização, adsorção, fermentação fracionária, etc. (MAKKAR; CAMEOTRA, 2002).

3.10 Utilização de Resíduos Industriais na Produção de Biossurfactantes

Os resíduos industriais têm despertado grande interesse dos pesquisadores como alternativa de substratos de baixo custo para a produção de biossurfactantes (MAKKAR; CAMEOTRA, 2002).

A seleção do resíduo deve ser conduzida de forma a garantir um certo balanço de nutrientes que suportem o crescimento microbiano e a produção do biossurfactante. Os resíduos industriais com elevado conteúdo de carboidratos ou lipídeos se destacam como substratos para produção de biossurfactantes (MAKKAR; CAMEOTRA, 1999; MERCADE et al., 1994).

Segundo Barros et al. (2007) a utilização de resíduos agroindustriais para produção de biossurfactantes é um dos passos para viabilização e implantação desses bioprocessos em escala industrial, sendo necessário o estudo de diferentes condicionantes para o desenvolvimento de condições adequadas para sua aplicação.

A literatura descreve a utilização de inúmeros resíduos para a produção de biossurfactantes, como óleos vegetais e efluentes oleosos (MERCADE et

al., 1993; SARUBBO et al., 2007; BATISTA et al., 2010), efluente de amido (FOX; BALA, 2000; THOMPSON et al., 2000), gordura animal (DESHPANDE; DANIELS, 1995; MANEERAT, 2005), gordura vegetal (GUSMÃO et al., 2010), resíduos de fritura de óleos vegetais (ALCANTARA et al., 2000; CVENGROS; CVENGROSOVA, 2004; HABA et al., 2000; MANEERAT, 2005), borra de sabão (SHABTAI, 1990; BENINCASA et al., 2002; MANEERAT, 2005), melaço de cana (GHURYE; VIPULANANADA, 1994; KALOGIANNIS et al., 2003; LAZARIDOU et al., 2002; MAKKAR; CAMEOTRA, 1997), resíduos da indústria de laticínios (soro de leite) (SUDHAKAR-BABU et al., 1996), milhocina (LUNA et al., 2011a; 2013; RUFINO et al., 2007; SOUZA SOBRINHO et al., 2008), manipueira (NITSHKE et al., 2004), resíduos de destilaria de óleos (LUNA et al., 2011a; 2013; RUFINO et al., 2007) e glicerina (SILVA et al., 2010).

3.11 Aplicações dos Biossurfactantes no Meio-Ambiente

Devido às diversas estruturas e propriedades, os biossurfactantes apresentam aplicação em vários processos industriais, além da possibilidade de novas aplicações para estas biomoléculas. Acredita-se que os biossurfactantes ficarão conhecidos como os “materiais multifuncionais” do novo século (MARCHANT; BANAT, 2012; MUTHUSAMY et al., 2008).

Até o presente momento, o maior mercado para os biossurfactantes é a indústria petrolífera, onde podem ser usados na limpeza de derramamento de óleos, na remoção de óleos de tanques de estocagem, na recuperação avançada de petróleo e na biorremediação de solos e águas (GAUTAM; TYAGI, 2006; SINGH et al., 2007).

A contaminação ambiental causada pela atividade industrial, muitas vezes, se deve à liberação acidental ou deliberada de compostos orgânicos e/ou inorgânicos para o ambiente. Tais compostos representam problemas para a remediação, uma vez que se ligam facilmente a partículas de solo ou se espalham em meio aquoso. A aplicação de biossurfactantes na

descontaminação de compostos orgânicos, tais como hidrocarbonetos, destina-se a aumentar a sua biodisponibilidade ou a mobilizar e remover os contaminantes por pseudossolubilização e emulsificação durante um tratamento de lavagem. A aplicação de biossurfactantes na recuperação de compostos inorgânicos, tais como metais pesados, por outro lado, envolve a ação quelante e remoção de tais íons durante a etapa de lavagem, a qual é facilitada pelas interações químicas entre os compostos anfífilos e os íons metálicos (BANAT et al., 2010; OLKOWSKA et al., 2012).

Aplicação na biorremediação

Biorremediação é a habilidade de organismos vivos em transformar ou mineralizar contaminantes orgânicos gerando substâncias menos nocivas, que possam ser integradas ao ciclo biogeoquímico natural. Contudo, a biodegradabilidade desses contaminantes é influenciada por fatores como oxigênio, pH, presença de macro e micro nutrientes, características físico-químicas do histórico da poluição do contaminante ambiental e das partículas de solo ou outras às quais os organismos e contaminantes possam estar adsorvidos (MARGESIN; SCHINNER, 2001).

Os compostos anfipáticos são capazes de alterar as condições físico-químicas nas interfaces que afetam a distribuição das substâncias químicas entre as fases (TIEHM, 1994). Por exemplo, um solo contaminado por um hidrocarboneto contém pelo menos seis fases: bactérias, partículas de solo, água, ar, líquido imiscível e hidrocarboneto sólido. Os hidrocarbonetos podem ser particionados entre os diferentes estados: solubilizados na fase de água, ad/absorvidos a partículas do solo, sorvidos às superfícies celulares e livres/insolúveis. Os biossurfactantes adicionados a este sistema podem interagir com ambas as partículas abióticas e as células bacterianas. Isto afeta os mecanismos de interação com os ambientes através da emulsificação dos contaminantes orgânicos. As interações entre as micelas surfactantes e as

células encontram-se entre as principais alterações que o componente bacteriano pode sofrer (VOLKERING et al., 1998). Estes fenômenos, de um lado, podem aumentar a biodisponibilidade dos contaminantes solúveis, aumentando assim a velocidade de biodegradação, ou, por outro lado, podem resultar na inibição da biodegradação.

Apesar das inúmeras aplicações bem sucedidas dos biossurfactantes, a literatura nesta área descreve resultados contrastantes. Por exemplo, os raminolipídios de *P. aeruginosa* podem estimular a degradação do n-hexadecano, mas não estimulam a degradação quando produzidos por *Rhodococcus*, mostrando a especificidade do micro-organismo. Em contraste, o biossurfactante de *R. erythropolis* 3C-9 aumentou significativamente a taxa de degradação de n-hexadecano. Portanto, o efeito da adição de biossurfactantes na biorremediação não é previsível, de modo que a eficácia de um dado surfactante precisa ser avaliada experimentalmente (FRANZETTI et al., 2008).

As interações entre as bactérias, os contaminantes e o biossurfactante podem ser interpretadas a partir de uma perspectiva funcional, considerando que o principal papel atribuído aos biossurfactantes seja seu envolvimento na captação de hidrocarbonetos (PERFUMO et al., 2006). Surfactantes microbianos podem promover o crescimento microbiano em hidrocarbonetos, aumentando a área superficial entre o óleo e a água e, por meio de emulsificação, aumentando a pseudosolubilidade de hidrocarbonetos por meio de partições em micelas (VOLKERING et al., 1998).

Os biossurfactantes de elevada massa molar (bioemulsificantes) têm um grande potencial de estabilizar emulsões de hidrocarbonetos líquidos e água, aumentando a área superficial disponível para a biodegradação bacteriana. No entanto, eles têm sido raramente testados como estimuladores da biodegradação de hidrocarbonetos em sistemas de biorremediação, sendo os resultados descritos na literatura muito contrastantes (BARKAY et al., 1999; FRANZETTI et al., 2009). Os biossurfactantes de baixa massa molar, acima da

concentração micelar crítica (CMC), particionam uma fração significativa dos contaminantes hidrofóbicos nos núcleos micelares. Em alguns casos, isto resulta no aumento geral da biodisponibilidade dos contaminantes para os micro-organismos degradadores. Aplicações bem sucedidas de raminolipídeo e surfactina em biorremediação foram avaliadas (MULLIGAN, 2009).

Pesquisas com consórcios microbianos e raminolipídeos demonstraram o potencial de biorremediação de hidrocarbonetos de petróleo. A aplicação do raminolipídeo de *Pseudomonas aeruginosa* DS10-129 aumentou a biorremediação de gasolina adsorvida em solo (RAHMAN et al., 2006).

Alguns estudos demonstraram o aumento da biodisponibilidade de compostos aromáticos pouco solúveis como os hidrocarbonetos aromáticos policíclicos (HPAS) pelo uso de biossurfactantes (MULLIGAN, 2005; SINGH et al., 2007).

A utilização de biossurfactantes na biodegradação de pesticidas vem sendo objeto de investigação. A degradação de hexaclorociclohexano por surfactantes produzidos por *Pseudomonas* foi primeiramente relatada, bem como a dos organoclorados como DDT e ciclodienos (KARANTH et al., 1999).

Os modos específicos de absorção de hidrocarbonetos, no entanto, não estão totalmente compreendidos, como relatados nas seções anteriores. Cameotra e Singh (2009) elucidaram o mecanismo de absorção de n-hexadecano mediado por raminolipídeos de *P. aeruginosa*. Os raminolipídeos produziram uma emulsão com hexadecano, facilitando assim o contato entre o hidrocarboneto e as bactérias. Observou-se, também, que ocorreu absorção das gotículas dos hidrocarbonetos revestidos pelo biossurfactante, o que sugere a ocorrência de um mecanismo de pinocitose, e não um processo de captação bacteriana, como relatado anteriormente. Em contraste, sabe-se bem que a presença de um surfactante pode afetar prejudicialmente a biodegradação. Núcleos micelares podem reter contaminantes orgânicos, criando uma barreira entre os micro-organismos e as moléculas orgânicas,

resultando na redução da disponibilidade do substrato. Por exemplo, o Witconol SN70, um surfactante não iônico de álcool etoxilado, reduziu a taxa de biodegradação de hexadecano (COLORES et al., 2000).

Outro papel proposto para os biossurfactantes na captação de hidrocarbonetos é a regulação da ligação de células a superfícies hidrofóbicas e hidrofílicas, alterando assim a hidrofobicidade de superfície celular (FRANZETTI et al., 2008; ROSENBERG et al., 1987). Este papel natural pode ser explorado através da adição de biossurfactantes para aumentar a hidrofobicidade de micro-organismos e facilitar o acesso das células aos substratos hidrofóbicos (SHREVE et al., 1995). Coimbra et al. (2009) descreveram estudos de hidrofobicidade com surfactantes de *Candida*. Chang et al. (2009) demonstraram o aumento da hidrofobicidade celular pelo acúmulo de diferentes ácidos graxos na superfície da célula durante o crescimento em hidrocarboneto com *R. erythropolis* NTU-1. A correlação entre a alteração da superfície da célula por saponinas e o grau de biodegradação de hidrocarbonetos foi relatada por Kaczorek et al. (2008).

