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RESÍDUOS INDUSTRIAIS COMO SUBSTRATOS ALTERNATIVOS PARA A PRODUÇÃO DE BIOSSURFACTANTES PARA APLICAÇÃO NA REMOÇÃO DE POLUENTES AMBIENTAIS GERADOS PELA INDÚSTRIA DE PETRÓLEO

Recife
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"Não sabendo que era impossível, foi lá e fez."
"Jean Cocteau"

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RESUMO

Os surfactantes são poderosos agentes anfipáticos com aplicação em vários segmentos industriais, como as indústrias petrolífera, alimentícia, e farmacêutica, entre outras. Muitos biossurfactantes têm sido produzidos, embora poucos sejam comercializados em virtude do alto custo de produção e principalmente no que se refere à utilização de substratos e aos processos de purificação. Neste sentido, a utilização dos resíduos industriais, milhocina e óleo de canola residual de fritura, como substratos alternativos para a produção de um biossurfactante por *Pseudomonas cepacia* CCT6659 foi avaliada com vistas à aplicação na área ambiental, As concentrações dos constituintes do meio foram estudadas utilizando um Delineamento Composto Central Rotacional (DCCR) como ferramenta estatística na redução da tensão superficial. O biossurfactante produzido no meio constituído por milhocina 3%, óleo de fritura (canola) 2% e $NaNO_3$ 0,2% reduziu a tensão superficial para 26 mN/m após 60 horas sob agitação de 250 rpm. No estudo da cinética observou-se que a produção do biossurfactante estava associada ao crescimento celular. O rendimento em biossurfactante isolado foi de 8,0 g/L. As propriedades do biossurfactante isolado foram investigadas e sua CMC calculada. A caracterização bioquímica preliminar revelou a natureza glicolipídica e aniônica do biossurfactante. O biossurfactante foi capaz de emulsificar 90% do óleo de motor e demonstrou estabilidade durante exposição a altas temperaturas (120ºC por 15 minutos), altas concentrações salinas (12% NaCl) e em ampla faixa de pH (2-12). Testes realizados com o biossurfactante bruto demonstraram ausência de toxicidade frente ao microcrustáceo *Artemia salina* e a duas espécies de repolho *Brassica oleracea*. O biossurfactante bruto também foi eficiente na remoção de 75% do óleo de motor adsorvido em amostras de areia e na dispersão do óleo 65%, além de ser capaz de remover 90% do óleo aderido à superfície sólida. Os resultados obtidos demonstram o potencial do biossurfactante para aplicação na indústria de petróleo, recuperação de óleo, limpeza de tanques de estocagem e na remediação de derramamentos de óleos em solos e água.

Palavras-chave: biossurfactante, *Pseudomonas cepacia*, resíduos industriais.

ABSTRACT

Surfactants are amphipathic powerful agents with application in various industries like oil industries, food, and pharmaceutical industries, among others. Several biosurfactants have been produced, but few are marketed due to the high cost of production and especially in relation to use of substrates and purification processes. In this sense, the use of industrial waste, corn steep liquor and residual canola oil frying, as alternative substrates for the production of a Pseudomonas cepacia CCT6659 biosurfactant was assessed in order to apply the environmental area, concentrations of constituents in the medium were studied using one central composite rotational design (DCCR) as a statistical tool to reduce surface tension. The biosurfactant produced in medium consisting of 3% corn steep liquor, cooking oil (canola) 2% NaNO3, and 0.2% reduced the surface tension to 26 mN / m after 60 hours under agitation of 250 rpm. In the study of the kinetics observed that the production of the biosurfactant was associated with cellular growth. The isolated biosurfactant yield was 8.0 g / L. The properties of the isolated biosurfactant were investigated and their CMC calculated. A preliminary biochemical characterization revealed the nature of the anion and glicolipídica biosurfactant. The biosurfactant was able to emulsify 90% engine oil and demonstrated stability during exposure to high temperatures (120 ° C for 15 minutes), high salt concentrations (12% NaCl) and in a wide pH range (2-12). Tests performed with crude biosurfactant showed no toxicity against the brine shrimp Artemia salina and two species of cabbage Brassica oleracea. The crude biosurfactant was also efficient in the removal of 75% of motor oil adsorbed on samples of sand and 65% oil dispersion, besides being able to remove 90% of the oil adhered to the solid surface. The results demonstrate the potential of biosurfactant for application in the oil industry, oil recovery, cleaning storage tanks and remediation of oil spills on land and water.

keywords: biosurfactant, *Pseudomonas cepacia*, industrial waste, oil pollution.

CAPÍTULO 1

1. INTRODUÇÃO

As refinarias de petróleo, assim como outros processos industriais de grande escala, são fontes potenciais de poluição ambiental. O derramamento de hidrocarbonetos provoca a contaminação ambiental, gerando conseqüências desastrosas para os organismos vivos (RAHMAN; GAKPE, 2008) Estima-se que 0,08%-0,4% da produção mundial de petróleo alcancem, eventualmente, os oceanos. Em 1998, 1200m^3 de óleo combustível foram derramados devido à corrosão de um oleoduto na cidade de Cubatão, no Estado de São Paulo. A corrosão de oleodutos também foi responsável pelo derramamento de 1300 m^3 de óleo combustível na Baía de Guanabara, que já havia sido contaminada anteriormente por outros derramamentos de petróleo (BENINCASA, 2007). Podemos citar também o acidente ocorrido no Golfo do México em 20 de abril de 2010, onde um incêndio na plataforma “Deepwater Horizon” ocasionou um vazamento de cerca de um milhão de litros de petróleo por dia durante 85 dias fazendo deste o maior acidente ambiental da história dos Estados Unidos. Para o controle do vazamento foram utilizadas mais de duas mil pessoas na tentativa de retirar o máximo possível do petróleo derramado (ESMERALDO, 2010).

No Brasil, os acidentes com derrames de petróleo e seus derivados passaram a ser mais freqüentes e tem causado sérios problemas ambientas, devido ao aumento da produção, à falta de manutenção preventiva das tubulações e à redução do quadro de profissionais especializados (ROCHA, 2010).

Acidentes como os citados acima têm intensificado o desenvolvimento de procedimentos e técnicas de combate à poluição ambiental por derivados de petróleo. Os solos contaminados por petróleo e derivados normalmente são tratados através de metodologias físicas, químicas ou biológicas. Entretanto, as novas diretrizes de recuperação de solos e águas têm restringido o uso de produtos químicos. Dentre as técnicas de remediação disponíveis, a biorremediação tem se destacado, embora a solubilidade reduzida dos hidrocarbonetos dificulte o acesso dos micro-organismos e a consequente biodegradação do poluente uma vez que esses compostos hidrofóbicos se ligam

às partículas do solo (MUKHERJEE et al., 2006). Nesse contexto, a utilização de compostos surfactantes surge como a tecnologia mais investigada para a resolução desse problema, permitindo a dessorção e consequente solubilização dos hidrocarbonetos, facilitando, assim, a assimilação desses compostos pelas células microbianas (CORTIS; GHEZZEHEI, 2007; LUNA et al., 2008; COIMBRA et al., 2009).

Os surfactantes são moléculas anfipáticas contendo porções hidrofílicas e hidrofóbicas que tendem a se localizar preferencialmente na interface entre fases fluidas com diferentes graus de polaridade e pontes de hidrogênio, como interfaces óleo-água ou ar-água. Estas propriedades permitem reduzir a tensão superficial e interfacial e formar microemulsões onde os hidrocarbonetos possam se solubilizar em água ou vice-versa (CALVO et al., 2008). A grande maioria dos surfactantes hoje disponível é sintetizada a partir de derivados de petróleo. Entretanto, as novas legislações de proteção ao meio ambiente, bem como a preocupação ambiental entre os consumidores, têm levado à procura por surfactantes naturais como alternativas aos produtos existentes. Nesse sentido, os biossurfactantes, metabólitos de bactérias, leveduras e fungos filamentosos ganham destaque e podem ser produzidos a partir de óleos, alcanos e resíduos industriais oleosos, formando estruturas como glicolipídeos, lipopeptídeos, fosforolipídeos, ácidos graxos ou heteropolímeros (CORTIS; GHEZZEHEI, 2007).

Há um crescente interesse industrial por surfactantes de origem microbiana, estudos realizados estimam que os biossurfactantes possam capturar em torno 10% do mercado dos surfactantes comerciais até 2010, o que representa um investimento de U$ 200 milhões (RUFINO et al., 2008). O maior mercado para os biossurfactantes é na indústria petrolífera, onde podem ser amplamente utilizados na recuperação avançada de petróleo (MEOR – microbial enhanced oil recovery), na remoção e mobilização de resíduos oleosos reduzindo os efeitos dos poluentes e na tecnologia da biorremediação (CALVO et al., 2008; PACWA-PLOCINICZAK et al., 2011).

A utilização de resíduos pode diminuir os custos de produção para níveis competitivos em relação aos similares obtidos por via petroquímica e, ao mesmo

tempo, reduzir os problemas ambientais relativos ao descarte e aos custos do tratamento. Considerando que o Brasil é um país essencialmente agrícola, a facilidade de acesso aos subprodutos e resíduos agroindustriais é bastante significativa, motivando as pesquisas nessa área. Neste contexto o resultado desse presente trabalho considerou a habilidade e o potencial de produção da *P. cepacia* (CCT6659) em produzir surfactantes atóxicos e biodegradáveis a partir de substratos alternativos de baixo custo para aplicação na despoluição de solos contaminados por derivados de petróleo, colaborando para o desenvolvimento tecnológico e sustentável da Região Nordeste e, em especial, do Estado de Pernambuco.

2. OBJETIVOS

2.1 Objetivo geral

Produzir um biossurfactante de baixo custo com potencial de aplicação na área ambiental.

2.2 Objetivos específicos

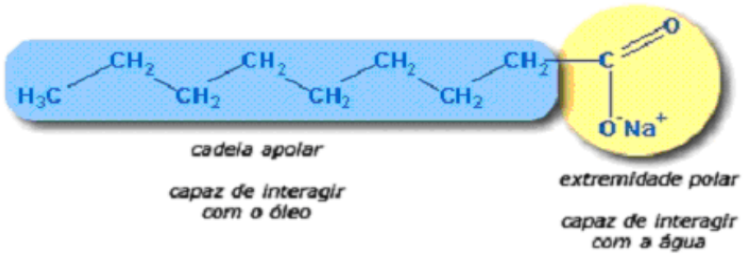
- Avaliar a *Pseudomonas cepacia* (CCT6659) como produtora de biossurfactante;
- Utilizar diferentes resíduos industriais como substratos para a produção do biossurfactante;
- Utilizar um Delineamento Composto Central Rotacional (DCCR) como ferramenta estatística para avaliar a influência das concentrações dos constituintes do meio na redução da tensão superficial;
- Descrever a cinética de crescimento do micro-organismo e da produção do biossurfactante a partir das condições de cultivo e do meio previamente estabelecidos;
- Isolar o biossurfactante e determinar o rendimento de produção;
- Determinar a Concentração Micelar Crítica do biossurfactante;
- Determinar a capacidade de emulsificação de diferentes hidrocarbonetos e óleos pelo biossurfactante;
- Caracterizar o biossurfactante quanto à estabilidade frente a condições específicas de temperatura, pH e presença de sal;
- Caracterizar o biossurfactante quanto à natureza iônica e composição bioquímica;
- Determinar a toxicidade do biossurfactante frente a um microcrustáceo e a sementes de vegetais;
- Avaliar o potencial de aplicação do biossurfactante na remoção de poluente hidrofóbico adsorvido em solos através de cultivo submerso;

- Avaliar a capacidade do biossurfactante em dispersar manchas de óleo em meio líquido.

3 . REVISÃO DE LITERATURA

3.1 Surfactantes

Os surfactantes são compostos anfipáticos contendo porções hidrofílicas e hidrofóbicas que se particionam, preferencialmente, na interface entre fases fluidas com diferentes graus de polaridade e pontes de hidrogênio, como interfaces óleo/água ou ar/água. A porção apolar é freqüentemente uma cadeia hidrocarbonada enquanto a porção polar pode ser iônica (catiônica ou aniônica), não-iônica ou anfotérica (SINGH et al., 2007; CALVO et al., 2009). Estas características permitem aos surfactantes reduzir a tensão superficial e interfacial e formar microemulsões onde os hidrocarbonetos possam se solubilizar em água ou onde a água possa se solubilizar em hidrocarbonetos (RON; ROSENBERG, 2002). Tais propriedades possibilitam uma ampla gama de aplicações industriais envolvendo detergência, emulsificação, lubrificação, capacidade espumante, capacidade molhante, solubilização e dispersão de fases. Contudo, nos últimos anos, a crescente preocupação com o controle e a preservação ambiental levou à procura por surfactantes naturais como alternativa à substituição dos produtos já existentes (BARROS et al., 2007; NITSCHKE; PASTORE, 2002; PERNA, 2010). A figura 1 representa a estrutura química de um monômero de surfactante.

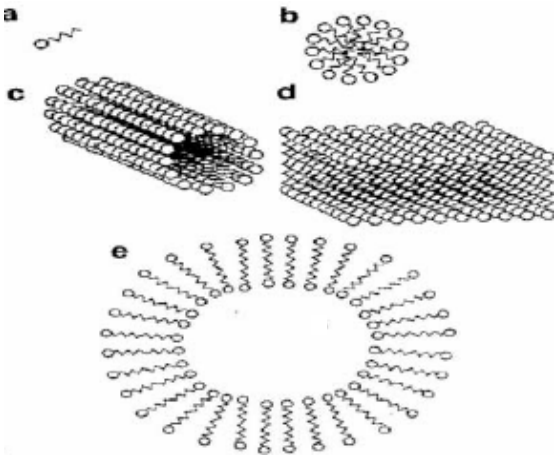


Fonte: http://www.virtuallaboratory.ne

Figura 1 – Estrutura Química representativa de um monômero surfactante

A tensão superficial é a força de atração existente entre as moléculas dos líquidos. Essa propriedade das substâncias diminui quando a concentração de surfactante no meio aquoso aumenta, ocorrendo a formação de micelas que são

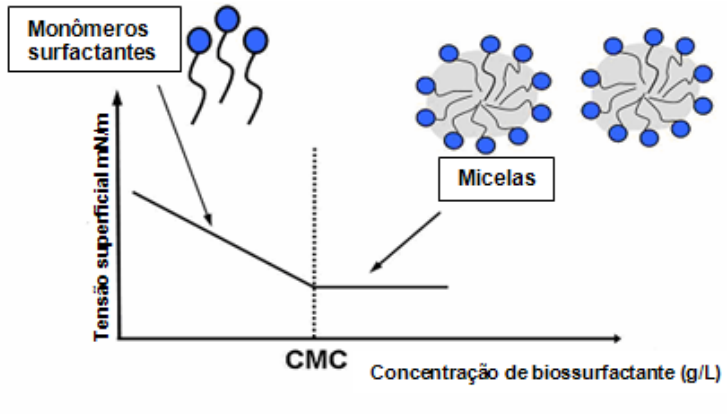
moléculas anfipáticas agregadas com as porções hidrofílicas posicionadas para a parte externa da molécula e as porções hidrofóbicas para a parte interna (CORTIS; GHEZZEHEI, 2007). A concentração dessas micelas forma a Concentração Micelar Crítica (CMC). Esta concentração corresponde à mínima concentração de surfactante necessária para que a tensão superficial seja reduzida ao máximo. Quando a CMC é atingida, várias micelas são formadas (VAN-HAMME et al., 2006). Figura 2 representa a formação de micelas de surfactantes.



Fonte: (LUNA, 2006)

Figura 2 – Surfactantes são caracterizados por uma estrutura anfipática. As propriedades hidrofóbicas e hidrofílicas dependem da carga do grupo polar (aniônico, catiônico, neutro ou anfotérico). (a) monômero surfactante, denotado por um círculo representando a cabeça hidrofílica ligada à cadeia de hidrocarboneto; (b) micela circular; (c) micela cilíndrica; (d) camada micelar; e (e) representação de uma vesícula (MAKKAR; CAMEOTRA, 2002; LUNA, 2006).

Eficiência e 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). A figura 3 representa a CMC em relação à concentração de biossurfactante.



Fonte: Whang, 2008.

Figura 3 – Formação de micelas na CMC

3.2 Biossurfactante

Vários compostos com propriedades tensoativas são sintetizados por organismos vivos, a partir de plantas (saponinas), microorganismos (glicolípidos) e por seres humanos (surfactante pulmonar), sendo considerados surfactantes naturais. Além disso, estes compostos têm sido produzidos através de processos biotecnológicos alargando sua diversidade e potenciais aplicações (LUNA et al., 2011).

A grande maioria dos surfactantes disponíveis comercialmente é sintetizada a partir de derivados de petróleo. No entanto, a preocupação ambiental entre os consumidores, combinada a novas legislações de controle do meio ambiente têm reforçado à procura por surfactantes naturais como alternativa aos produtos existentes (NITSCHKE; PASTORE, 2002; SILVA, 2009).

Vários compostos com propriedades tensoativas são sintetizados por organismos vivos, desde plantas (saponinas) até micro-organismos (glicolipídios) e também no organismo humano (sais biliares), sendo considerados surfactantes naturais (MANEERAT, 2005). Estes 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). Os biossurfactantes microbianos formam micro-emulsões onde

ocorre a formação de micelas capazes de solubilizar hidrocarbonetos em água ou água pode solubilizar em hidrocarbonetos (LUNA et al., 2012).

O uso eficaz de surfactantes sintéticos ou biológicos para melhorar a biodisponibilidade de contaminantes hidrofóbicos exige a otimização do surfactante/micro-organismos/ambiente alvo conjunto(s) e uma melhor compreensão do complexo de interações entre esses (ABOUSEOUD et al., 2010).

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 desses (VAN-HAMME et al., 2006). A possibilidade de produção dos biossurfactantes a partir de substratos renováveis e da utilização 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 competitividade com seus similares petroquímicos (CANET et al., 2002).

O principal fator que restringe o uso de biossurfactantes no mercado é seu custo de produção quando comparado com seus similares sintéticos. O sucesso para o desenvolvimento industrial da produção dos biossurfactantes depende do uso alternativo de substratos de baixo custo que não ultrapassem 10 – 30% do custo total, tais como resíduos agroindustriais, é uma importante estratégia para reduzir os custos associados ao processo (SARUBBO et al., 2007; RUFINO et al., 2008; COSTA, 2010).

3.2.1 Classificação

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 (RON; ROSENBERG, 2001; MANEERAT, 2005). 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 carboidrato, aminoácido, peptídeo cíclico, fosfato, um ácido carboxílico ou um álcool (BOGNOLO,1999; SOUZA-SOBRINHO, 2007). Os biossurfactantes são classificados bioquimicamente de acordo com a espécie microbiana produtora e com a utilização de hidrocarbonetos pela comunidade microbiana. Quanto à estrutura, podem ser classificados em cinco grandes grupos (SOUZA-SOBRINHO, 2007; RAHMAN; GAKPE, 2008; LUNA et al., 2012):

- Glicolipídeos, cujo grau de polaridade depende dos hidrocarbonetos utilizados como substratos;
- 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 *Acinotobacter calcoaceticus;*
- Lipopeptídeos, como a surfactina, produzida por *Bacillus subtilis*, um dos biossurfactantes mais efetivos já relatados na literatura;
- Fosfolipídeos, estruturas comuns a muitos micro-organismos, como o biossurfactante de *Corynebacterium lepus;*
- Ácidos graxos, lipídeos neutros (alguns classificados como glicolipídeos) e proteínas hidrofóbicas.

