Universidade Federal de Pernambuco
Centro de Biociências
Programa de Pós-Graduação em Ciências Biológicas

MILAGRE AMÉRICO PELE

SELEÇÃO DE AMOSTRAS DE *Rhizopus* spp. PRODUTORAS DE BIOSSURFACTANTES/BIOEMULSIFICANTES E APLICAÇÃO NA REMOÇÃO DE POLUENTES HIDROFÓBICOS

Recife
2017

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Dissertação Apresentada ao Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração Biotecnologia, da Universidade Federal de Pernambuco, como parte dos requesitos para a obtenção do título de Mestre em Ciências Biológicas.

Orientadora: Prof[a]. Dra. Galba Maria de Campos-Takaki

Co-orientador: Prof. Dr. André Luiz Cabral Monteiro de Azevedo Santiago

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BANCA EXAMINADORA:

_____

Profª. Drª. Galba Maria de Campos Takaki (Orientadora)
Universidade Católica de Pernambuco- UNICAP/PE

_____

Profª. Drª. Celuta Sales Alviano
Universidade Federal do Rio de Janeiro – UFRJ/RJ

_____

Profª. Drª. Luciana de Oliveira Franco
Universidade Federal Rural de Pernambuco – UFRPE/PE

Á minha mãe, Rosta Jossai Magomane,e
ao meu pai, Américo Bacela Phele,
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minha vida profissional e formação
acadêmica,
Dedico!

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RESUMO

Biossurfactantes e bioemulsificantes são moléculas anfifílicas que vêm conquistando a atenção de diferentes setores, devido às suas múltiplas aplicações e são mais sintetizados por bactérias e leveduras, e mais raramente, por fungos filamentosos. Neste contexto, investigações foram realizadas com quatro amostras de *Rhizopus* isoladas de solos do bioma Caatinga (região nordeste do Brasil), quanto ao potencial de produção de biossurfactantes, utilizando o meio (glutamato 1% e óleo de soja pós-fritura 5%) para selecionar a amostra de *Rhizopus* com maior produção de biossurfactante. A produção do biossurfactante foi detectada pela redução da tensão superficial, os testes parafilme, dispersão do óleo, índice de emulsificação e atividade hemolítica. A amostra *Rhizopus* sp. UCP 1607 foi selecionada considerando a redução da tensão superficial da água de 72 para 31,8 mN/m, capacidade dispersante de 66,4 cm^2 (ODA), formação de halo de 40mm em ágar sangue e teste de parafilme exibindo um diâmetro de 12mm. A amostra de *Rhizopus* sp. UCP 1607 foi identificada como *Rhizopus arrhizus*, através de características fenotípicas e confirmação molecular. Nos estudos com a bioconversão de resíduos agroindústriais (milhocina e glicerol residual), foram utilizadas concentrações estabelecidas por planejamentos fatoriais de 2^2, tendo como variável resposta a produção de biossurfactante e/ou bioemulsificante. Os resultados obtidos demonstraram que o *R. arrhizus* UCP 1607 apresentou excelente habilidade na produção de biossurfactante no ensaio 3 (3% de glicerol residual e 5% de milhocina), e o bioemulsificante no ensaio 4 (6% de glicerol residual e 3% de milhocina), respectivamente. O biossurfactante apresentou excelente habilidade de redução da tensão superficial da água de 72 para 28,8 mN/m, índice de emulsificação de 79% e capacidade dispersante de 53,4 cm^2 (ODA), usando como substrato o óleo queimado de motor. O bioemulsificante reduziu a tensão superficial de 72 para 36,5 mN/m e índice de emulsificação de 96,4% com o óleo queimado de motor e uma área de deslocamento do óleo de motor de 68.3 cm^2 (ODA). A caracterização bioquímica preliminar mostrou que o biossurfactante está constituído por proteínas (38%), carboidratos (35,4%) e lipídios (5,5%), e bioemulsificante de proteínas (40%), carboidratos (16,7%) e lipídios (39,6%). Ambas as biomoléculas apresentaram caráter aniônico, concentração micelar crítica (CMC) de 1,7% (biossurfactante) e 1,4% (bioemulsificante), e ausência de fitotoxicidade. A dinâmica de remoção de poluentes hidrofóbicos no mesmo suporte (óleo diesel impregnado no solo arenoso) mostrou que o biossurfactante bruto removeu 79,4%, enquanto que o bioemulsificante demonstrou uma eficiência de remoção de 90,6%. Os dados experimentais obtidos com o biossurfactante e o bioemulsificante produzidos por *Rhizopus arrhizus* UCP 1607 sugerem a possibilidade de produção independente das biomoléculas em meios com fontes renováveis, além da utilização na dispersão e remoção de poluentes hidrofóbicos de solos em processos de biorremediação.

Palavras-chaves: Bioconversão. Resíduos agroindustriais. *Rhizopus* spp. Tensoativos. Remediação de poluentes hidrofóbicos.

ABSTRACT

Biosurfactants and Bioemulsifiers are amphipathic molecules which have gained the attention of different sectors due to their many potential uses, and are mainly produced by bacteria, yeasts and, rarely by filamentous fungi. In this context, investigations were carried out with *Rhizopus strains* isolated from soil samples collected in biome Caatinga (Northeast of Brazil) for their potential in the production of biosurfactants and bioemulsifiers, using medium composed of 1% glutamate and 5% soybean oil residue, for selection of *Rhizopus* strain with potential for production of tensoactives. The production was detected using surface tension measurement, parafilm M test, oil displacement assay, hemolytic activity and emulsification index. The strain of *Rhizopus* sp. UCP 1607, isolated from soil sample of the State of Rio Grande do Norte, showed the best tensoactive properties resulting in the reduction of surface tension of water from 72 to 31.8 mN/m, dispersant capacity of 66.4 cm^2 (ODA), formation of a clear halo in the blood agar medium (40mm), and according to the parafilm M test the mycelia-free broth exhibited a diameter of 12mm. *Rhizopus* sp. UCP 1607 strain was identified as *Rhizopus arrhizus* using phenotypic characteristics and molecular confirmation. In the studies of bioconversion of agroindustrial residues (crude glycerol and corn steep liquor), were used the concentrations established through 2^2 factorial designs with biosurfactant and/or bioemulsifier production as response variable. The results demonstrated that *Rhizopus arrhizus* UCP 1607 showed excellent ability in the production of biosurfactant in the assay 4 (3% crude glycerol and 5% corn steep liquor), and bioemulsifier in the assay 3 (6% crude glycerol and 3% corn steep liquor). The biosurfactant showed an excellent reduction of surface tension of water from 72 to 28.8 mN/m, but also exhibited higher emulsification index of 79% and dispersant capacity of 53.4 cm^2 (ODA), using burnt motor oil as substrate. On the other hand, the bioemulsifier reduced the surface tension of water from 72 to 36.5 mN/m. Moreover, showed an excellent emulsification index of 96.4% against the burnt motor oil, and higher displacement activity of 68.3 cm^2 (ODA). The preliminar biochemical characterization of the isolated compounds demonstrated that the biosurfactant consisted of proteins (38%), carbohydrates (35,4%) and lipids (5,5%), and the bioemulsifier contained proteins (40%), carbohydrates (16,7%) and lipids (39,6%). Both the compounds presented an anionic character, with critical micelle concentrations (CMC) of 1.7% (biosurfactant) and 1.4% (bioemulsifier) and non-phytotoxicity. The applicability studies in the removal of hydrophobic pollutants from soil showed that the biosurfactant removed 79.4% of the diesel oil impregnated in the beach sand, whereas bioemulsifier demonstrated a removal rate of 90.6% of the contaminant from sand. The data obtained from experiments with biosurfactant and bioemulsifier produced by *Rhizopus arrhizus* UCP 1607 suggest the possibility of independent production of biomolecules in media composed of renewable resources, besides the use in the dispersion and removal of hydrophobic pollutants from soils.

Key-words: Bioconversion. Agroindustrial wastes. *Rhizopus* spp. Tensoactive agents. Remediation of hydrophobic pollutants.

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CAPÍTULO I

1. INTRODUÇÃO

O ser humano, desde os tempos remotos da história da humanidade, sempre fez uso dos micro-organismos e dos produtos do seu metabolismo tais como enzimas, ácidos orgânicos, pigmentos, antimicrobianos (DURÁN et al., 2010; PASTORE; MACEDO, 2010), quitosana, compostos tensoativos, entre outros (KLEEKAYAI; SUNTORNSUK, 2011; SHUKLA; BHANDARI, 2015).

Os surfactantes de origem microbiana (biossurfactantes) são moléculas promissoras oferecidas pela biotecnologia moderna (MULLIGAN et al., 2014). Estes compostos apresentam estruturas quimicamente diversificadas com propriedades amplamente bem distintas (REIS et al., 2013). Considerando a diversidade de moléculas, os biossurfactantes podem ser aplicados em diferentes áreas do conhecimento, em especial, a indústria de petróleo, em tecnologias de remediação ambiental, como agentes de bio-controle de parasitas, em cosméticos, e produtos de beleza (VIJAYAKUMAR; SARAVANAN, 2015; KHAN; BUTT, 2016). Potencialmente, as propriedades anti-adesivas e antimicrobianas são úteis para indústrias farmacêuticas e de alimentos (MULLIGAN; SHARMA; MUDHOO, 2014).

Portanto, os biossurfactantes são produtos altamente compatíveis do ponto de vista ecológico pois são biodegradáveis e menos tóxicos quando comparados com os sintéticos, são capazes de manter a sua atividade em condições de temperatura, salinidade e pH extremos, sendo a biocompatibilidade e digestibilidade características importantes. Além disso, existe uma ampla variedade de resíduos e subprodutos agroindustriais acesssíveis que podem ser usados como matérias-primas para a produção de biossurfactantes. Desta forma, a especificidade estrutural destes compostos permite a sua ampla aplicação em distintos problemas, tornando os biossurfactantes compostos mais atrativos em relação aos surfactantes químicos (MULLIGAN; SHARMA; MUDHOO, 2014; BRUMANO et al., 2016; USMAN et al., 2016).

As pesquisas sobre produção de compostos tensoativos por fungos demonstram que estes micro-organismos possuem um grande potencial de sintetizar diversos tipos destes compostos (BHARDWAJ ET AL., 2013; SHUKLA; BHANDARI, 2015), destacando-se as leveduras mais estudadas *Candida* (SARUBBO et al., 2007, LUNA et al., 2013, 2016; SOUZA et al., 2016), *Pseudozyma* (FARIA et al., 2014), e *Yarrovia* (FONTES et al., 2010; 2012). Contudo, os fungos filamentosos têm sido pouco explorados, embora alguns estudos relatem também seu elevado

potencial na síntese de biossurfactantes (KIRAN et al., 2009; ALEJANDRO et al., 2011), bem como a bioconversão de substratos alternativos e de baixo custo, como resíduos agroindustriais (ANDRADE SILVA et al., 2014; SILVA et al., 2014). Assim, a produção de bioemulsificantes e biossurfactantes produzidos por *Rhizopus* spp. isolados do solo da caatinga, surgem como uma alternativa promissora e sustentada pelo uso de resíduos agroindustriais, proporcionando o progresso, inovação e geração de tecnologia.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Isolar, caracterizar e investigar o potencial biotecnológico de estirpes de *Rhizopus* spp. na produção de biossurfactante utilizando resíduos agroindustriais e aplicar na remoção de poluentes hidrofóbicos.

2.2 OBJETIVOS ESPECÍFICOS

- Isolar estirpes do gênero *Rhizopus* de solo da caantiga da região do estado de Rio Grande do Norte;
- Selecionar espécies de *Rhizopus* produtores de biossurfactante;
- Realizar a identificação de *Rhizopus* sp. através de identificação morfológica e molecular;
- Selecionar as concentrações dos resíduos agroindustrais, utilizando um planejamento fatorial, visando a maximização da produção de biossurfactante/bioemulsificante pelo micro-organismo selecionado;
- Isolar e caracterizar o biossurfactante e bioemulsificante;
- Avaliar o potencial do biossurfactante e bioemulsificante na remoção de derivados de petróleo;
- Validar os resultados obtidos.

3. REVISÃO DE LITERATURA

3.1 CAATINGA

O bioma Caatinga localiza-se na região semi-árida no nordeste do Brasil, onde abrange parte dos estados de Ceará, Rio Grande do Norte, Paraíba, Pernambuco, Alagoas, Sergipe, Piauí, Bahia, e no sudeste do Brasil abrange uma pequena parte do estado de Minas Gerais (SILVA; COELHO; SILVA, 2015). A Caatinga é caracterizada por apresentar uma vegetação composta principalmente de pequenas árvores e arbustos baixos, muitos dos quais com espinhos, folhas pequenas e finas, e algumas características xerófilas e decíduas (PRADO, 2003; GORLACH-LIRA; COUTINHO, 2007). O nome "Caatinga" tem a sua origem na linguagem Tupi-Guarani e significa "floresta branca", em referência ao aspecto "acinzetado" que a vegetação toma no período seco (PRADO, 2003). O clima da Caatinga é o típico de uma região semi-árida com temperaturas altas, com as mínimas acima de 15° C e as máximas podendo atingir os 40° C, déficit de recursos hídricos, insolação intensa, e precipitações escassas, entre 280 a 800mm, com consequentes períodos prolongados de seca severa (GORLACH-LIRA; COUTINHO, 2007; ARAÚJO, 2011).

Os solos da Caatinga formados pela ação combinada de vários fatores com destaque para o clima e o material de origem (rocha-mãe) apresentam características geomorfológicas muito diversificadas (PRADO, 2003), ocorrendo latossolos, com textura de média a muito argilosos, profundos e bastante uniformes, com alto grau de intemperismo; argissolos, a textura varia de arenosa a argilosa na superfície e de média a muito argilosa em subsuperfície; ampla variabilidade de características morfológicas, físicas, químicas e mineralógicas; luvissolos, geralmente bastante profunda para solos rasos contendo argila; cambissolos, pouco evoluídos, podem ser rasos até muito profundos, pedregosos e não pedregosos (ARAÚJO FILHO, 2011).

O bioma da Caatinga sofreu intensa degradação de seus recursos naturais, em especial do solo, devido às ações humanas tais como desflorestação, com a consequente desertificação, agricultura de queima e corte, entre outros fatores (ARAÚJO, 2011; SILVA; COELHO; SILVA, 2015).

As condições ambientais adversas que caracterizam a região semi-árida da Caatinga, tais como temperaturas extremas, solos marcadamente argilosos e pedregosos, a baixa humidade, escassez de recursos hídricos, que levam a períodos prolongados de seca possuem influenciar

desfavoravelmente a atividade de micro-organismos no solo (GORLACH-LIRA; COUTINHO, 2007; SCHIMEL, 1995). Segundo Silva (2015) e seus colabodores, a população microbiana que habita os solos da Caatinga desenvolveu metabolismos típicos e adaptativos em resposta aos diversos fatores de estresse predominantes na região.

A diversidade de micro-organismos no solo da região semi-árida da Caatinga ainda não é suficientemente conhecida (GORLACH-LIRA; COUTINHO, 2007). Entretanto, um número significativo de pesquisas com micro-organismos isolados dos solos deste bioma tem sido desenvolvido, tendo em vista as suas potencialidades biotecnológicas (LUNA et al., 2011; 2013; SILVA; COELHO; SILVA, 2015; MONTERO-RODRÍGUEZ et al., 2015).

3.2. GÊNERO *RHIZOPUS*

O gênero *Rhizopus* pertence ao filo Mucoromycota, subfilo Mucoromycotina, ordem Mucorales (SPATAFORA et al., 2016), são sapróbios, podendo ser facilmente isolados de diversos ambientes tais como do solo, de excrementos de animais, frutos, vegetais e alimentos em decomposição (SANTIAGO et al, 2013). Além de serem conhecidos como contaminantes comuns, as espécies de *Rhizopus* são também causas de infeções graves e muitas vezes fatais em seres humanos, especialmente em pacientes imunocomprometidos (RIBES et al. 2000; SANTIAGO et al, 2013). Certas espécies deste gênero são patógenos das plantas (KWON et al. 2001).

As colónias de *Rhizopus* são inicialmente brancas e se tornam escuras com a idade, devido a esporulação, com crescimento abundante (RIBES et al., 2000; ZHENG et al., 2007). Assim como outros gêneros do filo, o gênero *Rhizopus* apresenta micélio cenocítico, porém distingue-se de outros gêneros pela formação de rizóides e estolões crescendo sempre opostos ao esporangióforo; em lâminas com corante Azul de Aman a columela frequentemente colapsa em forma de guarda-chuva; esporangióforos crescendo a partir de estolões, frequentemente a partir de rizoides e raramente a partir de hifas (ZHENG et al., 2007; PITT; HOCKING, 2009). Esporangióforos, rizóides e estolões normalmente marrom. Apófise sempre presente, evidente ou menos evidente. O esporângio é globoso ou sub-globoso com uma base um tanto achatada, castanho ou castanho-escuro, de tamanho variável, podendo sofrer deliquescência ou quebrar em água deixando ou não colarete. Os esporangiósporos são unicelulares, de textura lisa ou estriada

de variáveis formas e tamanhos (RIBES; VANOVER-SAMS; BAKER, 2000; ZHENG et al., 2007).

Vários estudos demonstram o potencial biotecnológico de *Rhizopus* sp. na produção de enzimas de interesse industrial. Behnam et al. (2016), obteve uma produção satisfatória de produção de xylanase (26 U/gbs) por *Rhizopus arrhizus*, utilizando xilana como substrato. Lipases produzidas por *Rhizopus arrhizus* têm atraído varios pesquisadores para a transesterificação enzimática de biodiesel (SATTARI; VAHABZADEH; AGHTAEI, 2015; MUKHTAR et al., 2016; VC et al., 2016). Produções significativas de fitase, enzima usada em nutrição animal, proteção ambiental e saúde humana (VOHRA; SATYANARAYANA, 2003), foram obtidas por espécies de *Rhizopus microsporus* e *Rhizopus oligosporus* (SATO et al., 2014; SURESH; RADHA, 2015).

Outro potencial biotecnológico do gênero *Rhizopus* é a produção de quitosana, um polissacarídeo de grande aplicabilidade (CARDOSO et al., 2012; BERGER et al., 2014; ZHANG et al., 2014; TASAR; ERDAL; TASKIN, 2016).

3.3. SURFACTANTES

Os compostos tensoativos integram uma grande variedade de produtos de uso cotidiano do ser humano tais como: produtos de limpeza doméstica e higiene pessoal, cosméticos, herbicidas ou pesticidas; em processos de biorremediação, agricultura, indústrias de alimentos, farmacêutica, de papel, de petróleo, entre outros (BANAT, et al., 2014b; GUDIÑA et al., 2015).

A demanda em escala industrial de surfactantes no mercado mundial é elevada, atingindo milhões de toneladas por ano (FRACCHIA et al., 2011; GEYS et al., 2014). Atualmente, essa demanda é respondida por surfactantes sintéticos, produzidos quimicamente com recurso à oleoquímicos e petroquímico **(**GEYS et al., 2014**;** KHAN; BUTT, 2016**)**. Todavia, esses compostos são considerados ambientalmente nefastos, devido ao fato de serem parcialmente biodegradáveis e elevada toxicidade (Kitamoto et al., 2009). Por isso, há tendência de substituir os surfactantes sintéticos pelos compostos similares de origem natural, produzidos a partir de recursos sustentáveis e renováveis (FRACCHIA et al., 2014).

3.4. BIOSSURFACTANTES/BIOEMULSIFICANTES

Os surfactantes de origem biológica, denominados de biossurfactantes e bioemulsificantes, vêm conquistando atenção de pesquisadores e de potenciais consumidores, devido a crescente preocupação ambiental, o surgimento de leis mais rigorosas visando a proteção ambiental. Com o avanço na área da biotecnologia, bem como, as propriedades distintas dos biossurfactantes indicam como alternativas aos disponíveis no mercado (KITAMOTO et al., 2009; HENKEL et al., 2012; REIS et al., 2013). Os biossurfactantes apresentam vantagens comparativamente aos surfactantes sintéticos, considerando a baixa toxicidade, elevada biodegradabilidade, biocompatibilidade, biodigestibilidade, eficiência em condições ambientais extremas de pH, temperatura e salinidade (MOUSSA et al. 2006; VECINO et al., 2015a). Além disso, os biossurfactantes podem ser obtidos a partir de recursos renováveis e de baixo custo (SARUBBO et al., 2007), e acresce-se também a possibilidade de modificação de sua estrutura e de propriedades físico-químicas, através de técnicas de engenharia genética ou bioquímicas, o que permite obter produtos para usos específicos (LUNA et al., 2009).

