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POTENCIAL BIOTECNOLÓGICO DE *Serratia marcescens* UCP/WFCC 1549 NA DEGRADAÇÃO DE COMBUSTÍVEIS, NA PRODUÇÃO DE LIPÍDEOS E DE BIOSSURFACTANTE

DAYANA MONTERO RODRÍGUEZ

Recife, 2015

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Univerdade Federal de Pernambuco, como parte dos requisitos para a obtenção do título de Mestre em Ciências Biológicas.

Área de Concentração: Biotecnologia

Orientadora: Profª. Dra. Galba Maria de Campos Takaki

Co-orientadora: Profª. Dra. Hélvia Waleska Casullo de Araújo

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“Si lo puedes soñar, lo puedes hacer”

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RESUMO

Serratia marcescens UCP/WFCC 1549, isolada do solo do semi-árido do Estado de Pernambuco - Brasil, foi investigada quanto o seu potencial de biodegradação de combustíveis, como também na produção de lipídeos e biossurfactante. A degradação de combustíveis foi avaliada utilizando o meio basal Bushnell Hass (BH), o indicador redox 2,6- diclorofenol – indofenol e a cepa de *S. marcescens* selvagem e aclimatada em diferentes concentrações do óleo diesel (2, 4, 6, 8, 10, 12 e 15%). Os resultados obtidos demonstraram que a bactéria aclimatada a 15% do óleo diesel apresentou os melhores índices de degradação, com valores de 79,63% para o biodiesel de algodão, 65,57% para o biodiesel de girassol, 60,50% para o diesel, 57,20% para gasolina e 39,26% para querosene. Além disso, *S. marcescens* demonstrou propriedade de crescer e acumular lipídeos (> 40%) utilizando resíduos agro-industriais (manipueira e óleos vegetais pós-fritura). Os lipídeos produzidos mostraram perfis de ácidos graxos com maior porcentagem em ácidos graxos monoinsaturados, sugerindo uma composição que corresponde às características requeridas para o biodiesel. Ao mesmo tempo, *S. marcescens* demonstrou habilidade para converter resíduos agro-industriais (manipueira e óleo de milho pós-fritura) em associação com lactose, na produção de biossurfactante, empregando um planejamento fatorial 2^3. A seleção da melhor condição do planejamento foi avaliada pela variável resposta tensão superficial. O melhor resultado foi obtido no meio constituido por 6% de manipueira e 7,5% de óleo de milho pós-fritura, na ausência de lactose, com uma redução da tensão superficial da água de 72 para 26,2 mN/m. O biossurfactante produzido apresentou propriedade emulsificante (EI_{24}), com valores superiores a 60% de emulsificação utilizando os óleos de soja, diesel, motor e motor queimado. Adicionalmente, o biossurfactante demonstrou estabilidade na redução da tensão superficial frente a diferentes valores de pH, temperatura e NaCl, e mostrou excelente eficiência na remoção de óleo de motor em água, areia de praia e sedimento de mangue (78%, 88,27% e 73,70%, respectivamente). Portanto, *S. marcescens* UCP/WFCC 1549 demonstrou seu elevado potencial biotecnológico para a produção de biodiesel de boa qualidade, assim como de biossurfactante com aplicação promissora em processos de biorremediação de ecossistemas contaminados com petróleo e seus derivados.

Palavras-chave: *Serratia marcescens*; biodegradação; lipídeos; biossurfactante; resíduos agro-industriais.