3.2.2 Micro-organismos produtores

Uma grande variedade de micro-organismos, incluindo bactérias, leveduras e fungos filamentosos quando crescem em diferentes substratos, variando desde carboidratos até hidrocarbonetos.é capaz de produzir biossurfactantes com diferentes estruturas moleculares (DELEU; PAQUOT, 2004). Estas mudanças podem ser benéficas quando se deseja propriedades específicas para uma aplicação direcionada (COOPER, 1987). Por evolução, as bactérias adaptaram-se à alimentação de substratos hidrofóbicos pela produção e utilização de um produto de superfície ativa que ajuda esses organismos para absorver,

emulsificar, molhar, dispersar ou solubilizar o material líquido (ABOUSEOUD et al., 2010). Diversos são os estudos realizados por vários autores (GUSMÃO et al., 2010; SILVA et al., 2010; SOBRINHO et al., 2008; LUNA et al., 2009; LUNA et al., 2008; SARUBBO et al., 2006) na produção de biossurfactantes, envolvendo propriedades físico-químicas. A Tabela 1 resume as principais classes de biossurfactantes e os respectivos micro-organismos produtores descritos na literatura.

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

Classe/Tipo de Biossurfactante	**Micro-organismo**
Glicolipídeos	
- raminolipídeos	*Pseudomonas aeruginosa*
- soforolipideos	*Torulopsis bombicola, T. apicola*
- trealolipídeos	*Rhodococcus erythropolis, Mycobacterium sp.*
Lipopeptídeos e lipoproteínas	
- Peptídeo-lipídeo	*Bacillus licheniformis*
- Viscosina	*Pseudomonas fluorescens*
- Serrawetina	*Serratia marcenscens*
- Surfactina	*Bacillus subtilis*
- Subtilisina	*Bacillus subtilis*
- Gramicidina	*Bacillus brevis*
- Polimixina	*Bacillus polymyxia*
Ácidos graxos, lipídeos neutros e fosfolipídeos	
- Ácidos graxos	*Corynebacterium lepus*
- Lipídeos neutros	*Nocardia erythropolis*
- Fosfolipídeos	*Thiobacillus thiooxidans*
Surfactantes poliméricos	
- emulsan	*Acinetobacter calcoaceticus*
- biodispersan	*Acinetobacter calcoaceticus*
- liposan	*Candida lipolytica*
- carboidrato-lipídeo-proteína	*Pseudomonas fluorescens*
- manana-lipídeo-proteína	*Candida tropicalis*
Surfactantes particulados	
- vesículas	*Acinetobacter calcoaceticus*
- células	Várias bactérias

Fonte: MUTHUSAMY et al., 2008.

As bactérias dos gêneros *Pseudomonas* e *Bacillus* são descritas na literatura como grandes produtoras de biossurfactantes. O gênero *Pseudomonas* é capaz de usar diferentes substratos, tais como glicerol, manitol, frutose, glicose,

n-parafinas e óleos vegetais, para a produção de biossurfactantes do tipo raminolipidio (ABOUSEOUD et al., 2008).

Os raminolipídeos produzidos por *Pseudomonas aeruginosa* (GUERRA-SANTOS et al., 1984) têm sido extensivamente estudados (ROBERT et al., 1989; SILVA, 2009). A composição e os rendimentos dependem do tipo do fermentador, do pH, da composição dos nutrientes, dos substratos e das temperaturas utilizadas (MULLIGAN, 2005).

Bacillus subtilis são produtores de lipopeptídeos, como a chamada surfactina, a qual contém sete aminoácidos ligados aos grupos carboxila e hidroxila do ácido C_{14} (KAKINUMA et al., 1969). Concentrações de surfactina menores que 0,005 % reduzem a tensão superficial para 27 mN/m, tornando a 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; SILVA, 2009).

Entre as leveduras, espécies de *Candida* têm sido largamente empregadas com sucesso na fermentação de hidrocarbonetos e, conseqüentemente, para produção de biossurfactantes. Sarubbo et al. (2006; 2007) demonstraram a possibilidade de combinação entre duas fontes, uma solúvel e outra insolúvel para a produção de biossurfactantes por espécies de *Candida* enquanto que Rufino et al (2007; 2008) e Coimbra et al., (2009) aplicaram com sucesso um resíduo industrial de óleo de soja na produção de um biossurfactante por *Candida lipolytica*.

3.2.3 Produção de Raminolipídios por *Pseudomonas aeruginosa*

A *Pseudomonas aeruginosa* é uma bactéria gram-negativa ambientalmente versátil a qual pode provocar doenças em indivíduos susceptíveis (oportunista) e é resistente a antibióticos. O gênero *Pseudomonas* é capaz de degradar hidrocarbonetos e de metabolizar vários compostos orgânicos de difícil assimilação por outros organismos, incluindo desde moléculas simples a compostos orgânicos complexos, o que a permite uma excepcional habilidade para colonizar nichos ecológicos onde há escassez de nutrientes (WARD et al.,

2003; CHANDRAN, 2011). Espécies deste gênero apresentam diversas aplicações biotecnológicas, em especial na área ambiental, pois são capazes de sintetizar uma grande variedade de enzimas sob as mais diversas condições de cultivo (WARD et al., 2003).

A *P. aeruginosa* secreta um grupo de biossurfactantes aniônicos de natureza glicolipídica, os raminolipídios (KUMAR ET AL., 2008). Os raminolipídios estão entre os surfactantes mais eficientes conhecidos. Eles possuem tensões superficiais reduzidas (32 - 27 mN/m), baixa concentração micelar crítica, atingindo 5 mg/L, elevada capacidade emulsificante, elevada afinidade por moléculas orgânicas hidrofóbicas, além de reduzir a tensão interfacial óleo/água de 43 mN/m para valores menores que 1 mN/m (LANG, WULLBRANDT, 1999).

A máxima produção de raminolipídeos é normalmente verificada no fim da fase exponencial de crescimento, sendo descrita por alguns autores como uma produção não associada ao crescimento. O aumento da concentração inicial da fonte de nitrogênio, nitrato, causa um aumento na concentração de biomassa e na produção de raminolipídeos. (KRONEMBERGER, 2007).

Os raminolipídeos são os glicolipídeos mais estudados. São formados por uma ou duas moléculas de ramnose ligadas a uma ou a duas moléculas de ácido β-hidroxidecanóico. A produção de glicolipídeos contendo ramnose por *Pseudomonas aeruginosa* foi primeiramente relatada por JARVIS e JOHNSON (1949). Os principais glicolipídeos produzidos por *P. aeruginosa* são os raminolipídeos dos tipos Lramnosil-β-hidroxidecanoil-β-hidroxidecanoato e L-ramnosil-L-ramnosil-β-hidroxidecanoil-β-hidroxidecanoato (KRONEMBERGER, 2007; NITSCHKE et al., 2011).

Tais propriedades conferem aos raminolipídios características atrativas para aplicação na despoluição de sistemas de solos. A gama de aplicações para raminolipídios, incluindo alimentos, cosméticos, fármacos e nas áreas ambiental e agrícola é potencialmente tão extensa quanto as suas propriedades. Outra vantagem frente aos biossurfactantes consiste na facilidade de isolamento dos líquidos metabólicos, uma vez que são extracelulares, além de poderem ser produzidos a partir de substratos hidrofóbicos e hidrofílicos de baixo custo,

incluindo hidrocarbonetos, óleos vegetais, carboidratos ou resíduos das indústrias de alimentos (MONTEIRO et al., 2007; NITSCHKE, 2011).

Vários autores reportam que os raminolipídios são produzidos por *P. aeruginosa* como misturas de diferentes homólogos onde o raminolipídio Rha-C_{10}-C_{10} predomina. A presença de outros raminolipídios com diferentes cadeias de ácidos graxos (C_{18}, C_{22} e C_{24}) em maior quantidade (ABDEL-MAWGOUD et al., 2009). De fato, mais de 28 homólogos têm sido reportados, com cadeias saturadas e insaturadas, variando de C8 a C14 e com porções de carboidratos acopladas (NITSCHKE et al., 2005; MULLIGAN, 2005). Figura 4 apresenta a estrutura molecular de raminolipídios.

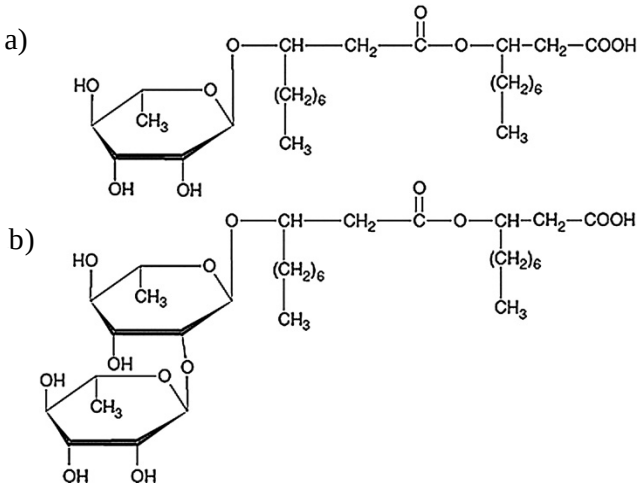


Figura 4 – Representação molecular a) mono e b) di-raminolipídeo

Enquanto um anel extra de raminose confere mais hidrofilicidade aos raminolipídeos (mono-raminolipídeos versus di-raminolipídeos), carbonos adicionais na cadeia de ácidos graxos podem aumentar a hidrofobicidade aos mesmos. Essas propriedades podem afetar a estabilidade dos raminolipídeos na fase aquosa (na forma monomérica ou como aglomerados micelares), suas capacidades em solubilizar compostos orgânicos hidrofóbicos e suas biodisponibilidades. Raminolipídeos mais hidrofílicos como Rha-C_{10} e Rha_2-C_{10} apresentam valores de CMC na faixa de 200 mg/L, enquanto que valores menores, entre 5 e 60 mg/L têm sido reportados para misturas contendo principalmente o monoraminolipídeo Rha-C_{10}-C_{10}. O diraminolipídeo Rha_2-C_{10}-C_{10}

apresenta valores intermediários de CMC, entre 40-65 mg/L. A literatura descreve que a composição da mistura de raminolipídeos irá influenciar de forma marcante sua performance como carreador de contaminantes (ABDEL-MAWGOUD et al., 2009).

Recentemente, com a busca pela utilização de substratos de baixo custo para a produção de biossurfactantes, diversos trabalhos foram publicados sobre a produção de raminolipídeos a partir de diversos óleos, como os de soja (RAZA et al., 2007, CHA et al., 2007), de oliva (WEI et al., 2005) e de castanha do Pará (COSTA et al., 2005). Nesses trabalhos, as fermentações foram conduzidas em frascos agitados com pequenos volumes e com o uso de diferentes cepas de *Pseudomonas aeruginosa*, com exceção de RAZA et al. (2007) que utilizaram uma cepa de *Pseudomonas putida* submetida à mutagênese com raios gama. Quando óleo de soja foi utilizado como principal fonte de carbono, os valores de produtividade volumétrica de raminolipídeos obtidos pelos autores variaram entre 15 mg/L.h (RAHMAN et al., 2002 b) e 50 mg/L.h (CHA et al., 2007). Quando a fonte de carbono foi óleo de oliva, a produtividade obtida foi de 26 mg/L.h (WEI et al., 2005). O melhor resultado foi obtido por (COSTA et al., 2005) com a utilização de óleo de castanha do Pará como fonte de carbono. Foi obtida uma concentração de 9,9 g/L de raminolipídeos, com uma produtividade igual a 83 mg/L.h (KRONEMBERGER, 2007).

3.2.4 Propriedades

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 desses agentes (DELEU; PAQUOT, 2004).

A CMC (concentração micelar crítica) é comumente usada para medir a eficiência do surfactante que geralmente 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 (NITSCHKE; PASTORE, 2002). Eficientes biossurfactantes têm

uma CMC baixa, o que significa que menos biossurfactante é necessário para diminuir a tensão superficial (PACWA-PLOCINICZAK et al., 2011).

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 do que os surfactantes convencionais, pois produzem menor tensão superficial a menores concentrações, a CMC dos biossurfactantes varia entre 1-2000 mg/L enquanto que a tensão interfacial (óleo/água) e superficial fica em torno de 1 e 30 mN/m;
- 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 pHs 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 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, cosméticos e 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 são 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.

Alguns pontos desfavoráveis podem ser citados, a despeito das vantagens, 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 de líquido metabólico;
- A existência de espécies superprodutoras é rara e as conhecidas não são capazes de produzir elevados rendimentos em surfactantes, além de necessitarem de 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.

3.2.5 Aplicações

A produção mundial de surfactantes somou 17 milhões de toneladas em 2000, esperando-se um aumento da ordem de 3-4% ao ano. 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 farmacêutica e de cosméticos, 3% na indústria de alimentos, 2% na agricultura e os 2% restantes em outras aplicações (MUTHUSAMY et al., 2008).

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 (MUTHUSAMY et al., 2008).

O derramamento de óleos ocorridos durante o seu transporte ou na construção de oleodutos afeta drasticamente as regiões costeiras e praias, sendo hoje uma das maiores causas de catástrofes ecológicas e sociais no mundo (MUTHUSAMY et al., 2008). Até o presente momento, os biossurfactantes têm sido usados principalmente nas indústrias de óleos, incluindo a limpeza de derramamento de óleos, a remoção de óleos de tanques de estocagem, a recuperação avançada de petróleo e a biorremediação de solos (GAUTAM; TYAGI, 2006; SINGH et al., 2007).

3.2.5.1 Aplicação de Biossurfactantes 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 ambiental por essas substâncias e das partículas de solo ou outras, às quais os organismos e contaminantes possam estar adsorvidos (MARGESIN; SCHINNER, 2002).

As substâncias contaminantes apresentam diferentes grupos funcionais tais como OH, Cl, NH_2, NO_2 e SO_3. Esses, por sua vez, comportam-se como doadores de elétrons sendo oxidados ou em alguns casos, mineralizados por diferentes espécies microbianas. Alguns dos metabólitos intermediários produzidos nessas reações são assimilados como fonte de carbono para o crescimento microbiano (MARGESIN; SCHINNER, 2002).

A biorremediação utilizando micro-organismos ou processos microbianos em ambientes contaminados tem inúmeras aplicações incluindo a limpeza de águas subterrâneas, solos, lagos e processos de tratamento de esgotos. Essa é

uma tecnologia bem aceita pela opinião pública na recuperação de ambientes poluídos não afetando o equilíbrio ecológico, já que as bactérias, os fungos filamentosos e as leveduras são agentes transformadores eficazes, face às suas habilidades em degradar uma ampla diversidade de substâncias orgânicas (DESAI; BANAT, 1997; CALVO et al. 2008).

Os biossurfactantes aumentam a interação água/óleo, aceleram a degradação de vários óleos por micro-organismos e promovem a biorremediação de águas e solos contaminados (MULLIGAN, 2005). A capacidade dos surfactantes em emulsificar e dispersar hidrocarbonetos em água aumenta a degradação desses compostos no ambiente. Os biossurfactantes também são úteis na biorremediação de locais contaminados com metais pesados tóxicos como urânio, cádmio e chumbo e na remoção de piche após a introdução de *Pseudomonas, Arhtrobacter*, e Bacillus *subtilis*, demonstrando resultados promissores (NITSCHKE; PASTORE, 2002).

Pesquisas com micro-organismos que produzem raminolipídeos demonstraram o potencial de biorremediação de hidrocarbonetos de petróleo (RAHMAN et al., 2006). A aplicação do raminolipídeo de *P. aeruginosa* DS10-129 aumentou a biorremediação de gasolina adsorvida em solo (RAHAMN et al., 2002). Algumas pesquisas 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; SILVA, 2009).

3.2.5.2 Aplicação de Biossurfactantes na Limpeza de Reservatórios de Óleos

A aplicação de biossurfactantes no tratamento de resíduos oleosos torna-se um dos pré-requisitos importantes para que ocorram interações entre os resíduos e

a célula microbiana, devido à redução da tensão superficial mediada entre o óleo e a fase aquosa (HUA et al., 2003; CALVO et al., 2008).

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, 2004). A remoção de resíduos e frações de óleos pesados requer lavagens com solventes ou mesmo manuais, ambas perigosas, demoradas e de custos elevados 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 crú, após quebra da emulsão (SINGH et al., 2007; MULLIGAN, 2004).

3.2.5.3. Aplicação de Biossurfactantes na Recuperação Avançada de Petróleo – “MEOR”

Segundo Desai e Banat (1997), a recuperação de óleos utilizando biossurfactantes constitui atualmente uma importante estratégia para a indústria do petróleo, uma vez que micro-organismos e produtos de seu metabolismo são utilizados para aumentar a recuperação do petróleo. Esse processo conhecido como “MEOR” (Microbial Oil Recovery Enhancement), recuperação avançada de óleo, apresenta vantagens importantes em relação aos métodos convencionais. Os surfactantes alteram algumas características físico-químicas do petróleo, facilitando ou aumentando sua remoção nos poços (SINGH et al., 2007).

3.2.5.4 Aplicação de Biossurfactantes na Indústria de Alimentos

Na indústria alimentícia, a emulsificação tem um papel importante na formação da consistência e textura, bem como na dispersão de fase e na solubilização de aromas (RAHMAN; GAKPE, 2008). De forma geral, a função dos emulsificantes em alimentos é promover a estabilidade da emulsão, controlando a aglomeração de glóbulos de gordura e estabilizando sistemas aerados (NITSCHKE; PASTORE, 2002).

Por definição, uma emulsão é um sistema heterogêneo, consistindo de ao menos um líquido imiscível (fase interna descontínua) disperso em outro (fase externa contínua) em forma de pequenas gotas (SARUBBO et al., 1999). Tais sistemas possuem uma estabilidade mínima, a qual pode ser aumentada por aditivos surfactantes, sólidos finamente divididos que atuam reduzindo a tensão interfacial, diminuindo a energia na superfície entre as duas fases e prevenindo a coalescência das partículas através da formação de barreiras estéricas e eletrostáticas. Exemplos de alimentos processados, que são emulsões: creme de leite, manteiga, margarina, maionese, molhos para salada, salsicha, recheios entre outros (VELIKONJA; KOSARIC, 1993; SILVA, 2009). Outras aplicações para os emulsificantes são descritas, entre elas melhorar a textura e vida de prateleira de produtos contendo amido, pela formação de complexos com os componentes destes, modificar as propriedades reológicas da farinha de trigo, pela interação com o glúten, melhorar a consistência e textura de produtos à base de gorduras, pelo controle de polimorfismo e da estrutura cristalina das gorduras (BANAT, 2000; SARUBBO et al., 1999).

Os biossurfactantes ainda podem ser utilizados como emulsificantes no processamento de matérias-primas, no controle da aglomeração de glóbulos de gordura, e na estabilização de sistemas aerados. O uso de raminolipídeos para melhorar as propriedades da manteiga, de croissants e de produtos de confeitaria congelados também foi reportado (MUTHUSAMY et al., 2008). O bioemulsificante produzido por *Candida utilis* tem sido utilizado em molhos prontos para saladas (LUNA, 2006).