Aplicação na Recuperação Avançada de Petróleo - MEOR

Os biossurfactantes também podem estar envolvidos na recuperação avançada de petróleo (MEOR - Microbial Oil Recovery Enhancement). Métodos de MEOR são usados para recuperar o óleo remanescente em reservatórios após os procedimentos de recuperação primária (mecânico) e secundária (físico) (SINGH et al., 2007; PACWA-PŁOCINICZAK et al., 2011). A recuperação avançada de petróleo consiste em um importante processo terciário no qual os micro-organismos ou um dos seus metabólitos, incluindo biossurfactantes, biopolímeros, biomassa, ácidos, solventes, gases e enzimas, também são utilizados para aumentar a recuperação de petróleo a partir de depósitos esgotados. A aplicação de biossurfactantes na recuperação avançada de petróleo é um dos métodos mais promissores para recuperar uma parte

substancial do óleo residual. O óleo remanescente fica muitas vezes localizado em regiões do reservatório que são de difícil acesso sendo o óleo aprisionado nos poros por pressão capilar. Os biossurfactantes reduzem a tensão interfacial entre o óleo / água e óleo/rocha. Isto reduz as forças capilares que impedem o óleo de mover-se através dos poros da rocha. Os biossurfactantes podem também ligar-se fortemente com a interface óleo-água formando uma emulsão. Isso estabiliza o óleo desorvido em água e permite a remoção de óleo, juntamente com a injeção de água (MARCHANT; BANAT, 2012). Bordoloi e Konwar (2008) investigaram a recuperação de petróleo bruto a partir de uma coluna de saturação sob condições de laboratório. Estudos laboratoriais sobre MEOR utilizam tipicamente colunas que contêm o substrato desejado, geralmente areia. Este substrato é utilizado para demonstrar a utilidade dos biossurfactantes na recuperação de óleo dos reservatórios. Para este efeito, uma coluna de vidro é embalada com areia seca; em seguida, a coluna é saturada com o petróleo bruto e uma solução aquosa de biossurfactante é vertida na coluna. O potencial de biossurfactantes em MEOR é estimado através da medição da quantidade de óleo liberado da coluna após a saída da solução aquosa de biossurfactante da coluna. O experimento deve ser realizado em temperatura ambiente e elevada, como 70-90 °C, para avaliar a influência da temperatura sobre a recuperação de óleo induzida pelo biossurfactante. Os biossurfactantes utilizados na experiência de Bordoloi e Konwar (2008) foram produzidos por bactérias isoladas de *P. aeruginosa* (MTCC7815, MTCC7814, MTCC7812 e MTCC8165). Os biossurfactantes das estirpes MTCC7815, MTCC7812 e MTCC8165 recuperaram cerca de 49-54 % de óleo bruto a partir da coluna empacotada à temperatura ambiente, 52-57% a 70 °C e 58-62 % a 90 °C. Bai et al. (1997) investigaram o potencial do raminolípido aniônico isolado de *P. aeruginosa* adsorvido em solo em colunas empacotadas. O biossurfactante foi capaz de remover 84 % de hexadecano adsorvido em areia de 20-30 mesh (0,6-0,85 mm), enquanto que 22 % de remoção foram obtidos quando areia de 40-50 mesh (0,3-0,42 mm) foi utilizada. A capacidade de remoção do raminolípido foi comparada com a capacidade

de remoção dos surfactantes sintéticos SDS (CMC de 2360 mg/L), também aniônico, e Tween 80 (mono oleato de sorbitana polioxietileno 20) (CMC de 13 mg/l), não iônico, utilizados em concentração de 500 mg/l, para areia de 40/50 mesh. O SDS (472 mg/L) e o Tween 80 (51 mg/L) removeram 0 e 6 % do hexadecano, respectivamente. Por outro lado, a capacidade do surfactante sintético SDS em remover diesel adsorvido em solo contido em coluna demonstrou que, enquanto a água, adicionada como controle, foi capaz de remover 24,7 % do diesel, o SDS removeu 97 % do combustível. Nesse trabalho foi observada a influência de fatores como a concentração de surfactante e o tempo de contato na cinética de remoção do poluente (KHALLADI et al., 2009). Concentrações elevadas (2,5 e 5,0 g/l) do biossurfactante isolado da *P. aeruginosa* 57SJ, que apresentou uma CMC de 400 mg/L, foram necessárias para remover 70% de pireno adsorvido em solo com tamanho de partículas de 2 mm (BORDAS et al., 2007). O líquido metabólico livre de células contendo os isolados de *P. aeruginosa* MTCC7815, MTCC7812 e MTCC8165 cultivados em 2 % de glicerol removeram cerca de 49-54 % do óleo bruto contido em colunas empacotadas (BORDOLOI; KONWAR, 2008).

Além das aplicações em MEOR, os biossurfactantes podem também ser explorados para outras aplicações na indústria do petróleo. As propriedades desemulsificantes de alguns biossurfactantes, por exemplo, podem ser utilizadas para quebrar emulsões que se formam em vários passos da extração e do processamento de petróleo, permitindo assim uma melhor recuperação do produto. A redução da tensão superficial provocada pelos surfactantes microbianos pode também ser usada para separar o óleo da parte inferior dos tanques (PERFUMO et al., 2006; SINGH et al., 2007).

Embora uma série de ensaios de campo de aplicações em “in situ” de MEOR sejam relatados na literatura (SEN, 2008), não está completamente elucidado se os micro-organismos introduzidos podem ser realmente eficazes na recuperação de óleo ou se eles competem com bactérias autóctones. A

incapacidade de se comparar os testes realizados com controles de poços submetidos a procedimentos de tratamento semelhantes, sem introdução de micro-organismos vivos ou de produtos dificulta traçar conclusões válidas. Wang et al. (2008), usando marcadores moleculares observaram mudanças na comunidade microbiana, em um reservatório de óleo durante um processo de MEOR, concluindo que tanto bactérias exógenas e autóctones parecem estimular o aumento da recuperação do petróleo.

Lavagem de Solos por Biossurfactantes

A aplicação de biossurfactantes para remover os contaminantes microbianos de solos é uma tecnologia menos conhecida do que a tecnologia de aplicação avançada de biossurfactantes na biorremediação, uma vez que a eficiência de remoção é impulsionada principalmente pelas propriedades físico-químicas dos biossurfactantes e não pelos seus efeitos sobre a atividade metabólica ou alterações das propriedades na superfície celular. No entanto, os mecanismos que afetam a mobilização ou a solubilização do hidrocarboneto em solos se assemelham aos envolvidos no aumento da biodisponibilidade para a biorremediação (BANAT et al., 2010; FRANZETTI et al., 2009; MULLIGAN, 2009).

Assim, os biossurfactantes podem aumentar a biorremediação de hidrocarbonetos através de dois mecanismos. O primeiro inclui o aumento da biodisponibilidade do substrato para os micro-organismos, enquanto que o outro envolve a interação com a superfície da célula o que aumenta a hidrofobicidade da superfície de substratos hidrofóbicos permitindo mais facilmente sua associação com as células bacterianas (PRIETO et al., 2008). Ao reduzir a tensão superficial e interfacial, os biossurfactantes aumentam as áreas de superfície de compostos insolúveis que conduzem a uma maior mobilidade e biodisponibilidade de hidrocarbonetos. Em consequência, os biossurfactantes aumentam a biodegradação e a remoção de hidrocarbonetos.

A adição de biossurfactantes pode aumentar a biodegradação de hidrocarbonetos por mobilização, solubilização ou emulsificação (PACWA-PŁOCINICZAK et al., 2011).

A capacidade de solubilização depende da habilidade do surfactante em aumentar a solubilidade dos constituintes hidrofóbicos na fase aquosa. Um grande aumento desta capacidade é observado acima da CMC, a qual é atribuída à partição do hidrocarboneto no sítio hidrofóbico das micelas surfactantes. Neste processo, maiores concentrações em surfactantes são normalmente requeridas uma vez que a solubilidade dos constituintes dos hidrocarbonetos na solução surfactante dependerá totalmente da concentração do surfactante (BAI et al., 1997).

A mobilização ocorre em concentrações abaixo da CMC e pode ser dividida em deslocamento e dispersão. O deslocamento consiste na liberação de gotas de hidrocarbonetos do meio poroso devido à redução na tensão interfacial. Partindo de uma explicação teórica, os hidrocarbonetos serão removidos se a tensão interfacial entre a fase aquosa e a fase oleosa for suficientemente reduzida a fim de superar as forças de capilaridade que causam a formação da saturação residual. A dispersão é o processo no qual o hidrocarboneto é disperso na fase aquosa como emulsões muito pequenas. As emulsões não são normalmente estáveis termodinamicamente. Contudo, elas podem permanecer estáveis por períodos significantes de tempo em função de restrições cinéticas. A dispersão está relacionada à tensão interfacial e à concentração do surfactante e difere do deslocamento uma vez que o processo de deslocamento está relacionado apenas à tensão interfacial entre as fases aquosa e hidrofóbica, sem formação de emulsões (BAI et al., 1997).

A eficiência de um surfactante na remoção de compostos hidrofóbicos depende também do pH e da força iônica da solução, que pode alterar a configuração dos agregados micelares e a sorção do surfactante ao solo. A sorção do surfactante ao solo limita, por sua vez, o transporte do hidrocarboneto pelo surfactante. Os raminolipídeos são considerados bons

exemplos de surfactantes aniônicos uma vez que são menos retidos pelo solo do que outros surfactantes não iônicos ou neutros devido à carga negativa superficial dos solos (BORDAS et al., 2007).

Vários trabalhos descrevem o uso de biossurfactantes para a lavagem de solos. Souza Sobrinho et al. (2008) demonstraram que o biossurfactante isolado de *C. sphaerica* foi capaz de remover 65 % do óleo de motor adsorvido em areia. O biossurfactante de *C. antarctica* demonstrou capacidade de remover cerca de 50 % de óleo adsorvido em areia (ADAMCZAC; BEDNARSKI, 2000), enquanto que a solução do biossurfactante isolado de *C. glabrata* removeu cerca de 84 % do óleo de motor adsorvido (LUNA et al., 2009). O biossurfactante de *C. lipolytica* cultivada em resíduos agroindustriais foi aplicado com sucesso na remoção de derivado de petróleo em solo da formação barreira (RUFINO et al., 2011). Resultados obtidos por Abu-Ruwaida et al. (1991) para o líquido metabólico livre de células contendo o biossurfactante produzido por *Rhodococcus* demonstraram remoções de 86 % de óleo bruto residual adsorvido em areia. O biossurfactante de *P. aeruginosa* UCP0992 cultivada em glicerina removeu percentuais elevados de diesel adsorvido em amostras de areia (SILVA et al., 2010). Mulligan (2009) analisou a aplicação de biossurfactantes na lavagem de solos contaminados por hidrocarbonetos e metal. Franzetti et al. (2009) relataram uma remoção eficiente de petróleo bruto a partir de solo usando um bioemulsificante extracelular produzido por *Gordonia* sp. BS29.

Aplicação na Limpeza de Reservatórios de Óleos

A remoção de resíduos e frações de óleos pesados requer lavagens com solventes ou mesmo manuais, ambas perigosas, demoradas, e caras já que os resíduos e as frações de óleos pesados que sedimentam no fundo dos tanques são altamente viscosos e podem não ser removidos através de bombeamento convencional. Um processo alternativo a esta limpeza é o uso de

biossurfactantes que promovem a diminuição na viscosidade e a formação de emulsões óleo/água, facilitando o bombeamento dos resíduos e a recuperação do óleo bruto, após quebra da emulsão (SINGH et al., 2007; MULLIGAN; WANG, 2004).

A utilização de biossurfactantes para a limpeza de tanques, em substituição aos surfactantes convencionais, promoveu a limpeza e recuperação de 90 % dos hidrocarbonetos presentes no resíduo (MULLIGAN et al., 2004).