ABSTRACT

Serratia marcescens UCP/WFCC 1549, isolated from soil of the semi-arid of state of Pernambuco, Brazil, was investigated with regard to their potential to fuel biodegradation as well as for the production of lipids and biosurfactant. The degradation was assessed using Bushnell Hass (BH) medium, the redox indicator 2,6-dichlorophenol – indophenol and *S. marcescens* wild-type and acclimatized in different concentrations of diesel (2, 4, 6, 8, 10, 12 and 15%). The obtained results showed that strain acclimatized in 15% diesel oil exhibited the best degradation index (79,63% of cotton biodiesel, 65,57% of sunflower biodiesel, 60,50% of diesel, 57,20% of gasoline and 39,26% of kerosene). Also, *S. marcescens* demonstrated the ability to grow and accumulate lipids (> 40%) using agro-industrial residues (cassava wastewater and waste vegetable oils). The produced lipids exhibited balanced profiles of fatty acids, mainly monounsaturated fatty acids which correspond with biodiesel requirements. In addition, *S. marcescens* showed ability to produce biosurfactant by bioconversion of agro-industrial residues (cassava wastewater and corn waste oil), in association with lactose, through a 2^3 factorial design. The best result was obtained in medium containing 6% cassava wastewater and 7,5% corn waste oil, in absence of lactose, with reduction of surface tension of water from 72 to 26,2 mN/m. The biosurfactant had good properties in the emulsification of hydrophobic compounds (EI_{24} > 60% of soybean oil, diesel oil, engine oil and burned engine oil). Moreover, the biosurfactant demonstrated stability in a wide range of pH, temperature and salinity. Also, it showed excellent efficiency on dispersion of engine oil in water (78%) as well as removing it in beach sand and mangrove sediment (88,27% and 73,70%, respectively). Then, *S. marcescens* UCP/WFCC 1549 demonstrated their high biotechnological potential for production of good quality biodiesel, as well as biosurfactant with promising application in bioremediation processes.

Keywords: *Serratia marcescens*; biodegradation; lipids; biosurfactant; agro-industrial wastes.

CAPÍTULO 1

1. INTRODUÇÃO

As bactérias do gênero *Serratia* sp. pertencem à família Enterobacteriaceae e se distinguem-se pela produção de três enzimas: DNAse, lipase e gelatinase (KHANAFARI et al., 2006; ARAÚJO et al., 2009). Dentre as espécies desse gênero, *S. marcescens* é a mais importante sendo saprófita e cosmopolita que habita diferentes nichos ecológicos e se caracteriza pela capacidade de produzir prodigiosina, pigmento de cor vermelha (KALIVODA et al., 2010). Além da prodigiosina, *S. marcescens* também é produtora de numerosas substâncias, destacando-se o biossurfactante conhecido como "serrawettina", além de proteases, nucleases e lipases (MONTANER et al., 2000; PÉREZ-TOMÁS, et al., 2003; ARAÚJO, 2010). Mais recentemente, *Serratia* sp. tem sido relatada como produtora de lipídeos com perfil químico similar ao biodiesel, aumentando assim o potencial de aplicação deste micro-organismo (BHARTI et al., 2014).

Os constantes acidentes ambientais envolvendo derramamentos de petróleo e seus derivados vêm desencadeando a preocupação, com o desenvolvimento de técnicas que objetivam a descontaminação das regiões impactadas (BENTO et al., 2008; ŁAWNICZAK et al., 2013). Assim, diversos métodos físicos, químicos e biológicos vêm sendo desenvolvidos na recuperação, remoção ou degradação de derivados do petróleo, e consequentemente, para a minimização dos efeitos sobre os ecossistemas. Dentre eles, a biorremediação destaca-se como uma alternativa viável e promissora para o tratamento desses ambientes impactados por petróleo e seus derivados (MADIGAN et al., 2000; MULLIGAN, 2009; AYED et al., 2015). Neste contexto, *S. marcescens* tem sido relatada como micro-organismo promissor na biodegradação de petróleo e derivados em processos de descontaminação ambiental (OKORO, 1999; WONGSA et al. 2004; OKORO et al., 2012; IBRAHIM et al., 2013).