A manoproteína produzida por *Saccharomyces cerevisiae* pode estabilizar emulsões água/óleo para produção de maionese, biscoitos, sorvetes, entre outros. É produzida através de um processo biotecnológico simples, de larga escala e baixo custo. Além de ser estável em uma larga faixa de pH, seu subproduto pode ser utilizado como ração animal (BANAT, 2000).

Apesar da aplicação potencial, a indústria de alimentos não utiliza ainda os biossurfactantes como aditivos em larga escala. Muitas propriedades dos biossurfactantes necessitam de aprovação para sua regulamentação como novo ingrediente para alimentos.

3.2.5.5 Aplicação de Biossurfactantes na Indústria Farmacêutica

Os biossurfactantes são amplamente utilizados em vários produtos, como em cosméticos e fármacos. Estima-se que num futuro próximo a maioria dos cosméticos seja "biocosméticos". Vários produtos necessitam de surfactantes em seus ingredientes, incluindo repelentes de insetos, antiácidos, soluções para lentes de contato, desodorantes, produtos para unhas, pasta de dentes, dentre outros (MAYER; SOBERON-CHAVEZ, 2000). Devido a compatibilidade com a pele, os biossurfactantes podem ser usados em produtos de higiene e cosméticos (NITSCHKE; PASTORE, 2002). Com essa finalidade, glicolipídeos obtidos de *Torulopsis bombicola* KSM 35 são usados no Japão, como agentes de limpeza facial (DELEU; PAQUOT, 2004). Soforolipídeos sofrem esterificação, resultando em um produto com aplicação em batons e como hidratante para pele e cabelos (NITSCHKE; PASTORE, 2002). A literatura também descreve a ação de soforolipídeos na estimulação da síntese de colágeno, podendo ser usados como medida preventiva do envelhecimento da pele (MUTHUSAMY et al., 2008).

3.2.5.6 Aplicação de Biossurfactantes 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 $CaSO_4$ e 30% para $CaCO_3$, ressaltando que, reagentes químicos convencionais são incapazes de separar estes dois minerais (NITSCHKE; PASTORE, 2002). O biodispersan, polissacarídeo aniônico produzido por *Acinetobacter calcoaceticus* A2 foi utilizado na prevenção da floculação e dispersão de misturas de pedra calcárea e água (RON; ROSENBER, 2002).

3.2.5.7 Aplicação de Biossurfactantes na Agricultura

Na agricultura, os biossurfactantes são utilizados na hidrofilização de solos argilosos para a obtenção de boa umidade e distribuição uniforme de fertilizantes (NITSCHKE et al., 2007). A formulação de herbicidas e pesticidas contendo bioemulsificantes também tem sido reportada (ROSENBERG; RON,

1999). Os compostos ativos dessas formulações são, geralmente, hidrofóbicos, sendo necessários agentes emulsificantes para dispersá-los em soluções aquosa. Stanguellini e Miller (1997) demonstraram a eficiência de raminolipídeos contra patógenos de plantas.

3.2.5.8 Aplicação de Biossurfactantes na Medicina

Os biossurfactantes também têm sido utilizados em várias aplicações biológicas (terapêuticas) como atividade fungicida, bactericida, inseticida e antiviral, agentes anti-adesivos e inibidores de enzimas (MEYLHEUC et al., 2001; MUTHUSAMY et al., 2008).

Atividade antibiótica já demonstrada por vários biossurfactantes, principalmente da classe dos lipopeptídeos e glicopeptídeos. Os raminolipídeos de *P. aeruginosa* e o surfactin de *B. subtilis*, funcionam como antibióticos solubilizando os principais componentes das membranas celulares microbianas, impedindo a excreção desses biossurfactantes no meio impossibilitando a sobrevivência dos micro-organismos através da competitividade na busca por nutrientes (LIN, 1996; RUFINO, 2006).

Vários raminolipídeos podem exibir atividades antibacteriana e antifitoviral. Abalos et al. (2001), identificaram seis raminolipídeos em culturas de *P. aeruginosa* AT10 produzidos a partir de resíduo de refinaria de óleo de soja e avaliaram as propriedades antimicrobianas da mistura. Esses raminolipídeos exibiram excelente propriedades antifúngicas contra vários fungos em concentrações variando de 16 µg/mL a 32 µg/mL (CAMEOTRA; MAKKAR, 2004).

A chamada atividade anti-aderente, ou seja, a capacidade de inibir a adesão de micro-organismos patogênicos em superfícies sólidas ou em sítios infecciosos também tem sido reportada em biossurfactantes, levando à diminuição de infecções hospitalares sem a necessidade de fármacos ou agentes químicos sintéticos (MUTHUSAMY et al., 2008). Meylheuc et al. (2001) estudaram um biossurfactante obtido de *P. fluorescens* dotado de propriedades inibidoras da adesão de *Listeria monocytogenes* LO28 às superfícies do politetrafluoroetileno e do aço inoxidável.

A deficiência do surfactante pulmonar, um complexo proteína-fosfolipídeo é responsável pela falência da respiração em bebês prematuros. O isolamento dos genes para as moléculas protéicas desse surfactante e a clonagem em bactérias permitiu sua produção fermentativa para aplicações médicas (MUTHUSAMY et al., 2008).

Soforolipídeos de *Candida bombicola* têm sido estudados por sua atividade espermicida, citotóxica e anti-HIV para reduzir a proliferação do vírus da Síndrome da Imunodeficiência Adquirida (AIDS) e a incidência de gravidez indesejada. Soforolipídeos também têm sido estudados como agentes antiinflamatórios para doenças imunológicas (MUTHUSAMY et al., 2008).

A iturina, lipopeptídio produzido por *B. subtilis*, demonstrou atividade antifúngica, afetando a morfologia e a estrutura da membrana celular de leveduras. Experimentos *in vitro* mostraram que a surfactina inativou eficazmente o vírus causador de herpes, assim como o retrovirus e outros vírus de RNA e DNA compactados. A atividade antiviral da surfactina foi determinada para uma larga gama de vírus. Além disso, foram examinados os efeitos da surfactina na absorção de insulina em pulmão de ratos (MUTHUSAMY et al., 2008).

3.2.5.9 Outras Aplicações de biossurfactantes

Outros campos de utilização dos biossurfactantes incluem as indústrias de papel, têxtil e cerâmica (DELEU; PAQUOT, 2004). A Tabela 2 descreve aplicações industriais dos biossurfactantes:

Tabela 2 – Aplicações industriais dos biossurfactantes

Indústria	**Aplicação**	**Papel dos biossurfactantes**
Petróleo	Recuperação avançada de óleos	Aumento da drenagem de óleo em poços perfurados; estimulação da liberação de óleo entranhado por forças capilares; redução da viscosidade de óleos; molhabilidade de superfícies sólidas; redução da tensão interfacial; solubilização de óleos

Ambiental	Desemulsificação	Desemulsificação de emulsões oleosas, seqüestro de metais pesados; redução da tensão interfacial; agente de molhabilidade
	Biorremediação	Emulsificação de hidrocarbonetos; redução da tensão interfacial; seqüestro de metais
	Remediação de solos	Emulsificação através da aderência a hidrocarbonetos; dispersantes; agentes espumantes; detergentes
Alimentos	Emulsificação e desemulsificação	Emulsificantes; estabilizantes; desemulsificantes; suspensão; solubilizantes; espumantes; inibidores de espumas; amaciantes; lubrificantes
	Ingrediente funcional	Interação com lipídeos, proteínas e carboidratos
Biológica	Microbiológica	Função fisiológica como mobilidade celular, acesso a nutrientes, competição célula-célula, patogênese em plantas e animais
	Farmacêutica e terapêutica	Antibacterianos; antifúngicos; agentes antivírus; vacinas; mecanismos de adesão
Agricultura	Biocontrole	Facilitadores de mecanismos de biocontrole de micróbios como parasitismo e competição
Bioprocessamento	“Downstream”	Biocatálise em sistemas bifásicos aquosos e em emulsões; biotransformações; recuperação de compostos intracelulares; aumento da produção de metabólitos fermentativos e enzimas extracelulares
Cosmética	Produtos de beleza e saúde	Emulsificantes; solubilizantes; espumantes; agentes microbianos, mediadores de ação enzimática

Fonte: MUTHUSAMY et al., 2008

3.3 Utilização de Resíduos Industriais na Produção de Biossurfactantes

A novo perfil da sociedade atual caracteriza-se pelo aumento do custo de vida, a necessidade de reutilizar materiais e com a preocupação ambiental. Conseqüentemente, vem dando uma ênfase maior a recuperação, reciclagem e reutilização de diversos resíduos. A necessidade de preservação ambiental leva à reutilização de diversos resíduos industriais. Isso particularmente é válido para os alimentos e as indústrias de produção de alimentos cujos resíduos, fluentes e co-produtos podem ser reutilizados. Essas indústrias produzem grandes volumes de resíduos sólidos e líquidos, resultantes da produção, preparação e consumo dos alimentos e quando descartados geram poluição e representam uma grande perda de nutrientes, particularmente das indústrias alimentícias. Tais resíduos sólidos e líquidos vêm sendo utilizados na bioconversão e chamando mais atenção devido à possibilidade de aplicação na produção de bioadsorventes (SINGH et al., 2007).

Uma variedade de subprodutos, incluindo derivados de óleo vegetais, resíduos de amido, resíduos de destilaria de óleos e substâncias lácteas têm sido utilizados na produção de muitos metabólitos microbianos. A disponibilidade e o tipo de matéria-prima podem contribuir consideravelmente para o custo de produção. Estima-se que 10% a 30% da matéria-prima representa o custo total de um produto biotecnológico (MUKHERJEE et al., 2006). Por outro lado, milhões de desperdícios em resíduos poluentes são jogados a cada ano por todo o mundo. O tratamento e a remoção destes resíduos também representam um alto custo para várias indústrias (PANDEY, et al., 2000). Nesse sentido, os resíduos industriais têm despertado grande interesse dos pesquisadores como alternativa para o fornecimento de substratos de baixo custo para a produção de biossurfactantes (GUSMÃO et al. 2010).

Muitos biossurfactantes têm sido produzidos de substratos agroindustriais, renováveis e de baixo custo, muito atrativos devido ao elevado valor de carboidratos ou lipídeos. Óleos vegetais, resíduos de fritura de óleos vegetais, resíduos de destilaria de óleos, resíduos da indústria de laticínios (soro de leite),

melaço de cana de açúcar e glicerina têm sido citados na literatura (MAKKAR; CAMEOTRA, 2002).

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

Nitschke e Pastore (2006) utilizaram com sucesso resíduos industriais de fritura de batata na produção de biossurfactantes por *B. subtilis.* Anteriormente, Nitschke et al. (2004) selecionaram micro-organismos para a produção de biopolímeros utilizando resíduos agroindustriais. Utilizaram melaço, soro de leite e manipueira obtendo baixos valores de tensão superficial em torno de 26 mN/m. Volbrecht et al. (1999) investigaram a produção de biossurfactantes usando óleo vegetal doméstico como substrato da bactéria *Tsukamurella spec* DSM 44370, conseguindo reduzir a tensão da água de 70 mN/m para 35 mN/m com CMC de 10 mg/L. Haba et al. (2000) compararam o uso de óleo de oliva e girassol para a produção de biopolímeros usando valores de tensão superficial até 40 mN/m como critério de seleção de micro-organismos potencialmente produtores. Recentemente, Rufino (2006) utilizou um resíduo de refinaria na produção de biossurfactante por *Candida lipolytica* obtendo resultados satisfatórios em termos de tensão superficial.

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

Diferentes elementos encontrados nos efluentes dos processos industriais também são considerados como fontes de nutrientes. Nitrogênio e ferro foram utilizados para aumentar o rendimento de biossurfactantes de *P. aeruginosa* BS-2 e *Ustilago maydis* (DUBEY et al., 2004). Amezcua-Veja et al. (2007) descreveram a importância da relação entre diferentes elementos como C e N, C e P, C e Fe ou C e Mg na produção de biopolímeros e na otimização de seus processos de obtenção.

A borra oleosa do fundo de tanques da Petrobrás, contendo querosene, óleo diesel e petróleo, foi utilizada como matéria-prima de baixo custo para a produção de biossurfactante pela bactéria *P. aeruginosa* isolada de solo contaminado por petróleo (PIRÔLLO, 2006). A Tabela 3 resume algumas matérias-primas de baixo custo e os respectivos micro-organismos utilizados na produção de biossurfactantes.

Tabela 3 - Matérias-primas de baixo custo e respectivos micro-organismos utilizados na produção de biossurfactantes

Matéria-prima de baixo custo ou resíduos	**Tipo de biossurfactante**	**Espécie microbiana produtora**	**Rendimento máximo (g/L)**
Óleo de babaçu	Glicolipídeo	*Candida lipolytica* IA 1055	---
Óleo de milho	Glicolipídeo	*Candida bombicola* ATCC 22214	4,0
Óleo de girassol e óleo de soja	Raminolipídeo	*Pseudomonas aeruginosa* DS10-129	4,31/2,98
	Lipídeo manosileritritol	*Candida* sp. SY16	95
Óleo residual de fritura (óleos de oliva e girassol)	Raminolipídeo	*Pseudomonas aeruginosa* 47T2 NCIB 40044	2,7
Resíduo de refinaria de óleo vegetal	Raminolipídeo	*Pseudomonas aeruginosa* LBI	11,72
Resíduo de refinaria de óleo de	Raminolipídeo	*Pseudomonas aeruginosa* LBI	16

girassol			
Resíduo de refinaria de óleo vegetal	Glicolipídeo	*Candida antactica* e/ou *Candida apicola*	10,5/13,4
Solo e resíduo de refinaria de óleo vegetal	Raminolipídeo	*Pseudomonas aeruginosa* AT10	0,92
Efluentes do processamento de batatas	Lipopeptídeo	*Bacillus subtilis*	---
Manipueira	Lipopeptídeo	*Bacillus subtilis* ATCC 21332 e *Bacillus subtilis* LB5a	2,2 – 3,0

Fonte: MUKHERJEE et al., 2006.

Os resíduos de óleos e gorduras comestíveis são considerados ótimas fonte de carbono, tornando o seu descarte um desperdício de fonte energética, contribuindo ainda para a poluição ambiental. É importante destacar que os micro-organismos são capazes de crescer nos óleos vegetais ou em gordura, produzindo novos produtos com potencial de aplicação industrial, tal como a lipase e o biodiesel (HABA et al., 2000a; ALCANTARA, et al., 2000; MANEERAT, 2005).

Haba e colaboradores (2000b) cozinharam o óleo de girassol e também o de oliva para utilizá-los como fonte de carbono na produção de biossurfactantes por 36 isolados bacterianos. Estirpes de *Pseudomonas* testadas mostraram crescimento satisfatório quando cultivadas em óleos de oliva e de girassol já utilizados, ou seja, cozido. Entretanto, o óleo de girassol não foi tão bom para o crescimento celular ou para a produção de biossurfactante quanto o óleo de oliva.

Nitschke *et al.,* (2011) observaram diferenças nas propriedades de raminolipídios produzidos por duas cepas diferentes do gênero *Pseudomonas* (LMI 6c e LMI7a), isoladas de solos de aterros pertencentes a indústrias de óleos, abatedouros de aves e postos de gasolina. Esses isolados foram crescidos utilizando quatro diferentes substratos insolúeis (óleo de soja usado, borra de soja, gordura de frango e gordura vegetal) como fonte de carbono. As diferenças

nas propriedades desses raminolipídios podem estar associadas a variações na composição dos óleos e na atividade de algumas enzimas bacterianas, como lipases. Os óleos vegetais constituem um dos mais importantes derivados das plantas, com uma produção nacional da ordem de 6 milhões de toneladas no ano de 2008. Cerca de dois terços dessa produção é usada em produtos alimentícios, sobretudo fritura de alimentos. Esses óleos também fazem parte da dieta humana, onde mais de 90% dos óleos produzidos são de origem vegetal, usados em produtos industrializados comestíveis (CARA, 2009).

A agroindustrialização de produtos a base de milho através de processamento úmido resulta em subprodutos sólidos e líquidos, que dispostos de forma inadequada tornam-se fontes de contaminação e agressão ao meio ambiente. A milhocina é um rejeito da água de lavagem e embebição dos grãos de milho quando do fracionamento em amido e germe (óleo), contendo 40% de sólidos. Possui entre 21 a 45% de proteínas, 20 a 26% de ácido lático, cerca de 8% de cinzas (contendo Ca^{2+}, Mg^{2+}, K^{+}), cerca de 3% de carboidratos e baixo teor de gordura (0,9% - 1,2 %). De acordo com a literatura, a milhocina é usada principalmente como suplemento alimentício para ruminantes, fonte de nutrientes para aves, na confecção de iscas atrativas para as moscas das frutas e fonte de nutrientes para o processo de fermentação industrial. A composição da milhocina é muito variável, dependendo da origem da matéria-prima e de seu processamento (FILIPOVIC e al., 2002; DOMINGOS, 2009).

3.4 Perspectivas de utilização

Muitas das potenciais aplicações dos biossurfactantes, bem como uma expansão da produção 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 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).

Segundo Bognolo (1999) os parâmetros que podem ser variados na tentativa de otimizar a produção de biossurfactantes incluem:

- seleção de matérias-primas de baixo custo, possibilitando o equilíbrio adequado de C, N, P e outros oligoelementos para maximização do rendimento e o desenvolvimento de cepas de micro-organismos capazes de metabolizar qualquer subproduto residual;
- bioprocessamento que pode ser otimizado por meio das condições operacionais do reator e da reciclagem do meio utilizado;
- 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, pode sem dúvida, conduzir a uma redução substancial de custos.

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).

3.5 Desenvolvimento de Bioprocessos para a Produção e Recuperação de Biossurfactantes

Um processo eficiente e econômico constitui a base de qualquer indústria biotecnológica com fins lucrativos; assim, o desenvolvimento de bioprocessos é o primeiro passo para a comercialização de todos os produtos biotecnológicos,

inclusive os biossurfactantes. Qualquer tentativa de aumentar o rendimento de um biossurfactante exige a adição de componentes do meio e a seleção das condições ótimas que conduzam à produtividade máxima (MUKHERJEE et al., 2006). De maneira semelhante, técnicas e métodos de processamento eficazes são necessários para a máxima recuperação do produto. Vários elementos, componentes do meio e precursores são mencionados como capazes de afetar o processo de produção dos biossurfactantes e a quantidade e a qualidade finais. Segundo a literatura, elementos como C:N, C:P, C:Fe ou C:Mg afetam a produção de biossurfactantes e a sua otimização qualifica a sua obtenção (AMEZCUA-VEGA et al., 2007).

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

Alguns métodos de recuperação não-convencionais foram utilizados nos últimos anos. Esses procedimentos tiram vantagem de algumas propriedades dos biossurfactantes – como a atividade superficial ou a capacidade de formar micelas e são particularmente adequados à recuperação contínua em larga escala de biossurfactantes extracelulares do líquido metabólico. Alguns exemplos dessas estratégias de recuperação de biossurfactantes incluem fracionamento de espuma (DAVIS et al., 2001; NOAH et al., 2005), ultrafiltração (SEN, SWAMINATHAN, 2005), adsorção-dessorção em resinas de poliestireno e cromatografia de troca iônica (REILING et al., 1986). Uma das principais

vantagens desses métodos é a capacidade de operar de modo contínuo na recuperação de biossurfactantes com um alto nível de pureza. Biossurfactantes com alto teor de pureza são exigidos em indústrias como a farmacêutica, alimentícia e cosmética, as quais exigem a aplicação dessas técnicas de recuperação. A Tabela 4 descreve os procedimentos de recuperação dos biossurfactantes e suas vantagens.