Aplicação na Remoção de Metais Pesados

Os poluentes inorgânicos presentes nos solos com maior potencial de risco ao homem são os metais pesados, que ocorrem naturalmente no ambiente e estão presentes em rochas, solos, plantas e animais. Os metais ocorrem em diferentes formas: como íons dissolvidos em água, vapor, ou sais minerais em rochas, areia e solo. Eles podem também estar ligados a moléculas orgânicas e inorgânicas ou atrelados por partículas no ar. Ambos os processos naturais e antropogênicos emitem metais para o ar e água (AGUIAR et al., 2002; JUWARKAR et al., 2007).

A contaminação por metais pesados surge como resultado das diversas atividades industriais, incluindo mineração, fundição de metais, produção de baterias automobilísticas, emissão de veículos e depósitos de resíduos industriais e a dispersão de cinzas provenientes dos processos de incineração (HONG et al., 2002; HASHIM et al., 2011). A presença de metais pesados nos solos provoca sérios problemas uma vez que os mesmos não podem ser biodegradados, levando à contaminação dos sistemas biológicos e do subsolo pelo processo de lixiviação (MORRISON, 1983). Nos Estados Unidos, por exemplo, o chumbo se encontra presente em 15 % dos terrenos contaminados, seguido de crômio, cádmio e cobre, encontrados em cerca de 7-11 % dos solos. Com a finalidade de reduzir os custos associados ao tratamento de solos

contaminados por metais pesados, diferentes tecnologias têm sido desenvolvidas e implantadas (PENG et al., 2008).

Existem duas tecnologias normalmente aplicadas ao tratamento de solos contaminados por metais. A primeira consiste em imobilizar os metais pesados numa matriz sólida fortemente ligada ao solo, minimizando a migração. Esta tecnologia, contudo, não consiste numa solução definitiva para o problema, considerando a impossibilidade de reaproveitamento do solo e a necessidade de monitoramento em longo prazo. A segunda tecnologia promove a mobilidade do metal e sua migração para a fase líquida por dessorção e solubilização. Esta tecnologia pode ser considerada uma solução permanente, permitindo ainda a reciclagem do solo remediado e consequentemente o reuso da terra (HASHIM et al., 2011). Normalmente, a lavagem do solo com ácidos e com agentes quelantes como o EDTA são as técnicas mais aplicadas. Contudo, a lavagem com ácidos leva a redução da fertilidade do solo e a alterações na composição química e física em virtude da dissolução de minerais (REED et al., 1996). A utilização de EDTA, por outro lado, é preocupante do ponto de vista salutar e de segurança, em função da sua degradação reduzida. A dificuldade de recuperação do metal pesado do complexo metal-EDTA também restringe o uso desta técnica.

Uma solução em potencial para a remediação de solos contaminados por metais e óleos consiste no uso de surfactantes, os quais podem ser adicionados em soluções, facilitando a solubilização, dispersão e dessorção dos contaminantes do solo, permitindo ainda sua reutilização (HASHIM et al., 2011).

Vários surfactantes sintéticos têm sido avaliados em testes de descontaminação (ASÇI et al., 2008; ELLIS et al., 1985; NASH et al., 1987). Por outro lado, a necessidade de substituição de compostos sintéticos por similares naturais tem levado a pesquisas para utilização de biossurfactantes (MULLIGAN, 2009). Vários trabalhos têm demonstrado o potencial de utilização de surfactantes biológicos, destacando-se os estudos com surfactina e

raminolípidios, de origem bacteriana, e de alguns soforolípidios originados de leveduras (MULLIGAN, 2009; HERMAN et al., 1995; MULLIGAN et al., 1999; NEILSON et al., 2003; OCHOA-LOZA et al., 2007; TAN et al., 1994). A natureza iônica desses agentes, bem como a biodegradabilidade, baixa toxicidade e excelentes propriedades de superfície os tornam candidatos em potencial para a remoção de metais pesados contidos em solos e sedimentos. Para Mulligan (2009) é possível a remoção de metais pesados pela utilização de várias concentrações de surfactante. Das et al. (2009) mostraram que a remoção de cádmio a partir de solução aquosa também ocorreu em concentrações menores do que a de CMC, embora com uma concentração de cinco vezes a CMC resultou na remoção quase completa de 100 ppm de íons metálicos. Wen et al. (2009) estudaram a degradação de um raminolípido em solos contaminados por Cd e Zn, sugerindo que o raminolípido pode permanecer por tempo suficiente no solo para aumentar a fito-extração metal.

Considera-se que a remoção de metais por biossurfactantes iônicos ocorre na seguinte sequência: (1) sorção do biossurfactante à superfície do solo e complexação com o metal, (2) destaque do metal do solo para a solução e (3) associação com as micelas do biossurfactante. Neste caso, os metais pesados ficam presos nas micelas através de interações eletrostáticas, podendo ser facilmente recuperados por precipitação ou técnicas de separação por membranas (KITAMOTO et al., 2002).

Os biossurfactantes aniônicos criam complexos não iônicos com os metais através de ligações iônicas. Essas ligações são mais fortes do que as ligações do metal com o solo, sendo os complexos metal-biossurfactante desorvidos da matriz do solo para a solução do solo, devido à redução da tensão interfacial. Os biossurfactantes catiônicos podem substituir os mesmos íons metálicos carregados por competição para algumas, mas não todas, as superfícies carregadas negativamente (troca de íons). Íons metálicos podem ser removidos da superfície do solo também pelas micelas surfactantes.

Os biossurfactantes possuem vantagens inquestionáveis uma vez que os micro-organismos capazes de produzir compostos surfactantes não precisam ter capacidade de sobrevivência no solo contaminado pelo metal pesado, embora o uso de biossurfactantes requer a adição contínua de novas porções destes compostos (PACWA-PLOCINIKZAC et al., 2011).

Os biossurfactantes também têm sido aplicados na mineração. Compostos tensoativos produzidos por *Pseudomonas* sp. e *Alcaligenes* sp. foram utilizados para flotação e separação de calcita e eschelita. A recuperação foi de 95 % para CaWO_4 e 30 % para CaCO_3 , ressaltando que reagentes químicos convencionais foram incapazes de separar estes dois minerais (NITSCHKE; PASTORE, 2002). Biossurfactantes produzidos por espécies de *Candida* têm sido aplicados com sucesso na flotação de metais pesados, sendo capazes de remover mais de 90 % dos cátions em colunas e em processos de Flotação por Ar Dissolvido (MENEZES et al., 2011; ALBUQUERQUE et al., 2012). O biodispersan, polissacarídeo aniônico produzido por *Acinetobacter calcoaceticus* A2 foi utilizado na prevenção da floculação e dispersão de misturas de pedra calcária e água (RON; ROSENBERG, 2001).

3.12 Aspectos Econômicos da Produção de Biossurfactantes

Muitas das potenciais aplicações dos biossurfactantes, bem como uma expansão dos poucos já firmados no mercado dependem da possibilidade de um processo de produção econômico. Muito trabalho ainda será necessário para a otimização de processos a nível biológico e de engenharia. Os custos típicos dos biossurfactantes variam de cerca de U.S 10 \$/mg para surfactina pura (98 % de pureza), utilizada em pesquisas médicas, a U.S. 24 \$/kg para fórmulas de emulsan propostas no início da década de 1980 para limpeza de tanques e/ou recuperação avançada de petróleo. Estimativas realizadas na década passada situaram os custos dos biossurfactantes em U.S. 3-20 \$/kg, enquanto o custo de produção de surfactantes sintéticos como etoxilatos e

alquil-poliglicosídeos pelas indústrias químicas estão na faixa de U.S. \$ 1-3/kg (BOGNOLO, 1999).

Embora se admita que o aperfeiçoamento da tecnologia de produção dos biossurfactantes já tenha possibilitado um aumento de 10–20 vezes da sua produtividade, é provável que novos e significativos progressos (ainda que de uma ordem de magnitude inferior) sejam necessários para tornar essa tecnologia comercialmente viável (GAUTAM; TYAGI, 2006).

Os parâmetros que podem ser variados na tentativa de otimizar a produção de biossurfactantes incluem:

- a) Seleção de matérias-primas de baixo custo, possibilitando o equilíbrio adequado de C, N, P e outros oligo-elementos para maximização do rendimento e o desenvolvimento de cepas de micro-organismos capazes de metabolizar qualquer subproduto residual.
- b) Bioprocessamento, que pode ser otimizado por meio das condições operacionais do reator e da reciclagem do meio utilizado.
- c) Isolamento/recuperação do produto: a maioria das tecnologias inicialmente propostas envolvia formas mais elaboradas de purificação e isolamento. A possibilidade de desenvolvimento *in-situ* ou a utilização de líquidos metabólicos, ou seja, do biossurfactante bruto, pode, sem dúvida, conduzir a uma redução substancial de custos (BOGNOLO, 1999).

3.13 Perspectivas

O sucesso da comercialização de um produto biotecnológico depende, em grande parte, da economia de seu bioprocessamento. Atualmente, os preços dos surfactantes microbianos não são competitivos com os preços dos agentes tensioativos químicos devido aos elevados custos de produção e aos rendimentos reduzidos em produto isolado. A fim de tornar a produção de

biossurfactantes comercialmente viável será importante otimizar os processos de produção a níveis biológico e de engenharia. Os avanços das pesquisas envolvendo o processo de produção de biossurfactantes já permitiram um aumento de 10 a 20 vezes na produtividade, apesar da necessidade de estudos mais aprofundados. O uso de substratos de baixo custo e o estabelecimento do crescimento microbiano em condições ideais de produção, juntamente com novos métodos de purificação e com a utilização de cepas microbianas hiperprodutoras pode tornar a produção de biossurfactantes economicamente viável. Embora um grande número de micro-organismos produtores de biossurfactantes seja relatado na literatura, as pesquisas relacionadas com o aumento de produção têm se concentrado na maioria das vezes a poucos gêneros de micro-organismos, tais como *Pseudomonas*, *Bacillus* e *Candida*. Os biossurfactantes são grandes candidatos para substituir os surfactantes sintéticos especialmente nas indústrias de óleos, sendo o investimento nas estratégias para aperfeiçoar o bioprocessamento dessas biomoléculas o caminho para a produção de biossurfactantes em larga escala.