Os micro-organismos na presença de substratos hidrofóbicos liberam biossurfactantes, promovendo o aumento da solubilidade desses compostos no ambiente (MULLIGAN, 2009; SOUZA et al., 2014). Os biossurfactantes ou biotensoativos são compostos anfipáticos, de origem microbiana, que atuam na redução da tensão superficial e interfacial, podendo também atuar na emulsificação e desemulsificação (BANAT et al., 2010; PETER et al., 2014). Considerando a crescente preocupação ambiental e econômica, a substituição dos surfactantes químicos pelos biológicos torna-se de grande interesse devido às características de baixa toxidade, biodegradabilidade e síntese a partir de fontes renováveis e de baixo custo, fatores esses que os caracteriza como compostos reconhecidamente seguros (CAMPOS-TAKAKI et al., 2010; UZOIGWE et al., 2015). Neste sentido, os biotensoativos apresentam ampla aplicabilidade destacando-se as indústrias de alimentos, farmacêutica, de cosméticos, na petroquímica e na área de

biorremediação (NITSCHKE e PASTORE, 2002; MARCHANT e BANAT, 2012; SILVA et al., 2014).

Por outro lado, atualmente existe um grande interesse em compostos lipídicos de origem microbiana, devido ao grande potencial de aplicação biotecnológica, como a produção de biodiesel, suplementos alimentares, atividade microbiana, entre outros (POLI et al., 2013). Em particular, os óleos microbianos constituem uma alternativa promissora para a obtenção de biodiesel, podendo alcançar elevados níveis de lipídeos não necessitando de terra arável (RATLEDGE, 2004; CERTIK et al., 2006). Diversos micro-organismos são capazes de acumular lipídeos, desempenhando papel fundamental na substituição de biodiesel vegetal (VICENTE et al., 2009; LIANG e JIANG, 2013). Por razões ambientais e econômicas, a obtenção de biocombustíveis a partir de biomassa microbiana é considerada uma possibilidade de integração dessas tecnologias nas plantas industriais atuais. Além disso, a produção destes micro-organismos não necessariamente compete com a produção de alimentos, visto que resíduos de biomassa podem ser utilizados como fontes de carbono (MA, 2006; THLIVEROS et al., 2014).

A demanda mundial de compostos de origem microbiana como biossurfactantes e lipídeos, tem aumentado nos últimos anos devido ao potencial de aplicação destas biomoléculas em diversas indústrias, além das vantagens, quando comparados com os de origem sintética (LEIVA-CANDIA et al., 2014; SILVA et al., 2014). No entanto, a produção industrial deles é limitada, considerando o elevado custo de produção, o que em parte é causado pelo valor dos meios de cultura (VICENTE et al., 2009; LUNA et al., 2011). Como consequência, são realizadas diversas pesquisas avaliando subprodutos e rejeitos agro-industriais como fontes nutricionais alternativas, tornando-se uma estratégia atraente e de baixo custo, e possibilitando a minimização dos problemas ambientais causados pelo descarte sem o tratamento prévio (MAKKAR et al., 2011; BERGER et al., 2014).

Estudos recentes com a *Serratia marcescens* UCP/WFCC 1549, isolada do semi-árido de Pernambuco, demonstraram seu potencial biotecnológico para produzir biossurfactante em meio contendo resíduos agro-industriais (ARAÚJO, 2010; ALVES et al., 2014). Contudo, a habilidade desta estirpe de remover e biodegradar combustíveis e utilizar rejeitos para produzir lipídeos visando à obtenção de biodiesel, não foram ainda explorados, apresentando-se como processos e produtos de interesse biotecnológico.

2. OBJETIVOS

2.1 Objetivo geral

Avaliar o potencial biotecnológico de *Serratia marcescens* UCP/WFCC 1549 na degradação de combustíveis, na acumulação de lipídeos e na produção de biossurfactante, utilizando resíduos agro-industriais como substratos alternativos.