Tabela 4 - Propriedades físico-químicas dos métodos de recuperação de biossurfactantes e suas vantagens relativas

Processo de recuperação	**Propriedade responsável pela seleção do método de separação**	**Instrumentação necessária**	**Vantagens**
Precipitação ácida	Biossurfactantes se tornam insolúveis a baixos pH	Não requer equipamentos	Baixo custo; eficiente na recuperação do surfactante bruto
Extração com solventes orgânicos	Biossurfactantes são solúveis em solventes orgânicos devido à presença da cadeia hidrofóbica	Não requer equipamentos	Eficiente na recuperação do surfactante bruto e na purificação parcial; natureza reutilizável
Precipitação por sulfato de amônio	Exclusão da fase saturada em sal pelo biossurfactante polimérico rico em proteínas	Não requer equipamentos	Efetiva no isolamento de determinados tipos de biossurfactantes Poliméricos
Centrifugação	Biossurfactantes insolúveis precipitam em função da força centrífuga	Necessidade de Centrífuga	Reutilizável; efetiva na recuperação do surfactante bruto
Fracionamento de espuma	Biossurfactantes, devido à atividade surfactante, formam e se particionam na espuma	Construção de biorreatores especiais que facilitem a recuperação da espuma durante a fermentação	Utilizado em processos contínuos de recuperação; alta pureza do produto
	Biossurfactantes	Unidades de	Rápido; recuperação

Ultrafiltração em membrana	formam micelas acima da CMC, as quais são retidas em membranas poliméricas	ultrafiltração com membrana polimérica porosa	em apenas uma etapa; alto grau de pureza
Adsorção em resinas de poliestireno	Biossurfactantes são adsorvidos em resinas poliméricas e podem ser desorvidos usando solvente orgânico	Resina de poliestireno empacotada em colunas de vidro	Rápido; recuperação em apenas uma etapa; alto grau de pureza; reutilizável
Adsorção em carbono ativo	Biossurfactantes são adsorvidos em carvão ativo e podem ser desorvidos usando solvente orgânico	Não requer equipamentos; pode ser adicionado ao meio de cultivo; também pode ser empregado em colunas de vidro	Pureza elevada do biossurfactante; baixo custo; reutilizável; recuperação em cultura contínua
Cromatografia de troca iônica	Biossurfactantes carregados se ligam a resinas trocadoras de íons e podem ser eluídos com um tampão específico	Resinas trocadoras de íons empregadas em colunas	Alta pureza, reutilização, rápida recuperação do produto
Extração por solvente (com MTBE)	Biossurfactantes são solúveis em solventes orgânicos devido à presença de cadeia hidrofóbica	Não requer equipamentos	Menos tóxico do que os solventes convencionais; baixo custo

Fonte: MUKHERJEE et al., 2006.

Novas pesquisas são necessárias para aperfeiçoar as fases de processamento já existentes, tornando-as mais competitivas em termos de custos. Muitas vezes, uma só técnica de processamento não é suficiente para a recuperação e purificação do produto. Nesses casos, uma estratégia de recuperação de múltiplas fases, utilizando uma seqüência de fases de concentração e purificação, é muito mais eficaz (REILING et al., 1986).

Num processo de recuperação de múltiplas etapas dos biossurfactantes, será possível obter o produto a qualquer grau de pureza desejado. Biossurfactantes brutos ou impuros obtidos nas fases iniciais do processo de

recuperação podem ser utilizados em aplicações ambientais e também na recuperação de petróleo e nas indústrias de tintas e têxtil e obtidos a custos mais baixos. Em alternativa, os biossurfactantes de elevado grau de pureza exigido pelas indústrias farmacêutica, alimentícia e cosmética podem ser obtidos por meio de novos estágios de purificação. Esse tipo de recuperação de fases múltiplas deverá ser útil nas indústrias que produzem biossurfactantes para uma vasta gama de aplicações (MUKHERJEE et al., 2006).

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CAPÍTULO 2

Manuscrito submetido para publicação na Revista Tenside Surfactants Detergents

ENHANCING OF BIOSURFACTANT PRODUCTION FROM *Pseudomonas cepacia* CCT 6659 THROUGH OPTIMIZATION OF NUTRITIONAL PARAMETERS USING A RESPONSE SURFACE METHODOLOGY

ENHANCING OF BIOSURFACTANT PRODUCTION FROM *Pseudomonas cepacia* CCT6659 THROUGH OPTIMIZATION OF NUTRITIONAL PARAMETERS USING A RESPONSE SURFACE METHODOLOGY

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Abstract

In this study, we have investigated the potential of a new strain of *Pseudomonas cepacia* CCT6659 for the production of a biosurfactant. Biosurfactant production was optimized by the combination of central composite rotatable design (CCRD) and response surface methodology (RSM). Surface tension was considered as the response variable. The factors selected for optimization of growth conditions were canola waste frying oil, corn steep liquor and $NaNO_3$ concentrations. The empirical model developed through RSM in terms of effective nutritional factors mentioned above was found to be adequate to describe the biosurfactant production. Through the analysis, all the factors studied were absolutely important within the ranges investigated. A maximum reduction in surface tension (26 mN/m) was obtained under the optimal conditions of 2% waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$ concentrations. The accumulation in isolated biosurfactant reached 8.0g/l under these conditions showing the suitability of the factorial design in increasing the productivity of the process.

Keywords: Biosurfactant; Optimization; *Pseudomonas*; Oil; RSM; Surface tension.

1. Introduction

Biosurfactants or microbial surfactants are surface active molecules that are produced from a variety of microorganisms. Due to its amphipathic nature, these biomolecules are capable of lowering the surface tension, interfacial tension and forming micro-emulsion to enable mixing of two immiscible solutions. Such properties exhibit excellent detergency, emulsifying and foaming, which can be applied in various industries. The features that make them commercially promising alternatives to chemically synthesized surfactants are their lower toxicity, higher biodegradability, better foaming properties, and greater stability towards temperature and pH [1].

Biosurfactans present varied chemical compositions consisting of various chemical structures, such as fatty acids, glycolipids, lipopeptides, lipopolysaccharides, lipoproteins and glycolipids which depend on the microorganism, raw matter and process condition, thus, characterization and production optimization studies are required [2]. The glycolipids comprise a class of biosurfactants that are composed of carbohydrates in combination with long-chain aliphatic or hydroxyl aliphatic fatty acids. Rhamnolipids from the genus *Pseudomonas* are the best known glycolipid surfactants, and their potential applications range from uses in cosmetics, food, pharmaceuticals, paper, metal and ceramics, to environmental uses such as in bioremediation [3].

Production economy is the major setback in biosurfactant production, as in the case with most biotechnological processes. Often the amount and type of a raw material can contribute considerably to the production cost; it is estimated that raw materials account for 10–30% of the total production cost in most biotechnological processes [4]. Thus to reduce this cost it is desirable to use low-cost raw materials for the production of biosurfactants [5]. Several elements, media components and precursors are reported to affect the process of biosurfactant production and the final quantity and quality [6]. A

variety of cheap raw materials, including plant-derived oils, starchy substances, lactic whey and distillery wastes have been reported to support biosurfactant production [7].

The identification and optimization of the cultivation conditions that affect the surfactant production represent key points for the development of a cost-competitive process [8]. There are a number of operating parameters controlling biosurfactant production, which are required to be maintained within a certain range in operating conditions whereby the activity of bacteria with the resultant of maximum production of biosurfactant can be optimized. In this regard, medium composition is of great importance for control and optimization of biosurfactant production. The amount of biosurfactant synthesis depends greatly on the availability of carbon sources and on the balance between carbon and other limiting nutrients [9]. Factors affecting surfactant biosynthesis have been studied extensively, but there is dirt of information about optimal conditions for biosurfactant production. Biosurfactant producers can only be effective if they are maintained at their optimal ambient conditions required for growth and activity. In this regard one of the best methodologies for designing the optimization experiments is response surface methodology (RSM). In statistical-based approaches, RSM has been extensively used in fermentation media optimization [10,11]. RSM is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions [12]. It is a statistically designed experimental protocol in which several factors are simultaneously varied [10]. In fact, the relationship between the response and the independent variables is usually unknown in a process; therefore the first step in RSM is to approximate the function (response) through analyzing factors (independent variables). In this study also the ambient growth conditions have been optimized by using this methodology.

The aims of this work were to investigate the production of a biosurfactant by a new strain of *Pseudomonas cepacia* by studying nutritional factors affecting the optimal composition of the growth medium for the production in flask-scale by using RSM.

2. Materials and methods

2.1. Materials

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

2.2. Bacterial strain and preparation of seed culture

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

2.3. Fermentation media

The components of the production medium were dissolved in a mineral medium containing 0.05% KH_2PO_4, 0.1% K_2HPO_4, 0.05% $MgSO_4.7H_2O$, 0.01% KCl and 0.001% $FeSO_4.7H_2O$ and the pH was adjusted to 7.0 by 1.0M HCl. Canola waste frying oil, corn steep liquor and $NaNO_3$ were added according to the factorial design. Aliquots at 2% (v/v)

of the cell suspension of 0.7 OD (optical density) at 600 nm, corresponding to an inoculum of 10^7 C.F.U./ml, were used to inoculate 500 ml Erlenmeyer flasks, containing 100 ml of sterile production medium. Cultivation was carried out at 28°C with shaking at 250 rpm for 60 h in a New Brunswick C-24 shaker (New Brunswick Scientific, NJ, USA). There was no adjustment of pH during cultivation. At the end of fermentation, samples were taken from the liquid culture to determine the surface tension. After selection of the best medium composition, the biosurfactant yield was determined as described below.

2.4. Optimization of culture conditions by RSM

The biosurfactant production was evaluated using an experimental design. A Central Composite Rotatable Design (CCRD) was carried out to verify the effects and interactions of medium constituent's on the production of the biosurfactant. Surface tension was considered as the response variable. Canola waste frying oil, corn steep liquor and $NaNO_3$ concentrations were considered as independent variables. In this design, a set of 20 experiments, with six replicates at the central points, were performed. The range and levels of the components (factors or independent variables) under study are given in Table 1. Each factor in the design was studied at five levels (-1.68, -1, 0 , +1 and +1.68). In this design, a set of all the variables were taken at the central coded value considered as zero. The values of the levels were based on results obtained in preliminary experiments. According to the factorial design matrix the surface tension was studied at various combinations of the medium constituents.

Please insert Table 1

The optimum values from the CCRD were obtained by solving the regression equation and also by analyzing the response surface contour plots [13,14]. To determine

the significance of effects, the analysis of variance (ANOVA) with 95% confidence limits was used. The effects and significance of the variables were graphically illustrated using the Pareto's chart. A Pareto's chart consists of bars with a length proportional to the absolute value of the estimated effects, divided by the standard error. In the Pareto's chart analysis of variance effect estimates are sorted from the largest absolute value to the smallest absolute value. The chart includes a vertical line at the critical t-value for an alpha of 0.05. Effects for which the bars are smaller than the critical t-value are considered as not significant and not affecting the response variables. Effects may be positive or negative.

Variance analysis, regressions coefficients determination and graphs were performed using Statistica® software version 7.0 (Statsoft.Inc, USA) [15].

2.5. Surface tension determination

Surface tension changes were carried out on the cell-free broth obtained by centrifuging the cultures at 5 000 g for 20 minutes by the ring method using a Sigma 70 Tensiometer (KSV Instruments LTD - Finland) at room temperature. Tensiometers determine the surface tension with the help of an optimally wettable ring suspended from a precision balance. In the Ring method the liquid is raised until contact with the surface is registered. The sample is then lowered again so that the liquid film produced beneath the liquid is stretched. As the film is stretched a maximum force is experienced, the force is measured and used to calculate the surface tension. The instrument was calibrated against Mill-Q-4 ultrapure distilled water (Millipore, Ilhinois, USA). Prior to use the platinum plate and all the glassware were sequentially washed with chromic acid, deionised water, acetone and finally flamed with a Bunsen burner.

2.6. Biosurfactant isolation

The biosurfactant was extracted from culture media after cell removal by centrifugation at 5 000g for 30 min. The supernatant pH was adjusted to 2.0 with 6.0 M HCl, and an equal volume of $CHCl_3/CH_3OH$ (2:1) was added. The mixture was vigorously shaken for 15 min and allowed to set until phase separation. The organic phase was removed and the operation was repeated twice again. The product was concentrated from the pooled organic phases using a rotary evaporator. The viscous yellowish product obtained was dissolved in methanol and concentrated again by evaporation of the solvent at 45°C [16].

3. Results and discussion

The application of statistical experimental design techniques in bioprocess development and optimization can result in enhanced product yields, closer conformance of the process output or response to target requirements and reduced process variability, development time and cost. Response surface methodology is a statistical technique employed for multiple regression analysis by using quantitative data obtained from properly designed experiments to solve multivariate equations simultaneously [17,18]. The graphical representations of these equations are called response surfaces, which can be used as tools to understand complex bioprocesses and to describe the individual, cumulative and interactive effects of the test variables on the process yield and hence the process economics. Medium composition, agitation speed, time of cultivation and inoculum size are examples of such parameters, which affect productivity of industrial processes.

A Central Composite Rotatable Design (CCRD) is a procedure with the purpose of screening important variables in the biosynthesis of surface-active compounds, by means of which optimum process conditions may be approached [13].

Thus, the initial results focused on the experimental design and the effects of the studied variables on biosurfactant production. Table 2 shows the experimental results of surface tension together with the process variables that were studied in the CCRD. Central point repetition was done aiming to provide the opportunity of detection of measurement errors and to subsequently use the deviation in the calculation of surface tension to obtain the variance [19].

An analysis of surface tension values in Table 2 shows that the respective maximum and minimum were obtained in runs 1 and 12, i.e., it occured a minimum of surface tension by passing conditions of minimal concentrations of the independent variables X_1, X_2 and X_3 to the average conditions of X_1 and X_3, and axial maximum X_2 condition. Therefore, it is necessary to further define the range of concentrations of corn steep liquor variable (X_3).

The summary of the analysis of variance (ANOVA) representing the results of the quadratic response surface model fitting is shown in Table 3. ANOVA is essential to test the significance and adequacy of the model. The p-value and *F*-value (with 95% confidence interval) were used as tools to check the significance of each studied variable and their interactions. This can be observed by the value of *F* or by the Pure Error. These parameters reached values much larger than 4. According to Box [20], Fisher variance ratio must be large enough to justify a very high degree of adequacy of the model and also to indicate that the treatment combinations are highly significant. The squared regression statistic (R^2), the determination coefficient, a measure of the goodness of fit of the model,

was very much significant at the level of 89%, meaning that the model was unable to explain only 11% of the total variations. The adjusted R^2 value (79%) also indicates the significance of the model.

Please insert Table 2

The effects of waste frying oil, corn steep liquor and $NaNO_3$ concentrations on surface tension are shown in Fig. 1. The Pareto's chart shows that all the linear and quadratic terms and the interaction between them are statistically significant ($p > 0.05$).

Waste frying oil concentration was the most important factor affecting the reduction of surface tension of cell-free culture broth, followed by corn steep liquor-$NaNO_3$ interaction. The waste frying oil shows a positive effect on surface tension reduction. This positive correlation that existed between oil concentration and surface tension implies that a higher amount is more effective in reducing surface tension values in the experimental limits chosen. Corn steep liquor and $NaNO_3$ also show their significance in the production process although at a much lower level. A negative effect of these nitrogen sources predicts a decrease in production upon increasing their concentration in the medium.

Please insert Fig. 1.

The application of response surface methodology on the basis of parameter estimate results in an empirical relationship between the surface tension values and the process variables. Thus, the following regression equation (1) shows the relative surface tension value (Y) as a function of the test variables (X_i) in coded units:

$$Y = 193.254 - 139.648 \cdot X_1 - 37.490 \cdot X_2 + 8.300 \cdot X_1 \cdot X_2 + \\ + 21.645 \cdot X_1^2 + 4.74 \cdot X_2^2 \qquad (1)$$

$$Y = 142.251 - 116.850 \cdot X_1 - 136.354 \cdot X_3 - 31.100 \cdot X_1 \cdot X_3 + \\ + 21.645 \cdot X_1^2 + 538.135 \cdot X_3^2 \quad (2)$$

$$Y = 109.442 - 41.730 \cdot X_2 - 403.350 \cdot X_3 + 102.40 \cdot X_2 \cdot X_3 + \\ + 4.740 \cdot X_2^2 + 538.135 \cdot X_3^2 \quad (3)$$

Where Y is the response, X_1, X_2 and X_3 are the coded values of the waste frying oil, corn steep liquor and $NaNO_3$, respectively. The coefficients of Eqs. (1), (2) and (3) were calculated using RSM.

The general equation involving the surface tension as a function of the independente variables is given by:

$$Y = 239.960 - 108.548 \cdot X_1 - 58.330 \cdot X_2 - 341.154 \cdot X_3 + 8.300 \cdot X_1 \cdot X_2 - \\ - 31.100 \cdot X_1 \cdot X_3 + 102.400 \cdot X_2 \cdot X_3 + 21.645 \cdot X_1^2 + 4.738 \cdot X_2^2 + \\ + 538.135 \cdot X_3^2 \quad (4)$$

Correlations between the experimental and calculated values in the range of 95% are shown in Table 3.

Please insert Table 3

Fig. 2 represents the relationship between the actual surface tension values and the predicted values determined by the general Eq. 4 for *P. cepacia* CCT6659. Clearly, most points were nearby the line adjustment, meaning that the experimentally determined value were similar to those determined by the model (R^2 = 89.14%).

Please insert Fig. 2.

Figure 3 shows the three dimensional plots for the minimum surface tension. RSM plots were generated using the data shown in Table 3, according to Eq, 1, 2 and 3. The Fig. 3(A) presents corn steep liquor and $NaNO_3$ concentrations effects on reduction of surface tension. As can be seen from Fig. 3(A) the minimal and maximum limits of these variables increase the surface tension of the medium, while the combination of a maximum value of corn steep liquor and a minimum of $NaNO_3$ bring the best value of surface tension in the conditions tested in this work. It can be observed from the level curves obtained by (xy) projection of the surface response plot a high interaction between the factors, i.e. it is not possible to predict the biosurfactant properties by modification of only one of these factors.

Fig. 3(B) shows corn steep liquor and canola waste frying oil concentrations effects on biosurfactants production. It can be seen from the graphic a very well delimited region reflection the optimized conditions of biosurfactant production around 2.5 % corn steep liquor and 2.3 % waste frying oil. In this region the absence of level curves show a considerable interaction between the factors (as a function of the parallelism between these curves in the most involved region). It would be not possible to make predictions about the surface tension from variations in the concentration of one of these factors.

Fig. 3(B) shows waste frying oil and $NaNO_3$ concentrations effects on biosurfactants production. A condition around 2.2 % waste frying oil and 0.2 % $NaNO_3$ produce a satisfactory surface tension. The level curves from this figure show a moderate interaction between these factors. It would be not impossible to make predictions about the surface tension from variations in the concentration of one of these factors.

Fig. 1(C) also shows a delimited region of optimized experimental conditions for the biosurfactant production from the concentrations of waste frying oil and $NaNO_3$. A

condition around 2.2 % waste frying oil and 0.2 % $NaNO_3$ produces a low and desirable surface tension. The level curves also show a weak interaction between the factors. Therefore, it becomes possible to make predictions about the surface tension based on the variation of one of these factors.