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CAPÍTULO 2

Artigo submetido para publicação na Revista Petroleum Science and Engineering

**Application of bacterial and yeast biosurfactants for enhanced removal
and biodegradation of motor oil from contaminated sand**

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Abstract

This study investigated potential application of two biosurfactants for enhanced removal capability and biodegradation of motor oil contaminated sand under laboratory conditions. The biosurfactants were produced by the yeast *Candida sphaerica* and by the bacterium *Bacillus* sp. cultivated in low-cost substrates. The ability of removing motor oil from soil by the two biosurfactants was identified and compared with that of the synthetic surfactants Tween 80 and Triton X-100. Both crude and isolated biosurfactants showed excellent effectiveness on motor oil removal from contaminated sand under kinetic conditions (70-90%), while the synthetic surfactants removed between 55 and 80% of the oil under the same conditions. The increase in biosurfactants and synthetic surfactants concentration did not enhance the removal of oil. A contact time of 5-10 min under agitation seemed to be enough for oil removal with the biosurfactants and synthetic surfactants tested. The crude and the isolated biosurfactant produced by *C. sphaerica* were able to remove high percentages of motor oil from packed columns (around 90%) when compared to the biosurfactant from *Bacillus* sp. (40%). For the degradation experiments conducted in motor oil contaminated sand enriched with sugar cane molasses, however, the oil degradation reached almost 100% after 90 days in the presence of *B. sp.* cells, while the percentage of oil degradation did not exceed 50 % in the presence of *C. sphaerica*. The presence of the biosurfactants increased the degradation rate in 10-20%, especially during the first 45 days of the experiments, indicating that biosurfactants acted as efficient enhancers for

hydrocarbon biodegradation. The results indicated the biosurfactants enhancing capability on both removal and rate of motor oil biodegradation in soil systems.

Keywords: *Candida sphaerica*; *Bacillus* sp.; bioremediation; petroleum; sand-packed column

1. Introduction

In recent years, much attention has been directed towards biosurfactants owing to their different advantages such as lower toxicity, higher biodegradability, better environmental capability, higher foaming, high selectivity, specific activity at extreme temperatures, pH and salinity, and the ability to be synthesized from renewable feed stocks (Silva et al., 2014a). Some disadvantages can be mentioned for the use of biosurfactants: at the time, a small amount of products is produced at industrial level. Many biosurfactants are yet in a laboratory scale level and some of them are quite expensive. The discovery of new biosurfactants, development of new fermentation and recovery processes and the use of cheap raw materials (specifically the use of agro-industry wastes as carbon sources) will allow that more inexpensive biosurfactants can be available for remediation process (Torres et al., 2011).

The major difficulty in bioremediation of oil-contaminated soil is the bioavailability or mass transfer limitation of the oil pollutants in the soil, causing

poor food-microorganism contact and thus poor biodegradation efficiency (Paria, 2008). Oil penetration through soil is an extremely complex process related to physical, chemical, and biological factors (Kuyukina et al., 2005). Petroleum hydrocarbons are highly hydrophobic material with low water solubility and those components attach to soil particles, reducing the bioavailability of oil compounds to microorganisms, thereby limiting the rate of mass transfer for biodegradation. The possible physical forms for oil contaminants in soil can be dissolved in pore water, adsorbed onto soil particles, absorbed into soil particles, or be present as a separate phase, which can be a liquid or a solid phase (Paria et al., 2008). The key process to enhance the bioavailability of the oil contaminant is to transport the pollutant to the aqueous bulk phase (Lai et al., 2009). One of the effective ways to increase the bioavailability (or solubility) of petroleum hydrocarbon pollutants in soil is using surfactants to enhance the desorption and solubilization of petroleum hydrocarbons, thereby facilitating their assimilation by microorganisms (Christofi and Ivshina, 2002; Lai et al, 2009; Mulligan et al. 2001).

Enhanced soil washing generally has been performed with synthetic surfactants, including anionic, nonionic, cationic and mixed surfactants, and some of them have shown great washing capabilities for hydrophobic organic compounds (HOCs) from contaminated soils and groundwater (Urum et al., 2006). Some synthetic surfactants, such as Triton X-100, Tween 80, Afonic 1412-7, are shown to be able to enhance the concentration of non polar compounds in the aqueous phase (Christofi and Ivshina, 2002; Lai et al., 2009).

However, the residual synthetic surfactants in soils and groundwater have the potential toxicity risk or hazard to environment and human health. So, an improved strategy for soil washing technology is to use biosurfactants (Zhou et al., 2013).

Therefore, biosurfactants seem to be better candidates for using in soil washing technology. However, literature data indicated that most of previous studies have focused on few biosurfactants (Christofi and Ivshina, 2002; Mulligan, 2005; 2009). More other biosurfactants should be investigated for their properties in enhancing soil washing because they may have more promising properties (Zhou et al. 2013).

At low concentrations, biosurfactants are soluble in water, and with increasing concentrations, they form micelle in solution. The concentration at which micelle begins to form is called the critical micelle concentration (CMC); above the CMC, biosurfactants can solubilize petroleum hydrocarbons in soil–water systems, but some biosurfactants may increase the water solubility of hydrocarbon molecules below the CMC. Therefore, biosurfactants may be useful in degradation of soil contaminating hydrocarbons (Bordoloi and Konwar, 2008).

In this work, two biosurfactants, i.e., a glycolipid produced by *Candida sphaerica* (Luna et al., 2013) and another biosurfactant produced by *Bacillus* sp. were used to remove motor oil from a laboratory oil-contaminated sand. The oil removal efficiency was examined using the two biosurfactants as well as two

commonly used synthetic surfactants (Tween 80, and Triton X-100) for comparison. Experiments were conducted through kinetic (flasks) and static assays (packeded columns). The effect of biosurfactant type and concentration, and the contact time on oil removal efficiency was also investigated. Additionally, potential application of the two biosurfactants for enhanced biodegradation of motor oil contaminated sand with a series of bench-scale experiments was evaluated. The outcome of this work is expected to provide a useful tool for screening the effective surfactants used in bioremediation of oil-polluted environments.

2. Materials and methods

2.1. Materials

All chemicals were of reagent grade. Growth media were purchased from Difco Laboratories (USA).

Three types of industrial waste were used as substrates to produce the biosurfactants. Ground nut oil refinery residue was obtained from ASA LTDA in the city of Recife, state of Pernambuco state, Brazil. Corn steep liquor was obtained from Corn Products do Brasil in the city of Cabo de Santo Agostinho, Pernambuco state, Brazil and sugar cane molasses was obtained from a local plant cane sugar in the city of Igarassu, Pernambuco state, Brazil.

Motor oil (15 cSt) was obtained from an automotive maintenance establishment in the city of Recife, Pernambuco, Brazil.

2.2. Sand

Samples of 100/50 mesh (0.15-0.3mm) of Brazilian standard sand NBR 7214 (ABNT, 1982) were used in the experiments. Laboratory impregnated sand samples with motor oil were prepared and left to stand at room temperature for subsequent use.

2.3. Synthetic surfactants used

Two chemically synthesized surfactants (namely, Tween 80 and Triton X-100) were also used for motor oil removal from contaminated soil to compare their performance with that from biosurfactants. Tween 80 (purchased from Sigma Chemical Co. St. Louis, MO, USA, is a nonionic surfactant and an oil-in-water emulsifier. The CMC of Tween 80 is about 0.0124% (w/v) and the surface tension is able to be reduced to 43.7 mN/m. Triton X-100, also obtained from Sigma Chemical Co. St. Louis, MO, USA, is a nonionic surfactant possessing a hydrophilic polyethylene oxide group and a hydrocarbon lipophilic or hydrophobic group. The CMC of Triton X-100 is about 0.0183% (w/v) % and the surface tension is able to be reduced to 32.7 mN/m.

2.4. Microorganisms and preparation of seed cultures

C. sphaerica UCP 0995 was obtained from the culture collection of the Universidade Católica de Pernambuco, Brazil. The microorganism was maintained at 5 °C on yeast mould agar slants. The *C. sphaerica* inoculum was

prepared by transferring cells grown on a slant to 50 ml of yeast mould broth. The seed culture was incubated at 28 °C and 150 rpm for 24 h.

The *Bacillus* sp., an indigenous bacterium, was isolated from a petroleum contaminated soil site located in Recife. The bacterium culture was maintained on nutrient agar slants at 4 °C. For pre-culture, the strain from a 24-h culture on nutrient agar was transferred to 50 ml of nutrient broth to prepare the seed culture. The cultivation conditions for the seed culture were 28 °C, 150 rpm and 10 to 14 h of incubation.

2.5. Production of biosurfactants

The microorganisms were cultivated in a submerged culture in a Marconi MA832 shaker (Marconi Ltda, Brazil).

The yeast biosurfactant was produced in a medium composed of 9% ground nut oil refinery residue and 9% corn steep liquor dissolved in distilled water. The final pH of the medium was 5.3 and the surface tension prior to inoculation was 50 mN/m. The inoculum (1%, v/v) was added to the cooled medium at the amount of 10^4 cells/ml. Fermentation was carried out in 500-ml Erlenmeyer flasks at 28 °C and 150 rpm for 144 h (Luna et al., 2013).

The bacterium biosurfactant was produced in Bushnell-Hass medium (Difco) composed by 0.1% KH_2PO_4 , 0.1% K_2HPO_4 , 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02% $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ and 0.005% $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$. The pH was adjusted to 7.0 by 1.0 M HCl. The surface tension prior to inoculation was 56 mN/m. Three percent sugar cane molasses and 3% corn steep liquor were added. Two percent aliquots

(v/v) of the cell suspension (0.7 optical density at 600 nm), corresponding to an inoculum of 10^7 CFU/ml, were used to inoculate 500-ml Erlenmeyer beakers containing 100 ml of sterile production medium. Cultivation was carried out at 27 °C with agitation at 200 rpm for 120 h.

2.6. Determination of surface tension

According to the literature, the CMC of *C. sphaerica* biosurfactant is about 0.025% (w/v) and the surface tension is about 25.0 mN/m (Luna et al., 2013) while the CMC of *Bacillus* sp. biosurfactant was determined as 0.5% (w/v) and the surface tension as about 29 mN/m.

Since the biosurfactant from *C. sphaerica* was produced according to the methods described above, measurements of the surface tension were conducted to control the quality of the biosurfactant obtained. Changes in surface tension were determined in the cell-free broth obtained by centrifuging the cultures at 5000 x g for 20 min. Surface tension was determined using a Sigma 700 Tensiometer (KSV Instruments LTD - Finland) at room temperature. Tensiometers determine the surface tension with the aid of an optimally wettable ring suspended from a precision scale. With the ring method, the liquid is raised until contact with the surface is registered. The sample is then lowered again so that the film produced beneath the liquid is stretched for the determination of maximum force, which is used to calculate the surface tension. The instrument was calibrated against Mill-Q-4 ultrapure distilled water (Millipore, Illinois, USA). Prior to use, the platinum plate and all glassware were

sequentially washed with chromic acid, deionised water and acetone and flamed with a Bunsen burner.

2.7. Isolation of biosurfactants

The two biosurfactants were extracted from the culture media after cell removal by centrifugation at 5000g for 30 min.

The cell-free culture broth from *C. sphaerica* was acidified with 6M HCl to pH 2.0 and precipitated with two volumes of methanol. After 24 h at 4 °C, samples were centrifuged at 5000 × g for 30 min, washed twice with cold methanol and dried at 37 °C for 24–48 h (Luna et al., 2013).

The cell-free culture broth from *P. sp.* pH was adjusted to 2.0 with 6.0 M HCl and an equal volume of CHCl₃/CH₃OH (2:1) was added. The mixture was vigorously shaken for 15 min and allowed to set until phase separation. The organic phase was removed and the operation was repeated twice. The product was concentrated from the pooled organic phases using a rotary evaporator. The viscous yellowish product obtained was dissolved in methanol and concentrated again by evaporation of the solvent at 45 °C (Silva et al., 2014b).