2.2 Objetivos específicos

- Avaliar o potencial de biodegradação de combustíveis derivados do petróleo e biocombustíveis por *S. marcescens* selvagem e aclimatada em diesel;
- Avaliar o potencial de *S. marcescens* de produzir lipídeos utilizando resíduos agro-industriais (manipueira e óleos vegetais pós-fritura);
- Avaliar a qualidade dos ácidos graxos produzidos por *S. marcescens* visando à similaridade com os ácidos graxos componentes do biodiesel;
- Avaliar o potencial de *S. marcescens* de produzir biossurfactante utilizando resíduos agro-industriais (manipueira e óleo de milho pós-fritura);
- Selecionar as melhores condições de produção do biossurfactante através de planejamento fatorial completo;
- Avaliar a eficiência do biossurfactante produzido através da remoção de derivados do petróleo adsorvidos em areia de praia e sedimento de mangue.

3. REVISÃO DA LITERATURA

3.1 *Serratia marcescens*

Serratia sp. apresenta-se sob a forma de bacilo Gram-negativo que pertence à família Enterobacteriaceae e dintingue-se pela produção de três enzimas: DNAse, lipase e gelatinase (KHANAFARI et al., 2006; ARAÚJO, 2010). Este gênero de micro-organismos está amplamente distribuído na natureza, encontrando-se no solo, água, plantas e no trato intestinal de seres humanos e animais homeotérmicos (KIM et al., 2009).

S. marcescens é o membro mais importante desse gênero, bactéria Gram-negativa em forma de bastonete, com diâmetro que varia de 0,9-2,0 µm de comprimento, anaeróbio facultativo, de crescimento quimioautotrófico e geralmente móvel devido ao flagelo peritriquio (ARAÚJO, 2010). *S. marcescens* é saprófita, cosmopolita, habita diversos nichos ecológico, sendo frequentemente encontrada em alimentos ricos em amido, como também é um patógeno oportunista, responsável por casos de infecções hospitalares (SOTO-CERRATO et al., 2007; IGUCHI et al., 2014). A bactéria apresenta perfil de elevada resistência, capaz de sobreviver em condições inóspitas, na presença de alguns desinfetantes e antissépticos (AUCKEN e PITT, 1998; DOI et al., 2004; IGUCHI et al., 2014). Também se caracteriza pela capacidade que têm algumas espécies de produzir o pigmento prodigiosina, de cor vermelha característica (KHANAFARI et al., 2006; KALIVODA et al., 2010) (Figura 1).

Figura 1- Cultivo de *Serratia marcescens* UCP 1549 em ágar Luria Bertani (LB).

Fonte: Autora.

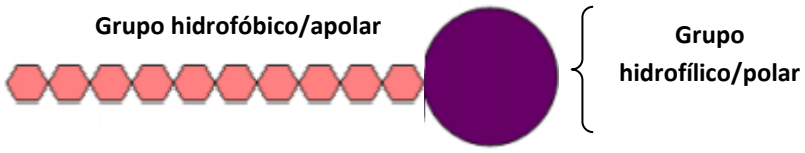
Além da prodigiosina, *S. marcescens* também é produtora de numerosas substâncias, como a serrawettina, um biossurfactante que lhe confere propriedade de aderência no processo de colonização de superfícies (MONTANER et al., 2000; PÉREZ-TOMÁS et al., 2003;

MATSUYAMA et al., 2011); enzimas como quitinases, lipases e cloroperoxidases (BAKKIYARAJ et al., 2012); e lipídeos com potencial aplicação na produção de biodiesel (BHARTI et al., 2014).

3.2 Surfactantes

A palavra surfactante deriva da expressão em inglês "surface active agents" (agentes de atividade superficial) por apresentarem atividade nas superfícies e interfaces dos líquidos (ROSEN e KUNJAPPU, 2012). Esta propriedade é dada aos surfactantes devido à sua estrutura química, a qual possui duas partes distintas, a primeira delas é uma região hidrofílica, usualmente chamada de cabeça, e a segunda região hidrofóbica, chamada de cauda (BANAT et al., 2000; ROSENBERG e RON, 2013) (Figura 2).