Os biossurfactantes, apesar possuirem diversas vantagens sobre os surfactantes químicos, ainda não podem competir comercialmente, devido aos altos custos de produção e baixos rendimentos (GOVINDAMMAL; PARTHASARATHI, 2013). A economia de produção é o principal empecilho para a aplicação dessas moléculas seja amplamente difundida (KIRAN et al., 2010b).

A viabilidade econômica na biossíntese de quaisquer metabólitos microbianos a escala comercial é determinada por três fatores básicos (MUKHERJEE et al., 2006), destacando-se: (i) custos de matéria-prima; (ii) procedimentos de produção e recuperação adequadas e econômicas; e (iii) o rendimento dos micro-organismos produtores. Neste sentido, para se ultrapassar as limitações na produção de biossurfactantes diversas estratégias têm sido estudadas por vários pesquisadores (MUKHERJEE et al., 2006; SAHARAN et al., 2011; MAKKAR et al., 2011), destacando-se

i. O uso de substratos renováveis de baixo custo e resíduos para formulação de meios fermentativos a fim de baixar os custos de matéria-prima inicial envolvida no processo;

ii. Desenvolvimento de processos eficientes, incluindo a otimização das condições de cultivo e processos de separação de rentáveis, para maximizar a produção e recuperação do biossurfactante;

iii. Desenvolvimento e uso de cepas mutantes ou recombinantes super-produtoras para se obter rendimentos melhorados.

Diversos estudos realizados a escala laboratorial buscaram explorar estas estratégias em processos de produção de biossurfactantes. Estes estudos concentram-se mais no uso de resíduos e subprodutos agroindustriais, bem como sobre a otimização das condições de produção e extração de biossurfactantes (RUFINO et al., 2008; KIRAN et al., 2010a; JAMAL et al., 2011; CHEN et al., 2012; PEREIRA et al., 2013; SHARMA et al., 2013; LIU et al., 2014; ZOUARI et al., 2014), e os resultados mostram que estas estratégias podem melhorar significativamente os rendimentos de produção de biossurfactantes.

3.4.1. Estrutura e Classificação

Os biossurfactantes constituem um amplo grupo de compostos produzidos por células vivas: plantas, animais e micro-organismos (KITAMOTO et al., 2009). Comumente, o termo é usado com referência aos surfactantes de origem microbiana, ou seja, produzidos por bactérias, leveduras e fungos filamentosos.

3.4.1.1. Estrutura de Biossurfactantes/Bioemulsificantes

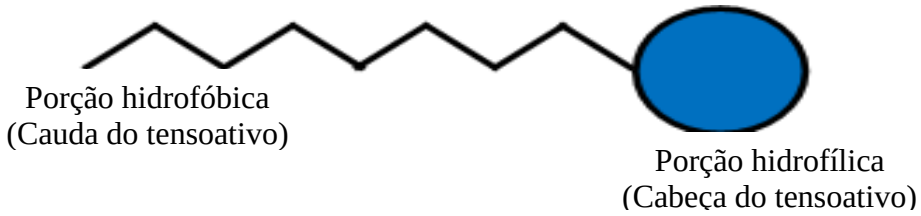
Biossurfactantes e bioemulsificantes são moléculas anfipáticas que possuem um domínio hidrofílico em uma de suas extremidades e um domínio hidrofóbico em outra (DESAI; BANAT, 1997), figura 1. A parte hidrofóbica, não-polar, é principalmente composta por uma cadeia hidrocarbonada, saturada ou insaturada, linear ou ramificada, de um ácido graxo ou derivados de ácidos graxos; mas também pode ser composta por uma proteína ou peptídeo com uma alta proporção de cadeias laterais hidrofóbicas (KŁOSOWSKA-CHOMICZEWSKA, MĘDRZYCKA, KARPENKO, 2009)). A cadeia é comumente constituída dentre 8 a 18 átomos de carbono (WALTER; SYLDATK; HAUSMANN, 2010).

A porção hidrofílica apresenta muitas variações, podendo consistir de anions ou cátions peptídicos, de carboidrato (mono-, di-, ou polissacarídeos), ou de ácido (BANAT; MAKKAR;

CAMEOTRA, 2000). A cauda hidrofóbica se liga ao grupo hidrofílico por meio de uma ligação éster glicosídico ou amida.

A maior parte de biossurfactantes/bioemulsificantes é de natureza neutra ou negativamente carregado e compreende ácidos graxos de baixo peso molecular e polímeros (CAMEOTRA et al., 2010).

Figura 1 - Organização estrutural de monómero de um biossurfactante/bioemulsificante.



Fonte - SOONGLERDSONGPHA; RONGSAYAMANONT; KHONDEE, 2014**.**

3.4.1.2. Classificação

Os biossurfactantes são categorizados, principalmente, de acordo a sua estrutura química e origem microbiana (GAUTAM; TYAGI, 2006). Com base na composição química, as principais classes de surfactantes microbianos são representadas por glicolipídeos, lipopeptídeos e lipoproteínas, ácidos graxos, fosfolipídeos e lipídeos neutros, surfactantes poliméricos e surfactantes particulados (DESAI; BANAT, 1997).

Os tensoativos naturais podem também ser divididos, de acordo com o peso molecular, em dois grandes grupos: compostos de baixo peso molecular, designados de biossurfactantes, e os de alto peso molecular, denominados bioemulsificantes (MATVYEYEVA et al., 2014). Assim, os biossurfactantes consistem de glicolipídeos e lipopeptídeos, e bioemulsificantes são moléculas poliméricas extracelulares (ANTONIOU et al., 2015). Do ponto de vista de suas propriedades tensoativas os biossurfactantes são caracterizados pela excelente capacidade de reduzir a tensão superficial na interface ar-água, ao passo que os bioemulsificantes apresentam grande habilidade na formação de emulsões estáveis entre materiais imiscíveis (UZOIGWE et al., 2015). No entanto, um biossurfactante possui tanto propriedades surfactantes quanto emulsificantes, mas os bioemulsificantes não reduzem necessariamente a tensão superficial (BATISTA et al., 2006).

Os biossurfactantes (em geral, compostos de baixo peso molecular) são constituídos de açúcares, aminoácidos, ácidos graxos e grupos funcionais tais como grupos carboxílicos.

Portanto, são representados por glicolipídeos e lipopeptídeos (MULLIGAN et al., 2014; UZOIGWE et al., 2015).

3.4.1.2.1 Glicolipídeos

Os glicolípidos constituem a classe mais comum de compostos tensoativos de baixo peso molecular produzidos por bactérias, leveduras como as do gênero *Candida* e fungos filamentosos (MATVYEYEVA et al., 2014). Estruturalmente, consistem de carboidratos (mono- ou oligossacarídeos) ligados a longas cadeias de ácidos alifáticos ou de ácidos hidroxi-alifáticos por um grupo éster (SATPUTE et al., 2010). A porção do carboidrato pode ser uma glicose, manose, galactose, galactose-sulfato, ácido glucurônico ou raminose. Os componentes lipídicos podem ser ácidos graxos saturados ou insaturados, hidroxilados ou álcoois graxos (HAUSMANN; SYLDATK, 2015).

Os glicolipídeos são produzidos por diversas espécies de bactérias e leveduras, como as do género *Candida*. Quatro grupos biotecnologicamente mais importantes de glicolípidos são conhecidos: raminolipídeos, soforolipídeos, trehalolipídeos, e manosileritritol-lipídeos (HAUSMANN; SYLDATK, 2015).

- *Raminolipídeos*: Um raminolipídeo consiste de um ácido graxo β-hidroxilado ligado a uma molécula de ramanose. Eles são produzidos por espécies de *Pseudomonas*, em particular por *Pseudomonas aeruginosa*. Classificam-se como mono- e di-raminolipídeos. Algumas bactérias são conhecidas como sendo produtoras de apenas mono-raminolipídeos, existindo algumas que produzem mono- e di-raminolipídeos (MARCHANT; BANAT, 2012a; RANDHAWA; RAHMAN, 2014).

- *Soforolipídeos*: São encontrados só em leveduras do gênero *Candida*, em especial as espécies *Candida bombicola* e *Candida apícola* são capazes de produzir consideráveis quantidades de soforolipídeos usando diversos substratos (KITAMOTO et al.,2002; HIRATA et al., 2009). Estas biomoléculas possuem na sua estrutura a soforose, um dissacarídeo de glicose. Os soforolipídeos podem apresentar-se ou sob a forma lactônica ou forma acídica aberta (HAUSMANN; SYLDATK, 2015). Na forma lactônica a extremidade carboxílica do ácido graxo está esterificado na posição-4′ ou na posição 6′ ou 6′ da soforose (MARCHANT; BANAT, 2012).

- *Trehalolipídeos:* Os trehalolipídeos são glicolipídeos associados com diversas espécies dos géneros *Mycobacterium*, *Rhodococcus*, *Arthrobacter*, *Nocardia* e *Gordonia* (VIJAYAKUMAR; SARAVANAN, 2015; FRANZETTI; TAMBURINI; BANAT, 2008). Sua estrutura possui a trehalose, que é formado por duas unidades de glicose. As cadeias hidrocarbonadas ligadas a trehalose variam no número, comprimento (KUGLER et al., 2014). Nos trehalolipídeos, maioritariamente, os lipídeos estão associados a parede celular e este fator contribui para sua baixa produtividade (KITAMOTO et al., 2002).

- *Manosileritritol-lipídeos* (MELs): As leveduras do género *Pseudozyma*, *P. rugulosa*, *P. aphidis*, *P. antarctica* e *P. parantarctica*, são excelentes produtores destes tipos moléculas (MORITA et al., 2013). Essas moléculas são também secretadas por fungos fuliginosos do género *Ustilago*, como *U. maydis* (ARUTCHELVI et al., 2008) e *U. scitaminea* (MORITA et al., 2009). Estas moléculas apresentam dois derivados de glicosil e vários ácidos graxos (YU et al., 2015).

3.4.1.2.2 Fosfolipídeos e Ácidos Graxos

Os fosfolipídeos e ácidos graxos são produzidos por várias bactérias e leveduras durante o crescimento em n-alcanos (COOPER et al., 1979; GAUTAM; TYAGI, 2006). A oxidação microbiana de alcanos pode produzir não só ácidos graxos de cadeias lineares, mas também ácidos graxos complexos contendo grupos hidroxila (OH) e ramificações alquila (KARANTH et al., 1999). Alguns desses ácidos complexos, por exemplo os ácidos corinomicólicos sintetizados por *Corynebacterium lepus*, exibem apreciável atividade surfactante (RAHMAN; GAKPE, 2008; COOPER et al., 1979). Semelhantes aos 2-hidroxi ácidos graxos, as propriedades superficiais dos ácidos corinomicólicos são relativamente insensíveis ao pH e salinidade (ROSENBERG; RON, 1999).

Os fosfolipídeos são produzidos por espécies dos gêneros *Acinetobacter*, *Corinebacterium*, *Thiobacillus*, *Micrococcus* e *Aspergillus* (SILVA et al., 2014; MATVYEYEVA et al., 2014; LIU et al., 2015). KARANTH et al., (1999), quando uma certa bactéria ou levedura capaz de degradar hidrocarbonetos é cultivada em substrato de alcanos o nível de fosfolipídeos aumenta drasticamente. Diferentemente dos ácidos graxos complexos, as propriedades tensoativas de fosfolipídeos são fortemente influenciadas por alterações no pH e força iónica (ROSENBERG; RON, 1999).

3.4.1.2.3 Lipopeptídeos

A classe de lipopeptídeos engloba um vasto número de biossurfactantes de baixo peso molecular consistindo de estruturas cíclicas (SHOEB et al., 2013), destacando-se os membros dos gêneros *Bacilllus* e *Pseudomonas* como os principais produtores de moléculas desta classe (GAUTAM; TYAGI, 2006; DHANARAJAN; SEN, 2014). Estruturalmente, consistem de uma cabeça hidrofílica composta por polipeptídeos, comumente entre 7 e 10 aminoácidos, ligada a uma estrutura hidrofóbica de um ácido graxo. A diferença entre eles reside no tipo de aminoácidos do anel peptídico, e também no comprimento e estrutura da cadeia hidrocarbonada (SMYTH et al., 2010; PATHAK; KEHARIA, 2013). Geralmente, os lipopeptídeos ocorrem como misturas de compostos intimamente relacionados (CAMEOTRA et al., 2010).

Três maiores grupos de lipopeptídeos produzidos por espécies de *Bacillus* amplamente estudados incluem a surfactina, a iturina, e a fengicina (DAS et al., 2008; PATHAK; KEHARIA, 2013; SINGH et al., 2014; RAUTELA et al., 2014; MNIF et al., 2015). Estas moléculas destacam-se pelos seus promissores usos na área médica e farmacêutica (MNIF; GHRIBI, 2015).

3.4.1.2.4 Biossurfactantes Poliméricos

Os biossurfactantes poliméricos são moléculas de elevado peso constituídos de lipoproteínas, proteínas, polissacarídeos, lipopolissacarídeos ou complexas misturas contendo vários destes biopolímeros (FRACCHIA et al., 2012). Biossurfactantes desta classe são produzidos por espécies bacterianas de diferentes gêneros, tais como *Acinetobacter, Pseudomonas* (SOBRINHO et al., 2014), e leveduras de género *Candida* (CIRIGLIANO; CARMAN, 1985; SILVA et al., 2014).

Os biossurfactantes poliméricos mais bem estudados incluem emulsan, liposan, e manoproteínas e outros complexos proteína-polissacarídeos. Rosenberg et al. (1979), extraíram o emulsan de *Acinotobacter calcoaceticus* RAG-1. Este polímero extracelular é um lipoheteropolissacarídeo polianiónico muito potente na emulsificação de hidrocarbonetos na água (RAHMAN; GAKPE, 2008; FRACCHIA et al., 2012).

Cirigliano e Carman (1985), isolaram e caracterizaram um bioemulsificante extracelular solúvel em água chamado liposan, sintetizado pela levedura *Candida lipolytica*. Esta macromolécula é constituída de 83% de carboidratos e 17% de proteínas (Cirigliano; Carman,

1985). A parte de carboidratos é um heteropolissacarídeo de glicose, galactose, galactosamina e ácido galacturónico (RAHMAN; GAKPE, 2008).

Manoproteínas podem ser produzidas por *Saccharomyces cerevisiae* (CAMERON et al., 1988; WALENCKA et al., 2007; DIKIT et al., 2010). Cameron et al., (1988), quando purificado e caracterizado, o polímero mostrou consistir de 44% de carboidratos (manose) e 17% de proteínas. Estes biopolímeros mostram ter excelentes propriedades bioemulsificantes (DIKIT et al., 2010), e antibiofilmes contra estafilococos (WALENCKA et al., 2007).

Os biopolímeros complexos consistindo de diversos grupos funcionais foram extraídos da levedura *Candida lipolytica* (RUFINO et al., 2007, e SARUBBO et al., 2007), de fungo filamentoso *Cunninghamella echinulata* (ANDRADE SILVA et al., 2014). A caracterização química preliminar revelou serem compostos de 50% de proteínas, 20% de lípidos e 8% de carboidratos (RUFINO et al., 2007), e 40% de lípidos, 35.2% de carboidratos e 20.3% de proteínas (ANDRADE SILVA et al., 2014).

3.4.1.2.5 Biossurfactantes particulados

Os biossurfactantes particulados encontrados, de acordo com KARANTH et al., (1999), podem ser de dois tipos: vesículas extracelulares e células microbianas com elevada hidrofobicidade na superfície celular.

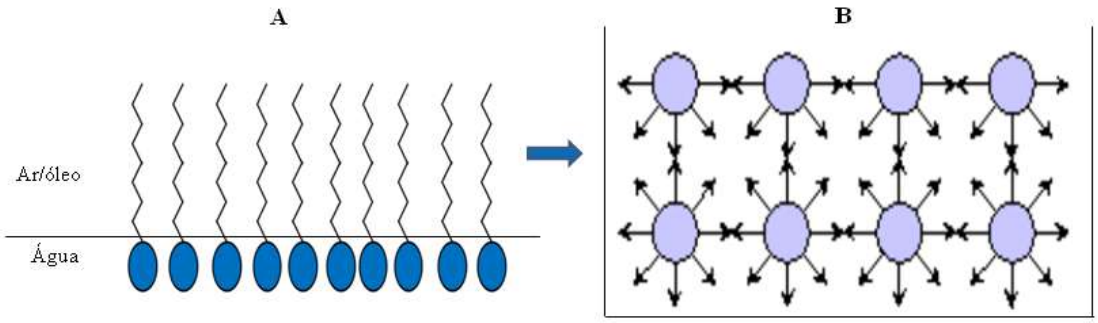
As Vesículas de membranas extracelulares podem emulsificar hidrocarbonetos, formando microemulsões que são importantes na assimilação de alcanos por células microbiana (DESAI; BANAT, 1997). Espécies de *Acinetobacter*, quando cultivadas em hexadecano, acumularam vesículas extracelulares de 20 a 50 mm de diâmetro com uma densidade de 1.158 g/cm^3. Estas vesículas parecem desempenhar um papel importante na captação de alcanos por *Acinetobacter sp*. HO1-N (OKOLIEGBE; AGARRY, 2012). As vesículas produzidas por *Acinetobacter sp*. são constituídas de proteínas, lipopolissacarídeos e fosfolipídeos (RAUTELA; CAMEOTRA, 2014).

Células microbianas inteiras que possuem alta hidrofobicidade superficial comportam-se, elas próprias, como agentes surfactantes (KARANTH et al., 1999.). Propriedades surfactantes são atribuídas a maioria de micro-organismos que degradam hidrocarbonetos, algumas espécies de *Cyanobacteria*, e alguns agentes patogénicos exibem uma forte afinidade pelas interfaces hidrocarboneto/água e água/ar (DESAI; BANAT, 1997; KARANTH et al., 1999).

3.4.2 Propriedades de Biossurfactantes

O carácter anfipático dos biossurfactantes, ou seja, são compostos complexos com uma cabeça hidrofílica e cauda hidrofóbica, faz delas moléculas capazes de se distribuir nas interfaces entre fases imiscíveis tais como óleo/água ou ar/água, o que leva à redução da tensão superficial e interfacial entre fases destes sistemas (DESAI; BANAT, 1997; FRACCHIA et al., 2015), figura 2. Para além de diminuir a tensão superficial e interfacial, outras propriedades importantes inerentes à organização estrutural destas moléculas incluem a sua capacidade molhante, de solubilização, de emulsificação e demulsificação, de dispersão de fases, de detergência, e lubrificante (MOUSSA et al., 2006; JAGTAP et al., 2010; FEMI-OLA et al., 2015).

Figura 2 - Distribuição de moléculas monoméricas de biossurfactantes/bioemulsificantes na interfaces (A) e seu efeito na redução de tensões superficiais e interfaciais (B).



Fonte - SOONGLERDSONGPHA; RONGSAYAMANONT; KHONDEE, 2014**.**

3.4.2.1 Tensão Superficial e Interfacial

A tensão superficial (TS, uma das fases é um gás) e tensão interfacial (TI) são propriedades de crucial importância com que estão associadas muitas outras propriedades funcionais dos biossurfactantes (NITSCHKE; PASTORE, 2002). De acordo com Al-Araji et al. (2007), tensão superficial é a força que atua sobre a superfície de um líquido levando a minimização da área desta superfície. Ela pode também ser definida como uma medida de energia livre por unidade da área associada com uma superfície ou interface (MNIF; GHRIBI, 2015). Para Rosen, 2004, a tensão superficial (ou interfacial) mede a diferença da natureza entre duas fases na interface. Quanto maior for a dissimilaridade da natureza das fases do sistema, maior a tensão interfacial (ou superfícial) entre eles (ROSEN, 2004).

A tensão superficial é um fenômeno que resulta de inúmeras forças de interação entre moléculas de um líquido. Os biossurfactantes quando se adsorvem na interface entre dois fluídos imiscíveis ou entre um fluído e um sólido diminuem a tensão superficial e interfacial e, assim,

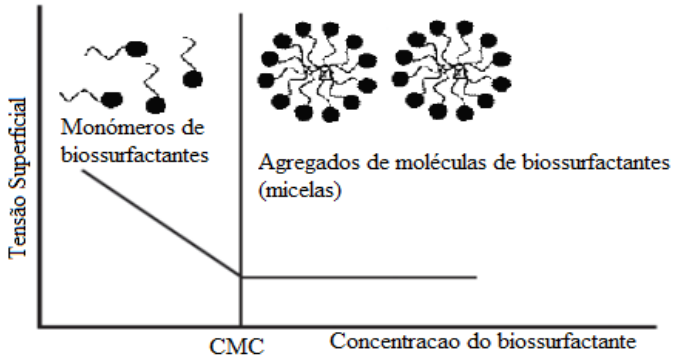
reduzem as forças de repulsão entre fases diferentes (entre a porções hodrofóbicas e moléculas de água) (PACWA-PŁOCINICZAK et al., 2011).