Please insert Fig. 3.

Microbial surfactants are not yet widely used due to high production costs, part of them associated with the use of expensive substrates. The problem of economic production of biosurfactants can be significantly reduced through the use of alternative sources of nutrients, readily available, low cost and enabling high concentrations of biosurfactant. In addition, the optimization of culture conditions allows the increase in production levels [20]. Data presented in the literature show that the production of rhamnolipids by bacteria of the genus *Pseudomonas* is strongly influenced by carbon and nitrogen sources and multivalent cations [21]. Based on these considerations, we have used Canola waste frying oil and corn steep liquor as the alternative sources of nutrient to study the production of a biosurfactant by the strain *P. cepacia* CCT6659 not studied before for biosurfactant production while $NaNO_3$ was used as the inorganic salt.

Different low cost substrates have been investigated for biosurfactants production and the results are described in the literature. Glycerol, cassava wastewater, waste cooking oil, hydrolyzed glycerin have been cited [21,22,23,24].

The preference of nitrate as a nitrogen source for the production of rhamnolipids by bacterias of the genus *Pseudomonas* is quite studied in the literature [25,26]. Based on these results, $NaNO_3$ was used in combination with Canola waste frying oil and corn steep liquor to study the effect of nutritional conditions on the production of the biosurfactants by *P. cepacia* CCT6659 according to the factorial design.

Thus, the medium formulated with 2% waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$ (run number 12), for which a surface tension of 26 mN/m was selected for biosurfactant production. A biosurfactant yield of 8g/l was obtained at these optimized medium constituents.

Conclusions

This work demonstrated the effectiveness and feasibility of using composite rotating designs (CCRD) to identify better medium composition for enhanced production of the biosurfactant from *P. cepacia*. Thus, it was found to be very useful in determining the relevant variables for further optimization, making it possible to consider a large number of variables and avoid information loss, both of which are essential in the optimization process. Canola waste frying oil, corn steep liquor and $NaNO_3$ were identified as important parameters for improving biosurfactant production. In conclusion, the methodology of CCRD proved to be very effective in improving biosurfactant production with increased production from 2 to 8g/L. By increasing the biosurfactant yield via this experimental design approach, the production cost of this biomolecule would markedly be reduced, enhancing feasibility of commercial application of this new and promising biosurfactant.

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Table 1

Experimental range and levels of the independent variables studied in the Central Composite Rotatable Design (CCRD)

Test variables	Range and levels				
	-1.68	**-1**	**0**	**+1**	**+1.68**
Waste frying oil (%), X_1	1.16	1.5	2.0	2.5	2.84
Corn steep liquor (%), X_2	1.16	1.5	2.0	2.5	2.84
$NaNO_3$ (%), X_3	0.12	0.15	0.20	0.25	0.28

Table 2

Experimental design matrix of biosurfactant production by *P. cepacia* CCT6659 according to the CCRD

Run Number	Waste frying Oil (%) X_1	Corn steep liquor (%) X_2	$NaNO_3$ (%) X_3	Surface tension (mN/m) Y
1	-1(1.5)	-1(1.5)	-1(0.15)	48.15
2	-1(1.5)	-1(1.5)	+1(0.25)	45.01
3	-1(1.5)	+1(2.5)	-1(0.15)	33.30
4	-1(1.5)	+1(2.5)	+1(0.25)	45.42
5	+1(2.5)	-1(1.5)	-1(0.15)	30.93
6	+1(2.5)	-1(1.5)	+1(0.25)	29.70
7	+1(2.50)	+1(2.50)	-1(0.15)	29.40
8	+1(2.50)	+1(2.50)	+1(0.25)	33.39
9	-1.68(1.16)	0(2.00)	0(0.20)	48.03
10	+1.68(2.84)	0(2.00)	0(0.20)	29.79
11	0(2.00)	-1.68(1.16)	0(0.20)	27.97
12	0(2.00)	+1.68(2.84)	0(0.20)	25.94
13	0(2.00)	0(2.00)	-1.68(0.12)	27.51
14	0(2.00)	0(2.00)	+1.68(0.28)	27.31
15	0(2.00)	0(2.00)	0(0.20)	27.01
16	0(2.00)	0(2.00)	0(0.20)	26.98
17	0(2.00)	0(2.00)	0(0.20)	26.78
18	0(2.00)	0(2.00)	0(0.20)	26.86
19	0(2.00)	0(2.00)	0(0.20)	26.94
20	0(2.00)	0(2.00)	0(0.20)	27.00

Table 3

Analysis of variance (ANOVA) for the quadratic model

Factor	Square sum (SS)	Degree of freedom	Mean square (MS)	*F-ratio*	*p*-value*
X_1 (L)	458.560	1	458.5603	8404.8	0.000000
X_1 (Q)	421.978	1	421.9784	7734.3	0.000000
X_2 (L)	18.035	1	18.0351	330.6	0.000000
X_2 (Q)	20.219	1	20.2186	370.6	0.000000
X_3 (L)	9.522	1	9.5222	174.5	0.000000
X_3 (Q)	26.083	1	26.0834	478.0	0.000000
X_1 by X_2	34.445	1	34.4450	631.3	0.000000
X_1 by X_3	4.836	1	4.8361	88.6	0.000002
X_2 by X_3	52.429	1	52.4288	960.9	0.000000
Lack of Fit	123.561	5	24.7122	592.6	0.000000
Pure Error	0.041	5	0.0083	-	-
Total SS	1137.749	19	-	-	-
Some important statistics:			R^2 (%) = 89.14; Adj., 79.36		

* $p \leq 0.05$ - significance at the 5% level; (L) – linear effect; (Q) – quadratic effect.

FIGURES CAPTIONS

Fig. 1. CCRD - Pareto's Chart of standardized effects for (X_1) canola waste frying oil, (X_2) corn steep liquor and (X_3) $NaNO_3$ using surface tension as response variable. The point at which the effect estimates were statistically significant ($p = 0.050$) is indicated by dashed line

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

Fig. 3. Three dimensional plots for the minimum surface tension (maximum biosurfactants production). RSM plots were generated using the data shown in Table 3. Inputs were the 20 experimental runs carried out under the conditions established by the CCD design. (A) Reduction in surface tension as a function of corn steep liquor and $NaNO_3$ concentrations. (B) Reduction in surface tension as a function of corn steep liquor and canola waste frying oil concentrations. (C) Reduction in surface tension as a function of canola waste frying oil and $NaNO_3$ concentrations.

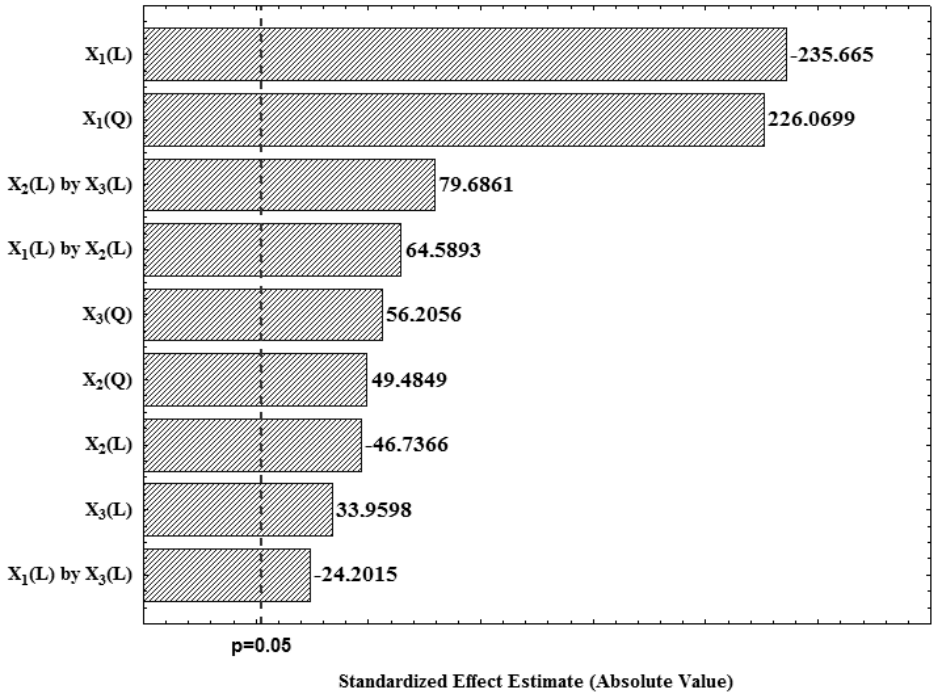
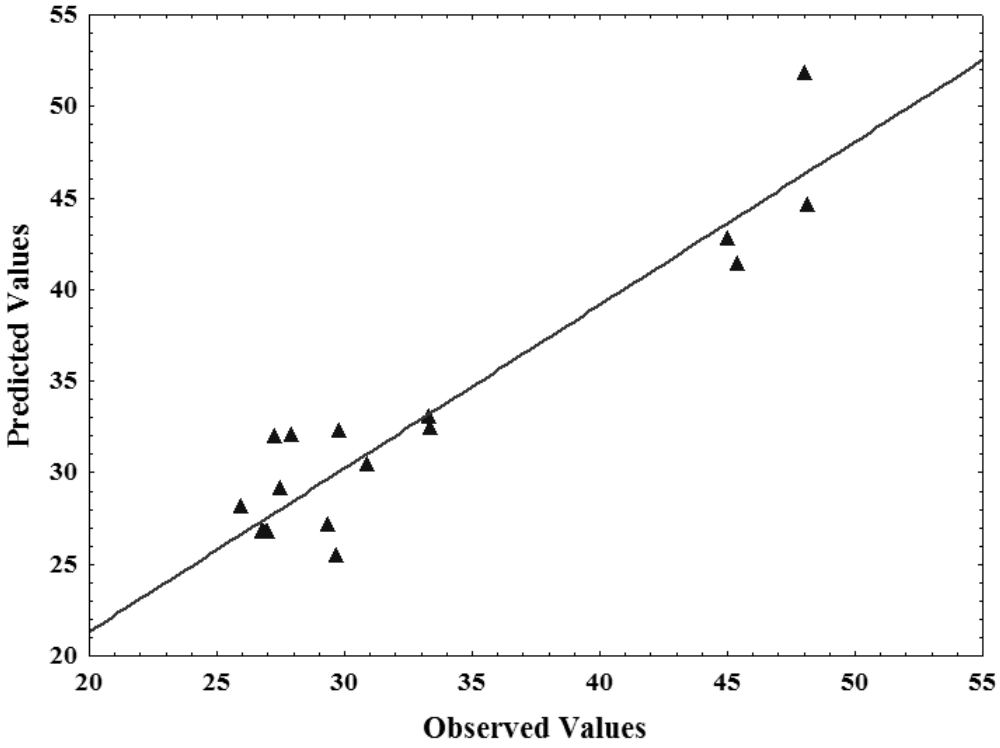

X1(L)
-235.665
X1(Q)
226.0699
X2(L) by X3(L)
79.6861
X1(L) by X2(L)
64.5893
X3(Q)
56.2056
X2(Q)
49.4849
X2(L)
-46.7366
X3(L)
33.9598
X1(L) by X3(L)
-24.2015
p=0.05
Standardized Effect Estimate (Absolute Value)

Figure 1


Predicted Values
Observed Values
55
50
45
40
35
30
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20
20
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50
55

Figure 2

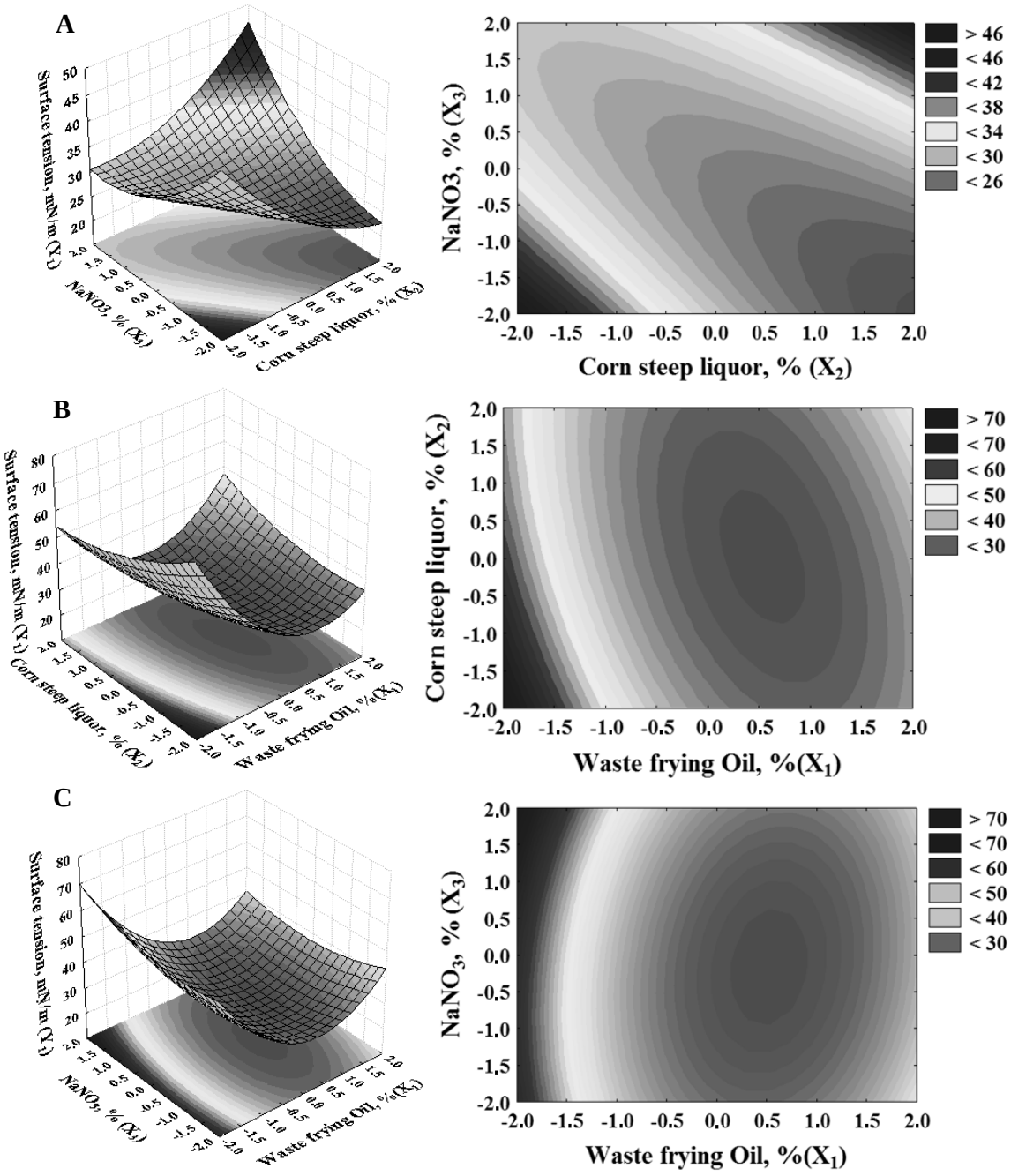
A
Surface tension, mN/m (Y1)
NaNO3, % (X3)
Corn steep liquor, % (X2)
NaNO3, % (X3)
Corn steep liquor, % (X2)
> 46
< 46
< 42
< 38
< 34
< 30
< 26
B
Surface tension, mN/m (Y1)
Corn steep liquor, % (X2)
Waste frying Oil, %(X1)
Corn steep liquor, % (X2)
Waste frying Oil, %(X1)
> 70
< 70
< 60
< 50
< 40
< 30
C
Surface tension, mN/m (Y1)
NaNO3, % (X3)
Waste frying Oil, %(X1)
NaNO3, % (X3)
Waste frying Oil, %(X1)
> 70
< 70
< 60
< 50
< 40
< 30

Figure 3

CAPÍTULO 3

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PRODUCTION OF A NEW BIOSURFACTANT BY *Pseudomonas cepacia* CCT6659 USING LOW-COST FERMENTATIVE MEDIUM

PRODUCTION OF A NEW BIOSURFACTANT BY *Pseudomonas cepacia* CCT6659 USING LOW-COST FERMENTATIVE MEDIUM

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Abstract

The production of a biosurfactant by *Pseudomonas cepacia* CCT6659 was studied in a low-cost medium formulated with 2% canola waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$ during 60 hours at 28ºC under 250 rpm. The production of biosurfactant was growth associated as indicated by the growth and biosurfactant production kinetics. The surface tension was reduced to below 27 mN/m. The properties of biosurfactant that was separated by acid precipitation and organic solvent extraction were investigated and its CMC determined. Preliminary chemical characterization revealed the anionic nature of the biosurfactant. Compositional analysis of the produced biosurfactant has been carried out by thin layer chromatography (TLC). The biosurfactant produced by the isolate was characterized as glycolipid derivative. It had a good surface tension reduction capacity and emulsifying activity against motor oil (up to 90%). It showed stability during exposure to high temperatures (up to 120°C for 15 min), high salinity (12% NaCl) and a wide range of pH (2-12). The crude biosurfactant did not show toxicity against the microcrustacean *Artemia salina* and against two cabbage species (*Brassica oleracea*). The cell-free broth (crude biosurfactant) was also effective in recovering up to 75% of the residual oil from oil-saturated sand samples and in oil displacement (65%). The crude biosurfactant from *P. cepacia* was also effective in recovery of up to 90% motor oil from the walls of beakers. These results indicate the potential value of the biosurfactant for application in the oil industry, especially in enhanced oil recovery, tank cleaning and in bioremediation of spills at seas and soils.

Keywords: Biosurfactant; Production; *Pseudomonas*; Characterization; Stability; Oil; Surface tension.

1. Introduction

Surfactants are amphiphilic molecules that partition at oil/water and air/water interfaces. This allows surfactants to reduce the surface tension and interfacial tension. As surfactant concentration increases, surface tension decreases from 72 mN/m (surface tension of pure water) to 25 to 35 mN/m when the critical micelle concentration (CMC) is reached. Surface tension does not decrease substantially at surfactant concentrations above the CMC. At concentrations below the CMC surfactants partition at the air-water interface to maximize contact of the hydrophilic moiety with water and minimize contact of the hydrophobic moiety with the water. At surfactant concentrations above the CMC all the surface sites are occupied and surfactants begin forming micelles, with the hydrophobic moieties in the center and the hydrophilic moieties on the exterior. The hydrophobic interior of micelles can partition nonaqueous phase liquids (NAPL), which allows their emulsion in water. Above the CMC, micelle content increases linearly with increasing surfactant concentration and so does the capacity to emulsify NAPL.

Biosurfactants are produced naturally by many microorganisms such as bacteria, yeasts, and fungi [1,2]. Biosurfactants consist of common cell material (e.g., glycolipids, lipopeptides, and fatty acids). Biosurfactants are widely used in different industries such as cosmetics, special chemicals, food, pharmaceutics, agriculture, cleansers and petroleum [3-8].

The most important advantage of biosurfactants over chemical surfactants is probably their ecological acceptability. Biosurfactants are biodegradable and thus problems of toxicity and accumulation in natural ecosystems are avoided In the environmental sector, bio- surfactants have potential applications in bioremediation and waste treatment because of their inherent degradability. [9,10].