2.8. Application of chemical surfactants and biosurfactants in removal of motor oil from sand through kinetic assay

The removal of motor oil from the laboratory contaminated sand was tested through the saturation of 50 g of the standard sand with 10% of motor oil as

described by Luna et al. (2011). The laboratory-contaminated soil was placed in 500-mL Erlenmeyer beakers, to which 100 mL of the crude biosurfactants (cell-free broth after fermentation) and isolated biosurfactants and chemical surfactants at 1/2 the CMC, the full CMC and twice the CMC were added. The beakers were shaken at 150 rpm for 5, 10 and 20 min during 24 h at 28 °C. The entire content was then centrifuged at 5000 rpm for 1200 s. Control assays were performed using distilled water at the same conditions. The amount of oil residing in the sand after the impact of biosurfactant was gravimetrically determined as the amount of material after extraction with hexane and the % of oil removal was calculated using the equation:

$$\text{Motor oil removed (\%)} = (O_i - O_r) / O_i \times 100\%$$

Where O_i is the initial motor oil in the soil (g) before washing and O_r is the motor oil remaining in the soil (g) after washing.

2.9. Application of chemical surfactants and biosurfactants in removal of motor oil from sand packed column through static assay

Glass columns measuring 55 cm in height x 6 cm in diameter were initially filled with approximately 200 g of a mixture containing the sand and 10% of motor oil. The surface was then inundated with 200 mL of the crude biosurfactants (cell-free broth after fermentation) and isolated biosurfactants and chemical surfactants at 1/2 the CMC, the full CMC and twice the CMC

under the action of gravity. Percolation of the biosurfactant solution was monitored for 24 h, when no further percolation of the solution was observed. Following the washing of the columns, the soil samples were washed with 20 mL of hexane for the removal of residual oil. The solvent was rotoevaporated at 50 °C and the amount of oil removed was determined by gravimetry as described in section 2.8 (Dahrazma and Mulligan, 2007; Rufino et al., 2013).

2.10. Evaluation of oil-degrading ability in sand

Samples of laboratory contaminated standard sand (10 g) were added to 100 mL of distilled water and the mixture was enriched with 1 mL of sugar cane molasses. Then, solutions of the isolated biosurfactants at their CMC and/or 15% of its microbial-producing species previously cultivated in yeast mould broth and/or nutrient broth (15% inoculum at the amount of 10^8 cells/ml for the yeast and 15% inoculum of 10^7 CFU/ml from a 0.7 optical density at 600 nm for the bacterium) were added and the medium was placed in a rotary shaker at 150 rpm and 28°C for 90 days (Table 1). Experiments were carried out in 250 ml Erlenmeyer flasks. At 15 days of experiment 1% molasses was added to the mixture, totaling five feeds (after 15, 30, 45, 60 and 75 days). Samples of 5 mL were removed every 15 days for hydrocarbons analysis, totaling 06 samples. The percentage of degradation of hydrocarbons was calculated as the concentration of hydrocarbon oil removed from a control prepared without the addition of microorganisms and biosurfactants and analyzed at time 0 (zero) (Joo et al., 2008).

2.11. Total motor oil biodegradation rate

The samples were drawn for estimation of motor oil degradation by gravimetric analysis. The residual motor oil was extracted in a preweighed beaker with hexane in a separating funnel. Extraction was repeated twice to ensure complete extraction. After extraction, hexane was evaporated in a hot air oven at 68–70 °C, the beaker was cooled down and weighed.

The % degradation was calculated as follows:

$$\text{Motor oil degradation (\%)} = (O_d - O_s) / O_d \times 100\%$$

where O_d is the amount of motor oil degraded (g) and O_s is the amount of motor oil added in the sand (g)

2.12. Statistical analysis

The analyses were performed in triplicates. The mean values and standard deviation (mean $\pm$ SD) were calculated and tested. Statistical analysis of variance (ANOVA) was performed on all values and tested for $p < 0.05$ for significance.

3. Results and discussion

3.1. Application of chemical surfactants and biosurfactants in removal of motor oil from sand through kinetic assay

Over decades, chemically synthesized surfactants have been used for enhanced oil recovery (EOR) and for oil spill clean-up. However, because of their toxicity and resistance to degradation, they have been replaced by biosurfactants (Kryachko et al., 2013; Mulligan, 2005).

3.1.1. Effect of biosurfactant concentration on motor oil removal efficiency

Figs. 1 and 2 displays the results of the experiments carried out in beakers for the removal of motor oil adsorbed to sand by the two biosurfactants.

Biosurfactant concentration is usually a critical factor for the removal of oil compounds from soil. To evaluate the performance of the two biosurfactants in removing motor oil from the contaminated soil, three biosurfactant concentrations were applied to wash the samples of sand i.e., under, at and above the CMC. High percentage removals of oil were observed for all solutions tested. The motor oil removal efficiency did not increase with an increase in both biosurfactants concentration. This finding is satisfactory from the environmental stand point, as high concentrations of some biosurfactants have a toxic effect on the native microbial population in the soil (Christofi and Ivshina, 2002). The biosurfactant from *Bacillus* sp. was able to remove a little more oil

than the biosurfactant from *C. sphaerica*. Both biosurfactants showed excellent effectiveness on motor oil removal from contaminated sand, thereby being suitable for future application for biostimulation of oil bioremediation in soil. Biosurfactants such as aescin, lecithin, tannin could not enhance the solubilization of crude oil in soil at concentrations greater than their CMC values (Urum and Pekdemir, 2003), which is consistent with our results. However, when rhamnolipids were used, the solubility of crude oil seemed to increase with an increase in rhamnolipids concentration (Lai et al., 2009).

Liu et al. (1995) showed that the increase in the apparent solubility of some PAHs in the presence of anionic and non-ionic surfactants increases significantly beyond the CMC. Lai et al. (2009) evaluated the performance of rhamnolipids and surfactin in removing hydrocarbons from soil, showing that the removal efficiency was positively correlated with the concentration of rhamnolipids and surfactin. The maximum oil removal efficiency of rhamnolipid and surfactin both occurred at 0.2 mass%, giving a removal percentage of 23.4 and 14.0, respectively while the isolated biosurfactants tested in our work removed around 70-80% of the oil. The biosurfactant produced by *Rhodococcus erythropolis* grown on glycerol removed 94% oil in shake flasks (Ciapina et al., 2006). The Rufisan biosurfactant from *C. lipolytica* at the CMC removed 98% of the oil from beakers in the kinetic assays and biosurfactant concentration exerted no influence on the oil removal rate (Rufino et al., 2013). On the other hand, the biosurfactant from *Candida sphaerica* at 0.1% solution removed 65% of motor oil adsorbed to soil, while the surfactant solution at the

CMC (0.08%) removed 55% of the oil and the solution at 0.05% removed approximately 30%, showing the influence of the biosurfactant concentration on the removal rates (Souza Sobrinho et al., 2008).

As described by Costa et al. (2010), two mechanisms are associated with the removal of oil in soils: mobilization and solubilization. Mobilization occurs at concentrations below the CMC and the phenomena associated with this mechanism include the reduction of surface and interfacial tension. Surfactants in contact with the soil/oil system increase the contact angle and reduce the capillary force holding oil and soil together due to the reduction of the interfacial force. Solubilization occurs above the surfactants CMC, as the apparent solubility of oil increases dramatically due to its aggregation within the surfactants micelles. Inside the micelles, the hydrophobic end of the surfactants molecules cluster together forming a hydrophobic environment capable to solubilize hydrophobic substances, while the hydrophilic end exposed to the aqueous phase on the exterior allow the whole structure to remain in solution (Costa et al., 2010).

The data observed in this work suggest that mobilization is the main mechanism associated with the removal of motor oil with the biosurfactants and the chemical surfactants, because the increase in (bio)surfactants concentration did not enhance the removal of oil. Besides biodegradability, the removal of oil contaminants without modifying the chemical nature of soil by mobilization is another advantage of biological surfactants over chemical surfactants, as stated by Lai et al. (2009).

The biosurfactant from *P. aeruginosa* UCP0992 also utilized the mechanism of mobilization to release the oils droplets from sand since the increase of the concentration did not improve the removal of the pollutants (Silva et al., 2010). On the other hand, solubilization was the main mechanism associated with the removal of crude oil with the rhamnolipid surfactants produced by *Pseudomonas aeruginosa* L2-1 from cassava wastewater added with waste cooking oil, because increasing rhamnolipid concentration enhanced the removal of crude oil, due the incorporation of these molecules into micelles (Costa et al., 2010).

In order to evaluate the use of the crude biosurfactants, the removal ability of the cell-free broth was also tested. The cell-free broth containing biosurfactants and the isolated biosurfactants are almost equally effective in the removal of the motor oil pollutant. Thus, cell-free broth containing biosurfactants can be directly used without purification steps, which would further reduce 30%-50% of the production cost of biosurfactants.

Silva et al.(2010) also observed that the cell-free broth containing the crude biosurfactant from *P. aeruginosa* was practically as effective as the isolated biosurfactant when removing 85% diesel oil from sand, thus indicating the possible use of the biosurfactant without purification steps, which would increase the production costs. The biosurfactant containing cell-free broth from *C. tropicalis* cultivated in waste frying oil removed approximately 78 to 97% of the petroleum and motor oil adsorbed in sand samples (Batista et al., 2010). Over 50% of the oil was extracted after rinsing of the sand with solutions of

biosurfactants from *C. antarctica* (Adamczak and Bednarski, 2000), while the crude biosurfactant from *C. guilliermondii* grown in industrial residues had removed approximately 90% of the motor oil adsorbed in sand samples (Coimbra et al., 2009). The cell-free broth containing the crude biosurfactant from *C. lipolytica* cultivated in medium containing animal fat and corn steep liquor was more effective in removing motor oil than the isolated biosurfactant (Santos et al., 2013). Satisfactory results were obtained for the removal of motor oil adsorbed to sand samples by the cell-free broth from *C. sphaerica* in comparison to the control (distilled water), with removal rates of 95% and 10%, respectively. The removal capacity can be affected also by the kind of soil as observed by Silva et al. (2014) since the cell-free broth from *Pseudomonas cepacia* grown in mineral medium supplemented with corn steep liquor and soybean waste frying oil achieved poorer than expected results regarding the removal of motor oil adsorbed to sand, whereas satisfactory results were achieved with clay soil, with removal rates surpassing 80%. Despite the greater permeability due to macro-pores between grains of sand, which facilitate the circulation of water and air, an interaction seems to have occurred between the biosurfactant and clay.

The samples prepared with distilled water (control) showed an interesting result, since it was possible to remove around 40% of the oil adsorbed in the sand. Our results are in accordance with the literature since Chang et al. (2000) found that, 73.6 up to 100% of polycyclic aromatic hydrocarbons (PAHs) were eliminated in the presence of sodium dodecyl sulfate (SDS), while 30–80%

when using only water. According to Khalladi et al. (2009) water washing of a diesel-polluted soil could eliminate up to 24% of *n*-alkanes. This low percentage is of great economic interest, especially for important quantity of polluted soil. Therefore, a water washing process can be recommended before any other remediation process to reduce the hydrocarbon soil content and subsequently the consumed surfactant quantity.

the *Pseudomonas* sp. 2B biosurfactant solution at 0.01% and 0.05% biosurfactant concentrations was capable to remove 89% and 92% of the oil adsorbed in the sand respectively, while the distilled water (control) and synthetic surfactant, sodium dodecyl sulfate (SDS), removed 48% and 63% of the contaminated oil respectively. 81% of crude residual oil was removed using the cell-free broth containing biosurfactant produced by *Pseudomonas* sp. 2B. Similar results were obtained by Abu-Ruwaida et al. (1991) for the cell-free broth containing a biosurfactant produced by *Rhodococcus* cells; 86% of crude residual oil adsorbed in the sand was removed.