A eficácia de um biossurfactante é tradicionalmente avaliada pela sua capacidade de reduzir a tensão superficial e interfacial (LANG, 2002; MULLIGAN, 2005; COHEN; EXEROWA, 2007). Conforme Soberón-Chávez e Maier (2011), um biossurfactante eficiente pode baixar a tensão superficial da água de 72 para 35mN/m, e a tensão interfacial entre n-hexadecano e água de 40 para 1mN/m. Vários métodos podem ser aplicados para medir directamente a tensão superficial e interfacial em líquidos metabólicos de culturas microbianas (MNIF; GHRIBI, 2015). Entre eles, o método de anel de Du Nouy é de longe o mais usado na pesquisa de biossurfactantes (KIM et al., 1997; ABOUSEOUD et al., 2007; PEREIRA et al., 2013; SARI et al., 2014). Este método mede a força necessária para separar/puxar um anel fino de platina da interface ar/líquido ou líquido/líquido (BODOUR; MILLER-MAIER, 1998), e pode facilmente ser executado usando um tensiômetro automático.

3.4.2.2 Concentração Micelar Crítica (CMC) e Formação de Emulsões

Os biossurfactantes quando presentes em concentrações baixas em uma solução suas moléculas se apresentam na forma monomérica, distribuindo-se paralelamente na superfície (SOBERÓN-CHÁVEZ; MAIER, 2011). Entretanto, acréscimos de monómeros do biossurfactante no meio diminuem a tensão superficial até uma concentração crítica em que adições subsequentes do composto no meio não terão efeito no decréscimo da tensão (SÁENZ-MARTA et al., 2015). Este ponto corresponde a concentração micelar crítica (CMC), figura 2, definida como a concentração mímina necessária para se alcançar a tensão superficial mais baixa (URUM; PEKDEMIR, 2004). À CMC, os monómeros do surfactante começam a se associar espontaneamente formando estruturas supramoleculares que podem ser micelas (de forma esférica ou lamelar) ou vesículas (SATPUTE et al., 2010, PACWA-PŁOCINICZAK et al., 2011). De acordo com Campos et al. (2013), micelas são agregados de moléculas anfipáticas com a parte hidrofílica voltada para água e parte hidrofóbica voltada para o óleo. Estas estruturas decorrem de interacções químicas entre grupos de cabeças polares e grupos de caudas apolares incluindo interacções hidrofóbicas e electrostáticas, forças de Van der Waals e ligações de hidrogénio (RAZA et al., 2010).

Figura 3 - Orientação de moléculas de biossurfactantes na interface água/óleo. A parte hidrofóbica orienta-se no sentido de minimizar o contato com água.



Fonte - PACWA-PŁOCINICZAK et al., 2011; BUSTAMANTE et al., 2012.

A concentração micelar crítica é convencionalmente aplicada para avaliar a eficiência de um composto tensoativo (MULLIGAN, 2005), ou seja, quanto mais baixa for a CMC mais eficiente é o biossurfactante na diminuição da tensão superficial (PACWA-PŁOCINICZAK et al., 2011). A CMC para um biossurfactante depende da estrutura da molécula (URUM; PEKDEMIR, 2004; SÁNCHEZ et al., 2007), além de que pode ser afectada pelo pH, força iónica e temperatura da solução (ÖZDEMIR et al., 2004; COHEN; EXEROWA, 2007; RAZA et al., 2010; Mendes et al., 2015), uma vez que estes fatores podem interferir com a organização estrutural da molécula do biossurfactante (ÖZDEMIR et al., 2004).

A CMC possui um papel determinante em relação a muitas propriedades físico-químicas usadas para caracterizar os biossurfactantes, incluindo a formação de emulsões, solubilização do óleo, formação de espuma e detergência, assim como a tensão superficial e interfacial (Urum; Pekdemir, 2004).

A redução da tensão superficial por formação de micelas na interface de um sistema óleo/água ou hidrocarboneto/água permite a formação de emulsões ou microemulsões, melhorando a interacção entre os componentes da mistura (NGUYEN et al., 2010; SÁENZ-MARTA et al., 2015).

3.4.2.3 Equilíbrio Hidrofílico/Lipofílico (HLB)

A formação de emulsões do tipo óleo-em-água ou água-em-óleo é definida pelas propriedades físico-químicas do biossurfactante, expressas essencialmente pelo equilíbrio hidrofílico/lipofílico (OLIVEIRA et al., 2004; MUTHUSAMY et al., 2008).

O equilíbrio hidrofílico-lipofílico indica a capacidade de um biossurfactante promover a formação de emulsões óleo-em-água ou água-em-óleo (MULLIGAN et al. 2014). Esta propriedade é representada por uma escala de arbitrária de 0-20 (SATPUTE et al., 2010a). O HLB é comumente usado como um parámetro para correlacionar a estrutura, a funcionalidade interfacial e a CMC (ÁLVAREZ VANEGAS et al., 2013); e conforme sugerem Muthusamy et al., (2008), o HLB está diretamente relacionado com o comprimento da cadeia hidrocarbonada na estrutura do biossurfactante. Agentes emulsificantes com valores menores que 6 favorecem a estabilização de emulsificação água-em-óleo, ao passo que emulsificantes com valores de HLB entre 10 e 18 favorece a emulsificação do tipo óleo-em-água (MANEERAT, 2005). O HLB mede a afinidade do surfactante para uma das fases na emulsão (ÁLVAREZ VANEGAS et al., 2013).

3.4.2.4 Propriedade de Emulsificação

Uma emulsão é um sistema coloidal de dois líquidos imiscíveis em que um deles se encontra disperso em outro em forma de gotículas (OLIVEIRA et al., 2004; FRANGE; GARCIA, 2009; CAMPOS et al., 2013). A distinção entre estas duas estruturas é baseada fundamentalmente na sua estabilidade, sendo que as microemulsões são sistemas termodinamicamente estáveis, microscópicos com tamanho de gotículas entre 10-100nm de diâmetro; ao passo que as emulsões não verdadeiramente estáveis, e o diâmetro da gota é na ordem de micrómetros, ou seja, maior que 0,1µm (SATPUTE et al., 2010a).

Em uma emulsão óleo/água, as gotículas do óleo são incorporadas no interior da micela em contacto com extremidades hidrofóbicas das moléculas do biossurfactante (FRANZETTI; TAMBURINI; BANAT, 2008). E o processo de incorpração dessas moléculas na micela é conhecido como solubilização (URUM; PEKDEMIR, 2004).

Os biossurfactantes são capazes de melhorar a estabilidade das emulsões (MUTHUSAMY et al., 2008). De modo geral, quando estes compostos possuem elevado peso molecular são melhores agentes emulsificantes do que os de baixo peso molecular (UZOIGWE et al., 2015). A formação de emulsões estáveis é de particular importância uma vez que confere detergência, alta capacidade dispersante e espumante aos biossurfactantes (DESAI; BANAT, 1997).

3.4.2.5 Propriedade de Dispersão

A propriedade dispersante de biossurfactantes reside na sua capacidade de reduzir as forças coesivas entre partículas similares de um compostos hidrofóbicos (KLEINDIENS et al.,

2015; MNIF; GHRIBI, 2015). Este fenómeno evita que enormes agregados de partículas insolúveis se formem, mantendo-os em suspensão coloidal em um líquido (NYANKSON et al., 2016). Por exemplo, quando um biossurfactante com atividade dispersante é aplicado ao sistema óleo/água, a camada do óleo é quebrada e dispersa em pequenas gotículas no interior de micelas, devido a redução da tensão superficial (MACÍAS-ZAMORA et al., 2014; NYANKSON et al., 2016).

A capacidade de biossurfactantes de dispersar materiais hidrofóbicos pode resultar na desorção de moléculas destes compostos presas em superfícies rochosas ou de solo, além de aumentar a área superficial do óleo como resultado de sua dispersão em pequenas gotículas (SAEKI et al., 2009; MACÍAS-ZAMORA et al., 2014). O fato dos biossurfactantes apresentarem propriedades dispersantes permite a sua aplicação prática em processos que envolvem mobilização, tais como a lavagem de diversos materiais e seu uso ambientes contaminados com compostos hidrofóbicos (YU; XIAO; WANG, 2014; HALLMANN; KRYSTYNA, 2015; NYANKSON et al., 2016).

3.4.3 Micro-organismos Produtores de Biossurfactantes/Bioemulsificantes

Os micro-organismos produtores de biossurfactantes têm sido isolados de diversos habitats tais como ambientes marinhos, locais contaminados e não contaminados como águas e sedimentos (DHAIL; JASUJA, 2012; HASSANSHAHIAN *et al*.,, 2012a; 2012b; DHASAYAN *et al*., 2014), de ambientes terrestres (águas e solos) contaminados com derivados de petróleo (Bento *et al*., 2005; BATISTA *et al*., 2006; CHEN *et al*., 2012), com metais (VIJAYANAND; DIVYASHREE, 2015) bem como de locais sob condições extremas de temperatura (MALAVENDA *et al*., 2015; ELAZZAZY *et al*., 2015), de pH (TAMBEKAR; DHUNDALE, 2012; TAMBEKAR *et al*., 2013), de alta pressão osmótica (BELGACEM et al., 2015), de ambientes salinos e hipersalinos (SARAFIN et al., 2014; COUTO et al., 2015), de reservatórios de petróleo (TABATABAEE; ASSADI, 2005), até de órgãos de animais como pele (JAGTAP *et al*., 2010; EBRAHIMI *et al*., 2012), e intestino (SHARMA *et al*., 2016).

O estudo de micro-organismos com potencial de produzir biossurfactantes isolados de ambientes extremos é uma estratégia de particular interesse para aplicações industriais uma vez que as adaptações específicas dos seus metabólitos podem contribuir para sua estabilidade em ambientes adversos (COUTO *et al*., 2015; BELGACEM *et al*., 2015).

A produção de biossurfactantes por bactérias usando materiais residuais tem sido a mais explorada (BRUMANO et al., 2016). As bactérias dos gêneros *Pseudomonas* (BENINCASA *et al*., 2004; ROCHA E SILVA et al., 2014), *Bacillus* (RAUTELA *et al*., 2014; FEMI-OLA *et al*., 2015), *Rhodococcus* (KRETSCHMER *et al*., 1982; PACHECO *et al*., 2010), *Arthrobacter* (FATIMAH *et al*., 2016), *Acinetobacter* (BAO *et al*., 2014), *Enterobacter* (SARAFZADEH *et al*., 2013; HOŠKOVÁ *et al*., 2013), estão entre as mais reportadas. Dentre bactérias, propriedades e aplicações de biossurfactantes de espécies dos gêneros *Pseudomonas* e *Bacillus* são as que foram amplamente estudadas e são principalmente conhecidos por produzir biossurfactantes de baixo peso molecular como glicolipídeos (raminolipídeos) e lipopeptídeos (surfactina), respectivamente (SOBRINHO *et al*., 2014; BRUMANO *et al*., 2016).

A produção de compostos tensoativos por fungos usando substratos renováveis e de baixo custo ainda não foi amplamente estudada (BRUMANO et al., 2016). Dentro deste grupo, biossurfactantes derivados de leveduras do gênero *Candida*, tais como *Candida lypolitica* (SARUBBO *et al*., 2007; RUFINO *et al*., 2008; 2012; SANTOS *et al*., 2013), *Candida sphaerica* (SOBRINHO et al., 2008; LUNA et al., 2011; 2013), *Candida bombicola* (CASAS *et al*., 1997; ROELANTS *et al*., 2013; ELSHAFIE *et al*., 2015), *Candida apícola* (KURTZMAN *et al*., 2010; VEGA-ALVARADO *et al*., 2015), *Candida shiwadae* (THANOMSUB et al., 2004), *Candida batistae* (KONISHI *et al*., 2008), e do gênero *Pseudozyma* (MORITA *et al*., 2007; AMARAL *et al*., 2010; KATEMAI, 2011), têm sido mais explorados.

Fungos filamentosos como a dos gêneros *Ustilago*, *Absidia*, *Aspergillus* (BATRAKOV et al., 2001; HEWALD et al., 2005), mostram grande capacidade de produzir vários tipos de biossurfactantes. Batrakov *et al.* (2003), extraíram e caracterizaram glicolipídeos extracelulares do micélio de *Absidia corymbifera* F-295. Num trabalho anterior Batrakov et al. (2001), haviam isolado ácidos graxos, glicolipídeos e fosfolipídeos de uma outra cepa desse fungo filamentoso (*Absidia corymbifera* VKMF-965). Hewald *et al*. (2005), fizeram a análise genética do mecanismo de biossíntese de glicolipídos por *Ustilago maydis*, que é capaz de produzir grandes quantidades destas moléculas sob condições de privação de nitrogênio. Usando óleos de soja e de peixe Alejandro *et al*. (2011), também obtiveram glicolipídeos por *Ustilago maydis* FBD12 com propriedades antioxidantes e antimicrobianas. Kiran *et al*. (2009), usando um meio sintético (Zobell Marine broth), avaliaram as propriedades de uma glicoproteína de *Aspergillus ustus* MSF3, que mostrou um largo espectro de atividade antimicrobiana contra patôgenos de humanos

como *Candida albicans, E. coli, Micrococcus luteus, Staphylococcus epidermidis, Enterococcus faecalis, Klebsiella pneumoniae,* e que pode ser usado em processos de recuperação melhorada de petróleo. Qazi et al. (2014), obteve uma tensão superficial de 32 mN/m de um lipopetídeo produzido por *Fusarium* sp. BS-8 em meio suplementado com sacarose e extrato de levedura.

A habilidade dos fungos filamentosos na produção de biossurfactantes usando substratos alternativos de baixo custo como os resíduos agroindustriais foi reportada por alguns cientistas (ANDRADE SILVA *et al*., 2014; SILVA et al., 2014). Assim, Castiglioni *et al*. (2009), sintetizaram um biossurfactante por *Aspergillus fumigatus* com recurso a casca e farelo de arroz em fermentação submersa e sólida. Andrade Silva *et al*. (2014), usou óleo de soja pós-fritura e milhocina para produzir por *Cunninghamella echinulata*, um biossurfactante polimérico capaz de reduzir a tensão superficial da água de 72 para 36 mN/m. Este composto mostrou possuir propriedades que sugerem sua aplicação em diversos setores como indústria de petróleo e remediação ambiental. O fungo *Mucor circinelloides* (SILVA *et al*., 2014) produziu um potente biossurfactante com 26m N/m de tensão superficial e 86% de índice emulsificante em meio suplementado com milhocina e óleo de soja pós-fritura. Um outro composto caracterizado como glicolipídeo foi obtido por Oje *et al*., (2016) em cultura submersa de *Mucor indicus* usando casca de arroz como substrato.

3.4.4 Uso de Substratos Agroindustriais para Produção de Biossurfactantes

O uso de substratos alternativos para formulação de meios de cultura para síntese de biossurfactantes coloca o problema essencial de selecionar um resíduo apropriado com um equilíbrio certo de nutrientes que favoreça um ótimo crescimento do micro-organismo e produção do metabólito (NITSCHKE et al., 2004; THAVASI et al., 2008; SHARMA et al., 2014). A aplicação de resíduos ou subprodutos agroindustriais pode superar esse problema uma vez que geralmente estes materiais possuem níveis elevados de carboidratos e/ou lipídeos requeridos para suportar o crescimento e biossíntese de surfactantes (NITSCHKE et al., 2005). Conforme enfatiza Helmy et al. (2011), a seleção de um resíduo que contenha altos níveis de carboidratos, nitrogênio e lipídeos cumpre as exigências para ser usado como substrato para produção de surfactantes microbianos.

O uso de resíduos agroindustriais como matéria-prima de baixo de custo para a produção microbiana de biossurfactantes é uma estratégia atual e bastante explorada (JAIN et al., 2013).

Muitos destes resíduos tais como milhocina e manipueira (MONTERO-RODRÍGUEZ et al., 2015; (SARUBBO et al., 2015), melaço de cana-de-açúcar (LOBATO et al., 2013), farelo de trigo (PRADEEP et al., 2015), palha de arroz (ZHU et al., 2012; 2013), farelo de arroz (BHARDWAJ et al. 2015), casca da soja (MARTI et al., 2015), são ricos em matérias orgânicas essenciais para o crescimento microbiano.

3.4.4.1 Milhocina

No caso da milhocina, constitui o principal subproduto remanescente da indústria de processamento úmido do milho (LIGGETT; KOFFLER, 1948; XIAO et al., 2012). Este substrato representa aproximadamente 40-50% (w/w) do peso seco do milho (VECINO et al, 2015a), e possui uma mistura de carboidratos, aminoácidos livres (XIAO et al., 2012), peptídeos, ácidos orgânicos (ex.: ácido láctico), vitaminas, sais minerais (magnésio, fósforo, cálcio, potássio, cloreto, sódio, enxofre), fosfato de mio-inositol (HULL et al., 1996; SHAHBAZ QAMAR, et al., 2015)

Devido ao seu elevado teor nutricional a milhocina tem sido aplicada a biossíntese de diversos produtos tais como ácido glutâmico, penicilina, ácidos láctico e hialurónico, celulase (XIAO et al., 2012; NASCIMENTO et al., 2009). Vários trabalhos na área de biossurfactantes sugerem que este resíduo é muito apropriado para a produção destes metabólitos (VECINO et al., 2014; 2015b). SHARMA et al. (2013), testaram diversos resíduos agroindustriais nomeadamente óleos pós-fritura de arroz, da soja, de semente de algodão, da mostarda e milhocina para sintetizar biossurfactante pulullan, e entre os estes residuos a milhocina revelou-se substrato adequada para esse fim.

O uso de milhocina em processos biotecnológicos como fonte de nitrogênio e de carbono depende grandemente de sua composição química (XIAO et al., 2012). A composição química da milhocina é variável, e pode depender da condição e tipo do grão do milho utilizado e de outras variáveis envolvidas no processamento (LIGGETT; KOFFLER, 1948). A qualidade da milhocina e do seu nitrogénio orgânico são de grande importância em processos fermentativos (XIAO et al., 2012).

3.4.4.2 Glicerol

Glicerol é um álcool simples 1,2,3-propanotriol, também conhecido como glicerina, abundante na natureza, uma vez que é principal componente estrutural de lipídeos (SILVA et al., 2009). Tradicionalmente, o glicerol é liberado como um subproduto durante a hidrólise de gorduras (WANG et al., 2001; SILVA et al., 2009). Este resíduo é gerado em largas quantidades no processo de fabricação de biodiesel durante a transesterificação de óleos vegetais e gorduras animais (MOHAMMAD et al., 2002; SOUSA et al., 2011; MA et al., 2012; WEN, 2012). O glicerol tem sido aplicado com diferentes fins nas indústrias de cosméticos, de alimentos, farmacêutica, de tabaco, téxtil, papel e pintura, ou como matéria-prima para produção de vários químicos (WANG et al., 2001). Por outro lado, a utilização de glicerol como substrato em processos fermentativos aumentou nos últimos anos devido ao incremento da produção de biodísiel e outros oleoquímicos (SILVA et al., 2010). Desta forma, este resíduo é usado fundamentalmente como fonte de carbono na indústria biotecnológica para síntese de diferentes produtos microbianos (PUTRI; HERDATI, 2015). Putri e Herdati (2015), e Silva et al., (2010), avaliaram a produção de biossurfactantes por, respectivamente, *Pseudomonas stutzeri* BK-AB12 e *Pseudomonas aeruginosa* UCP0992, usando glicerol como única fonte de carbono, e obtiveram biossurfactante do tipo raminolipídeo, que mostrou ótimas propriedades surfactantes. A levedura *Candida antarctica* foi capaz de render altos níveis de biossurfactante, com 13,6/L de produto final (ACCORSINI et al., 2012).

3.4.5 Aplicações de Biossurfactantes

A diversidade estrutural e as propriedades funcionais excepcionais que os biossurfactantes apresentam fazem deles compostos atrativos para serem utlizadas em uma variedade de processos e operações industriais e ambientais (THAVASI et al., 2011; FRACCHIA et al., 2014; WAGHMODE et al., 2014).