A major obstacle on the way of wide-scale industrial application of biosurfactant is the high production cost coupled with less production rate as compared to commercially available synthetic surfactants. Therefore, if the production cost becomes competitive with the synthetic surfactants, and as the commercial availability of biosurfactant increases, the industrial use of biosurfactant can be expected to grow tremendously in the coming decade. To achieve this goal, during the recent years, efforts have been directed to explore the means to reduce the biosurfactant production costs through improving the yield, and the use of either cost-free or low-cost feed stocks or agricultural byproducts as substrate(s) for biosurfactant production. Many of the cheaper byproducts such as peat hydrolysate [11], olive-oil mill effluent [12], soapstock and waste-water from sunflower oil [13], de-proteinized whey [14], vegetable oil refinery residue and corn steep liquor [15-17], waste frying oil [18], vegetable fat waste [19], wheat bran and okara [20, 21], molasses [22] and potato effluent [23] have been targeted as sole source of carbon for biosurfactant production by microbes in submerged fermentation.

Apart from the industrial applications of biosurfactants envisage, their application in the oil industry is one of the potential uses which requires lower purity specifications so that whole cell broth could be used, eliminating the purification steps that represent almost 60% of the total production costs [24].

Various studies on the production and characterization of rhamnolipids produced by *Pseudomonas* genus using low-cost and renewable raw material have been reported in literature. However, to our knowledge, no reports have been published on biosurfactant production from industrial residues by the *P. capacia* strain.

Thus, environmental and economic issues have motivated the completion of this study that presents biosurfactant production by a *P. cepacia* strain, coded as *P. cepacia* CCT6659, using a previously optimized mineral low-cost medium supplemented with

waste frying oil and corn steep liquor as substrates [25]. This study also describes the kintetics of biosurfactant production, its characterization, surface active properties, emulsifying capacity and toxicity. The application of the biosurfactant in the environment was also investigated.

2. Materials and methods

2.1. Materials

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

2.2. Bacterial strain and preparation of seed culture

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

2.3. Fermentation media

Production media that was used for liquid submerged fermentation have the following composition (%): canola waste frying oil (2), corn steep liquor(3), $NaNO_3$ (0.2),

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

The kinetics of microorganism growth and biosurfactant production were monitored along fermentation. At regular intervals, different process parameters such as growth, pH, surface tension, and biosurfactant concentration were evaluated.

2.4. Biomass determination

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

2.5. Emulsifying activity with different hydrophobic compounds

Emulsification index (EI) was measured using the method described by Cooper and Goldenberg (1987) [26] whereby 2 ml of a liquid hydrophobic compound (motor oil, lubricating oil, diesel, kerosene, n-hexadecane and vegetables oils) was added to 2 ml of the culture broth free of cells in a graduated screwcap test tube, and vortexed at high speed for 2 min. The emulsion stability was determined after 24 h and the emulsification index

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

2.6. Surface tension and CMC determination

The surface tension of the culture supernatants obtained by centrifuging the cultures at 5 000 g for 20 minutes was measured using a Sigma 700 digital surface tensiometer (KSV Instruments LTD - Finland) working on the principle of the Du Nuoy ring method. Ten milliliters volume of each sample was transferred into a clean 20 ml beaker and placed onto the tensiometer platform. A platinum wire ring was submerged into the solution and then slowly pulled through the liquid–air interface, to measure the surface tension (mN/m). Between each measurement, the platinum wire ring was rinsed with chromic acid, deionised water, acetone and finally flamed and was allowed to dry. The calibration was done using Mill-Q-4 ultrapure distilled water (surface tension =71.5 mN/m ±0.5) before taking samples measurement.

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

2.7. Effect of environmental factors on biosurfactant activity

The effect of addition of different concentrations of NaCl on the activity of the biosurfactant was investigated in the cell-free broth. A specific concentration of NaCl (2-

12%, w/v) was added and surface tension and emulsification activity were determined as previously stated. The cell-free broth was also maintained at a constant temperature (0, 5, 28, 70, 100 and 120°C) for 15 min and used for surface tension and emulsification measurements. The effect of pH on surface tension and emulsification was evaluated after adjustment of the broth pH to 2, 4, 6, 8, 10 and 12 with 6.0 M NaOH or HCl.

2.8. Biosurfactant isolation

The biosurfactants was extracted from culture media after cell removal by centrifugation at 5 000g for 30 min. The supernatant pH was adjusted to 2.0 with 6.0 M HCl, and an equal volume of $CHCl_3$/CH_3OH (2:1) was added. The mixture was vigorously shaken for 15 min and allowed to set until phase separation. The organic phase was removed and the operation was repeated twice again. The product was concentrated from the pooled organic phases using a rotary evaporator. The viscous yellowish product obtained was dissolved in methanol and concentrated again by evaporation of the solvent at 45°C [27].

2.9. Biosurfactant characterization by thin-layer chromatography

After isolating the biosurfactant, a sample of 0.1g was dissolved in methanol and analysed by thin layer chromatography (TLC) on silica gel plates (G60; Merck, Germany). Chromatograms were developed with chloroform: methanol:acetic acid (65:15:2, v/v) and the detection was done by the following methods: (1) exposure to iodine vapours for lipid stains, (2) exposure to the Molish reagent for sugar detection and (3) exposure to 1% ninhydrin solution for free amino groups. The reagents were sprayed, and the plates were heated for 30 to 40 min at 110°C until the appearance of the respective colour [28, 29].

2.10. Determination of biosurfactant ionic character

The ionic charge of the biosurfactant was determined using the agar double diffusion technique [30].Two regularly spaced rows of wells were made in an agar of low hardness (1% agar). Wells of one row were filled with the biosurfactant solution and wells of the other were filled a pure compound of known ionic charge.

The anionic substance chosen was sodium dodecyl sulphate (SDS) 20 Mm and the cationic one was barium chloride, 50 mM. The appearance of precipitation lines between the wells, indicative of the ionic character of the biosurfactant, was monitored over a 48-h period at ambient temperature.

2.11. Phytotoxicity assay

The phytotoxicity of the biosurfactant was evaluated in static test by seed germination and root elongation of two cabbages species (*Brassica oleracea* var. *botrytis* L. *and Brassica oleracea* var. *capitata*) according to Tiquia et al. (1996) [31]. Solutions of the isolated biosurfactant were prepared with distilled water in concentrations of 0.8, 1.6 and 3.2%. The toxicity was determined in sterilized Petri dishes (1x10cm) containing Whatman Nº 1 filter paper. The seeds were pre-treated with Sodium hypochlorite and 10 seeds were inoculated in each Petri dish which was inoculated with 5 ml of the test solution at 27ºC. After five days of incubation in the dark, the seed germination, root elongation (≥ 5 mm) and germination index (GI, a factor of relative seed germination and relative root elongation) were determined as follows:

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

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

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

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

2.12. Artemia assay

The toxicity assay was performed with the isolated biosurfactant using brine shrimp (the microcrustacean *Artemia salina*) as the toxicity indicator. Brine shrimp eggs were obtained in a local story. Larvae were used within 1 day of hatching.

Following dilutions of a biosurfactant solution at the CMC (1.6%) with saline water (33 g/l) to give concentrations of 0.8, 1.6 and 3.2%, the assays were conducted in penicillin tubes of 10 ml capacity containing 10 brine shrimp larvae in 5 ml of saline water per tube. The brine shrimp larvae in each tube were tested using 5 ml per concentration level of biosurfactant solution. They were observed for 24 hours to calculate mortality [32].The toxicity threshold concentration, expressed as biosurfactant concentration per 100 ml of saline water, was defined as the lowest concentration that kills all tested brine shrimp within 24 hours. Each test was run in triplicate, and saline water was used as the control.

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

Biosurfactant suitability for enhanced oil recovery was carried out using artificially contaminated sand with 10% of motor oil. Samples of 50 g of 40/50 mesh (0.3-0.42mm) and 20/30 mesh (0.6-0.85mm) fractions of the contaminated Brazilian standard sand NBR

7214 [33] were transferred to 250-ml Erlenmeyer flasks, which were submitted to the following treatments: addition of 50 ml distilled water (control) or 50 ml of the cell-free broth or 50 ml of a solution of the isolated biosurfactant at 0.8, 1.6 and 3.2%. The samples were incubated on a rotary shaker (150 rev/min) for 24 h at 27 °C and then were centrifuged at 5 000 g for 20 minutes for separation of the laundering solution and the sand. The pH of the samples was also measured before and after the treatment. The amount of oil residing in the sand after the impact of biosurfactant was gravimetrically determined as the amount of material extracted from the sand by hexane [34].

2.14. Application of the biosurfactant in hydrophobic contaminant spreading

The oil displacement test was carried out slowly by dropping of 15 µl of motor oil onto the surface of 40 ml of distilled water layer contained in a Petri dish (15 cm in diameter) that spread all over the water surface area. This was followed with the addition of 10 µl of the cell-free broth or aqueous solutions containing the isolated surfactant at 0.8, 1.6 and 3.2% onto the surface of the oil layer. The average value of the diameters of the clear zones of triplicate experiments was measured and recorded then calculated as percentage of the Petri dish diameter [20].

2.15. Application of the biosurfactant in hydrophobic contaminant cleaning test

As a means to check the cleaning ability of the biosurfactant, the inner walls of a set of beakers were coated with motor oil. To remove the adhered oil, 50.0 mL of the cell-free broth or wash solutions containing 0.8, 1.6 and 3.2% aqueous solution of the isolated biosurfactant was added to each beaker, vortexed for 1.0 min, and allowed to stand for 6 h [35].

2.16. Statistical analysis

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

3. Results and discussion

3.1. Biosurfactant production and growth kinetics

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

Fig. 1 shows the pattern of biosurfactant formation and cell growth of *P. cepacia* CCT6659 in the mineral previously optimized medium [25] containing 2% waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$ at 28 ºC during 60 hours under agitation speed of 250 rpm. *P cepacia* started to produce biosurfactant soon after inoculation along with cell growth. Surface tension measurements were used as an indirect measure of surfactant production and to evaluate the efficiency of the produced biosurfactant. The culture broth surface tension reached the minimum value of 27 mN/m after 12 h, while the accumulation of biosurfactant was gradually increasing. The maximum biosurfactant production (8 g/L) occurred during the stationary phase of the culture (48-60h). At this point, it was observed not only the maximum biosurfactant accumulation, but also the highest biomass concentration (about 15.0 g/L dry weight). Biosurfactant production by *P. cepacia* was growth-associated. There was an almost parallel relationship between biosurfactant production, cell growth and surface tension reduction.

In fermentation without pH control, its value is the result of the microorganism metabolism. For the cultivation of *P. cepacia* CCT6659, the pH showed small variations between 6.0 and 7.0 especially during the first 24 hours, remaining more stable after 36 h around 7.0 until the end of cultivation.

Please insert Fig.1.

The literature describes the occurrence of different kinetic profiles for biosurfactant production. The growth-associated production of biosurfactants had been reported from several researches. [36] for example, observed that the production of the biosurfactant from *P. aeruginosa* cultivated in an optimized medium containing 2% acidified soybean oil was found to be a function of cell growth. Biosurfactant was produced at a concentration of 5.0 g/L, with a cell concentration of 25 g/L.[37], on the other hand, observed that the production of 9.2 g/L rhamnolipid biosurfactants using orange fruit peelings from *P aeruginosa* MTCC2297 was growth independent. A surface tension reduction up to 31.1 mN/m was obtained.

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

Wu et al. [38] described the production of 3.7 and 2.6 g/L of biosurfactant for olive oil and soybean oil, respectively, from *P. aeruginosa* EM1, while Souza et al. (2011) obtained 1.2 g/L biosurfactant from *P. aeruginosa* MSIC02 grown in hydrolyzed glycerin for a surface tension value of 29.3 mN/m.

Oliveira et al. [39] used experimental design tools to study the effects of process conditions on surfactant production during batch tests conducted using a strain of *P.*

alcaligenes growing on palm oil. The authors obtained 2.3 g/L biosurfactant after 48 h of bioprocess, with a surface tension of 31 mN/m. The use of sequencing batch reactors for biosurfactant production from *P. aeruginosa* SP4 growing in palm oil and glucose during 48 h showed a surface tension reduction to 28-30 mN/m [40].

3.2. Biosurfactant emulsification capacity

A practical measurement of a surface-active compound utility is its ability to turn immiscible liquids into stable emulsions. Table 1 presents the hydrophobic substrates tested for emulsification by the cell-free broth containing the biosurfactant from *P cepacia* CCT6659. Motor oil of car engine was the best substrate while n-hexadecane and soybean vegetable oil were the poorest. The cell-free broth containing the biosurfactant obtained after 60 hours of cultivation was able to emulsify 90% motor oil. The water-oil emulsions showed to be compact and remained stable for more than six months at room temperature, suggesting that the addition of such biosurfactant into a remediation process may enhance the availability of the recalcitrant hydrocarbon.

The vegetable oils were particularly not good substrates for emulsification by the biosurfactant from *P. cepacia* (data not shown).

Most microbial surfactants are substrate specific, solubilizing or emulsifying different hydrocarbons at different rates. Poor emulsification of some of the hydrocarbons might be due to the inability of the biosurfactants to stabilize the microscopic droplets. In addition to surface and interfacial tension, stabilisation of an oil and water emulsion is commonly used as a surface activity indicator, although the ability of a molecule in forming a stable emulsion is not always associated with the capacity in reducing the surface tension [41].

Please insert Table 1

3.3. Biosurfactant stability related to surface tension and emulsification

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

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

The effect of salinities on the activity of the biosurfactant was evaluated to investigate its applicability in the bioremediation of contaminated marines. Considering that the highest sea salinity in the world is of 3%, the biosurfactant from *P. cepacia* CCT6659 could be applied in saline environments.

The stability of the biosurfactant was also tested over a wide temperature range. The surface tension of the biosurfactant showed to be stable during incubation for 1 h at temperatures ranging from 0 to 120 °C (Table 2). The emulsification indexes of the motor oil were thermally stable, while small variations around 10% were observed for the emulsification of lubricating oil. The surface tension reducing activity and emulsification activity were, in a general mode, quite stable at these used temperatures, indicating the usefulness of the biosurfactant in industries where heating to achieve sterility is of paramount importance.

The stability of the biosurfactant at different pH values is also an important issue that can affect its application spectrum. Studies on reduction of surface tension showed that the best value of surface tension was found at pH 8.0, which is near the natural

biosurfactant pH (7.0). Under alkaline conditions the surface tension was maintained around 28-29 mN/m, while a small increase in the value of surface tension has been observed in the acid region, reaching 32 mN/m at pH 2.0. Regarding the capacity of emulsification by the cell-free broth containing the biosurfactant it was also observed an increase in the emulsification of motor oil by increasing the pH, especially at pH 10.0 and 12.0, for which an emulsification index of 100% was obtained. For the emulsification of lubricating oil, on the other hand, higher emulsification indexes of 100% were obtained in the pH range of 2-6.

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

Considering that the purification accounts for up to 60% of the total production cost of biosurfactants and the economic considerations in the oil industry, most biosurfactants would require either whole-cell culture broths or crude preparations. Therefore, the use of the biosurfactant from *P. cepacia* CCT6659 in its crude form can be considered another advantage of this new biomolecule in the petroleum market.

Please insert Table 2

3.4. Biosurfactant characterization

Most of the reported research on biosurfactant production by *Pseudomonas* strains has focused on the obtainment of the glycolipids rhamnolipids [37]Rhamnolipids comprise a mixture of homologous species R1, R2, R3 and R4, although a great variation of other homologues had been detected. It is known that the properties of rhamnolipids depend on the distribution of these homologues, i.e., on their composition and distribution that vary according to bacterial strain, culture condition, and medium composition Another

possibility to explain these differences can be the presence of impurities such as extracted non-metabolized fatty acids from the culture broth that could influence the surface-active properties.

The crude extract form *P. cepacia* CCT6659 appeared as viscous sticky oily residue with brown colour. The biosurfactant was soluble in aqueous solution and in organic solvents like methanol, chloroform and ethylacetate. Similar physical properties had been described for other rhamnolipids surfactants [24].

Agar double diffusion tests revealed the appearance of precipitation lines between the biosurfactant produced by *P. cepacia* CCT6659 and the cationic compound selected (barium chloride), while no lines had formed between the biosurfactant and the anionic compound (SDS). Under the experimental conditions of this work, this very simple test confirmed the anionic character of the biosurfactant produced. A similar result had been observed for the biosurfactant from *P. aeruginosa* UCP0992 [42] and *P. fluorescens* 495, both submitted to the same test [30].

The biosurfactant extracted from the cell-free broth was developed by thin-layer chromatography (TLC) and visualized with specific reagents and produced a spot with a R*f* (retention factor) of 0.9. The spot showed positive reactions for sugars with Molish reagent and for lipids with iodine vapours, but negative reactions for amino groups with ninhydrin. The presence of both glycosyl units and lipid moieties on the same spots indicated that the sample was a glycolipid.

George and Jayachandran [37], studying the production of biosurfactant by *P.aeruginosa* MTCC2297 cultivated in orange fruit peelings detected spots with R*f* values of 0.19 (dirhamnolipids), 0.36 (monorhamnolipids), 0.59 and 0.71 (various rhamnolipid forms), 0,82 and 0,98 in silica plates eluted with the same system used in his work. [43], on the other hand, observed that *Pseudomonas* sp. cultivated in medium supplemented with

used vegetable oils produced a mixture of two rhamnolipids with a R*f* of 0.7 and 0.45. Arino et al. [44] characterized the rhamnolipid mixture produced by *P. aeruginosa* GL1. The R*f* values for different spots were calculated and it corresponds to R1 0.72 (Rha-$C_{10}C_{10}$,), R2 0.40 (Rha-C_{10}), R3 0.32 (Rha-Rha-$C_{10}C_{10}$) and R4 0.13 (Rha-Rha-C_{10}).

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

The biosurfactant produced by *P. cepacia* CCT6659 is able to reduce the surface tension of supernatant significantly. As seen in Fig. 1, the surface tension of supernatant in all cultures has been drastically decreased from 70 to about 27 mN/m. It has been happened even by the early taken samples that show the production of biosurfactant has taken place at early stage of culture. For further investigation we determined CMC values for the biosurfactant. The presence of the biosurfactant reduced the surface tension, which was proportional to biosurfactant concentration in solutions, until it reached the CMC concentration. The profile of changes in surface tension versus concentration has been presented in Fig. 2. The surface tension of water decreased gradually with increasing biosurfactant concentration from 70 mN/m to 33 mN/m, with a biosurfactant concentration of 1.6% (16000 mg/l), and then remained constant.

This CMC value differs greatly from that of 700 mg/l reported for the rhamnolipids produced by *P aeruginosa* UCP0992 on glycerol [42], or 230 mg/l found for a mixture of seven homologues [45], or 120 mg/l found for a mixture of six homologues from *P. aeruginosa* LB1 cultivated in soapstock [46].

The different CMC values may have resulted from differences in purity and composition of the biosurfactant. In this study, the low purity of the test glycolipid may be responsible for the difference in CMC values between the test and other glycolipids like rhamnolipids described in the literature [24]. Due to the intrinsic variability of the

rhamnolipids accumulated and the complexity of its composition, number and proportions of homologues, presence of unsaturated bonds, branching and length of the aliphatic chain of the rhamnolipid can all affect the CMC and surface tension values between the rhamnolipids produced. The proportions of the homologues produced can also differ with the bacterium, medium and cultivation conditions [38].