3.1.2. *Effect of contact time on motor oil removal efficiency*

The contact time is also an important parameter affecting the efficiency of oil removal, as a sufficient contact time is required for effective oil removal. In this study, we investigated the effectiveness of oil removal at 5, 10, 20 and 1440 min. As indicated in Figs. 1 and 2, irrespective of the biosurfactant type and biosurfactant concentration, an increase in contact time from 5 to 1440 min in

general led to either a similar motor oil removal efficiency or a slightly decrease in oil removal performance. These results indicate that a contact time of 5-10 min under agitation seemed to be enough for oil removal with the biosurfactants applied. Lai et al. (2009) tested the the removal efficiency of rhamnolipids and surfactin during 7 days, showing that 1 day was sufficient for solubilisation of the hydrocarbons to the mobile phase.

3.1.3. Comparison of motor oil removal efficiency between biosurfactants and synthetic surfactants

For practical application of biosurfactants on oil removal from sand, it is of great interest to compare the performance of biosurfactants with that of two commonly used chemical surfactants (i.e., Tween 80 and TritonX-100). After adding different concentrations of surfactants for 1440 min, it was observed that the contact time of 5-10 min under agitation was also enough for oil removal with the chemical surfactants (Figs. 3 and 4). It could be observed that the biosurfactants were more effective than the commercially available surfactants. The results indicated the superior performance of 10% of the biosurfactants over chemical surfactants in terms of mobilization of oil pollutants from the contaminated soil and thus the two biosurfactants examined in this work have the potential to be used as biostimulation agents for bioremediation of oil-polluted soils.

Our results are consistent with the results obtained by Lai et al. (2009) for two biosurfactants compared to the same chemical surfactants used in this work. The biosurfactant from *Klebsiella* sp. strain RJ-03 grown in sucrose removed about 90% of oil compared to 57 - 67% recovery by chemical surfactants in shake flasks (Jain et al., 2012). Three biosurfactants from *Bacillus subtilis* strains isolated from Brazilian crude oils at a concentration of 1 g/l recovered between 19% and 22% of oil, whereas the recoveries obtained with the chemical surfactants at the same concentration were between 9% and 12% (Pereira et al., 2013). Urum et al. (2006) also investigated the efficiency of different surfactant solutions in the removal of oil from contaminated soils and found higher rates with the sodium dodecyl sulphate (SDS) and rhamnolipid biosurfactants (46% and 44%, respectively) in comparison to natural saponin surfactants (27%). Another study also investigated the enhanced soil washing of the plant derived natural biosurfactant of *Sapindus* saponin for phenanthrene from contaminated soil. *Sapindus* saponin could effectively remove phenanthrene from contaminated soil with a maximum removal percentage of about 87.4%, which was only slightly less than that of Tween 80 (Zhou et al., 2013). Liu et al. (2015) showed that surfactin and the chemical surfactants SDS and polyethylene glycol monododecyl ether (PGME) could remove more than 95% of artificial crude oil from sand.

3.2. Application of chemical surfactants and biosurfactants in removal of motor oil from sand packed column through static assay

Laboratory studies on MEOR typically use sand-packed columns, which provide a suitable bench-scale approach to evaluate oil recovery for several reasons: it is an economic model; a battery of columns can be set up simultaneously; and they can simulate the oil recovery operations usually conducted in reservoirs (Suthar et al., 2008).

In this work, a sand-packed column was used to study the effect of two biosurfactants and two chemical surfactants on solubilization of entrapped oil.

The crude and the isolated biosurfactant produced by *C. sphaerica* were able to remove high percentages of motor oil from packed columns when compared to the biosurfactant from *Bacillus* sp. (Table 2). Based on its high surface activity, the biosurfactant from *C. sphaerica* seems to have the potential for the use in mobilizing crude oil in biostimulation processes. It was also observed that the use of the crude biosurfactant is sufficient to reach the best removal percentages values and that the biosurfactants concentrations did not influence the removal rates of motor oil. Studies carried out by Urum et al. (2003) demonstrated that the mobilization or solubilization of hydrophobic compounds by surfactants in sand-packed columns may or may not vary depending on the concentration employed. Some surfactants of a vegetal origin, such as aescin, lecithin and tannin, were not capable of enhancing the solubilization of hydrophobic compounds at concentrations above the CMC.

Cell-free broths containing *Pseudomonas aeruginosa* isolates cultivated in glycerol removed 49–54% of crude oil contained in packed columns (Bordoloi

and Konwar, 2008). High concentrations (2.5 and 5.0 g/l) of a biosurfactant isolated from *P. aeruginosa* 57SJ (CMC 400mg/l) were needed to remove 70% of pyrene adsorbed to soil (Bordas et al., 2007).

The removal of motor oil in the static assay in packed glass columns by the biosurfactant from *C. lipolytica*, on the other hand, showed the influence of biosurfactant concentration since removal rates of the percolating liquids obeyed the following increasing order: distilled water (7%), Tween 80 (12%), cell-free metabolic broth (26%), biosurfactant at the CMC (33%) and biosurfactant at three times the CMC (37%) (Rufino et al., 2013).

The biosurfactants produced by *Bacillus* species cultivated in residues of molasses and cheese whey removed about 30% of the oil contained in a packed column (Joshi et al., 2008). The oil removal activity of surfactin had been evaluated by sand packed test with fresh kerosene contaminated soil, showing a 34–62% oil recovery by flushing with 0.1 mass % surfactin solution (Makkar and Cameotra 1997; 1998).

Cameotra and Makkar (1998) had demonstrated that the biosurfactant isolated from *Pseudomonas aeruginosa* was able to recover 56% of the oil adsorbed to the sand contained in a column.

It is interesting to observe that the experiments under static conditions allowed removal percentages similar to the experiments in flasks, showing that the agitation did not increase the interaction between the biosurfactant from *C. sphaerica* and the contaminant. Such behaviour was not observed for the *B. sp.*

biosurfactant and chemical surfactants since the kinetic experiments allowed better removal rates compared to sand packed columns. Lee et al. (2002) obtained a removal ratio of 73 and 95% in batch and column experiments, respectively.

The performance of water in the removal of motor oil was negligible as shown in Table 2. Khalladi et al., (2009), on the other hand, showed that the performance of water in the removal of diesel fuel was found to be non-negligible, while water contributed by 24.7% in the global elimination of *n*-alkanes. The biosurfactant produced by a crude oil degrading bacteria was tested for oil recovery in sand packed column showing an oil recovery efficiency of 76% compared to the control in which only 30% of the oil was recovered over the same period (Ibrahim et al., 2013).

According to Zhou et al., (2013) sorption of surfactants onto soil would decrease the effective concentrations of surfactant in aqueous solution to solubilize hydrophobic organic contaminants (HOCs), and the soil-sorbed surfactants can also enhance soil retardation capability for HOCs, both of which would reduce soil washing efficiency and result in an increase in remediation times and costs. The results obtained in this work suggests that the two biosurfactants studied did not show a strong interaction with the soil.

The chemical surfactant Triton X-100 removed similar quantities of motor oil in both kinetic and static experiments. It is interesting to observe that the removal efficiency was positively correlated with the concentration of Triton X-

100 under static conditions while the agitation allowed no difference between the rate of removal under kinetic experiments. Tween 80 on the other hand, removed practically half the oil removed when applied in the sand packed column when compared to the experiments under kinetic conditions. The increase in concentration of the surfactant did not improve the oil removal rates, as observed under the kinetic assays.

In general, biosurfactants exhibit more ability to remove hydrophobic contaminants under static conditions than chemical surfactants, although results may vary depending on the type of surfactant, its concentration and the kind of soil, which can potentialize the interaction with the surfactant more than the interaction between surfactant and oil.

Microbially produced biosurfactants were studied to enhance crude oil desorption and mobilization in model soil column systems. The ability of biosurfactants from *Rhodococcus ruber* to remove the oil from the soil core was 1.4-2.3 times greater than that of a synthetic surfactant of suitable properties, Tween 60. Biosurfactant was less adsorbed to soil components than synthetic surfactant, thus rapidly penetrating through the soil column and effectively removing 65%-82% of crude oil (Kuyukina et al., 2005).

Souza Sobrinho et al. (2013) observed removals around 75% and 92% depending on the soil type with the crude biosurfactant from *C. sphaerica* cultivated in industrial residues, while percentages removal between 30% and 50% were obtained for the isolated biosurfactant in the soils contained in

packed columns. The synthetic surfactant Tween 20 and the distilled water removed around 20% of the oil in the soils tested.

The washing process of a soil column by the ionic surfactant sodium dodecyl sulfate (SDS) was investigated. The effect of SDS was significant beyond a concentration of 8mM. The soil washing process had removed 97% of the diesel fuel (Khalladi et al., 2009).

Like the cell-free broth from *C. sphaerica*, the culture broth from *Rhodococcus* sp. strain TA6 grown on sucrose was effective in recovering up to 70% of the residual oil from oil-saturated sand packeds. Comparison of the results (SDS 0%, spolene 63% and petroleum sulfunato 58%) with residual oil recovery obtained by TA6 culture broth indicated the potential value of the biosurfactant for MEOR (Shavandi et al., 2011).

Jain et al. (2012) investigated the potential use of two biosurfactants in removing oil in glass columns compared to synthetic surfactants. The results showed the efficiency of biosurfactants produced by *B. subtilis* PT2 and *P. aeruginosa* SP4 in removing oil. They exhibited values of 68% and 57%, respectively, compared to the synthetic surfactants Tween 80 (52%), SDBS (51%) and Alfoterra 5PO-145 (55%).

Bai et al. (1997) investigated the potential of an anionic rhamnolipid isolated from *P.aeruginosa* for the removal of hydrocarbons adsorbed to soil in packeded columns. The biosurfactant was able to remove 84% of hexadecane absorbed to sand with particles measuring 0.6–0.85 mm (mesh20/30), where as

a 22% removal rate was found for sand particles measuring 0.3–0.42 mm (mesh40/50). The removal capacity of the rhamnolipid using 40/50 meshes was compared with that of two synthetic surfactants: the anionic sodium dodecylsulfate (SDS) (CMC 2360mg/l) and the non-ionic Tween 80 (CMC 13 mg/l). SDS (472mg/l) and Tween80 (51mg/l) removed 0% and 6% of the hexadecane, respectively.

3.3. Motor oil biodegradation

Five different sets were used to study motor oil biodegradation. The results were recorded on 15, 30, 45, 60, 75 and 90th day for each set as shown in Fig. 5.

The addition of molasses provided required nutrients for enhanced biodegradation of the petroleum derivate. Molasses is a co-product of sugar production, both from sugar cane as well as from sugar beet industry in Brazil. Molasses is rich in carbon, organic nitrogen and mineral compounds required for growth of microorganisms. Therefore, molasses was added to the mixtures of contaminated sand along the experiments.