3.4.5.1 Aplicação Ambiental

Propriedades de compostos tensoativos de origem microbiana tais como emulsificação e demulsificação, habilidade de dispersar hidrocarbonetos, capacidade molhante, espumante e de detergência podem ser aproveitadas em processos de biorremediação de ambientes contaminados com compostos orgânicos e inorgânicos (SOUZA et al., 2014; IVSHINA et al., 2016). Além disso, o fato dos biossurfactantes serem ecologicamente compatíveis torna-lhes moléculas com

elevado potencial para serem utilizadas na remoção de diversos agentes contaminantes tanto em ambientes terrestres como aquáticos sem colocar riscos de possível contaminação secundária quando comparados aos seus análogos sintéticos (LAI et al., 2009).

3.4.5.1.1 Aplicação de Biossurfactantes na Biorremediação

A biorremediação baseia-se na capacidade que os micro-organismos possuem de degradar uma enorme variedade de poluentes orgânicos hidrofóbicos em compostos mais simples e não tóxicos. Os micro-organismos apresentam grande versatilidade metabólica e adaptabilidade a diferentes fontes de energia e carbono (AGARWAL; LIU, 2015). Diversos estudos relatam a capacidade das bactérias (FRANZETTI; TAMBURINI; BANAT, 2008; WANG et al., 2011; MONTERO-RODRIGUEZ, et al., 2015; SIANIPAR et al., 2016), leveduras (LUNA et al., 2011; SARUBBO et al., 2016) e fungos filamentosos (POTIN et al., 2004; MANCERA-LÓPEZ et al., 2008) de degradar uma grande variedade de hidrocarbonetos em diferentes ambientes.

A biorremediação, embora seja referido como um método relativamente despendioso no tempo e não rentável para o tratamento de elevados volumes de materiais poluidos, é considerado um método eficiente e ambientalmente aceitável (HASHEMI et al., 2016). Entretanto, a eficiência do processo de biorremediação pode ser influenciada grandemente pela acessibilidade do composto hidrofóbico aos micro-organismos (FRANZETTI; TAMBURINI; BANAT, 2008; CHAPRÃO et al., 2015). As caracteríticas físico-qumicas do composto poluente (polaridade, solubilidade, estrutura molecular) e do ambiente (por ex.: teor de matéria orgânica e inorgânica no solo, tipo de solo), a relação contaminantes/micro-organismos, a interação de contaminantes com matérias orgânica e inorgânica, as propriedades fisiológicas da célula, são fatores que podem afectar a biodisponibilidade do composto para os micro-organismos (BUSTAMANTE et al., 2012; SÁENZ-MARTA et al., 2015).

A baixa solubilidade que os compostos hidrofóbicos apresentam em água aliada ao facto de que eles podem se adsorver sobre as partículas do solo, são fatores que limitam grandemente a interacção do substrato com as células microbianas que captam e utilizam moléculas em solução aquosa para seu metabolismo (LAI et al., 2009). Nesse sentido, os biossurfactantes podem ser aplicados para auxiliar eficientemente a dessorção de compostos insolúveis adsorvidos/absorvidos sobre o solo (CHAPRÃO et al., 2015), como um passo integrante tanto no

processo de biorremediação ou de lavagem melhorada do solo (LAI et al., 2009; BUSTAMANTE et al., 2012).

Na biorremediação de contaminantes orgânicos insolúveis em água os biossurfactantes podem exercer funções importantes com base em seguintes mecanismos (MULLIGAN; GIBBS, 2004):

i. Aumentam a biodisponibilidade de substratos facilitando a sua captação e degradação por células microbianas presentes em ambientes poluídos tais como solo e água;

ii. Interacção com a superfície celular que aumenta a hidrofobicidade da superfície, fazendo com que os substratos hidrofóbicos se associem mais facilmente com os células microbianas.

A finalidade do uso de biossurfactantes em processos de biorremediacão de locais contaminados com compostos hidrofóbicos é tornar a taxa de biodegradação dos contaminantes mais rápida e, por conseguinte, melhorar a remoção de poluentes hidrofóbicos do ambiente (AGARWAL; LIU, 2015).

Os biossurfactantes, dado o seu carácter anfipático, quando em contacto com sistemas óleo/água ou solo/água tendem a se distribuir nas interfaces, reduzindo as tensões superficial e interfacial. Este fenómeno leva ao aumento da área interfacial óleo/água e melhora a transferência da massa do substrato insolúvel para a fase aquosa permitindo que mais micro-organismos possam aceder o substrato (MATVYEYEVA et al., 2014). Portanto, a biodisponibilidade do substrato pode ocorrer com base em seguintes mecanismos: formação de micelas e emulsificação (MOTEVASEL, 2014).

A solubilização ocorre a uma concentração acima de CMC de soluções do biossurfactante. As moléculas se agregam para formar micelas e, assim, aumentam grandemente a solubilidade do óleo em água. As extremidades hidrofóbicas das moléculas do biossurfactante são encapsuladas dentro da estrutura da micela, com a parte hidrofílica ficando exposta à fase aquosa no exterior. O processo de incorporação dessas moléculas na micela é conhecido como pseudosolubilização (FRANZETTI; TAMBURINI; BANAT, 2008).

Por outro lado, na interação biossurfactantes/micro-organismos, estas moléculas desempenham o papel de regular a adesão de micro-organismos à superfícies hidrofóbicas e

hidrofílicas. Os biossurfactantes ligam-se a superfície celular do micro-organismo expondo diferentes partes de suas moléculas alterando, assim, a hidrofobicidade da superfície celular, de acordo com as necessidades do micro-organismo (BANAT et al., 2010; SÁENZ-MARTA et al., 2015). Na remoção de compostos hidrofóbicos do ambiente, esta função fisiológica pode ser explorada por adição de biossurfactantes para aumentar a hidrofobicidade da superfície celular de micro-organismos que permite às células poderem aceder facilmente aos substratos hidrofóbicos (FRANZETTI; TAMBURINI; BANAT, 2008; MOTEVASEL, 2014). Elevada hidrofobicidade celular permite que os micro-organismos tenham contacto direto com as gotas do óleo ao passo que a baixa hidrofobicidade facilita a sua adesão à micelas ou óleos emulsificados (PACWA-PŁOCINICZAK et al., 2011; LIM et al., 2016).

3.4.5.2 Aplicação na Indústria de Petróleo

O principal mercado potencial para utilização de biossurfactantes no setor industrial é o ramo petrolífero, onde tem sido aplicados na recuperação melhorada de petróleo (NITSCHKE; PASTORE, 2002; BANAT et al., 2010). Devido a sua alta capacidade de emulsificação e detergência, os biosuurfactantes podem desempenhar um papel em processos de extração, transporte de petróleo crú, refinamento e produção de petroquímicos (OKOLIEGBE; AGARRY, 2012).

3.4.5.2.1 Recuperação Melhorada de Petróleo

As tecnologias convencionais de recuperação primária e secundária de petróleo podem extrair parcialmente o petróleo, com uma eficiência aproximada de 30-40% da totalidade de petróleo inicialmente disponível no reservatório. Tal eficiência poderá decrescer com a depleção gradual de reservatórios de óleo leve no reservatório (PERFUMO; RANCICH; BANAT, 2008). Assim, processos “terciários” ou recuperação melhorada de petróleo (MEOR) apresentam-se como métodos economicamente atrativos e ambietalmente compatíveis (SEN, 2008; BANAT et al., 2010), que utilizam micro-organismos e/ou produtos de seu metabolismo para recuperação de óleo residual contido nas rochas de reservatórios e que não pode ser extraído pelos métodos convencionais (MARCHANT; BANAT, 2012).

Os micro-organismos sintetizam uma variedade de metabólitos importantes para aplicação na MEOR incluindo gases, ácidos, solventes, biomassa, biossurfactantes/bioemulsificantes. Os biossurfactantes em particular têm o papel de reduzir a tensão interfacial óleo/água e óleo/rocha,

reduzindo as forças capilares que impedem a movimentação do óleo através dos poros da rocha, formando emulsões, melhorando a eficiência do processo (BANAT et al., 2010; PERFUMO; RANCICH; BANAT, 2008).

As estratégias até agora identificadas para a MEOR usando biossurfactantes incluem (NITSCHKE; PASTORE, 2002): injeção no reservatório de biossurfactantes produzidos *ex situ*; injeção no reservatório de micro-organismos produtores de biossurfactantes; estimular o crescimento de micro-organismos selvagens para produzir biossurfactantes *in situ* através do suprimento de nutrientes apropriados.

Entre os micro-organismos, somente bactérias são consideradas candidatas promissoras para MEOR. O principal obstáculo para o desenvolvimento da estratégia de produção *in situ* relaciona-se com dificuldade de isolar cepas microbianas adaptadas ao ambiente adverso de reservatórios, caracterizado por baixa tensão de oxigênio, alta pressão e salinidade, temperaturas até 85ºC e pH extremos (FRACCHIA et al., 2012). Alguns operadores quando injetaram micro-organismos nos reservatórios de petróleo, experimentaram problemas de obstrução e corrosão. Por esta razão, a adição de biossurfactantes produzidos *in situ* foi recentemente proposta como uma opção para tal aplicação (FRACCHIA et al., 2012).

3.4.5.2.2 Limpeza de Tanques de Armazenamento de Petrólleo

Borras e resíduos de óleos pesados que se acumulam no fundo e nas paredes de tanques usados no transporte e estocagem do óleo são altamente difíceis de removê-los por meio de bombeamento convencional. A remoção periódica requerida envolve a lavagem com solventes ou limpeza manual e ambos são procedimentos perigosos, demorados, laboriosos e caros (MULLIGAN; SHARMA; MUDHOO, 2014). Porém, os biossurfactantes podem ser aplicados para limpeza de tanques de estocagem de óleo pesado. Estas moléculas promovem a formação de emulsões óleo/água reduzindo a viscosidade de resíduos do óleo, que pode ser facilmente bombeados e as frações do petróleo podem ser recuparadas após a quebra da emulsão (BANAT et al., 1991).

3.4.5.3 Aplicação na Indústria Alimentar

Os biossurfactantes possuem uma variedade de propriedades potencialmente úteis para a indústria de alimentos especialmente como agentes emulsificantes, solubilizantes, molhantes (NITSCHKE; COSTA, 2007; RANASALVA; SUNIL; POOVARASAN, 2014), bem como no

controle de crescimento, adesão e formação de biofilmes de patôgenos microbianos (CAMPOS et al., 2013). A emulsificação tem papel importante na consistência e textura de alimentos bem como dispersão de fase e a solubilização de aromas (CAMPOS et al., 2013; SANTOS et al., 2016). A função geral de um emulsificante é estabilizar a emulsão controlando a agregação de glóbulos de gorduras e estabilização de sistemas gaseificados (SANTOS et al., 2016). Exemplos de alimentos que são emulsões compreendem maionese, licor de creme, sorvete, manteiga, margarina, etc. (SANTOS et al., 2016). Além de sua aplicação principal na formação e estabilização de emulsões, os bioemulsificantes seriam usados também para melhorar a textura e validade de produtos contendo amido, modificar as propriedades reológicas de massa para pão de trigo, melhorar a consistência e textura de produtos à base de gordura (FRACCHIA et al., 2012).

O aumento da consciência entre os consumidores sobre as vantagens que os surfactantes de origem microbiana oferecem comparativamente aos sintéticos, levou a um elevado interesse em encontrar fontes naturais alternativas de ingredientes e aditivos apropriados para uso em indústrias de alimentos, reduzindo assim, o uso de compostos artificiais ou quimicamente sintetizados (FRACCHIA et al., 2012). Os emulsificantes comercialmente disponíveis usados como ingredientes nas indústrias de alimentos e de bebidas compreendem dois tipos principais: a lecitina, derivada de soja e ovo, e uma variedade de emulsificantes produzidos a partir de fontes sintéticas. Não obstante as propriedades que os biossurfactantes apresentam, seu uso na indústria de alimentos ainda não é difundido (SANTOS et al., 2016).

3.4.5.4 Aplicações Biomédicas e Terapêuticas

As propriedades bioativas de biossurfactantes e suas potenciais aplicações medicinais têm sido objeto de estudo em muitos trabalhos de pesquisa (RUFINO et al., 2012; SAMBANTHAMOORTHY et al., 2014; MEENA; KANWAR, 2015). O interesse pelo uso de biossurfactantes na área biomédica tem crescido continuamente devido às suas variadas atividades biológicas que incluem atividades antimicrobiana, antiviral, anti-adesiva, antitumoral, imunomoduladora ou inibidora de toxinas e enzimas específicas (FRANZETTI; TAMBURINI; BANAT, 2008; FARIQ; SAEED, 2016). Além disso, o uso dessas biomoléculas para estes propósitos é visto como uma estratégia promissora e alternativa às drogas convencionais disponíveis no mercado face a resistência demonstrada por patôgenos microbianos contra muitos destes antimicrobianos (DAS; MUKHERJEE; SEN, 2008; FARIQ; SAEED, 2016).

As diversas habilidades que os biossurfactantes exibem, fazem deles biomoléculas com possibilidade de serem aplicados para o fabrico de agentes terapêuticos visando combater doenças causadas por micro-organismos e diversas outras aplicações biomédicas (KALYANI; BISHWAMBHAR; SUNEETHA, 2011).

3.4.5.4.1 Atividade Antimicrobiana

Os biossurfactantes mostram atividade contra diferentes micro-organismos: bactérias, leveduras, fungos filamentosos, algas e vírus (BENINCASA et al., 2004; RANASALVA; SUNIL; POOVARASAN, 2014). A forma como os agentes antimicrobianos agem contra os patôgenos é diversificada e é determinada pela sua composição química (GHARAEI-FATHABAD, 2011; PRASAD et al., 2015), podendo ser por inibição de síntese de proteínas, de parede celular ou de ácidos nucléicos, rutura de membranas bacterianas, e em alguns casos interferência com vias metabólicas (FARIQ; SAEED, 2016).

Muitos biossurfactantes exibem um largo espectro de atividade na sua ação contra os micro-organismos (GHARAEI-FHATABAD, 2011). Kalyani et al., 2014, estudaram as propriedades antibióticas de um biossurfactante produzido por *Streptomyces matensis* PLS-1, e os seus resultados mostraram potente atividade contra duas espécies bacterianas, *Escherichia coli* (Gram-negativa) e *Staphylococcus aureus* (Gram-positiva). Biossurfactantes produzidos por duas bactérias probióticas, *Lactococcus lactis 53* e *Streptococus thermophilus*, mostraram bioatividade em pequenas concentrações contra bactérias e leveduras (PRASAD et al., 2015). Rufino et al., 2012, relataram as atividades antimicrobianas de dois biossurfactantes isolados de *Candida lipolytica* UCP 0988 e *Candica sphaerica*, com capacidade de inibir o crescimento de vários micro-organismos tais como *C. albicans*, *Staphylococcus. aureus*, *Streptococcus oralis*, *E. coli*, entre outros tantos.

As potenciais aplicações biomédicas e modo de ação de biossurfactantes produzidos por bactérias e leveduras são os que têm sido mais reportados na literatura (RODRIGUES; TEIXEIRA, 2008; CORTÉS-SÁNCHEZ et al., 2013; FRACCHIA et al., 2015). Entre biossurfactantes, os pertencentes às classes de glicolipídeos e lipopeptídeos são os que tem sido mais estudados (RODRIGUES et al., 2006). Neste sentido, as propriedades antimicrobianas de raminolipídeos isolados de espécies do gênero *Pseudomonas* contra vários patôgenos foram relatadas por vários investigadores (BENINCASA et al., 2004;; SOTIROVA; SPASOVA;

GALABOVA, 2009; ARAUJO et al., 2016). Em diversos estudos demonstrou-se que estas moléculas possuem excelentes propriedades antifúngica contra por exemplo, *Aspergillus niger* e *Penicillium chrysogenum* (ABALOS et al., 2001), e também exercem ação antibacteriana (ARAUJO et al., 2016). Os biossurfactantes manosileritritol-lipídeos (KITAMOTO; ISODA; NAKAHARA, 2002), sofolipídeos (MORYA et al., 2013) e trealose-lipídeos (ZARAGOZA et al., 2009); VARVARESOU; IAKOVOU, 2015) são outros glicolipídeos que mostram enorme potencial clínico no combate aos diversos agentes patogênicos. Manosileritritol-lipídeos produzidos por *Candida antarctica* apresentou forte atividade contra, principlamente, bactérias Gram-positivas (KITAMOTO, 1993).

Para a classe de lipopeptídeos têm sido reportadas propriedades antimicrobianas em particular para surfactina e iturina produzidos por bactérias de gênero *Bacillus* (FERNANDES et al., 2007; RODRIGUES; TEIXEIRA, 2008; SHEKHAR; SUNDARAMANICKAM; BALASUBRAMANIAN, 2015). Seu largo espectro de ação antibiótica inclui micro-organismos resistentes à múltiplas drogas (SHEKHAR; SUNDARAMANICKAM; BALASUBRAMANIAN, 2015). A surfactina possui importância antimicrobiana muito promissora devido às suas versáteis propriedades bioativas incluindo ação antifúngica, antibacteriana, antimicoplasma e antiviral (MEENA; KANWAR, 2015). Outros lipopeptídeos com ação antibiótica e antiviral são iturina e a fengicina (DAS; MUKHERJEE; SEN, 2008; MEENA; KANWAR, 2015).

3.4.5.4.2 Atividade Anti-adesiva

A atividade antiadesiva é outra característica peculiar de biossurfactantes também com possível aplicação na área médica. Ela está relacionada com a capacidade que estas moléculas possuem de inibir a adesão de micro-organismos causadores de várias doenças à superfícies de diferentes materiais bióticos e abióticos (FRACCHIA et al., 2012, 2015; PRASAD, 2015; MNIF; GHRIBI, 2015b). Biossurfactantes produzidos por micro-organismos isolados de diferentes locais tais como ambientes marinhos (THAVASI, R; BANAT, 2014; GUDIÑA; TEIXEIRA; RODRIGUES, 2016), solos contaminados com metais (LUNA et al., 2011) e probióticos (FARIQ; SAEED, 2016), exibiram poderoso efeito anti-adesivo contra vários patôgenos testados.

O glicolipídeo trealoselipídeo produzido por *Rhodococcus ruber* IEGM 231 (KUYUKINA et al., 2016), teve ação contra adesão e formação de biofilmes de bactérias Gram-positivas e Gram-negativas tanto em estado latente como em fase exponencial de crescimento

sobre superfície de microplacas de poliestireno. Este glicolipídeo foi mais ativo contra células bacterianas em crescimento do que em estado de dormência (KUYUKINA et al., 2016). Da mesma forma, um lipopeptídeo produzido por *Bacillus licheniformis*, foi notavelmente eficiente no pré-tratamento da superfície de poliestireno para evitar a adesão e consequente desenvolvimeno de biofilmes de *Staphylococcus aureus* resistente à meticilina (MRSA) e *Candida albicans*. O mesmo foi também muito ativo na remoção de biofilmes do MRSA e *Yersinia enterocolitica* (CORONEL-LEÓN et al., 2016).

Os biossurfactantes produzidos por leveduras mostram também propriedades anti-adesivas contra bactérias e fungos (RUFINO et al., 2011; 2012). Um biossurfactante isolado de *Candida sphaerica* UCP0995 (LUNA et al., 2011), inibiu a adesão de *Pseudomonas aeruginosa*, *Streptococcus agalactiae*, *S. sanguis* 12 sobre material plástico. O mesmo mostrou ser um potente antimicrobiano contra bactérias (LUNA et al., 2011).

Os micro-organismos, na maior parte dos casos, ocorrem na natureza sob a forma de associações multiespecíficas crescendo sobre diferentes tipos de substratos designadas biofilmes (MIQUEL et al., 2016). A formação de um biofilme consiste em uma sequência de fases que envolvem: (i) aderência de micro-organismos sobre o substrato; (ii) formação de colónias e produção da matriz polimérica (consistindo de polissacarídeos, proteínas, fosfolipídeos e ácidos nucléicos), (iii) maturação do biofilme e (iv) separação e dispersão de células que podem atacar outras superfícies (MULLIGAN et al., 2014; CUKKEMANE et al., 2015). Nestas associações estruturadas os biofilmes apresentam uma fisiologia que lhes permite perceber e responder a própria densidade celular e comunicam-se através da sinalização molecular, este fenômeno é conhecido como quorum-sensing (MULLIGAN et al., 2014).