Please insert Fig. 2.

3.6. Biosurfactant toxicity

The biosurfactant from *P. cepacia* was tested for its toxicity in a short term bioassay using brine shrimp, as shown in Table 3. The mortality rate of larvae increased with increasing the isolated biosurfactant concentration after 24 hours, while no lethality was observed with the cell-free broth. The acute toxicity tests of the surfactant JE1058BS produced by the bacterium *Gordonia* sp. against two species of marine larvae, *Mysidopsis bahia* (shrimp) and *Menidia beryllina* (fish), also showed the low toxicity of this biosurfactant [47].

Please insert Table 3

The germination index (GI), which combines measures of relative seed germination and relative root elongation, has been used to evaluate the toxicity of the biosurfactant on two cabbages species (*Brassica oleracea*). Considering that a GI value of 80% has been used as an indicator of the disappearance of phytotoxicity [31], the results obtained indicated that solutions of the cell-free broth containing the biosurfactant tested did not show inhibitory effects on the seed germination and root elongation of cabbage, while increasing the concentration of the surfactant reduced the percentage of seed germination (Table 4).

Please insert Table 4

3.7. Application of the biosurfactant in hydrophobic contaminant removal

Oil is one of the most important resources of energy in the modern industrial world. As long as oil is explored, transported, stored and used there will be the risk of a spillage. Oil spills impose a major problem on the environment [48].Various processes have been developed to remove oil from contaminated areas. Among them mechanical recovery of oil by oil sorbents is one of the most promising countermeasures. This process includes the transfer of oil from the contaminated area to some transportable form of temporary storage with the help of oil sorbents [49]. However, in this process most of the used sorbents end up in landfills and incineration, which either produces another source of pollution or increase the oil recovery cost. There is an increased interest in promoting environmental responsibility through cleaning products that have traditionally been discarded after a single use.

Biosurfactants, biologically produced, have been increasingly used in soil washing and oil removal from contaminated areas [50, 7, 51]

In order to investigate the application of the biosurfactant produced by *P. cepacia* CCT6659 in contaminant removal, a preliminary experiment using the cell-free broth containing the surfactant and solutions of the isolated surfactant under, at and above the CMC were performed to verify the removal of a hydrocarbon from sand samples (Table 5).

High percentage removals of motor oil were observed for all solutions. The particle size of the sands did not exercised great influence on the percentage removal of the pollutant, neither the biosurfactant concentration. It could be observed that the cell-free broth containing the crude biosurfactant was very effective in removing motor oil, thus indicating the possible use of the biosurfactant without purification steps, which would increase the production costs.

Please insert Table 5

3.8. Application of the biosurfactant in hydrophobic contaminant spreading

The cell-free broth containing the biosurfactant produced by *P. cepacia* CCT6659 gave a high oil spreading efficiency (65% oil displacement). This was more effective than the aqueous solutions of the isolated biosurfactant at 0.8, 1.6 and 3.2%, which displaced 30, 47 and 50% of the oil. According to Sitohy et al.[52] the biosurfactant produced by *B. subtilis* NRRL B-94*C* (0.1%) gave an oil spreading efficiency of 57% while the well-known industrial surfactant Triton X-100 displaced 80% of the oil at the same concentration.

3.9. Cleaning test

Of the several envisioned industrial applications of the biosurfactants, the greatest potential use is in the MEOR. The cell-free broth from *P. cepacia* CCT6659 was effective in recovery of up to 90% oil from the walls of the beakers, while the aqueous solutions of the isolated biosurfactant at 0.8, 1.6 and 3.2% recovered 78, 80 and 85% of the motor oil, respectively. These results suggest the suitability of the biosurfactant from *P. cepacia* to remove the sticky crude oil from the walls of containers.

Conclusions

Apart from the industrial application of biosurfactant envisaged, their application in oil industry is one of the potentials where lower purity biosurfactant preparations or whole cell broth can be used. However, the biosurfactants need to be stable under the extreme environmental conditions encountered in the oil reservoir such as high temperature, pressure and salinity. The biosurfactant produced by *P. cepacia* showed stability under extreme conditions of pH, temperature and salinity. The crude biosurfactant could reduce the surface tension of the medium to 27mN/m. Our preliminary lab scale results showed

that besides the potent surface activity the crude biosurfactant has high emulsifying activities, capacity to remove hydrophobic contaminants and did not show toxicity. In conclusion, the biosurfactant produced by *P.cepacia* was a kind of preferable surface-active substance, having potential application in bioremediation of hydrocarbons contamination.

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Table 1

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

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

Table 2

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

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

[a] Emulsification index of motor oil

[b] Emulsification index of lubricating oil

Table 3

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

Biosurfactant concentration in saline water (%)	**Mortality of brine shrimp larvae (%)**
0.2	40±0.30
0.4	50±0.13
0.8	60±0.21
1.2	70±0.30
1.6	100±0.10

Table 4

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

Cabbage Seeds	**Germination index (GI) (%)**			
	Cell-free broth	**Isolated biosurfactant at 0.8%**	**Isolated biosurfactant at 1.6%**	**Isolated biosurfactant at 3.2%**
Brassica oleracea var. botrytis L.	80±0.51	50±0.45	NG	NG
Brassica oleracea var.capitata	80±0.61	35±0.39	30±0.29	NG

NG: no germitation

Table 5

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

Removal agent	**Motor oil removal from sand (%)**	
	40/50 mesh (0.3-0.42mm)	**20/30 mesh (0.6-0.85mm)**
Biosurfactant (cell-free broth)	84.0±0.6	76.1±0.3
Solution of the isolated biosurfactant at 0.8%	82.0±0.3	94.8±0.3
Solution of the isolated biosurfactant at 1.6%	92.8±0.6	92.3±0.5
Solution of the isolated biosurfactant at 3.2%	95.0±0.7	96.3±0.3
Control (distilled water)	50.0±0.1	45.2±0.2

FIGURES CAPTIONS

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

Fig. 2. Surface tension versus concentration of the isolated biosurfactant from *P. cepacia* CCT6659 cultivated in mineral medium supplemented with 2% canola waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$ during 60 hours at 250 rpm and 28ºC

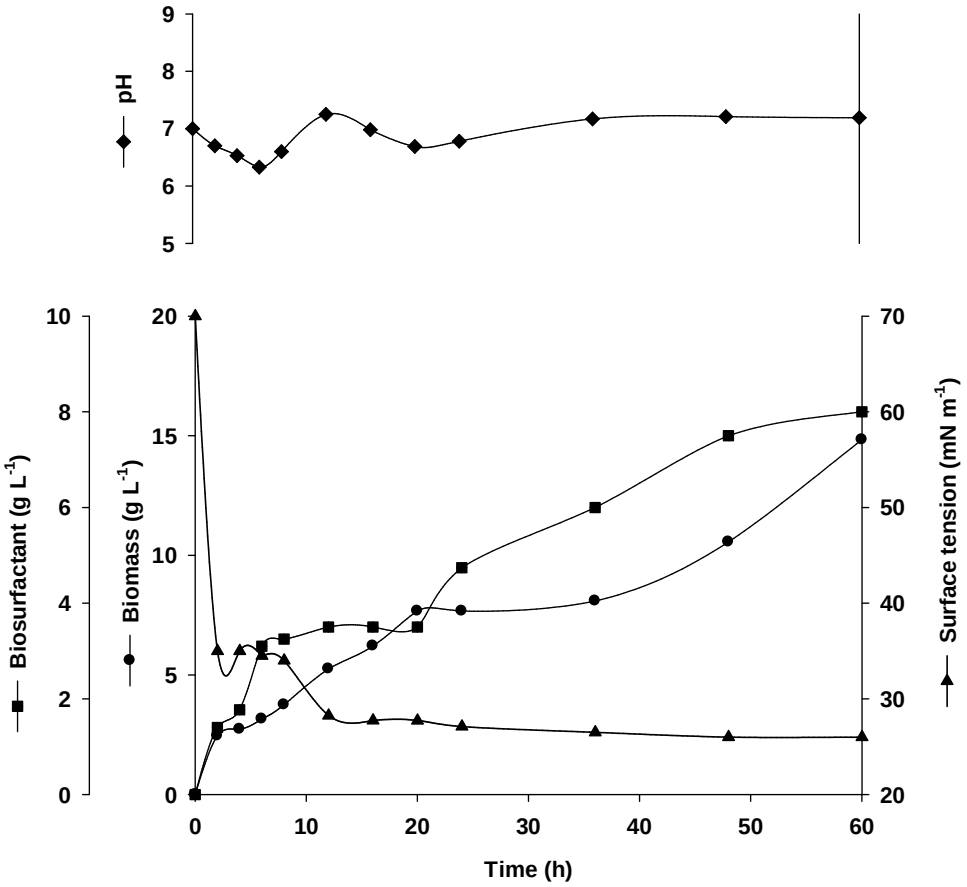
pH
Biosurfactant (g L-1)
Biomass (g L-1)
Surface tension (mN m-1)
Time (h)

Figure 1

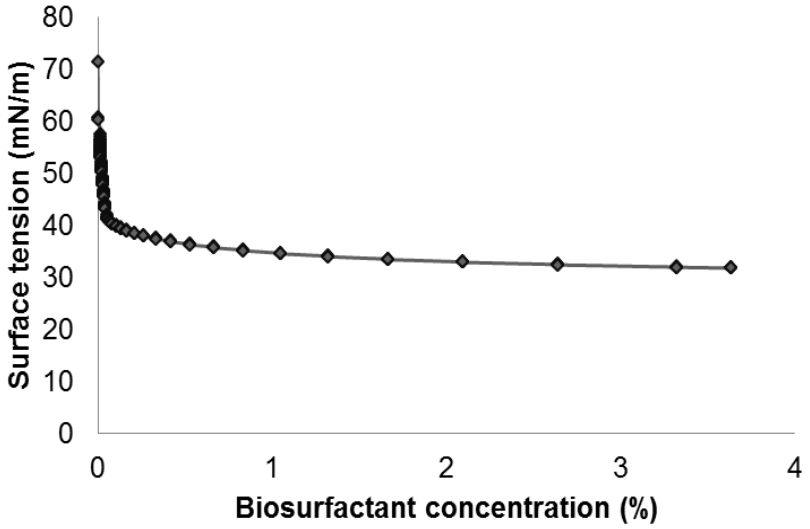
Surface tension (mN/m)
80
70
60
50
40
30
20
10
0
0
1
2
3
4
Biosurfactant concentration (%)

Figure 2

CAPÍTULO 4

CONCLUSÕES GERAIS

Os estudos realizados com a linhagem de *Pseudomonas cepacia* CCT6659 permitem as seguintes conclusões:

- A *P. cepacia* CCT6659 apresenta potencial como micro-organismo produtor de compostos com atividades surfactante e emulsificante.

- A utilização dos resíduos industriais milhocina e óleo de canola residual de fritura apresenta-se como alternativa promissora na formulação de um meio de baixo custo para a produção de um novo biossurfactante.

- A utilização do DCCR (Delineamento Composto Central Rotacional) como ferramenta estatística foi de grande importância para a redução do número de experimentos e consequente otimização da produção do biossurfactante.

- O biossurfactante produzido por *P. cepacia* reduz consideravelmente a tensão superficial da água.

- O biossurfactante produzido por *P. cepacia* apresenta estabilidade em condições extremas de temperatura, pH e concentrações de NaCl.

- O biossurfactante bruto produzido demonstra atividade de superfície satisfatória e elevada atividade emulsionante para derivado de petróleo.

- O biossurfactante bruto não apresenta toxicidade nas condições testadas, enquanto que o biossurfactante isolado pode apresentar toxicidade em função da concentração utilizada.

- A caracterização bioquímica preliminar revelou a natureza glicolipídica e aniônica do biossurfactante.

- O biossurfactante produzido demonstra potencial de utilização como agente surfactante e emulsificante para indústria de petróleo, especialmente na recuperação de óleo, na limpeza de tanques de estocagem e na remediação de derramamentos de óleos em solos e água.

ANEXOS

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ENHANCING OF BIOSURFACTANT PRODUCTION FROM *Pseudomonas cepacia* CCT 6659 THROUGH OPTIMIZATION OF NUTRITIONAL PARAMETERS USING A RESPONSE SURFACE METHODOLOGY

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Enhancement of Biosurfactant Production from *Pseudomonas cepacia* CCT6659 Through Optimisation of Nutritional Parameters Using Response Surface Methodology

The aim of the present study was to optimise the production of a biosurfactant by a new strain of *Pseudomonas cepacia* CCT6659 with aid of a combination of central composite rotatable design (CCRD) and response surface methodology (RSM). The factors selected for optimisation of the growth conditions were canola waste frying oil, corn steep liquor and $NaNO_3$ substrate concentrations. Surface tension was chosen as the response variable. All factors studied were important within the ranges investigated. The empirical forecast model developed through RSM regarding effective nutritional factors was adequate for explaining 89% of the variation observed in biosurfactant production. Maximal reduction in surface tension of 26 mN m^{-1} was obtained under the optimal conditions of 2% waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$. The accumulation of isolated biosurfactant increased from 2 g L^{-1} to 8.0 g L^{-1} under these conditions, demonstrating that the factorial design is adequate for identifying the optimal conditions for biosurfactant production.

Key words: Biosurfactant, optimisation, *Pseudomonas*, RSM, surface activity

Steigerung der Biotensidproduktion aus *Pseudomonas cepacia* CCT6659 über die Optimierung der Ernährungsparameter mit Hilfe der Antwortflächenmethodik. Ziel dieser Untersuchung war, die Produktion eines Biotensids aus dem neuen Stamm *Pseudomonas cepacia* CCT6659 mit Hilfe des „Central Composite Rotatable Design" (CCRD) und der „Response Surface Methodology" (RSM) zu optimieren. Die für die Optimierung der Wachstumsbedingungen ausgewählte Faktoren waren die Konzentrationen des Substrats aus gebrauchtem Fritieraltöl aus Raps, Maisquellwasser und $NaNO_3$. Als Antwortvariable wurde die Oberflächenspannung gewählt. Alle untersuchten Faktoren waren innerhalb des untersuchten Bereichs wichtig. Das mit Hilfe der RSM entwickelte Vorhersagemodell für die effektiven Ernährungsparameter eigente sich zur Erklärung von 89% der beobachteten Variation in der Biotensidproduktion. Man erhielt eine maximale Reduktion der Oberflächenspannng bei folgender optimaler Bedingung: 2% Frittiertaltöl, 3% Maisquellwasser und 0,2% $NaNO_3$. Unter diesen Bedingugnen lag die Anreicherung des isolierten Biotensids zwischen 2 g L^{-1} bis 8,0 g L^{-1}, womit gezeigt werden konnte, dass die faktorielle Versuchsplanung geeignet ist, die optimalen Bedingugnen der Biotensidproduktion zu ermitteln.

Stichwörter: Biotensid, Optimierung, *Pseudomonas*, RSM, Oberflächenaktivität

1 Introduction

Biosurfactants (microbial surfactants) are surface active molecules that are produced from a variety of microorganisms. Due to their amphipathic nature, these biomolecules are capable of lowering surface and interfacial tensions and forming micro-emulsions to enable the blending of two immiscible solutions. Biosurfactants exhibit excellent detergency, emulsifying and foaming properties and are applied in different industrial processes. The features that make biosurfactants commercially promising alternatives to chemically synthesised surfactants are their lower toxicity, higher biodegradability, better foaming properties and greater stability within a wide temperature and pH range [1].

Biosurfactants consist of a variety of chemical structures, such as fatty acids, glycolipids, lipopeptides, lipopolysaccharides, lipoproteins and glycolipids, which depend on the microorganism, raw materials and processing conditions employed. Thus, characterisation and production optimisation studies are required [2]. Glycolipids comprise a class of biosurfactants composed of carbohydrates in combination with long-chain aliphatic or hydroxyl aliphatic fatty acids. Rhamnolipids of the genus *Pseudomonas* are the best known glycolipid surfactants and their potential applications range from the cosmetic, food, pharmaceutical, paper, metal and ceramic industries to environmental uses, such as in bioremediation [3].

As with most biotechnological processes, production costs are the major drawback in biosurfactant production. It is estimated that raw materials account for 10 to 30% of the total production cost in most biotechnological processes [4]. It is therefore desirable to use low-cost raw materials for the production of biosurfactants [5]. A number of elements, medium components and precursors affect the biosurfactant production process as well as the final quantity and quality of the product [6]. A variety of cheap raw materials are reported to support biosurfactant production, including plant-derived oils, starchy substances, lactic whey and distillery wastes [7].

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The identification and optimisation of the cultivation conditions that affect biosurfactant production are key points in the development of a cost-competitive process [8], as the different parameters that control biosurfactant production must be maintained within a certain range in order to optimise the activity of the bacteria and maximise biosurfactant production. The composition of the medium is of considerable importance to the optimisation of biosurfactant production. The amount of biosurfactant synthesis depends greatly on the availability of carbon sources and the balance between carbon and other limiting nutrients [9]. Factors affecting surfactant biosynthesis have been studied extensively, but information on optimal production conditions is scarce. Such studies are important, as biosurfactant-producing microorganisms are more effective when maintained under the optimal ambient conditions required for their growth and activity.

The response surface methodology (RSM) is one of the best methods for designing optimisation experiments. In statistical approaches, this method has been extensively used in the optimisation of fermentation media [10, 11]. RSM is a collection of statistical techniques for designing experiments, building models, simultaneously evaluating the effects of factors and establishing optimal conditions [12]. Indeed, the relationship between the response and independent variables is generally unknown in a process. Thus, the first step in RSM is to approximate the function (response) through analysing factors (independent variables). Ambient growth conditions can also be optimised using this methodology.

The aim of the present study was to investigate nutritional factors that affect the composition of the growth medium for biosurfactant production by a new strain of *Pseudomonas cepacia* on a flask scale using RSM.

1 Materials and Methods

2.1 Materials

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

2.2 Bacterial strain and preparation of seed culture

A strain of *P. cepacia* CCT669 was provided from the culture collection of the Fundação André Tosello de Pesquisa e Tecnologia, city of Campinas, state of São Paulo, Brazil. The cultures were maintained on nutrient agar slants at 4 °C. For pre-culture, the strain from a 24-hour 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.

2.3 Fermentation media

The components of the production medium were dissolved in a mineral medium containing 0.05% KH_2PO_4, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% KCl and 0.001% $FeSO_4 \cdot 7H_2O$ and the pH was adjusted to 7.0 by 1.0 M HCl. Canola waste frying oil, corn steep liquor and $NaNO_3$ were added according to the factorial design. Two percent aliquots (v/v) of the cell suspension (0.7 optical density at 600 nm), corresponding to an inoculum of 10^7 colony-forming units/ml, were used to inoculate 500 ml Erlenmeyer flasks containing 100 ml of sterile production medium. Cultivation was carried out at 27 °C with agitation at 200 rpm for 120 h in a New Brunswick C-24 shaker (New Brunswick Scientific, NJ, USA). No adjustment of pH was performed during cultivation. At the end of fermentation, samples were taken from the liquid culture to determine the surface tension. After the selection of the best medium composition, the biosurfactant yield was determined, as described below.