In the first set of experiment (Contaminated sand + sugar cane molasses + *C. sphaerica*), the oil degradation reached 50% after 90 days. The same percentage was obtained in the presence of the biosurfactant (Set 4), which accelerated the oil degradation during the first 75 days. On the other hand, the percentage of degradation in the second Set was much higher, reaching almost

100% in the presence of *B. sp.* cells. The presence of the biosurfactant produced by *B. sp.* also accelerated the degradation process in the first 45 days of the experiment (Set 5), i.e., the biosurfactant increased the degradation rate in 10%, indicating that biosurfactant acted as an efficient enhancer for hydrocarbon biodegradation. It may be due to i) increase in the surface area of hydrophobic water-insoluble substrates and ii) increase in the bioavailability of hydrophobic compounds (Jadhav et al., 2013). The presence of both microorganisms, namely yeast and bacterium used together (Set 3) was not efficient in the degradation of the oil, which did not exceed 50 %. As described by Luna et al. (2011), the biosurfactant from *C. sphaerica* expressed antimicrobial properties against a variety of microorganisms, suggesting the possible inhibition of the growth of *B. sp.* by the biosurfactant produced by the yeast. Degradation was not observed in the control set of experiment (Contaminated sand + sugar cane molasses).

Variable results have been shown concerning the utility of using biosurfactants in hydrocarbon solubilization and biodegradation (Bordas et al. 2007; Chang et al., 2004). According to Zhang et al., (2000), the solubilizing capacity of a specific surfactant is determined only by its intrinsic micelles property and thus enhancing its solubilizing capacity is usually very difficult. Therefore, continuing efforts have been made to search for new surfactants or biosurfactants with much higher solubilizing efficacy, lower cost and low microbial toxicity.

Oberbremer et al. (1990) used a mixed soil population to assess hydrocarbon degradation in a model oil system. They reported a statistically significant enhancement in hydrocarbon degradation when sophorose lipids were added to the system containing 10% soil and a 1.35% hydrocarbon mixture in the mineral salt medium. In the absence of surfactant, 81% of the hydrocarbon mixture was degraded within 114 h, while in the presence of biosurfactant up to 90% of the hydrocarbon mixture was degraded within 79 h.

The biosurfactant from *Oceanobacillus* sp. BRI 10 was tested in crude oil biodegradation experiments. The percent degradation reached 63% in the first set of experiment (basal salt medium + crude oil + bacterial cells) on the 27th day. On the other hand, it was around 90% in the second set of experiment (basal salt medium + crude oil + bacterial cells + biosurfactant) (Jadhav et al., 2013).

Two biosurfactants, surfactin and rhamnolipid were applied for enhanced biodegradation of diesel contaminated water and soil with a series of bench-scale experiments. The addition of surfactin near its CMC increased diesel biodegradation percentage (94%), compared to batch experiments with no surfactin addition (40% biodegradation percentage). Addition of surfactin more than 40 mg/L, however, decreased diesel biodegradation efficiency. Addition of rhamnolipid to diesel/water systems from 0 to 80 mg/L (CMC at 50 mg/L) substantially increased diesel biodegradation percentage from 40 to 100%, respectively. Rhamnolipid addition at a concentration of 160 mg/L provided similar results to those of an 80 mg/L addition (Whang et al., 2008).

The effects of the addition of the biosurfactant from *P. cepacia* alone and with cells of the bacterium in the biodegradation process of HOCs adsorbed to soil was studied during 60 days. Results indicated the efficiency of both the biosurfactant and its producing species in degrading high percentages of the HOCs adsorbed to the soil samples (Silva et al., 2014).

Youssef et al. (2013) described the injection of a glucose-nitrate-mineral nutrient mixture and two lipopeptide biosurfactant producing *Bacillus* strains into two wells to correlate in-situ metabolism with oil recovery. Analysis of production water indicated in-situ growth of the injected strains and other heterotrophic fermenting bacteria, metabolism of the nutrients, and biosurfactant production.

Most studies describe the use of bacteria in the degradation of HOCs although the efficiency of yeast has also been demonstrated. The efficacy of *Candida catenulata* CM1 on petroleum hydrocarbon degradation was evaluated during composting of a mixture containing 23% food waste and 77% diesel contaminated soil including 2% (w/w) diesel. After 13 days of composting, 84% of the initial petroleum hydrocarbon was degraded (Joo et al., 2008).

4. Conclusions

It could be observed in the present study that the two biosurfactants were more effective than the commercially available surfactants tested. The cell free broth containing biosurfactants and the isolated biosurfactants are almost

equally effective in the removal of the oil pollutant. Thus, cell free broth containing biosurfactants can be directly used without purification steps, which would further reduce the cost of production of the biosurfactants. The biosurfactant produced by *Candida sphaerica* could be applied in enhanced oil recovery operations, while the biosurfactant produced by *Bacillus* sp. should more suited for enhanced biodegradation of petroleum derivatives in soil systems.

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FIGURES LEGENDS

Fig.1. Removal of motor oil adsorbed to sand through kinetic assay by the biosurfactant from *Candida sphaerica*. Error bars show the corresponding standard error.

Fig.2. Removal of motor oil adsorbed to sand through kinetic assay by the biosurfactant from *Bacillus* sp. Error bars show the corresponding standard error.

Fig.3. Removal of motor oil adsorbed to sand through kinetic assay by the synthetic surfactant TritonX-100. Error bars show the corresponding standard error.

Fig.4. Removal of motor oil adsorbed to sand through kinetic assay by the synthetic surfactant Tween 80. Error bars show the corresponding standard error.

Fig. 5. Biodegradation of motor oil. Set 1 - Contaminated sand + sugar cane molasses + *C. sphaerica*; Set 2 - Contaminated sand + sugar cane molasses + *Bacillus* sp.; Set 3 - Contaminated sand + sugar cane molasses + *C. sphaerica* + *Bacillus* sp.; Set 4 - Contaminated sand + sugar cane molasses + *C. sphaerica* biosurfactant + *C. sphaerica*; Set 5 - Contaminated sand + sugar cane molasses + *Bacillus* sp. biosurfactant + *Bacillus* sp. Error bars show the corresponding standard error.

Table 1

Formulated mixtures for motor oil biodegradation experiments in sand

Experiment	Composition
Set 1	Contaminated sand + sugar cane molasses + <i>C. sphaerica</i>
Set 2	Contaminated sand + sugar cane molasses + <i>Bacillus</i> sp.
Set 3	Contaminated sand + sugar cane molasses + <i>C. sphaerica</i> + <i>Bacillus</i> sp.
Set 4	Contaminated sand + sugar cane molasses + <i>C. sphaerica</i> biosurfactant + <i>C. sphaerica</i>
Set 5	Contaminated sand + sugar cane molasses + <i>Bacillus</i> sp. biosurfactant + <i>Bacillus</i> sp.
Control	Contaminated sand + sugar cane molasses

Table 2 Removal of motor oil adsorbed to sand in packedded columns (static assay) by the biosurfactants produced by *C. sphaerica* and *Bacillus* sp. and by the chemical surfactants Tween 80 and Triton X-100

Removal of motor oil by percolating liquids (%)				
Surfactants	Crude biosurfactant	Surfactant (1/2 CMC)	Surfactant (CMC)	Surfactant (2 x CMC)
Produced by				
<i>Candida</i>				
<i>sphaerica</i>	93±3.9	87±3.2	92±2.7	91±2.8
Produced by				
<i>Bacillus</i> sp.	43±3.0	15±2.1	30±1.9	40±2.5
Tween 80	-	45±2.1	45±2.0	40±1.8
Triton X-100	-	60±1.0	70±2.1	80±1.5
Distilled water				
(control)	6±1.0	-	-	-

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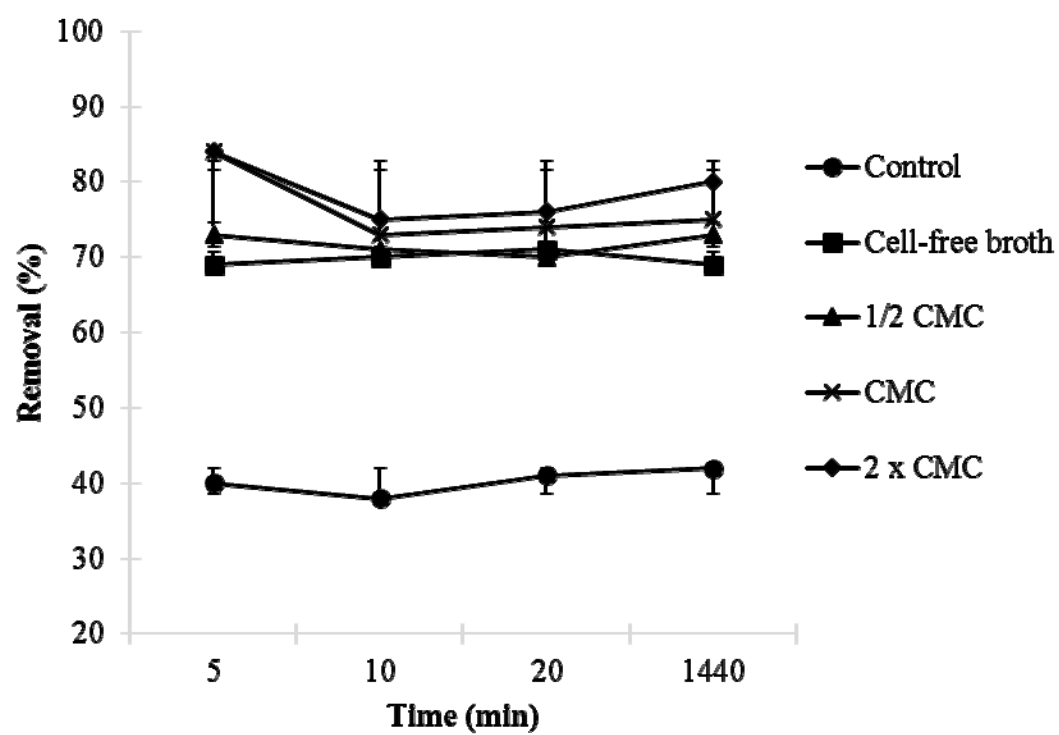


Fig.1.