O desenvolvimento descontrolado de biofilmes não-desejados representa uma enorme preocupação em diferentes setores de atividades, pois uma vez presentes podem ser importantes fontes de contaminação e degradação de materiais, de transmissão de doenças e podem afetar sobremaneira a economia de processos industriais (CORONEL-LEÓN et al., 2016; ARAUJO et al., 2016). Por exemplo, agentes patogênicos *Lysteria monocytogenes* (MEYLHEUC; VANOSS; BELLON-FONTAINE, 2001; ARAUJO et al., 2011), *Staphylococcus aureus* (WALENCKA et al., 2007); *Salmonela enterica* e *S. enteriditis* (PENG et al., 2015), são implicados com infeções humanas transmitidas por alimentos. Biofilmes constituem também de objeto elevada atenção na

área médica. Infecções hospitalares transmitidas por espécies patogénicas tipos tais como dos gênero *Candida* (BULGASEM et al., 2016) e *Streptococcus* (CUKKEMANE et al., 2015; CORTÉS-SÁNCHEZ et al., 2016), são uma questão de preocupação, pois têm habilidade de formar biofilmes em diferentes objetos de uso e tratamento médicos.

O controle de formação de biofilmes prejudiciais em diversos ambientes de atividade tem sido principalmente com recurso a limpeza e uso desinfectantes. Estas estratégias, embora possam ser sucedidos na remoção ou inibição de crescimento de células planctônicas, as mesmas mostram-se ineficientes para eliminar as células protegidas dentro de biofilmes (CORONEL-LEÓN et al., 2016). O surgimento de estirpes de certos micróbios resistentes às drogas convencionais é outro aspecto que reforça grandemente a dificuldade para eliminação de biofilmes microbianos (PENG et al., 2015). Os micro-organismos associados em biofilmes são mais resistentes a uma larga variedade de estresses ambientais em relação às células correspondentes na forma planctónica (BANAT et al., 2014a).

A adesão microbiana a uma superfície é o primeiro passo importante na formação de biofilmes. Este fenómeno pode ser afetado pelo tipo do micro-organismo, disponibilidade de nutrientes e factores ambientais, capacidade do micro-organismo para produzir polímeros extracelulares, a hidrofobicidade da superfície celular e cargas eléctricas da superfície (RUFINO et al., 2014; CORTÉS-SÁCHEZ et al., 2013; VIJAYAKUMAR; SARAVANAN, 2015; KUYUKINA et al., 2016). São muitos os estudos pelo mundo fora que reportam as propriedades anti-adesivas e antibiofilmes dos vários tipos de biossurfactantes contra diferentes patôgenos micronianos. Os mecanismos de ação da propriedade anti-adesiva destas moléculas está relacionada com sua capacidade de modificar a superfície celular do micro-organismo interferindo no processo adesivo (SAMBANTHAMOORTHY et al., 2014), e também de destruir membranas celulares de micro-organismos sésseis (BANAT et al., 2014a). Neste contexto, esta propriedade é considerada promissora para ser aplicada no desenvolvimento de novos agentes com ação antibiofilmes (RUFINO et al., 2011; LUNA et al., 2011; KUYUKINA et al., 2016; ARAUJO et al., 2016), além de que são mais seguros e menos tóxicos (SINGH; CAMEOTRA, 2004).

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CAPÍTULO II

Artigo I

Development and Improved Selected Markers to Biosurfactant and Bioemulsifier Production by *Rhizopus* Strains Isolated from Caatinga Soil

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Development and Improved Selected Markers to Biosurfactant and Bioemulsifier Production by *Rhizopus* Strains Isolated from Caatinga Soil

Milagre A. Pele [1,6], Dayana Montero-Rodriguez [1,6], Daylin Rubio-Ribeaux [1,6], Adriana F. Souza [2,6], Marcos A. C. Luna [2,6],Michele F. Santiago[3], Rosileide F. S. Andrade [4,6], Thayse A. Lima e Silva [4,6], André L. C. M. A. Santiago[5], Galba M. Campos-Takaki [6,*]

[1] Biological Sciences Post-Graduation Program, Federal University of Pernambuco, 50670-420 Recife-PE, Brazil. phelema@yahoo.com; dayanamontero87@gmail.com; drubioribeaux@gmail.com

[2] Northeast Network for Biotechnology, Federal Rural University of Pernambuco, 52171-900 Recife-PE, Brazil. adrife.souza@gmail.com ; macluna@bol.com.br

[3] Agronomic Institute of Pernambuco (IPA), 50761-000 Recife-PE, Brazil. miccafs@hotmail.com

[4] Post-Doctorate National Program-CAPES, Catholic University of Pernambuco, 50050-900 Recife-PE, Brazil. rosileide_fontenele@yahoo.com.br ; ithalv@yahoo.com.br

[5]Department of Mycology, Federal University of Pernambuco, 50670-901 Recife-PE, Brazil. andrelcabral@msn.com

[6] Nucleus of Research in Environmental Sciences and Biotechnology, Catholic University of Pernambuco, 50050-590, Recife-PE, Pernambuco, Brazil. galba_takaki@yahoo.com.br

[*] Correspondence: galba_takaki@yahoo.com.br ; Phone.: +51-81-21194044; Fax: +55-81-21194043.

Abstract

This study was screened four *Rhizopus* species to biosurfactant-producing using different markers. First of all *Rhizopus* sp. UCP 1607 was identified as *R. arrhizus* by morphological and molecular methods. The production of biosurfactant/bioemulsifier was investigated by submerged fermentation using soybean post-frying oil (5% v/v) and sodium glutamate (1% w/v) medium. The primary markers hemolysis and parafilm M tests showed that *R. arrhizus* UCP1607 strain producing higher hemolytic activity (49 mm of clear zone) on sheep blood agar, and parafilm M test exhibited a larger drop diameter (12 mm) on parafilm hydrophobic surface. The experimental results showed the most promising biosurfactant production by *R. arrhizus* UCP 1607 strain resulting in the reduction of surface tension of 31.8 mN/m, and the measurement of the diameter of the oil spreading of the 66.4 cm^2. The strains *R. microsporus* var. *chinensis* UCP1296, *R. microsporus* var. *microsporus* UCP1304 and *R.arrhizus* UCP1607 were capable of forming stable emulsions corresponding to 91.7%, 94.8%, and 82.6%, respectively in crude oil.

Key words: Tensio-active agent, bioemulsifier, screening of *Rhizopus* strains, submerged fermentation.

1. Introduction

Caatinga is a biome that comprises an extensive semi-arid area of 969.589,4 km^2, located in the Northeast of Brazil. A prominent feature of the biome Caatinga is the climate markedly characterized by severe environmental conditions where high temperatures with the minimum above 15°C and the maximum around 40°C, intense insolation, scanty water resources, and the annual rainfall in the area is estimated to be lower than 1000 mm, leading to prolonged periods of serious drought. Moreover, the predominantly shallow soils present low natural fertility [1,2]. These environmental conditions of the semi-arid region of Caatinga biome have a direct influence on soil microbial life. Thus, microorganisms to survive in these stressful factors have developed adaptive mechanisms of response, synthesizing appropriate metabolites [2,3].

Studies aiming to explore the biotechnological potential of genus *Rhizopus* have demonstrated that species of this genus are able to produce different types of compounds of an enormous industrial importance namely enzymes [4], organic acids [5], chitin and chitosan [6], including biosurfactant production [7].

Biosurfactants are products of the metabolism of living cells especially of bacteria, yeasts and filamentous fungi that may be produced extracellularly or as part of cell membranes [8].

Structurally biosurfactants are amphipathic molecules possessing hydrophobic and hydrophilic domains [9]. Their complex structural organization gives them important physico-chemical properties such as lowering surface and interfacial tensions between immiscible phase systems, promoting the formation of micelles through which hydrophobic compounds can be solubilized in water or vice-versa [10]. In addition, these compounds are known to be efficient dispersing and emulsifying agents, exhibit higher foaming and wetting abilities, displaying low critical micelle concentration (CMC) [11]. These properties make biosurfactants molecules with a wide range of practical applications in the bioremediation of contaminated environments, enhanced oil recovery, as ingredients in the food processing industry, cosmetics and pharmaceutical industry [11,12].

The natural origin of these molecules turn them more interesting compounds, along with non/lower toxicity, higher biodegradability, effectiveness at extreme conditions (of

pH, temperature, and salinity), biocompatibility, and specificity in their function [12]. Due to their advantages and numerous possible uses in different areas microbial surfactants has been de central point of diverse studies aiming to identify potential microorganism producers of these molecules [13]. However, the majority of screening studies has been carried out using bacteria [14,15,16,17], and outgrows by far those evaluating the fungi producing potential [18,19].

With this in mind, it is attractive the discovery of biosurfactant producing microorganisms capable of inhabiting environments featured by adverse typical conditions such as extreme salinity, higher temperatures, scanty humidity [20,15,21].
In this work, was done the identification of *Rhizopus* sp. UCP 1607, as well as the new efficient biosurfactant-producing strains were screened by primary markers (hemolysis and parafilm M tests), and properties of the biosurfactant including, surface tension reduction, oil spreading activity and emulsification ability were studied.

2. Materials and Methods

2.1 Micro-organisms

Four *Rhizopus* strains from the semi-arid region of Caatinga biome were screened for biosurfactant/bioemulsifier production. Three of them named *Rhizopus arrhizus* var. *arrhizus* UCP 1295, *Rhizopus microsporus* var. *chinensis* UCP 1296, and *Rhizopus microsporus* var. *microsporus* UCP 1304 were kindly provided by the culture collection of Nucleus for Research in Environmental Sciences and Biotechnology, Catholic University of Pernambuco, Recife-PE, Brazil which is registered in the World Federation for Culture Collections (WFCC).

2.2 Fungus isolation

The new *Rhizopus* sp. Strains was isolated from Caatinga soil sample collected in Rio Grande do Norte state, Northeast of Brazil were used following media (g/L): wheat germ agar medium (wheat germ 15; glucose 5 and agar 15, supplemented with chloramphenicol 0.1), malt extract agar (MEA) (malt extract 20; agar 20), Sabouraud dextrose agar (SDA) (peptone 10; glucose 40 and agar 20). The isolation of the fungus was carried out by soil sprinkling technique according to Benny [22]. Briefly, 5 mg of soil

sample were weighed using a precision balance, and then were spread onto wheat germ agar medium plates and incubated at 28°C until sporulation. Then after that, using a sterile syringe mature sporangiospores were transferred directly from the colonies to MEA plates and then incubated at 28°C for 7 days. Pure culture of the isolate was maintained on SDA slants and stored at 4^{o}C in a refrigerator. Transfers were done to fresh SDA slants each three months to maintain the isolate viable.

2.2 Morphological and molecular identification

The macroscopic and microscopic identification was conducted according to Zheng et al. [23]. The macroscopic morphology (colony size, aspect and color) was attained by naked eye examination of 5-7 days old culture grown on potato dextrose agar (PDA) medium (g/L peeled potato 200, dextrose 20, and agar 15). The microscopic morphology was observed by light microscopy using Lactophenol Cotton Blue staining and distilled water.

The genomic DNA was extracted from mycelium grown on PDA for 72 hours, at 28^{o}C by the CTAB DNA Extraction Protocol method, adapted from Doyle and Doyle [24]. The ribosomal DNA ITS1-5.8S-ITS2 and LSU regions were amplified by polymerase chain reaction (PCR) on a Peltier PTC-100® thermocycler (MJ Research, Inc., USA) in a total volume of 25 µL of sample. The rDNA ITS regions were amplified using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS2 (3'-GCTGCGTTCTTCATGAGC-5'), [25]. The D1/D2 LSU region of the rDNA was sequenced using primers NL1 (5' GCATATCAATAAGCGGAGGA-3') and NL4 (5'-GGTCCGTGTTTCAAGACGGGTCG-3') [26]. The amplicons purified with *PureLink - PCR Purification Kit C/50rxn Columns - Invitrogen* and sequenced by ACT Gene Molecular Analyzes, Alvorada-RS.

2.3 Screening for biosurfactants production

2.3.1 Primary Screening: Hemolysis and Parafilm M tests

Preliminary identification of the potentially biosurfactant-producing *Rhizopus* strains was performed by the hemolytic activity test [27]. Spores of *Rhizopus* strains was inoculated on the central part of the agar plate containing 5% (v/v) of defibrinated sheep blood and incubated at 28°C for four days. The experiments were monitored for

observation of hemolytic activity which was detected by appearance of clear zone on blood agar plate.

Parafilm M assay is a rapid and simple test that does not require specialized equipment and can be carried out with small sample volumes. The test consisted of placing 25 µL of mycelia-free metabolic liquid on hydrophobic surface of the parafilm M strip. The shape of the drops was examined after 1 min, and its diameters were measured using a caliper. The presence of the surface active compounds in the mycelia-free metabolic liquid was detected by the flat shape of the drop, while the semispherical shape indicates the absence of biosurfactant/bioemulsifier. The medium without microorganism was used as control [18].

2.4 Biosurfactant/bioemulsifier production

The strains *R. arrhizus* var. *arrhizus* UCP 1295, *R. microsporus* var. *chinensis* UCP 1296, *R. microsporus* var. *microsporus* UCP 1304, and *Rhizopus* sp. UCP 1607 were grown on PDA for 96 hours at 28ºC. Spore suspensions were prepared in the sterile water and adjusted to 10^7spores/mL, and 5% of suspensions were inoculated in Erlenmeyer flasks containing 100 mL of the medium constituted by soybean post-frying oil (5% v/v), sodium glutamate (1% w/v), and salt solution (g/L): **(**NH_4NO_3 1.0, KH_2PO_4 0.2, and $MgSO_4.7H_2O$ 0,2), and the pH was adjusted to 5.5. The flasks were incubated in orbital shaker at 150 rpm, at 28ºC during 96h. The net metabolic liquid containing biosurfactant was obtained by filtration followed by centrifugation (10.000x *g* for 15 min), and was used to secondary screening.

2.4 Secondary screening

2.4.1 Surface tension determination

The measurement of surface tension in the mycelia-free broth was performed using an automatic Tensiometer (model Sigma 70 KSV Ltd, Finland), utilizing the Du Nouy ring method as described by Kuyukina et al. [28]. The results were reported as the average of measurements in triplicate.

2.4.2 Oil spreading assay

In order to determine the biosurfactant dispersing ability was undertaken the oil spreading test by placing 40 mL of distilled water in a Petri dish (15 cm of diameter), and this was followed by addition of 1.0 mL of burnt motor oil onto water layer surface. After that, 0.5 mL of metabolic liquid (A), 0.5 mL of commercial detergent (B), 0.5 mL of chemical surfactant SDS (C), and 0.5 mL of distilled water (D) were placed in the center of the oil film. The presence of the biosurfactant/bioemulsifier in the mycelia-free broths was detected by the spreading of oil resulting in the formation of oil displacement areas. The clear zone diameters were measured and the respective oil displacement areas (ODA) were determined and expressed in cm^2 using the equation below [29]. The experiments were performed in triplicate. ODA = 3.14 x r2

2.4.3 Emulsification index (E_{24})

The emulsifyer properties of the biosurfactant in crude extracts produced by *Rhizopus* strains was evaluated by emulsification index assay. For determination of emulsification index 1.0 mL of mycelia-free metabolic liquid containing biosurfactant and 1.0 mL of burnt motor oil were mixed together in a test tube, and then homogenized vigorously for 2 min at room temperature (25^{o}C). After 24 hours measurements were performed through the equation:

$$E_{24}\ (\%) = He/Ht \times 100,$$

where He = emulsion height; Ht = mixture total height [30].

3. Results and Discussion

3.1 Isolation, phenotypic and molecular Identification

The isolation of *Rhizopus* sp. UCP 1607 from the Caatinga semi-arid region soil sample was accomplished on basis of colony morphology (Figure 1). The isolate showed rapid growth on PDA plates at temperature of 28^{o}C, covering the entire Petri plate of 9 cm in diameters with abundant mycelia, and the colonies were cottony, initially white and later turned gray blackish (A). Under light microscope straight and opposite sporangiophores arising from rhizoids were observed (B). Rhizoids finger-like branched (C). Straight to substraight, 2-3 in groups and opposite sporangiophores arising from

rhizoids, 285 to 840-1000 µm long, 8-17 µm diameters. Sporangia black, globose to slightly depressed globose, 72-168 µm diameter. Columellae subglobose, 50-114 x 78-149 µm, light grayish-brown. Sporangiospores ovoid, the mostly regular in shape and size, 5-7 x 4-6.5 µm, light gray the solitary (D). The fungus was identified as belonging to *R. arrhizus* [23].

Species of *Rhizopus* are worldwide distributed inhabiting different environments, so they may be isolated from soil, dung, decaying organic material and mature fruits and a variety of food products [31]. Some species of this genus live as pathogens causing diseases in humans, animals and plants [32].

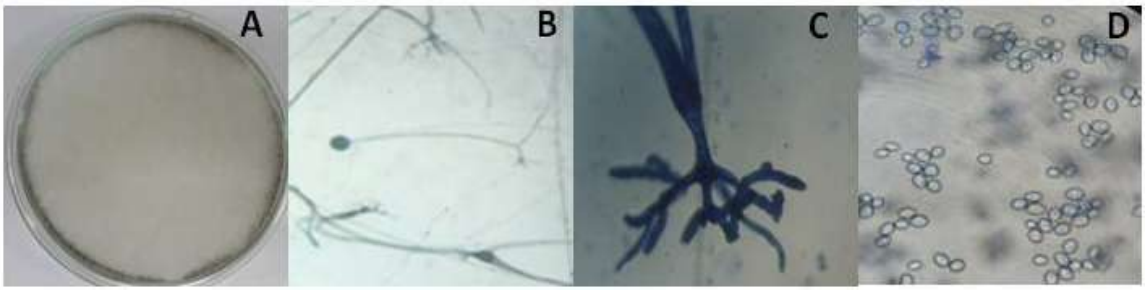

Figure 1: Photographs of macroscopic characteristics of *Rhizopus* sp. UCP 1607 grown on potato dextrose agar (A), under light microscope straight and opposite sporangiophores arising from rhizoids were observed (B). Rhizoids finger-like branched (C). Sporangiospores (D).

There are various studies on isolation and assessment of diversity of tropical areas filamentous fungi including Brazil [33,34], only few reports are referred to isolates from semi-arid environments [35,36,37]. In this context, little is known about filamentous soil mycota of the Caatinga biome.

Considering this fact Cavalcanti et al. [38], studied the diversity of soil filamentous fungi in Xingó, state of Bahia, a region with typical Caatinga ecosystem. Among Zygomycota two *Rhizopus* species were isolated and identified as *R. microsporus* var. *chinensis* and *R. microsporus* var. *microspores.* Santiago et al . [31], worked with soil samples of three different semi-arid areas of the state of Pernambuco to evaluate the distribution of Mucorales order. The authors reported the *R. microsporus* var. *microsporus* (10.19%) and *R. arrhizus* var. *arrhizus* (7.41%) as one of the most frequent genus in the three areas. In this study *R. stolonifer* and *R. microsporus* var. *chinensis* were isolated from the same area. Oliveira et al. [39], assessed the diversity of

filamentous fungi from soil in the same state and identified same species as *R. microsporus* var. *microsporus* and *R. arrhizus*.

We observe that molecular identification of the isolate, using the nucleotide sequence found was compared to those deposited in NCBI (National Center for Biotechnology Information website) using the BLAST program. The results identified homology of 95% of similarity to *Rhizopus arrhizus*. In the current study, the phenotypic characteristics of the *R. arrhizus* matched with the molecular analysis for the definitive identification of the fungus. Different rDNA regions have been frequently used for the identification of Mucorales. Moreover, the 18S regions of the small ribosomal subunit, the region of the internal transcribed spacer (ITS), and the large ribosomal subunit (LSU) region are the most commonly used as markers [40,41,42,43].

3.2 Detection of biosurfactant-producing Rhizopus strains

According to Thavasi et al. [44], microorganisms with positive hemolytic activity for production of biosurfactants showed a clear zone in the blood agar plates. In this context, only the strain *R. arrhizus* UCP1607 produced extracellular compound on the blood agar and formed higher halo (40 mm), during the radial growth (72h). The hemolytic activity demonstrated by *R. arrhizus* strain was superior to that presented by *Aspergillus* sp. MSF1, which clear zone showed diameter of 7 mm on blood agar medium as described Kiran et al. [27]. The hemolytic activity was employed by several authors as initial criterion for selection of biosurfactant producers [14,16]. However, Satpute et al. [45], recommend the use of more than one screening method for indication of biosurfactant-producing microorganisms.