2.4 Optimisation of culture conditions by RSM

Biosurfactant production was evaluated using an experimental design. A central composite rotatable design (CCRD) was used to determine the effects and interactions of the medium components regarding the production of biosurfactant. Canola waste frying oil, corn steep liquor and $NaNO_3$ concentrations were the independent variables. Surface tension was the response variable. In this design, a set of 20 experiments was performed, with six replicates at the central points. The statistical analysis of the six replicates gives an indication of the experimental error of the production technique. The range and levels of the components (factors or independent variables) are given in Table 1. Each factor in the design was studied on five levels (–1.68, –1.0, 0, +1 and +1.68), with zero as the central coded value. These levels were based on results obtained in preliminary experiments. Based on the factorial design matrix, surface tension was studied with different combinations of the medium constituents.

The optimal values from the CCRD were obtained by solving the regression equation and analysing the response surface contour plots [13, 14]. Analysis of variance (ANOVA) with 95% confidence limits was used to determine the significance of the effects. The effects and significance of the variables were graphically illustrated using Pareto charts. A Pareto chart consists of bars with a length proportional to the absolute value of the estimated effects divided by the standard error. On this chart, ANOVA effect estimates are arranged from the largest to smallest absolute value. The chart includes a vertical line at the critical p-value of 0.05. Effects for which the bars are smaller than the critical p-value are considered non-significant and do not have an effect on the response variables. The effects are either positive or negative.

ANOVA, the determination of regression coefficients and the construction of graphs were performed using the Statistica® program, version 7.0 (Statsoft Inc, USA) [15].

2.5 Determination of surface tension

Changes in surface tension were performed on the cell-free broth obtained by centrifuging the cultures at 5000 × g for

Test variables	Range and levels				
	–1.68	–1	0	+1	+1.68
Waste frying oil (%), X_1	1.16	1.5	2.0	2.5	2.84
Corn steep liquor (%), X_2	1.16	1.5	2.0	2.5	2.84
$NaNO_3$ (%), X_3	0.12	0.15	0.20	0.25	0.28

Table 1 Experimental range and levels of the independent variables studied in the Central Composite Rotatable Design (CCRD)

20 min. Surface tension was determined using a Sigma 70 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 liquid film produced beneath the liquid is stretched. Maximal force is determined as the film is stretched; this force is measured and used to calculate the surface tension. The instrument was calibrated against Mill-Q-4 ultrapure distilled water (Millipore, Illinois, USA). Prior to use, the platinum plate and all glassware were sequentially washed with chromic acid, deionised water and acetone and flamed with a Bunsen burner.

2.6 Biosurfactant isolation

The biosurfactant was extracted from the culture media after cell removal by centrifugation at 5000 × g for 30 min. The supernatant pH was adjusted to 2.0 with 6.0 M HCl and an equal volume of $CHCl_3/CH_3OH$ (2:1) 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 [16].

3 Results and Discussion

The application of statistical experimental designs in the development and optimisation of bioprocesses results in enhanced product yield, closer conformance of the process output or response to target requirements as well as reductions in process variability, development time and costs. RSM is a statistical technique employed for multiple regression analysis using quantitative data obtained from properly designed experiments to solve multivariate equations simultaneously [17, 18]. The graphic representations of these equations are denominated response surfaces, which can be used to understand complex bioprocesses and describe the individual, cumulative and interactive effects of the test variables on the process yield and, consequently, the process economics. Medium composition, agitation speed, cultivation time and inoculum size are examples of parameters that affect productivity in industrial processes. CCRD is a procedure used to screen important variables in the biosynthesis of surface-active compounds, by means of which optimal process conditions can be determined [13]. Thus, the initial results focused on the experimental design and the effects of the variables on biosurfactant production. Table 2 displays the experimental results of surface tension together with the process variables studied in the CCRD. Central point repetition was performed to allow the detection of measurement errors and subsequently use the deviation in the calculation of surface tension to obtain the variance [19].

An analysis of the surface tension values listed in Table 2 reveals that the maximum and minimum were obtained in runs 1 and 12, respectively, i.e., minimum surface tension occurred by going from conditions of minimum concentrations of the independent variables X_1, X_2 and X_3 to the average conditions of X_1 and X_3 and axial maximum condition of X_2. Therefore, it is necessary to define the range of concentrations of the corn steep liquor variable (X_3) further.

Table 3 displays a summary of ANOVA representing the results of the fit of the quadratic response surface model. ANOVA is essential to testing the significance and adequacy of a model. The p-value and *F*-value (with 95% confidence interval) were used to check the significance of each variable and respective interactions. This can be observed by the *F*-value or the pure error, which reached values much larger than 4. According to Box [20], Fisher's variance ratio must be large enough to justify a very high degree of adequacy of the model and indicate that the treatment combinations are highly significant. In this table, the value of the explained variance ($R^2 = 89.14\%$) ensures adequate fit ($R = 0.944$), thereby validating the forecast model.

A Pareto chart is another tool used to confirm the results predicted in Table 3, as shown in Figure 1. This Pareto chart shows that all the linear and quadratic terms as well as the interaction between them were statistically significant ($p < 0.05$). The concentration of waste frying oil was the most important factor to the reduction in the surface tension of the cell-free culture broth, followed by the interaction between corn steep liquor and $NaNO_3$. The correlation between waste frying oil concentration and surface tension demonstrates that a greater amount of oil is more effective at reducing surface tension within the experimental limits chosen. Corn steep liquor and $NaNO_3$ were also significant in the production process, albeit to a much lesser degree. A negative effect denotes a decrease in production with the increase in the concentration of these nitrogen sources in the medium.

The application of RSM for the estimation of the optimal parameters results in an empirical relationship between the surface tension values and process variables. The following regression equations (1, 2 and 3) of the forecast models

Run Number	Waste frying Oil (%) X_1	Corn steep liquor, % X_2	$NaNO_3$, % X_3	Surface tension, mN m^{-1} Y
1	−1(1.5)	−1(1.5)	−1(0.15)	48.15
2	−1(1.5)	−1(1.5)	+1(0.25)	45.01
3	−1(1.5)	+1(2.5)	−1(0.15)	33.30
4	−1(1.5)	+1(2.5)	+1(0.25)	45.42
5	+1(2.5)	−1(1.5)	−1(0.15)	30.93
6	+1(2.5)	−1(1.5)	+1(0.25)	29.70
7	+1(2.50)	+1(2.50)	−1(0.15)	29.40
8	+1(2.50)	+1(2.50)	+1(0.25)	33.39
9	−1.68(1.16)	0(2.00)	0(0.20)	48.03
10	+1.68(2.84)	0(2.00)	0(0.20)	29.79
11	0(2.00)	−1.68(1.16)	0(0.20)	27.97
12	0(2.00)	+1.68(2.84)	0(0.20)	25.94
13	0(2.00)	0(2.00)	−1.68(0.12)	27.51
14	0(2.00)	0(2.00)	+1.68(0.28)	27.31
15	0(2.00)	0(2.00)	0(0.20)	27.01
16	0(2.00)	0(2.00)	0(0.20)	26.98
17	0(2.00)	0(2.00)	0(0.20)	26.78
18	0(2.00)	0(2.00)	0(0.20)	26.86
19	0(2.00)	0(2.00)	0(0.20)	26.94
20	0(2.00)	0(2.00)	0(0.20)	27.00

Table 2 Experimental design matrix of biosurfactant production by *P. cepacia* CCT6659 according to the CCRD

show relative surface tension (Y) as a function of the test variables (X_i):

$$Y = 193.254 - 139.648 \cdot X_1 - 37.490 \cdot X_2 + 8.300 \cdot X_1 \cdot X_2 + 21.645 \cdot X_1^2 + 4.74 \cdot X_2^2 \quad (1)$$

$$Y = 142.251 - 116.850 \cdot X_1 - 136.354 \cdot X_3 - 31.100 \cdot X_1 \cdot X_3 + 21.645 \cdot X_1^2 + 538.135 \cdot X_3^2 \quad (2)$$

$$Y = 109.442 - 41.730 \cdot X_2 - 403.350 \cdot X_3 + 102.40 \cdot X_2 \cdot X_3 + 4.740 \cdot X_2^2 + 538.135 \cdot X_3^2 \quad (3)$$

in which Y is the response and X_1, X_2 and X_3 are the coded values of the waste frying oil, corn steep liquor and $NaNO_3$, respectively. The coefficients of Eqs. (1, 2 and 3) were calculated using the Statistica® program, as described in the Materials and Methods section.

The general equation (4) also obtained from the regression coefficients using the Statistica Software is given by:

$$Y = 239.960 - 108.548 \cdot X_1 - 58.330 \cdot X_2 - 341.154 \cdot X_3 + 8.300 \cdot X_1 \cdot X_2 - 31.100 \cdot X_1 \cdot X_3 + 102.400 \cdot X_2 \cdot X_3 + 21.645 \cdot X_1^2 + 4.738 \cdot X_2^2 + 538.135 \cdot X_3^2 \quad (4)$$

Figure 2 shows the three-dimensional plots for minimum surface tension. RSM plots were generated according to Eqs. 1–3. Fig. 2(a) displays the effects of corn steep liquor and $NaNO_3$ concentrations on the reduction in surface tension. The minimum and maximum limits of these variables increased the surface tension of the medium, while the combination of a maximum corn steep liquor value and minimum $NaNO_3$ led to the best surface tension value under the conditions tested in this study (local minimum in the studied region). A high degree of interaction between these factors is seen from the level curves obtained by the xy pro-

Factor	Square sum (SS)	Degree of freedom	Mean square (MS)	F-ratio	*p*-value*
X_1 (L)	458.560	1	458.5603	8404.8	0.000000
X_1 (Q)	421.978	1	421.9784	7734.3	0.000000
X_2 (L)	18.035	1	18.0351	330.6	0.000000
X_2 (Q)	20.219	1	20.2186	370.6	0.000000
X_3 (L)	9.522	1	9.5222	174.5	0.000000
X_3 (Q)	26.083	1	26.0834	478.0	0.000000
X_1 by X_2	34.445	1	34.4450	631.3	0.000000
X_1 by X_3	4.836	1	4.8361	88.6	0.000002
X_2 by X_3	52.429	1	52.4288	960.9	0.000000
Lack of Fit	123.561	5	24.7122	592.6	0.000000
Pure Error	0.041	5	0.0083	–	–
Total SS	1137.749	19	–	–	–
Some important statistics:	R^2 (%) = 89.14; Adj., 79.36				

* $p \leq 0.05$ – significance at the 5% level; (L) – linear effect; (Q) – quadratic effect

Table 3 Analysis of variance (ANOVA) for the quadratic model

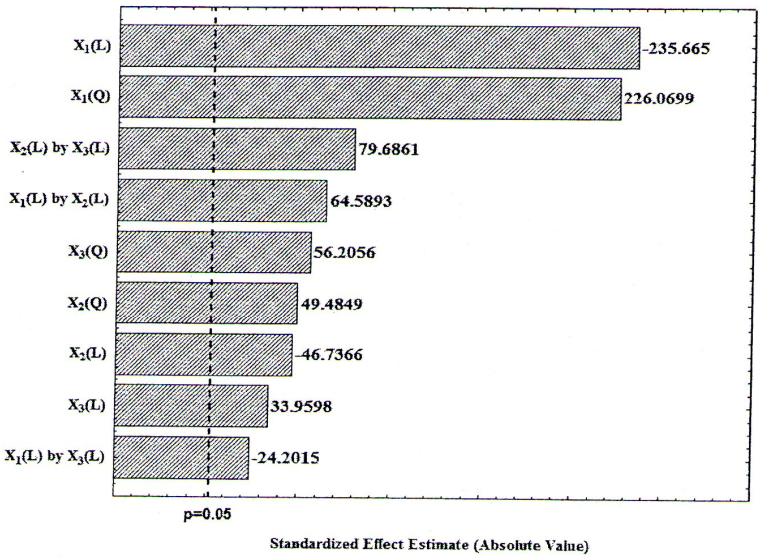

Figure 1 CCRD – Pareto chart of standardised effects for (X_1) waste frying oil, (X_2) corn steep liquor and (X_3) $NaNO_3$ using surface tension as response variable; Dashed line = point at which effect estimates are statistically significant ($p = 0.05$)

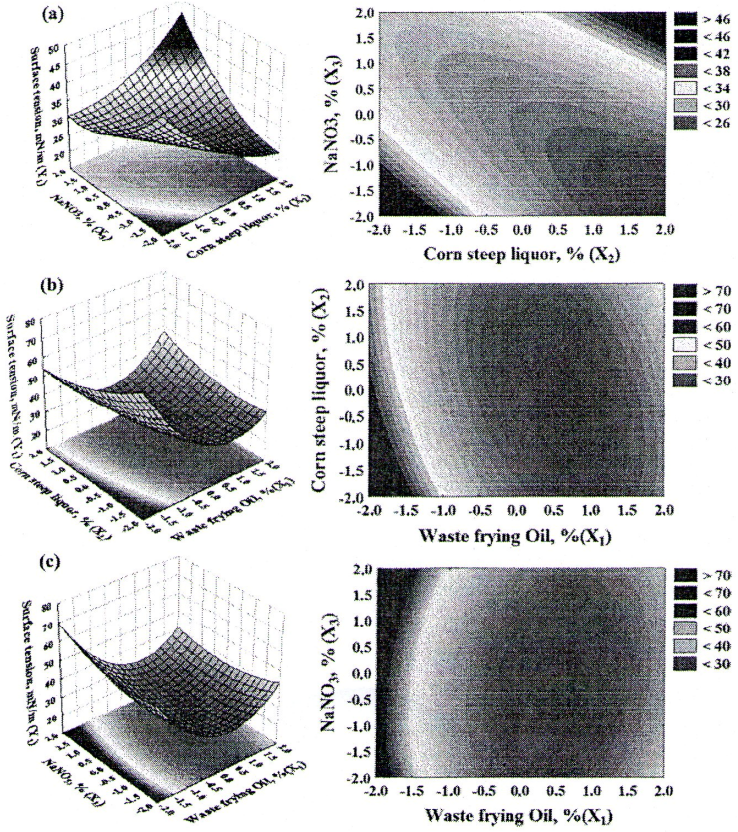

Figure 2 Three-dimensional plots for minimum surface tension (maximum biosurfactant production); RSM plots generated using data shown in Table 3; Inputs = 20 experimental runs carried out under conditions established by CCRD; (a) Reduction in surface tension as function of corn steep liquor and $NaNO_3$ concentrations; (b) Reduction in surface tension as function of corn steep liquor and waste frying oil concentrations; (c) Reduction in surface tension as function of waste frying oil and $NaNO_3$ concentrations

jection of the surface response plot, i.e., it is not possible to predict the biosurfactant properties by modifying only one of these factors.

Fig. 2(b) displays the effects of corn steep liquor and waste frying oil concentrations on biosurfactant production. A very well-delimited region is seen in the graph, reflecting the optimised conditions of biosurfactant production at around 2.5% corn steep liquor and 2.3% waste frying oil. In this region, the absence of parallelism between the curves demonstrates considerable interaction between the factors. Thus, it is not possible to make predictions regarding surface tension from variations in the concentration of only one of these factors.

Fig. 2(c) displays the effects of waste frying oil and $NaNO_3$ concentrations on biosurfactant production. A condition with around 2.2% waste frying oil and 0.2% $NaNO_3$ produces satisfactory surface tension. The level curves in this figure demonstrate a considerable parallelism between the factors and, consequently, weak interaction. Thus, it is possible to make predictions regarding surface tension from variations in the concentration of only one of these factors.

Microbial surfactants are not yet widely used due to the high production cost, part of which stems from the use of expensive substrates. The problem regarding the economic production of biosurfactants can be significantly reduced through the use of alternative, readily available, low-cost nutrients that enable high concentrations of biosurfactant. Moreover, the optimisation of the culture conditions also allows an increase in production levels [20]. Data presented in the literature demonstrate that the production of rhamnolipids by bacteria of the genus *Pseudomonas* is strongly influenced by the carbon and nitrogen sources as well as multivalent cations [21]. Different low-cost substrates have been investigated for biosurfactant production, such as glycerol, cassava wastewater, waste cooking oil and hydrolysed glycerin [21–24]. The preference for nitrate as a nitrogen source for the production of rhamnolipids by bacteria of the genus *Pseudomonas* has been widely studied [25, 26].

Based on these considerations, canola waste frying oil and corn steep liquor were employed in the present study as alternative sources of nutrients to study the production of a biosurfactant by the strain *P. cepacia* CCT6659, which has not previously been studied for this purpose, while $NaNO_3$ was used as the inorganic salt. Based on the results of the factorial design, the medium formulated with 2% waste frying oil, 3% corn steep liquor and 0.2% $NaNO_3$ (run number 12, for which surface tension was 26 mN m^{-1}) was selected for biosurfactant production. A biosurfactant yield of 8 g l^{-1} was obtained with these optimised levels of the medium constituents.

4 Conclusions

The present study demonstrated the effectiveness and feasibility of using a composite rotating design (CCRD) to identify the best medium composition for the enhanced produc-

tion of a biosurfactant from *P. cepacia*. This method was very useful in determining important variables for further optimisation, allowing the consideration of a large number of variables and the avoidance of information loss, both of which are essential to the optimisation process. Waste frying oil, corn steep liquor and $NaNO_3$ concentrations were identified as important parameters for improving biosurfactant production. Thus, CCRD proved very effective in improving biosurfactant production, with an increase in production from 2 to 8 g l^{-1}. By increasing the biosurfactant yield using CCRD, the production cost of this biomolecule could be markedly reduced, enhancing the feasibility of the commercial application of this promising new biosurfactant.

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[2] *Claesson, P.M., Ederth, T., Bergeron, V.* and *Rutland, M. W.*: Adv. Colloid Interface Sci. *67*

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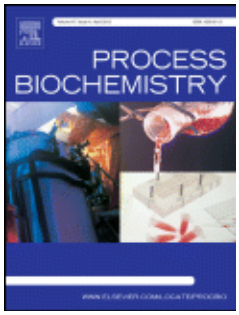

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Process Biochemistry is an application-orientated research journal devoted to reporting advances with originality and novelty, in the science and technology of the processes involving bioactive molecules and living organisms. These processes concern the production of useful metabolites or materials, or the removal of toxic compounds using tools and methods of current biology and engineering. Its main areas of interest include novel bioprocesses and enabling technologies (such as nanobiotechnology, tissue engineering, directed evolution, metabolic engineering, systems biology, and synthetic biology) applicable in food (nutraceutical), healthcare (medical, pharmaceutical, cosmetic), energy (biofuels), environmental, and biorefinery industries and their underlying biological and engineering principles. Main topics covered include, with most of possible aspects and domains of application:

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Process Biochemistry is an application-orientated research journal devoted to reporting advances with originality and novelty, in the science and technology of the processes involving bioactive molecules and living organisms. These processes concern the production of useful metabolites or materials, or the removal of toxic compounds using tools and methods of current biology and engineering. Its main areas of interest include novel bioprocesses and enabling technologies (such as nanobiotechnology, tissue engineering, directed evolution, metabolic engineering, systems biology, and synthetic biology) applicable in food (nutraceutical), healthcare (medical, pharmaceutical, cosmetic), energy (biofuels), environmental, and biorefinery industries and their underlying biological and engineering principles. Main topics covered include, with most of possible aspects and domains of application: cell culture and fermentation, biochemical and bioreactor engineering; biotechnology processes and their life science aspects; biocatalysis, enzyme engineering and biotransformation; and downstream processing. Manuscripts and data using response surface methodology (RSM) which are mainly descriptive, without any physiological or systemic explanation or correlations are not suitable for submission to the journal.

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