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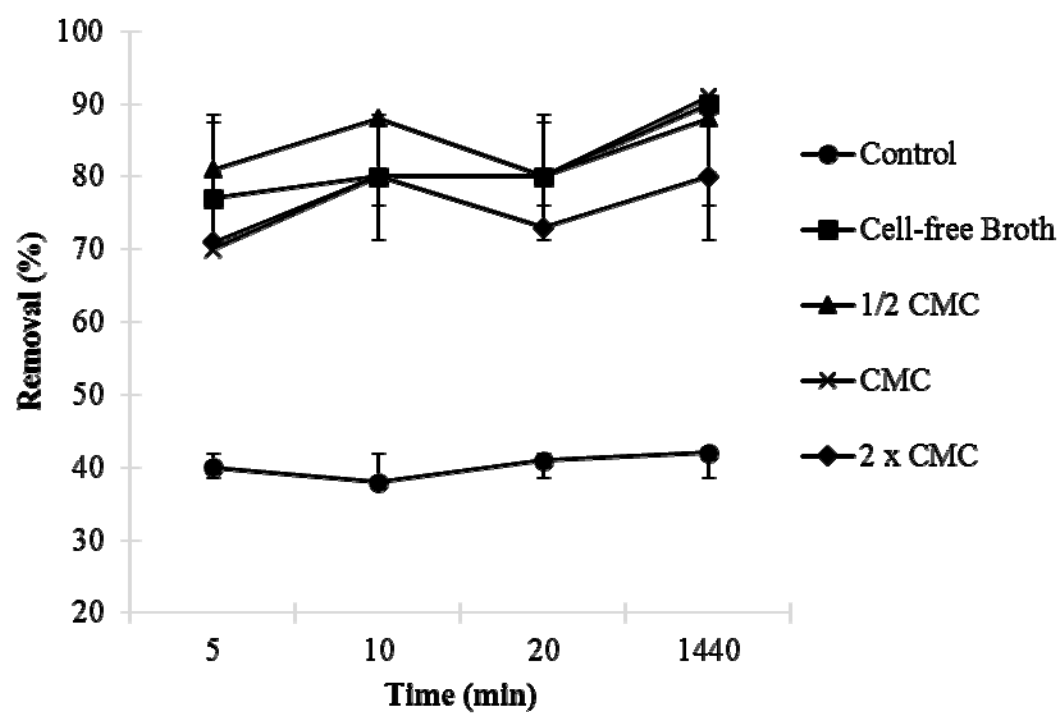


Fig.2.

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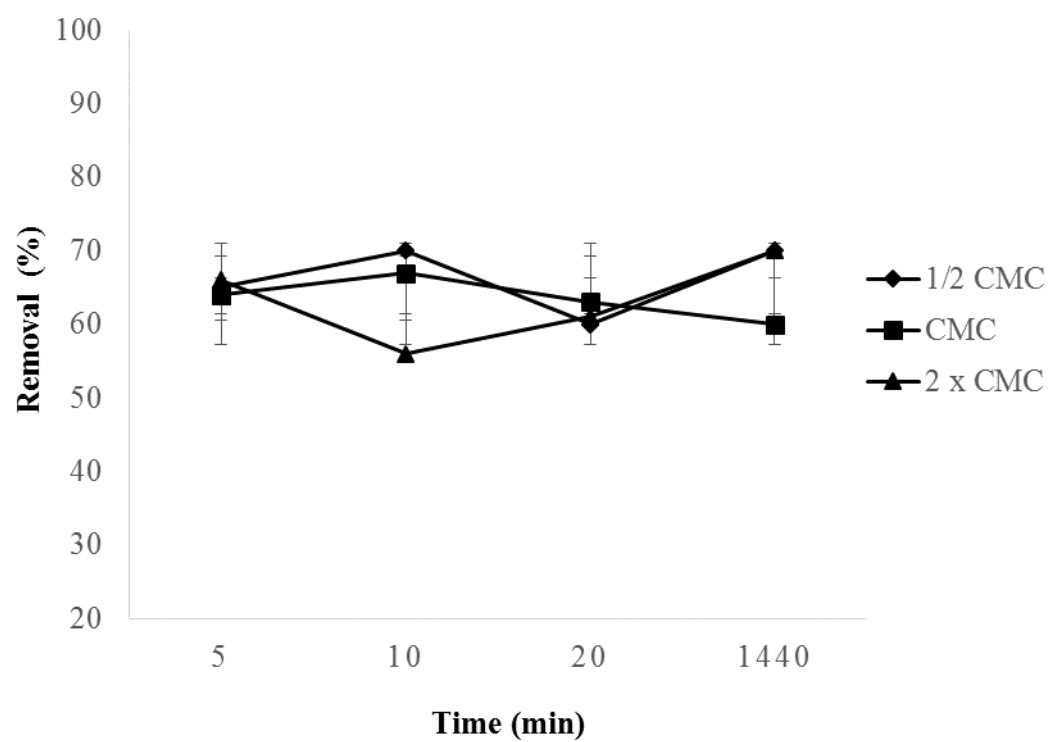


Fig.3.

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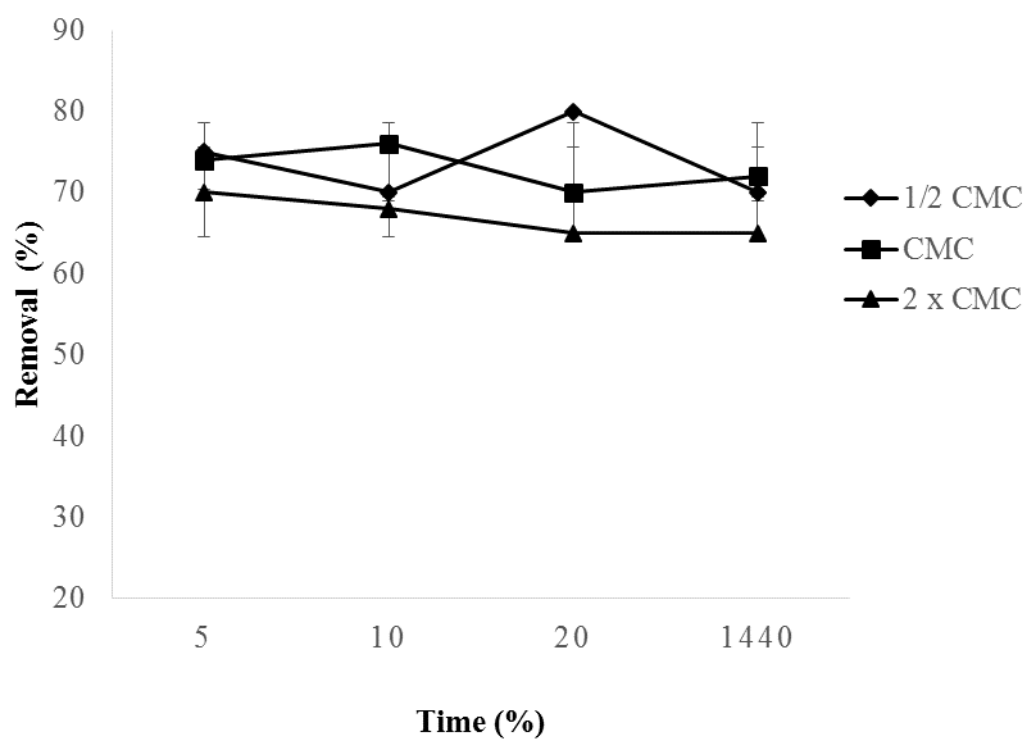


Fig.4.

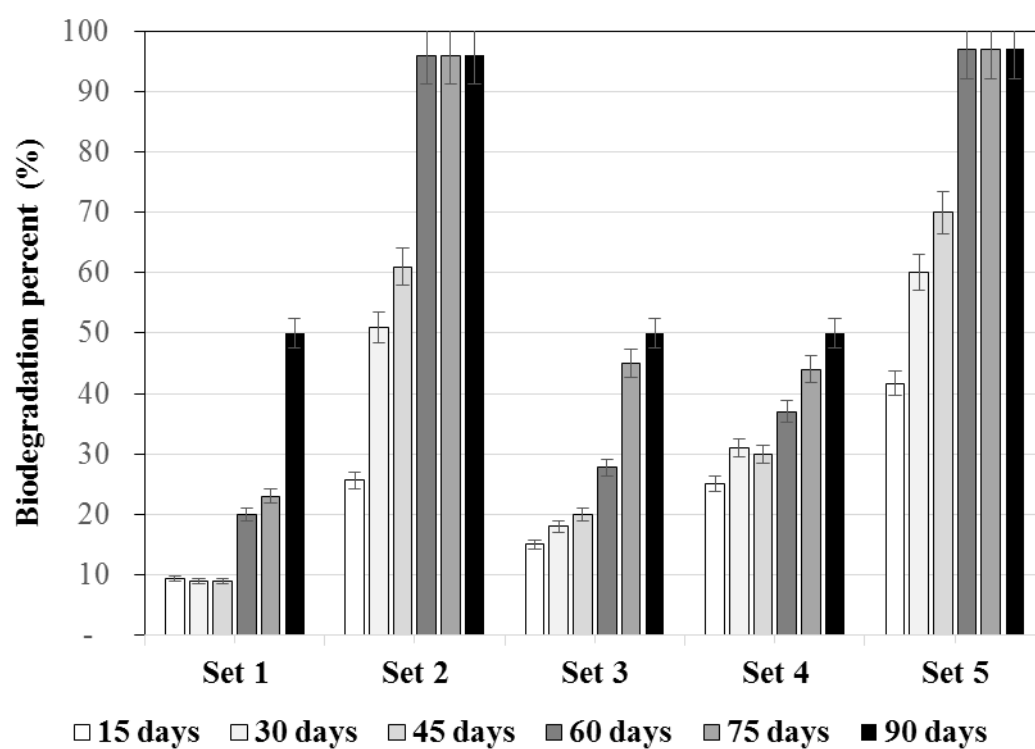


Fig.5.

HIGHLIGHTS

1. The ability of motor oil remediation by two biosurfactants in sand was evaluated.
2. The biosurfactants removed 70-90% of motor oil under kinetic conditions.
3. The biosurfactant from *C. sphaerica* removed 90% of motor oil from pack columns.
4. Oil degradation reached 97% in the presence of *Bacillus* sp. and its biosurfactant.
5. The biosurfactants acted as enhancers for motor oil biodegradation in soil.

CAPÍTULO 3

CONCLUSÕES GERAIS

Os estudos realizados nessa pesquisa permitem as seguintes conclusões:

- Os micro-organismos *C. spahaerica* e *Bacillus* sp. apresentam potencial como produtores de compostos com atividade surfactante.
- Ambos os biossurfactantes, nas formas bruta e isolada, mostraram eficiência na remoção do óleo de motor da areia contaminada em condições cinéticas.
- Os surfactantes sintéticos Tween 80 e Triton X-100 apresentaram eficiência inferior aos biossurfactantes sob condições cinéticas.
- Os biossurfactantes isolados foram eficientes em concentrações reduzidas.
- Os biossurfactantes e os surfactantes sintéticos apresentaram uma rápida eficiência de remoção do poluente testado sob condições cinéticas.
- O biossurfactante bruto e isolado de *C. sphaerica* foi eficaz sob condições estáticas, sugerindo-se seu uso na recuperação avançada de petróleo.
- A presença de ambos os biossurfactantes acelerou a taxa de degradação dos hidrocarbonetos do óleo de motor adsorvido na areia.
- O *Bacillus* sp. foi capaz de aumentar a degradação do poluente em solo.

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- Os biossurfactantes testados são promissores como agentes de remediação, podendo agir não só na remoção de óleos como também no aumento da capacidade biodegradação de poluentes hidrofóbicos em solos.

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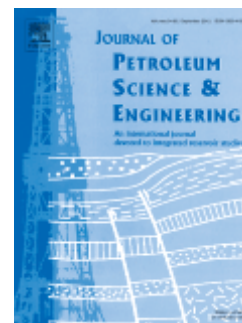
ANEXOS

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