Parafilm test and surface tension determination are both physical methods widely applied for identification of biosurfactant-producing microorganisms [18,46]. The results from parafilm M assay showed that *Rhizopus* strains tested in current study were able to produce biosurfactants with different surface-active properties, since the droplet diameter of the metabolic liquid of each strain was larger, compared with fresh culture medium (Table 1). However, the best results were exhibited by the new strain of *R. arrhizus* UCP 1607 forming largest drop (12 mm diameter) on the hydrophobic surface (parafilm M strip) (Figure 2).

The surface tension of the cell free broth from *R. arrhizus* UCP1607 reached lowest value (31.8 mN/m). Correlations between the drop diameters and the reduction of surface tension and the drop spreading value from *Rhizopus* strains were observed (Table 1).

Sari et al. [18], evaluated the capability of isolates of *Pseudozyma* strains for biosurfactants production, and found the surface tensions varying 35.8 – 44.3 mN/m. Therefore, it concluded that the biosurfactants produced by all *Rhizopus* strains were their primary metabolite, due to the production of growth-associated biosurfactant.

According to Sharma et al. [47], a microorganism is considered a good surface-active compounds producer if its net metabolic liquid is able to reduce the surface tension of water from 72 mN/m to 35 mN/m or below this value. Similar criterion for biosurfactants-producing microorganism detection was applied by Ariech and Guchi [48], considering surface-active potential biomolecule reduced the surface tension below 40 mN/N.

Table 1: Evaluation of tenso-active produced by *Rhizopus* strains using parafilm M test and surface tension determination.

Strain	Parafilm M Test (mm)	Surface Tension (mN/m)
Rhizopus arrhizus var. *arrhizus* UCP 1295	8	35.0
Rhizopus microsporus var. *chinensis* UCP 1296	10	33.3
Rhizopus microsporus var. *microsporus* UCP 1304	9	34.7
Rhizopus sp. UCP 1607	12	31.8
Fresh medium (control)	6	----------

Figure 2: Tensoactivity of crude biosurfactants of the *Rhizopus* strains on parafilm M hydrophobic surface.

3.3 Dispersing capacity and Emulsification Activity of the Rhizopus strains

The oil displacement assay requires no specialized equipment, and also the method is rapid and simple which can be undertaken with small volumes of sample [13]. The Table 2 showed the results for dispersing ability of the crude biosurfactant extracts produced by *Rhizopus* strains. Thus, significant oil displacement activities were demonstrated by biosurfactants produced by *R. microsporus* var. *microsporu*s UCP1304 and R. *microsporus* var. *chinensis* UCP1296 corresponding to 39.6 cm^2 and 56.7 cm^2 of oil displacement areas, respectively. However, the biosurfactant produced by *R. arrhizus* UCP1607 exhibited excellent potential in the dispersion of burnt motor oil on water surface that resulted in 66.4 cm^2 of oil displacement area (ODA). The results showed that the oil displacement area of the biosurfactant produced by *R. arrhizus* UCP1607 (Figure 3A) was superior to dispersion induced by commercial detergent (44.2 cm^2) (Figure 3B). Synthetic surfactant dodecyl sulphate (SDS) showed the best dispersion (75.4 cm^2) (Figure 3C) as positive control, as well as the negative control was used the burnt motor oil with distilled water (Figure 3D).

Table 2: Oil displacement area by biosurfactants/bioemulsifiers produced by different Rhizopus atrains compared with chemical surfactant and commercial detergent.

Strain	Oil displacement area - ODA (cm^2)
Rhizopus arrhizus var. *arrhizus* UCP 1295	22.0
R. microsporus var. *chinensis* UCP 1296	56.7
R. microsporus var. *microsporus* UCP 1304	39.6
Rhizopus sp. UCP 1607	66.4
Commercial detergent	44.2
Chemical surfactant (SDS)	75.4
Distilled water	Negative

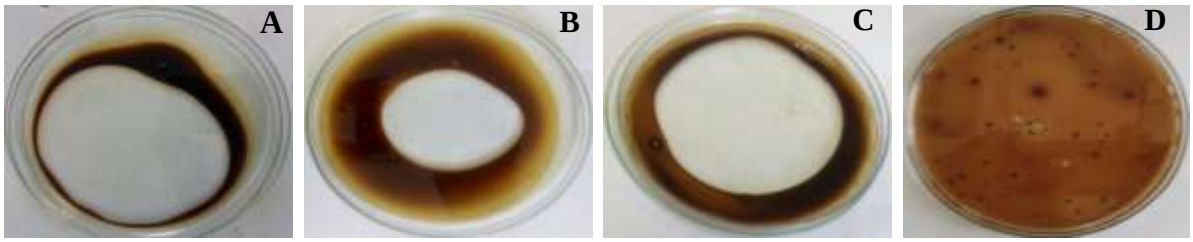

Figure 3: Oil displacement area (ODA) for: (A) Biosurfactant produced by *Rhizopus arrhizus* UCP1607, (B) Commercial detergent, (C) Chemical surfactant (SDS), (D) Distilled water (negative control).

The data obtained in this study using *Rhizopus* strains were superior to dispersion capacity of the biosurfactant produced by *Pleurotus ostreatus* (12.56 cm^2) of oil displacement area [49], and the bioemulsifier production by *Cunninghamella echinulata* (37.36cm^2) [29].

According Walter et al. [13], the emulsification index is a reliable method for detection of bioemulsifier producers. The *Rhizopus* strains showed emulsifying capacity of biosurfactants produced (Figure 4).

The significant positive values are considering above 50% after 24 hours of emulsion formed [15]. Thus, the best emulsification property against burnt motor oil were observed by *R. microsporus* var. *microsporus* UCP 1304 (94.8%), followed *R. microsporus* var. *chinensis* UCP 1296 (91.7%) and *Rhizopus arrhizus* UCP 1607 (82.6%) of emulsification.

All results obtained in this this study were higher than the biosurfactants produced by *Aspergillus niger* CF12 (18.5%) and *R. nigricans* CF3 (21.66%), as described Lodha et al [19].

In addition, the current study suggested the importance of the screening methods mainly based on primary and secondary assays led to isolate biosurfactant and bioemulsifying agents using mycelia-free broths, in particular from filamentous fungi. Those assays considered the important properties of higher dispersion oil activity, lower surface tension of the tension active agent, and bioemulsification. According to Uzoigwe [50], emulsification index test is a suitable screening method for detection of bioemulsifier producing by microorganisms.

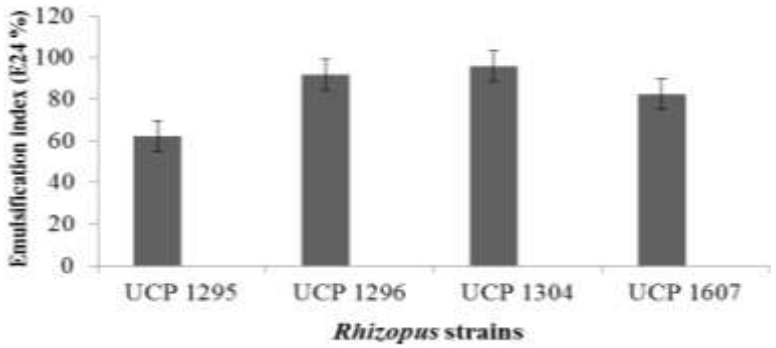

Figure 4: Emulsification index (E_{24}) of burnt motor oil with biosurfactants produced by *Rhizopus* strains**.**

Most of the surfactants compounds are chemically synthesized. However, the main drawback for the biosurfactants commercialization is associated with non-economical production and is not yet competitive with the synthetics products. The renewable substrates used, especially from industrial wastes as soybean post-frying oil, supplemented with sodium glutamate showed excellent results to biosurfactant and bioemulsifier production at an experimental scale. The agro-industrial waste soybean post-frying oil is considered as the promising substrate for reduction of the cost of production to tenso-active and emulsifier molecules.

4. Conclusion

In this work, the four biosurfactant/bioemulsifier-producing strains were isolated from Caatinga soil of Brazil, named *Rhizopus arrhizus* var. *arrhizus* UCP 1295, *R. microsporus* var. *chinensis* UCP 1296, *R. microsporus* var. *microsporus* UCP 1304 and *Rhizopus* sp.UCP1607, identified as *R. arrhizus*. The experimental result showed among the four strains, the best results was achieved when using *R. arrhizus* UCP 1607 had the biosurfactant activity. The biosurfactant produced by *R. arrhizus* UCP 1607 strain had a large hemolytic activity, parafilm drope and the oil-spreading diameter, low surface tension, and the best emulsifying activity was observed by *R. microsporus* var. *microsporus* UCP1304. The screening methods mainly based on surface tension determination have led in many cases to elimination of bioemulsifying agents. The promising physico-chemical results showed that evaluation of emulsifying activities from mycelia-free broths demonstrated great possibility for production of bioemulsifiers with powerful potential to induce stable emulsion. The better surface active properties confirmed with its great effectiveness in the lowering of surface tension and oil dispersion in water by the new strain of *R. arrhizus*. UCP 1607. Further studies are under way to scale up growth conditions and to optimize biosurfactant and bioemulsifier productions.

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CAPÍTULO III

Artigo II

Biosurfactant and Bioemulsifier Productions by *Rhizopus arrhizus* UCP1607 from Renewable Resources and Removal of Hydrophobic Pollutants

Trabalho submetido para publicação no períodico **Journal of Colloid and Interface Science - A2**

Biosurfactant and Bioemulsifier Productions by *Rhizopus arrhizus* UCP1607 from Renewable Resources and Removal of Hydrophobic Pollutants

Milagre A. Pele [a,e], Daylin Rubio-Ribeaux [a,e], Dayana Montero-Rodriguez [a,e], Adriana F. Souza [b,e], Marcos A.C. Luna [b,e], Rosileide F.S. Andrade [c,e], Thayse A. L. Silva [c,e], André L.C.M.A. Santiago [d], Galba M. Campos-Takaki [e,*]

[a] Biological Sciences Post-Graduation Program, Federal University of Pernambuco, 50670-420 Recife-PE, Brazil. phelema@yahoo.com; drubioribeaux@gmail.com; dayanamontero87@gmail.com
[b] Northeast Network for Biotechnology, Federal Rural University of Pernambuco, 52171-900 Recife-PE, Brazil. adrife.souza@gmail.com; macluna@bol.com.br
[c] Post-Doctorate National Program-CAPES, Catholic University of Pernambuco, 50050-900 Recife-PE, Brazil. rosileide_fontenele@yahoo.com.br ; ithalv@yahoo.com.br
[d] Department of Mycology, Federal University of Pernambuco, 50670-901 Recife-PE, Brazil. andrelcabral@msn.com
[e] Nucleus of Research in Environmental Sciences and Biotechnology, Catholic University of Pernambuco, 50050-590, Recife-PE, Pernambuco, Brazil. galba_takaki@yahoo.com.br

[*] Corresponding author: galba_takaki@yahoo.com.br ; Phone: +51-81-21194044; Fax: +55-81-21194043.

Abstract

The use of agroindustrial wastes for production of high-value added biomolecules like surface active compounds is a promising approach for lowering the total production costs. The objective of this study was to produce biosurfactant and bioemulsifier by *Rhizopus arrhizus* UCP1607 using crude glycerol and corn steep liquor as substrates. Two 2^2 factorial designs were applied, and the best condition for production of biosurfactant was obtained in the assay 4 (3% crude glycerol and 5% corn steep liquor), and for bioemulsifier in the condition 3 (6% crude glycerol and 3% corn steep liquor). The biosurfactant showed an excellent water surface tension reduction from 72 to 28.8 mN/m, whereas for bioemulsifier was 36.5 mN/m. The preliminary biochemical characterization showed that biosurfactant consisted of proteins (38%), carbohydrates (35,4%) and lipids (5,5%); bioemulsifier consisted of proteins (40%), carbohydrates (16.7%) and lipids (39.6%). Both compounds presented an anionic character, non-toxicity and stability over the conditions tested. The removal efficiency of biosurfactant was 79.4% of the diesel impregnated in beach sand, whereas bioemulsifier demonstrated a removal rate of 90.6 %. The data suggested that the biosurfactant and bioemulsifier from *R. arrhizus* UCP1607 have promising potential for application in enhanced bioremediation of hydrophobic contaminants in environment.

Key-words: Tensoactive agents, hydrophobic compounds removal, filamentous fungi.

Introduction

The quality of soil environment ensures the suitable development of humans, animals and plants. Due to this reason, one of the main concerns of the modern society in the soil contamination by hydrophobic compounds such crude oil and its derivatives, owing to various negative impacts it can cause on soil biological, physical and chemical properties [1,2,3].

In this context, the development of innovative and cost-effective technology which remove petroleum hydrocarbons from contaminated soil ecosystems is a priority [4]. Different treatment strategies such as physical, chemical or biological methods have been used for remediation of hydrocarbon polluted sites. However, the use of cleanup technologies involving synthetic chemicals for the treatment of hydrocarbons contaminated soil are considered environmentally incompatible due to higher toxicity and not cost effective when compared to microbial bioremediation [1,5]. Alternatively, biological treatment approach offers more environmentally friendly as well as low cost techniques [6].

Over the last years, microbial surface active compounds commonly known as biosurfactants have gained the special attention of researchers [7]. This vast group of molecules is mainly produced by microorganisms. Despite the large diversity in the compositional structure, biosurfactants and bioemulsifiers are recognized universally as amphiphilic compounds which have both hydrophilic and hydrophobic domains [8]. Due to this particular organization, these compounds may concentrate at interfaces between immiscible phases such as air/water, oil/water, leading to reduction of surface and interfacial tensions [9].

The surface and interfacial tension reduction abilities of biosurfactants and bioemulsifiers define their different physico-chemical properties such as emulsifying capability, mobilizing and solubilizing agents, detergency, foaming, wetting ability, and phase dispersion [10,9]. These properties make them attractive molecules with potential applications in many industrial sectors including oil and food industries, domestic activities, environmental remediation of contaminated sites, among others [5].

Biosurfactants and bioemulsifiers possess many advantages over their chemical counterpart such as ecological acceptability, biocompatibility and digestibility, efficiency in extreme conditions of

pH, temperature and salinity. In addition to their functional specificity and the possibility to be synthesized using renewable and less costly substrates [11,12].

A huge number of researches throughout the world have been directed to microbial tensoactives agents since when they were discovered [13]. However, the majority of the studies have exploited the ability of bacteria and yeasts in synthesizing biosurfactants and bioemulsifier using low-cost substrates [14,15,12,16]. The potential of filamentous fungi in producing biosurfactants and bioemulsifiers have also been reported by a few scientists[17,18,19]. Therefore, this study aims to evaluate the biotechnological potential of *Rhizopus arrhizus* UCP 1607 isolated from Caatinga soil samples in the production of biosurfactants and bioemulsifier using two agro-industrial wastes as low-cost substrates.

Materials and Methods

Microorganism

The filamentous fungus *Rhizopus arrhizus* UCP1607 was isolated from a Caatinga soil sample collected in Rio Grande do Norte state, Northeast of Brazil. The strain was maintained on Sabouraud dextrose agar (SDA) slants and stored at 4^{o}C in refrigerator. Transfers were done to fresh SDA slants each three months to maintain the fungus viable.

Agroindustrial Substrates, Culture Condition and Biosurfactant/Bioemulsifier Production

The agroindustrial substrates used were kindly provided by agro-processing industries: corn steep liquor - from corn wet-processing industry (Cabo-PE, Brazil), and crude glycerol - from the biodiesel production from cotton oil (CETENE-PE, MCT, Brazil). The experiments were carried out in 250 mL Erlenmeyer flasks containing 100 mL of the medium for biosurfactant/bioemulsifier production consisting of a salt solution as following (g/L): (KH_2PO_4 0.2 g, $MgSO_4.7H_2O$ 0.2 g, distilled water – 1000 mL), added with 1 mL/L of trace elements solution ($FeSO_4 \cdot 7H_2O$ 0.63 mg, $MnSO_4$ 0.01 mg, $ZnSO_4$ 0.62 mg) [17]. Crude glycerol and corn steep liquor were used as carbon and nitrogen sources, respectively [20,21] in varying concentrations according to two 2^2 full factorial designs. The pH of the production medium in the flasks were adjusted to 5.5±0.03 and sterilized by autoclaving at 121°C for 15 min. Then, a sporangiospores suspension of the *Rhizopus arrhizus* UCP 1607 grown on potato dextrose agar (PDA) medium (g/L): (peeled potato 200, dextrose 20, and agar 15), at 28°C for 96 h, was

prepared and adjusted to 10^7 spores/mL, and 5% (*v*/*v*) of the spores suspension were inoculated into each flask containing the production medium and incubated in an orbital shaker at 150 rpm, at 28°C for 96 h. The mycelia-free broths were removed by filtration followed by cold centrifugation at 10,000× g at 5°C for 15 min, and then was used in the screening analyses for biosurfactant/bioemulsifier production.

Full Factorial Design (FFD)

In order to analyze the effects of crude glycerol-CG and corn steep liquor-CSL, and the interactions between them on biosurfactant and bioemulsifier production by *Rhizopus arrhizus* UCP 1607, two 2^2 full factorial designs were developed using surface tension and emulsification index as response variables, respectively. A set of eight assays with four replicates in the central points for each factorial design was carried out (Table 1). The statistical analysis of the data obtained from the experiments were performed using version 7.0 STATISTICA software package (Statsoft Inc., Tulsa, OK, USA), and the significances of the results were tested at ($p \leq 0.05$).

Table 1. Levels and variables applied for the factorial designs.

First 22 full factorial design applied for bioemulsifier production

Variables	**Levels**		
	-1	0	+1
Crude glycerol (%v/v)	2	4	6
Corn steep liquor (%v/v)	3	6	9

Second 2^2 full factorial design applied for biosurfactant production

Variables	**Levels**		
	-1	0	+1
Crude glycerol (%v/v)	2	2.5	3
Corn steep liquor (%v/v)	3	4	5

Surface Tension Determination

Surface tension was determined on mycelia-free metabolic liquid with a Tensiometer model Sigma 70 (KSV Instruments Ltd., Finland) using the Du Nouy ring method at room temperature (±28°C). Measurements of surface tension from distilled water were used as control [22].

Emulsification Index Test (%E_{24})

Emulsification capacity of the bioemulsifier was assessed by adding 2 mL of the mycelia-free broth to equal volume of hydrocarbons (diesel, kerosene, hexadecane, gasoline, motor oil and

burnt motor oil), and shaken hardily with a vortex for 2 min. The mixture was left over the bench at room temperature. The emulsification index was calculated after 24 h as the height of the emulsion layer divided by the total mixture height, and the results were expressed in percentage [23]. The measurements were conducted in triplicates.

Extraction of Bioemulsifier

Both the biosurfactant and bioemulsifier produced by *Rhizopus arrhizus* UCP 1607 were extracted from the supernatants by organic solvent precipitation method with acetone 1:1 (v/v). The mixture was allowed to stand for 24 h at 4^{o}C, afterwards the precipitated materials were centrifuged at 5000x g for 15 min, at 5^{o}C. The supernatant was discarded and the isolated material was submitted to dialysis against deionized water, which was changed every 3 h, for 96 h at 5°C [17].

Biochemical Characterization of the Tensoactives

The protein concentrations in the isolated biosurfactant and bioemulsifier were determined using the total protein test kit from Labtest Diagnóstica S.A., Brazil. The total carbohydrate contents were estimated with the use of phenol-sulfuric acid method [24]. The lipid contents determined in accordance with Manocha et al. [25].

Ionic Charge Determination

The ionic charge of the biomolecules was determined by using a Zeta potentiometer model ZM3-D-G, Zeta Meter System 3.0+, with direct images to the video of the Zeta Meter, San Francisco, CA, USA [14].

Critical Micelle Concentration (CMC)

For the CMC determination different concentrations of partially purified biosurfactant and bioemulsifier were diluted in distilled water. With an automatic Tensiometer (model Sigma 70 KSV Ltd., Helsinki, Finland) using the DuNouy ring method, surface tensions of the aqueous solutions of the compounds were measured up to a value at witch surface tension remained constant. A graph of the surface tension reduction as a function of biosurfactant or bioemulsifier concentration in percentage (%) was plotted [26].

Phytotoxicity Assay

The phytotoxicity of the both biosurfactant and bioemulsifer produced by *Rhizopus arrhizus* UCP1607 was assessed on basis of seed germination and root elongation of lettuce (*Lactuca sativa* L.), and cabbage (*Brassica oleracea*), following the methodology described by [27]. Solutions of the isolated compound were prepared with distilled water at concentrations 1.0, 1.7 and 2.15 g/L of biosurfactant, and 0.7, 1.4 and 2.1 g/L for bioemulsifier. The phytotoxicity of the biomolecules against ten seeds of each plant was determined in sterilized Petri dishes containing Whatman no. 1 filter paper. 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 the control) x 100

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

Germination index = (% of seed germination) x (% of root growth)/100%

Controls were prepared with distilled water. The mean and standard deviation of triplicate experiments from each concentration were calculated.

Stability Analysis of the Biosurfactant and Bioemulsifier

The metabolic liquid without mycelia obtained through filtration followed by centrifuging the culture broth at 10000×*g* for 15 min was used to assess the stability of the biosurfactant in relation to surface tension, and bioemulsifier to emulsification index. Forty milliliters of the supernatant culture were heated at 0, 5, 70, 100 and 120^{o}C for 15 min, and cooled to room temperature, after that the surface tension and emulsification index were determined. In the case of the stability to pH, the mycelia-free broths pH were adjusted to different values (2-12) by adding 1.0M HCl or 1.0M NaOH to solutions, and the surface tension and emulsification index were measured. The effect of NaCl concentrations (2.0%–14%) was also determined. All experiments were performed in triplicate [12].

Oil Displacement Area (ODA)

The oil spreading test was performed by placing 40 ml of distilled water in a Petri dish (15 cm in diameter), followed with the addition of 1 mL of burnt motor oil onto the surface of the water layer. Afterwards, 0,5 mL of the cell free metabolic liquid was gently dropped onto the thin

surface of the oil layer. The positive control for oil displacement was the anionic synthetic surfactant Sodium Dodecyl Sulfate (SDS) and negative control was the water. The average values of the diameters of the clear zones of experiments in triplicate was measured and calculated as an oil displacement area (ODA) according to Morikawa et al. [28], using the following equation: ODA = 22/7 $(radius)^2$ cm^2.

Application of the Tensoactives in Removal of Diesel Oil from Contaminated Sand

The ability of both the biosurfactant and bioemulsifier in the removal of hydrophobic compounds was assessed by using 20.0 g of beach sand impregnated with 2.0 g of diesel oil, and then the mixtures were left to stand for 7 days at room temperature. Afterwards, the fractions of 20.0 g of the contaminated sand were transferred to 250 mL Erlenmeyer flasks, followed by addition of 40.0 mL of the mycelia-free broths produced by *Rhizopus arrhizus* UCP 1607, and 40 mL of distilled water, as negative control. The samples were incubated on a rotary shaker (150 rpm) for 24 h at 28°C, and then were centrifuged at 5000x g for 10 min for separation of the washing solution and the sand. 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 [29].

Results and Discussion

Production of Biosurfactant and Bioemulsifier by *Rhizopus arrhizus* UCP 1607 Using Crude Glycerol and Corn Steep Liquor

In this study two agroindustrial byproducts crude glycerol and corn steep liquor were used as inexpensive culture media components for production of biosurfactant and bioemulsifier by *Rhizopus arrhizus* UCP 1607. Thus, aiming to find the best conditions for production of both tensoactive agents experiments were undertaken varying the combination of concentrations of crude glycerol - CG and corn steep liquor - CSL, in accordance with the respective 2^2 full factorial designs (Table 1). Results in the Table 2 showed that *Rhizopus arrhizus* UCP 1607 had the ability to reduce surface tension and to form consistent emulsions. The lower surface tension of 28.8 mN/m was achieved in the condition 4 corresponding to the medium consisting of 3% CG and 5% CSL of the second FFD, and the higher emulsification index of 95.5% was detected in the condition 3 with 6% CG and 3% CSL from the first FFD. Therefore, these conditions were selected as the best ones for the respective growth media formulation for production of

biosurfactant and bioemulsifier by *Rhizopus arrhizus* UCP 1607.

The measurement of surface tension has often been used as a rapid method to detect tensoactive agents in the culture media [30]. However, this method does not permit to identify surface active compounds which do not possess significant ability in the reduction of surface tension [31]. Thus, emulsification index is seen as suitable method to identify bioemulsifier which are markedly characterized by their excellent emulsion-stabilizing capacity without necessarily reducing the surface tension [32,31]. That was why both the techniques were performed for further detection of tensoactives in culture media.

According to Mnif and Ghribi [5], the most powerful biosurfactants are those capable of reducing the surface tension of water to values equals or below 30 mN/m, and are referred to in the literature as being mostly produced by bacteria such as *Pseudomona aeruginosa* and *Bacillus subtilis*. However, the biosurfactant produced by *Rhizopus arrhizus* UCP 1607 in the condition 4 demonstrated a greater effectiveness in reducing the surface tension of water. Similar results were reported for a biosurfactant from the filamentous fungus *Aspergillus* sp. MSF that showed a reduction of surface tension of water from 66 to 28 mN/m [33]. Recently, Silva et al. [34] observed that the biosurfactant produced by *Mucor circinelloides* showed a surface tension reduction of 26 mN/m, and the biosurfactant produced by *Rhizopus arrhizus* reduced the surface tension of water to 26.5 mN/m [35], corroborating the ability of genus *Rhizopus* in the synthesis of surface active compounds.

Table 2. Surface tension and emulsification index ($\%E_{24}$) values for the two 2^2 full factorial designs applied for the production of biosurfactant and bioemulsifier by *Rhizopus arrhizus* UCP 1607.

Condition	Crude Glycerol (%)	Corn steep liquor (%)	Surface Tension (mN/m)	Emulsification Index ($\%E_{24}$)
1	-1	-1	32.1	78.3
2	-1	+1	33	62.5
3	+1	-1	30	95.5
4	+1	+1	28.8	60.9
5	0	0	34.5	62.5
6	0	0	34.1	63.4
7	0	0	34.6	62.0
8	0	0	34.4	62.0

The effects of concentrations of crude glycerol and corn steep liquor as well as their interaction

on surface tension and emulsification index as dependent variables are shown in Figures 1-A and 1-B. The statistical analysis of results from factorial designs by Pareto chart indicates that crude glycerol was the independent variable which had more influence on surface tension reduction, followed by interaction between the byproducts. The diagram also showed that corn steep liquor had no effect as independent variable. Contrarily, corn steep liquor was the independent variable that more influenced in the formation and stabilization of emulsions than crude glycerol. These results suggest that crude glycerol and corn steep liquor are suitable alternative substrates for production of biosurfactants and bioemulsifiers by *Rhizopus arrhizus* UCP 1607.

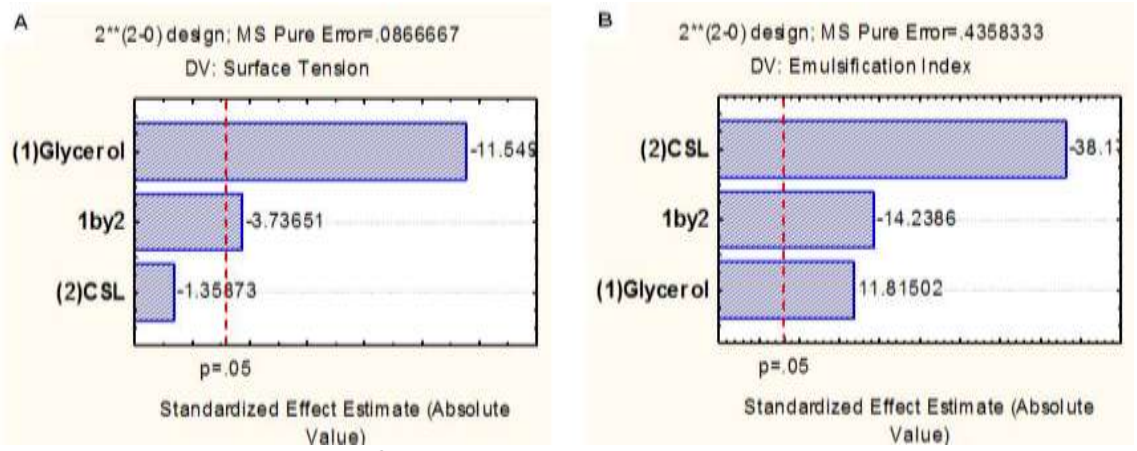

Figure 1. Pareto chart for the 2^2 full factorial design of standardized effects for (1) crude glycerol and (2) corn steep liquor - CSL with surface tension (A) and emulsification index (B) as dependent variables. The point at which the effect estimates were statistically significant (at $p = 0.05$) is indicated by the vertical broken line.

According to Helmy et al. [36], the fact that crude glycerol and corn steep liquor consist of a appreciable diversity of organic materials and minerals that can work as essential supplements for microbial metabolism render them attractive byproducts for microbial production of tensoactive agents. Moreover, they are plentifully generated in the respective industries [37]. In this regard, the role of both byproducts in the surface active compounds production has been broadly studied in particular for bacteria [38,39,29].

However, the potential of these agroindustrial wastes in the biosurfactant/bioemulsifier production has not yet been sufficiently studied for filamentous fungi. So far reports on surface active compounds production through crude glycerol bioconversion among fungi are still more scarce than regarding corn steep liquor [30,38,27].

Growth Kinetics

Different parameters related to the growth profile and production of biosurfactant and bioemulsifier by *Rhizopus arrhizus* UCP 1607 cultivated at temperature of 28^{o}C, during 96 h of incubation under orbital shaking at 150 rpm are summarized in the Figure 2 and Figure 3. According to Okpokwasili and Nweke [40], the growth of a microorganism results from its capacity to utilize the essential nutrients available in the culture medium, and this has the consequent in releasing of microbial metabolites. In this regard, *Rhizopus arrhizus* UCP 1607 showed great ability to use crude glycerol and corn steep liquor as alternative substrates, bioconverting them into different essential materials for its growth, but also resulted in the production of biosurfactant and bioemulsifier [41]. According to the data, the fungus demonstrated a profile of growth more active in the first 48 h, with slight changes until the end of cultivation time.

Thus, the growth of *Rhizopus arrhizus* UCP 1607 in the medium consisting of 3% crude glycerol and 5% corn steep liquor showed a rapid increase in biomass (14.8 g/L) in the first 48 h, and continuously increased achieving 19.84 g/L at end of 96 h. In parallel, the surface tension decreased clearly in the first 48 h of growth reaching 34.5 mN/m, and gradually decreased to around 28.8 mN/m at the end of 96 hours of cultivation.

The fungus showed a similar pattern of growth profile in the other medium composed of 6% CG and 3% CSL with rapid increase in the biomass in the first 48 h (7.5 g/L), that remained increasing until 96 h of cultivation when the biomass reached 9.45 g/L, whereas the surface tension dropped from around 71 to 38.3 mN/m, and then a slight decrease to 36.5 mN/m occurred at the end of cultivation period. Both biosurfactant and bioemulsifier showed a growth-associate production, that were similar to those produced by *Cunninghamella echinulata* grown in soybean oil residue and corn steep liquor [17], and by *C. lipolytica* cultivated in glucose and canola oil as substrates [42].

The pH of the both culture media did not undergo significant changes throughout the growth of *Rhizopus arrhizus* UCP 1607. Several studies have shown that the medium acidity is related with a minor surfactant production [41,43]. Thus, as the pH remained almost unchanged throughout the cultivation it may have not exerted negative influence on performance of the biosurfactant synthesis by *Rhizopus arrhizus* UCP 1607.

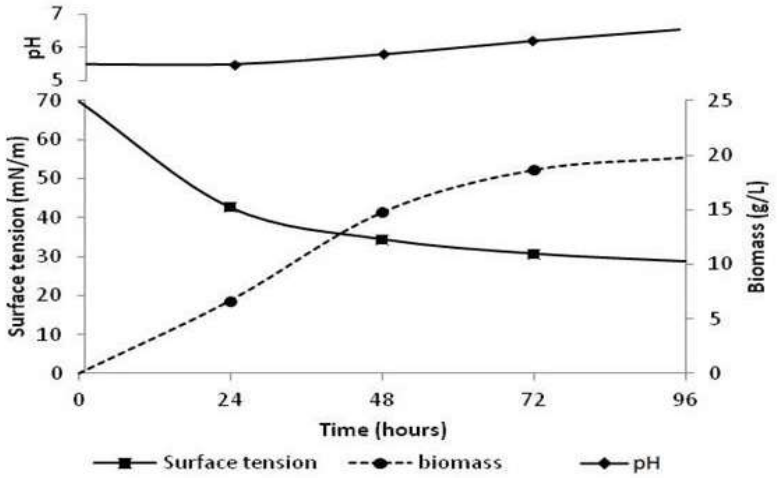

Figure 2. Parameters of growth and production of biosurfactant by *Rhizopus arrhizus* UCP 1607 at 28°C, during 96 h incubation in the culture medium supplemented with 3% crude glycerol and 5% corn steep liquor.

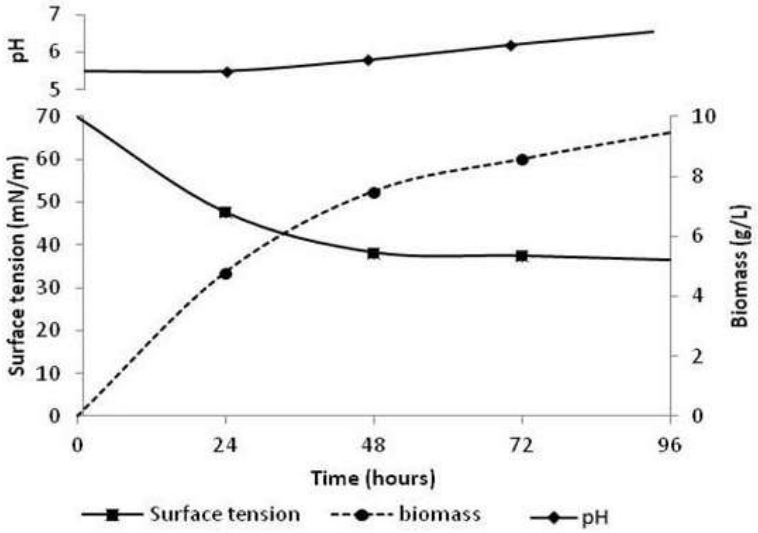

Figure 3. Parameters of growth and production of bioemulsifier by Rhizopus arrhizus UCP 1607 at 28°C, during 96 h incubation in the culture medium supplemented with 6% crude glycerol and 3% corn steep liquor.

Biosurfactant and Bioemulsifier Yields

The yields of the isolated biosurfactant and bioemulsifier produced by *Rhizopus arrhizus* UCP 1607 grown in crude glycerol and corn steep liquor during the 96 h of incubation were 1.74 g/L, and 1.05 g/L, respectively. Biosurfactants from *Ustilago maydis* FBD12 [44], showed a production yield of 0.183 g/L and 0.096 g/L in the fish oil and soy oil as substrates, during 9 days of cultivation. Andrade Silva et al. [17], reported a higher yield of 4.5 g/L for a bioemulsifier produced by *C. echiculata* grown in a medium consisting of soybean oil residue and corn steep

liquor. Although water-soluble substrates are referred to as possessing lower performance yield of biosurfactant production in comparison with hydrophobic materials [45], these results suggested the potential of the crude glycerol and corn steep liquor for their use as suitable alternative sources for tensoactive agents production. Besides, the results showed that the biosurfactant yield is dependent not only on the nature of medium composition but also on ability of the microorganism for biosurfactants production, as has been shown by *Rhizopus arrhizus* UCP 1607.

Critical Micelle Concentration (CMC)

From a practical point of view critical micelle concentration (CMC) is the minimum concentration of a biosurfactant/bioemulsifier required to attain the maximum water surface tension diminution, and it measures the efficiency of a biosurfactant/bioemulsifier [46]. In this context, with the increase of the isolated biosurfactant concentration gradual decrease of surface tension of water from 72 to 28.8 mN/m was reached with a CMC of 1.7%, and from this critical point no further decrease in the surface tension was observed with the increase of biosurfactant concentration, indicating that the CMC had been attained (Figure 4 - A). In turn, isolated bioemulsifier showed a CMC of 1.4% towards the surface tension of 36.5 mN/m (Figure 4 - B).

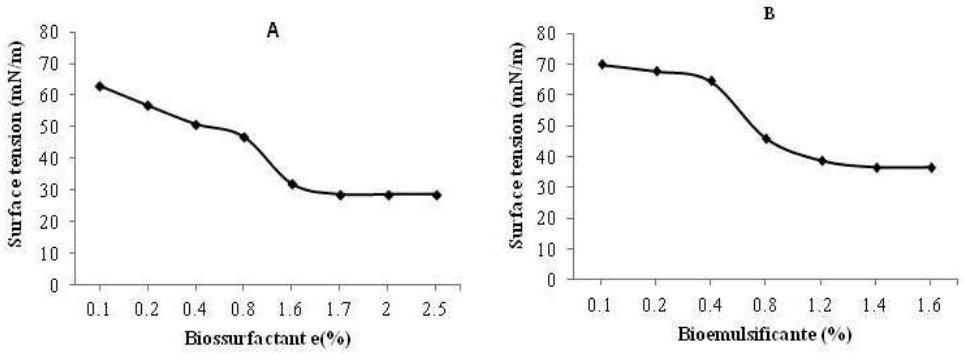

Figure 4. Surface tension versus concentration of isolated biosurfactant (A) and bioemulsifier (B) produced by *Rhizopus* sp. UCP 1607 using crude glycerol and corn steep liquor.

These results reveal that the biosurfactant produced by *Rhizopus arrhizus* UCP 1607 has a greater capacity to reduce tension when in comparison to those from other fungi, *Candida glabrata* UCP 1002 (31 mN/m) [47], *C. lipolytica* (32 mN/m) [48], and *Fusarium* sp. BS-8 (32 mN/m) [18]. Furthermore, both biosurfactant and bioemulsifier synthesized by *Rhizopus arrhizus* UCP 1607 exhibited lower CMC values than those given by other fungi tensoactive agents, when

considering the CMC of 2 and 2,5% for bioemulsifier and biosurfactant from *Cunninghamella echinulata* [17] and *C. glabrata* UCP1002 [47], respectively.

Ionic Charge and Biochemical Composition of Biosurfactant and Bioemulsifier

Both biomolecules produced by *Rhizopus arrhizus* UCP 1607 demonstrated an anionic character by Zeta meter. Biosurfactant with -31,28±0.29 ZPmv, 147 µS/cm at 27.4^{o}C, full scale, and bioemulsifier heads are negatively charged [10]. Biosurfactants from other fungi showed anionic profile when subjected to Zeta meter analysis [17,14].

The preliminary biochemical characterizations demonstrated that biosurfactant consisted of proteins (38.%), carbohydrates (35.4%) and lipids (5.5%), whereas bioemulsifier showed a polymeric structure consisting of proteins (40%), lipids (39.6%) and carbohydrates (16.7%). The biopolymer from *C. echinulata* cultivated in soybean oil residue and corn steep liquor showed lipids (40.0%), carbohydrates (35.2%) and proteins (20.3%) [17].

Stability analysis

Emulsion stabilization and surface tension reduction are biosurfactants/bioemulsifiers physico-chemical properties that occur on account of micelles formation in the different phase systems, when these compound exist at CMC [49]. However, the intermolecular interactions which lead to micellization phenomenon of biosurfactants and bioemulsifiers may be affected by environmental factors such as pH, temperature and salinity, thus, influencing the stability and activity of microbial surfactants. Therefore, it is important to analysis the effect of these variables in order to validate the applicability of these compounds when considering their various environmental and industrial usage [50].

Figure 5 and Figure 6 shows the influence of different levels of pH, temperature, and salinity monitored on surface tension of biosurfactant and emulsification index of bioemulsifier produced by *Rhizopus arrhizus* UCP 1607.

According to the data, the biosurfactant maintained its surface tension diminution capacity over a wide range of temperature (0-100^{o}C), pH (2-12) and NaCl concentration (2-10%). The biosurfactant activity was slightly affected when the temperature was raised up to 120^{o}C (34.4 mN/m) and salinity to 14% NaCl (42.8 mN/m). The emulsification index of the mycelia-free

broth containing bioemulsifier also showed a great stability during exposure to a wide range of temperature (0-120^{o}C), pH (2-12) and salinity (2-14%). These results suggest the feasibility of the biomolecules for application in industries that work under extreme conditions of salinity, pH, and temperature, and are comparable to reported for other biosurfactants from fungi [32,52].

According to Khopade et al. [51] and Santos et al. [52], biosurfactants are able to tolerate wide ranges of temperature, pH, and salt concentration when compared to the synthetic ones which at 70^{o}C and 2% NaCl can get inactivated with significant loss of their surfactant stability.

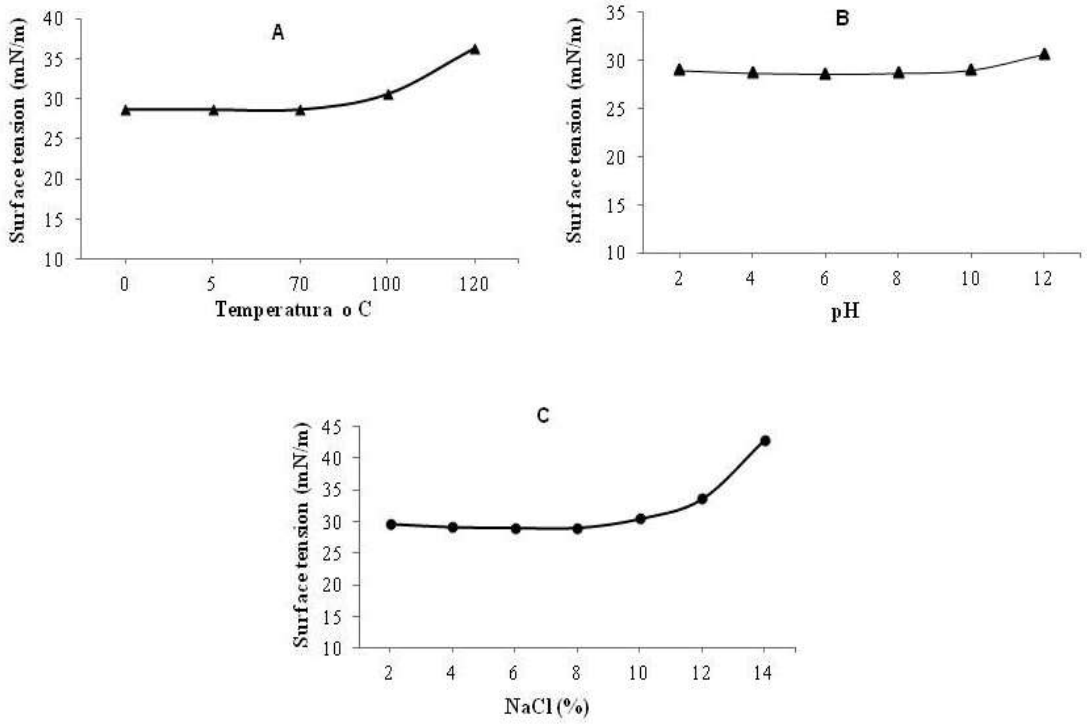

Figure 5. Effect of temperature (A), pH (B) and sodium chloride concentrations (NaCl) (C) on surface tension (mN/m) of biosurfactant produced by *Rhizopus arrhizus* UCP 1607 using crude glycerol (3%) and corn steep liquor (5%) as substrates.

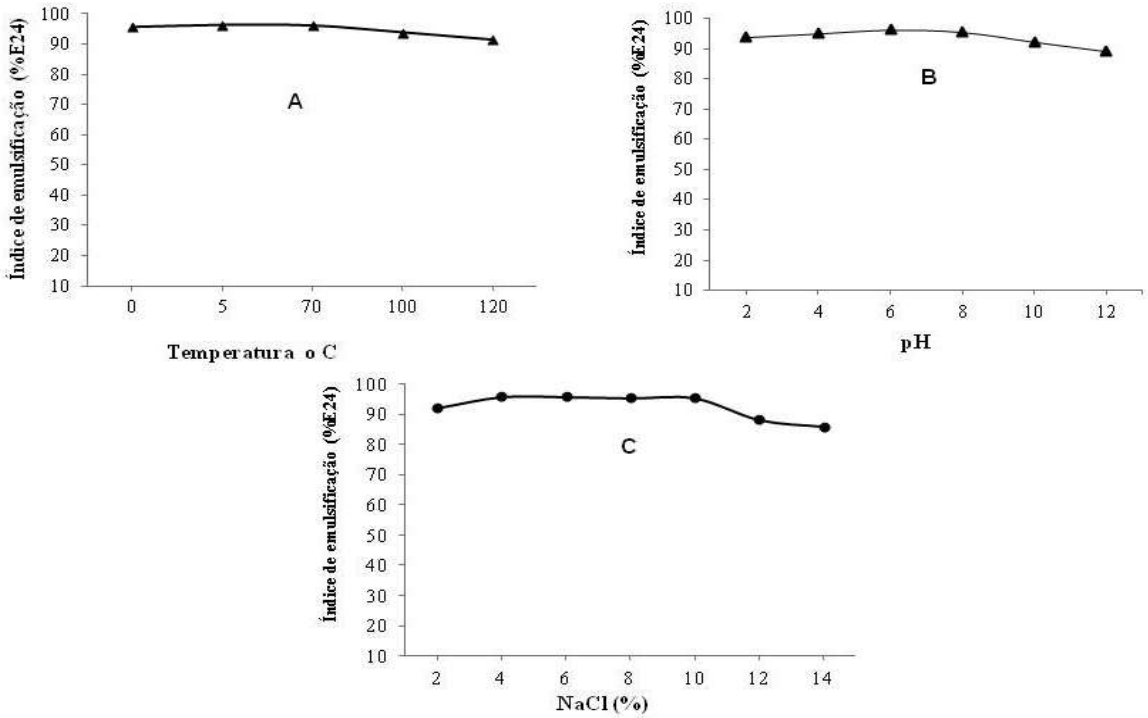

Figure 6. Effect of temperature (A), pH (B) and sodium chloride concentrations (NaCl) (C) on emulsification index ($\%E_{24}$) of bioemulsifier produced by *Rhizopus* sp. UCP 1607 using crude glycerol (6%) and corn steep liquor (3%) as substrates**.**

Biosurfactant and Bioemulsifier Toxicity

Plant bioassays play a crucial role since they allow predicting the effect of chemical in the ecosystems [53]. Therefore, the toxicity assessment of a new biosurfactant/bioemulsifier in the context of its applications in diverse environmental bioremediation processes is of a great importance in order to safeguard that its potential application trigger no harmful effects to terrestrial environmental [56,57]. In the current study, the evaluation of both biosurfactant and bioemulsifier for their phytotoxicity against cabbage (*Brassica oleracea)* and lettuce (*Lactuca sativa* L.), showed non-inhibitory effect of the tensoactive solutions towards seed germination or root elongation in the vegetables tested, since the germination index values attained during the growth of the plants were above the value of 80% considered the indicator of the absence of phytotoxicity [27]. These results indicate that the biosurfactant and bioemulsifier from *Rhizopus arrhizus* UCP 1607 are non-toxic compounds. In addition, elongation of secondary roots and leaf growth occurred under all conditions tested for both biomolecules (Tables 3 and 4).

Table 3. Phytotoxicity of biosurfactant isolated from *Rhizopus arrhizus* UCP 1607 grown in 3% crude glycerol and 5% corn steep liquor against *Brassica oleracea* and *Lactuca sativa* L. The results represent means of the experiments undertaken in duplicate.

Biosurfactant (g/L^{-1})	Seed germination (%)		Root elongation (%)		Germination index (%)	
	B. olaracea	*L. sativa*	*B. olaracea*	*L. sativa*	*B. olaracea*	*L. sativa*
1.0	100	100	100	104	100	104
1.7	100	100	96	102	96	102
2.5	99	97	102	106	101	103

Table 4. Phytotoxicity of bioemulsifier isolated from *Rhizopus arrhizus* UCP 1607 grown in 6% crude glycerol and 3% corn steep liquor against *Brassica oleracea* and *Lactuca sativa* L. The results represent means of the experiments undertaken in duplicate.

Bioemulsifier (g/L^{-1})	Seed germination (%)		Root elongation (%)		Germination index (%)	
	B. olaracea	*L. sativa*	*B. olaracea*	*L. sativa*	*B. olaracea*	*L. sativa*
0.7	100	100	100	109	100	108
1.4	100	100	110	100	110	100
2.1	99	99	108	90	107	89

Oil Displacement Areas (ODA)

Oil displacement assay is a rapid and reliable technique used not only to indicate surface active compounds production [56], but also to assess the dispersing activities of biosurfactants and bioemulsifiers, as this property is crucial for a biosurfactant to be applicable in environmental and industrial purposes [10]. According to Mnif and Ghribi [5], in the dispersion process the biosurfactant or bioemulsifier reduces the cohesive forces between similar hydrophobic particles, preventing to form large aggregations with each other. With this, hydrophobic compounds are dispersed into aqueous phase as small emulsion droplets [57], leading to desorption of hydrocarbons from rock surfaces, and increase in surface area of hydrophobic activity [58].

Figure 7 illustrates the burnt motor oil layer dispersing capacities for both tensoactives from *Rhizopus arrhizus* UCP 1607, as indicated with clearing zones formation on water surface. According to the data, no dispersion activity was observed with distilled water (Figure 7 A); when commercial detergent (1%) was used it displaced the oil in 44.2 cm ODA (Figure 7 B), and the **s**odium dodecil sulphate SDS (1%) resulted in 72.7 cm^2 ODA (Figure 7 C). The use of biosurfactant crude extract induced a clear zone formation of 53.4 cm^2 ODA (Figure 7 D).

However, larger burnt motor oil displacement area was achieved when bioemulsifier was applied resulting in 68.3 cm^2 (ODA) (Figure 7 E).

Souza et al. [21], evaluated the dispersing activity of isolated bioemulsifier produced by *Candida lipolytica* UCP 0998 that showed 45.34 cm^2 of oil displacement area. The dispersion rate of biosurfactant produced by *R. arrhizus* UCP 1607 is superior to that reported for biosurfactants from other fungi, as described by Poomtien et al. [59] (44,5 cm^2 ODA). These results suggest their potential applications in enhanced oil recovery and oil spill bioremediation [5].

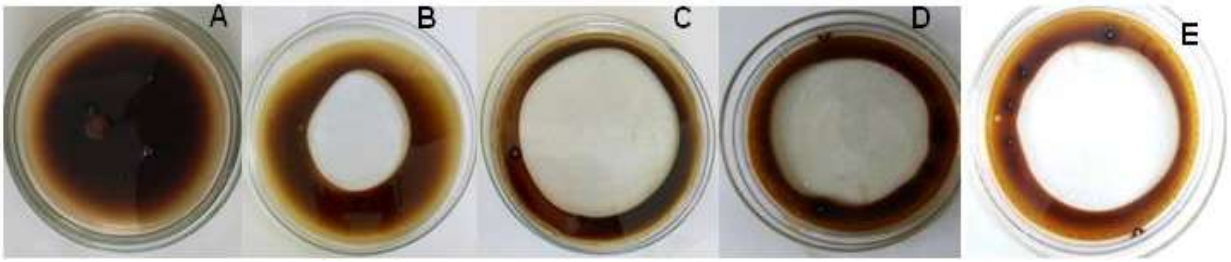

Figure 7. Burnt motor oil displacement areas (ODA) obtained in accordance with the dispersant: (A) water; (B) commercial detergent; (C) chemical surfactant (SDS); (D) biosurfactant; (E) bioemulsifier.

Emulsification Index (%E_{24})

Emulsification capacity has been frequently used as a suitable technique in the detection of surface-active compounds producing strains, as it correlates to biosurfactant concentration [30], however, this functional property also has been exploited as a powerful measuring tool to determine biosurfactant/bioemulsifier ability in forming and stabilizing emulsions of different hydrophobic substrates, when considering their potential practical applications in many interesting areas, particularly in the enhanced environmental protection processes and petroleum processing industry [60].

According to Kebbouche-Gana et al. [61] the emulsion-stabilizing capacity of a surface-active compound is assessed by its ability to maintain at least 50% of the original emulsion volume after 24 h of its formation. Considering this criterion, the tensoactives produced by *Rhizopus arrhizus* UCP 1607 were able to emulsify a variety of hydrocarbons, as shown in Figure 8. In this regard, the biosurfactant demonstrated great ability of forming stable emulsions for kerosene (58.3%), motor oil (50%), and burnt motor oil (69%), as higher emulsification (E_{24}). In turn, bioemulsifier displayed significant emulsion-stabilizing capacity for kerosene (51.4%), hexadecane (52.3%), gasoline (58%), and motor oil (72%). However, the greatest emulsion-stabilizing performance of

bioemulsifier was verified towards burnt motor oil resulting in 96.4% of emulsification ($\%E_{24}$). As can be observed in the graph, both biosurfactant and bioemulsifier showed greater emulsion-stabilizing capacity for burnt motor oil, and less stable emulsion with diesel.

Similar results to biosurfactant produced by *Rhizopus arrhizus* UCP 1607 were reported by Elshafie et al. [60], who tested the efficacy of a biosurfactant from *Candida bombicola* ATCC 22214, which showed emulsification values ranging from 23.8-68.75% with various petroderivates. Also, Rubio-Ribeaux et al. [62] reported a novel biosurfactant from *Candida* sp. strain which formed significantly stable emulsions with diesel (79%) and motor oil (67%). The emulsion-stabilizing capacity against motor and burnt motor oils demonstrated by the bioemulsifier produced by *Rhizopus arrhizus* UCP 1607 was comparable to those reported for bioemulsifiers from *Cunninghamella echinulata* (85%) [17], and *Candida lipolytica* UCP 0998 (96.66%) [21]. The higher $\%E_{24}$ values shown by both biosurfactant and bioemulsifier produced by *Rhizopus arrhizus* UCP 1607 suggest their potential application in enhanced oil recovery, and bioremediation of hydrophobic contaminated environments [5].

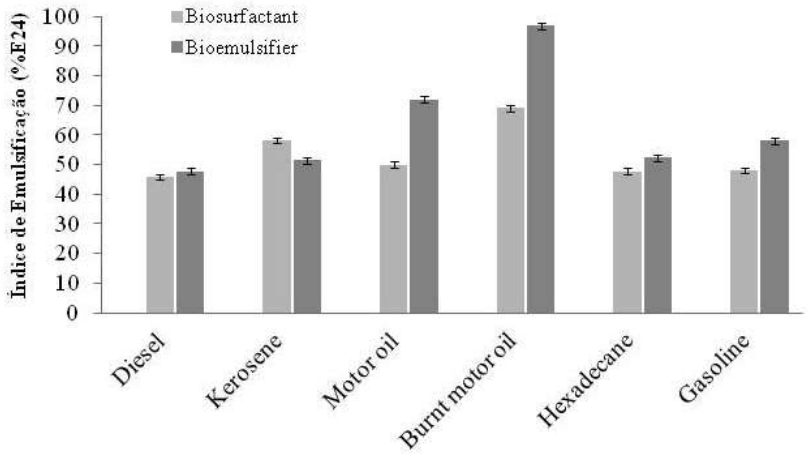

Figure 8. Emulsifying capacities of tensoatives produced by *Rhizopus arrhizus* UCP 1607 towards various hydrocarbon substrates.

Application of Biosurfactant and Bioemulsifier on Removal of Diesel Oil

The hydrophobic contaminants once adsorbed to soil, their removal becomes difficult due to low solubility in water, creating a long-term source of contamination [57]. Thus, the addition of biosurfactant solutions in hydrocarbon contaminated soils can be effective in enhancing the solubilization and mobilization of hydrophobic organic compounds entrapped in soil particles, facilitating desorption and removal of the pollutants from the soil [63,64].

Considering this fact, the crude extracts (mycelia-free broths) of the tensoactives produced by *Rhizopus arrhizus* UCP 1607 were evaluated for their efficiency in the removing of hydrophobic contaminant adsorbed to sand. The results obtained showed the biosurfactant removed 79.4% of the diesel impregnated to beach sand, whereas crude bioemulsifier demonstrated a removal efficacy of 90.6 % of the contaminant from sand (results illustrated in supplementary material).

Andrade et al. [14], investigated the effectiveness of cell-free broth containing biosurfactant from *Candida glabrata* that showed a removal capacity of 95.7% of burnt motor oil impregnated in sand. As reported in the literature, the washing efficiency of crude biosurfactant solutions from *C. antarctica* removes around 50% of the oil adsorbed in the sand [65,21]. On the other hand, the crude bioemulsifier produced by *C. lipolytica* UCP 0998 removed 92.3% of diesel oil from sand [21], comparable to that displayed by the bioemulsifier produced by *Rhizopus arrhizus* UCP 1607. These results, confirm that biosurfactant and bioemulsifier are promising candidates for applications in enhanced removal of hydrophobic contaminant from polluted soil.

According to SILVA et al. [53], the crude biosurfactants and the isolated biosurfactants are almost equally effective in the removal of the motor oil pollutant. The same authors identify advantage in this, since cell-free broth containing biosurfactants can be directly used without purification steps, which would further reduce 30%–50% of the production cost of biosurfactants.

Conclusions

The new strain of *Rhizopus arrhizus* UCP 1607 isolated from soil sample from Rio Grande do Norte state demonstrated a great potential in the synthesis of biosurfactants and bioemulsifier using low-cost substrates. The biosurfactant produced in this study, besides possessing a good emulsifying capacity, has excellent tensoactive properties. The bioemulsifier produced by *Rhizopus arrhizus* UCP 1607 possesses excellent emulsifying properties towards different hydrophobic substrates. The both tensoactives are promising candidates for application in the enhanced oil recovery and bioremediation of hydrocarbons contaminated sites.

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4. CONCLUSÕES GERAIS

- O solo da região semi-árida do bioma Caatinga apresenta-se como fonte de isolamento de diversidade de microorganismos com grande potencial biotecnológico.

- A cepa isolada do solo da região semi-árida da Caatinga do estado de Rio Grande do Norte foi identificada como *Rhizopus arrhizus* UCP 1607 e depositada no banco de cultura do NPCIAMB/UNICAP.

- O *Rhizopus arrhizus* UCP 1607 demonstrou capacidade de produção biossurfactantes e bioemulsificantes bioconvertendo substratos renováveis e de baixo custo como os resíduos agraoindustrais (glicerol resdual e milhocina).

- O glicerol residual e milhocina podem ser utilizados fontes alternativas para formulação de meios de cultivo para a biossíntese de biossurfactantes/bioemulsificantes.

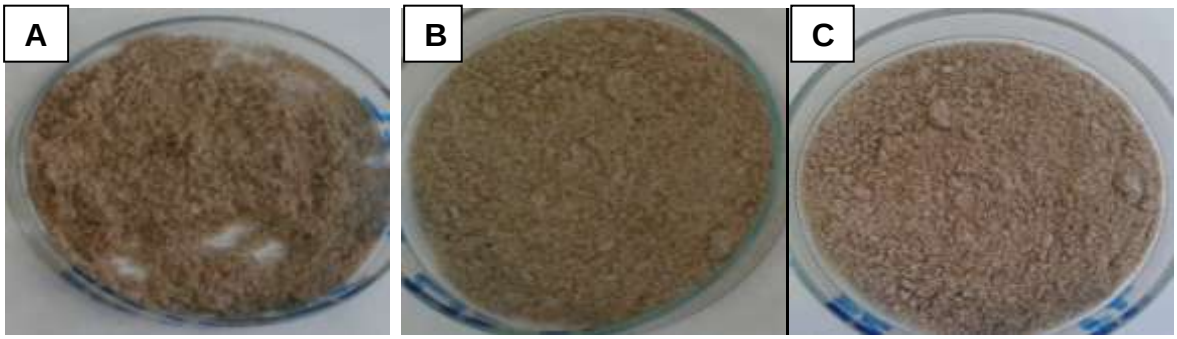
- O *R. arrhizus* UCP 1607 produziu biossurfactante no meio consistindo de 3% de glicerol e 5% de milhocina, e bioemulsificante no meio feito de 6% glicerol residual e 3% de milhocina.

- O biossurfactante produzido por *R. arrhizus* UCP 1607 possui potentes propriedades tensoativas, baixando a tensão superficial da água de 72 para 28.8 mN/m. O bioemulsificante destacou-se pela sua excelente capacidade de formar emulsões estáveis.

- As biomoléculas além de possuir grande estabilidade contra condições extremas de temperatura, pH e salinidade, também são atóxicos para o ambiente.

- Os dados obtidos do estudo da capacidade dispersante de substratos hidrofóbicos na água e de remoção de poluentes hidrofóbicos do solo sugerem o grande potencial tanto do biossurfactante quanto do bioemulsificante para aplicação na biorremediação ambiental melhorada.

5. APÊNDICE: Supplementar Material



Removal of diesel oil impregnated in beach sand using biosurfactant and bioemulsifier crude extracts produced by *Rhizopus arrhizus* UCP 1607. (A) Beach sand with remaining diesel oil after contact with distilled water; (B) beach sand with remaining diesel oil after contact with crude biosurfactant, and (C) beach sand with remaining diesel oil after contact with crude bioemulsifier.