Figura 2- Representação esquemática dos grupos hidrofílico e hidrofóbico do surfactante.



Fonte: ANDRADE, 2014

A parte hidrofóbica ou região apolar da molécula do surfactante é usualmente constituída por hidrocarbonetos (SABATINI et al., 2006), possuindo a cadeia hidrocarbônica de 10 a 20 átomos de carbono, que podem ser aromáticos ou alifáticos, linear ou ramificada e carbonos reduzidos (CHU e CHAN, 2003). Embora não seja o grupo que caracteriza o surfactante, o grupo hidrofóbico influencia na concentração micelar crítica (CMC), uma propriedade das moléculas tensoativas (FERREIRA, 2004).

O grupo hidrofílico categoriza o surfactante, podendo ser iônico (catiônico ou aniônico), não-iônico ou anfotérico (BANAT et al., 2000; ROSENBERG e RON, 2013). Os catiônicos são constituídos por sais de amônio, enquanto os aniônicos possuem a parte hidrofílica constituída por grupo carboxilato, hidroxi, sulfato ou fosfato. Surfactantes não-iônicos não contêm grupos com carga e, nos surfactantes denominados anfotéricos, a parte hidrofílica é constituída por grupos que contêm uma carga negativa e uma positiva, o que lhes confere propriedades Zwiteriônicas, dependendo do pH (WOODS e CHARLES, 2004).

Quase todos os surfactantes atualmente utilizados são quimicamente derivados do petróleo (UZOIGWE et al., 2015). Entretanto, o interesse por surfactantes biológicos tem aumentado nos

últimos anos, devido às suas inúmeras características: possibilidade de produção através de fermentações, potenciais aplicações em áreas como a proteção ambiental, recuperação de resíduos e indústrias de processamento de alimentos e cosméticos (CASTIGLIONE et al., 2009; SAHARAN et al., 2011; JAMAL et al., 2012; PATHAK e KEHARIA, 2014).

3.2.1 Biossurfactantes

Os biossurfactantes foram descobertos na década de 60 como compostos extracelulares anfifílicos em pesquisas de fermentações de hidrocarbonetos (SOBERÓN-CHÁVEZ e MAIER, 2011). São produzidos, principalmente, pelo crescimento aeróbio de micro-organismos como leveduras, fungos filamentosos e por bactérias (DESAI e BANAT, 1997; MARCHANT e BANAT, 2012; SILVA et al.,2014). A produção microbiológica de biossurfactantes é considerada promissora devido ao curto tempo de geração quando comparados ao crescimento de plantas e animais (BANAT et al., 2010).

Os biossurfactantes são capazes de reduzir as forças de repulsão entre fases diferentes, interface ou superfície, e permitem que as duas fases se misturem mais facilmente (LUNA et al., 2011). Assim sendo, quanto menor a força de atração existente entre as moléculas do líquido menor será a tensão superficial, ocorrendo menor viscosidade e maior tendência a espalhar-se (SHARMA e SAHARAN, 2014). O tipo de biossurfactante é muito especifico podendo variar de espécie para espécie de micro-organismo (NIE et al., 2010; JARA et al., 2013).

3.2.1.1 Classificação

Diferente dos surfactantes sintetizados por via química, que são classificados de acordo com a natureza do seu grupamento polar, os biossurfactantes são classificados pela sua composição química e origem microbiana (MAKKAR e CAMEOTRA, 2002). As principais classes destes metabólitos secundários com baixo peso molecular incluem os glicolipídeos, os lipopeptideos e os fosfolipídeos (PIRÔLLO, 2006; SOBERÓN-CHÁVEZ e MAIER 2011). Por outro lado, os biossurfactantes de alto peso molecular incluem os polissacarídeos, as proteínas, os lipopolissacarídios, as lipoproteínas ou os complexos de misturas desses biopolímeros (Tabela 1) (SINGH, 2012).

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

Classe	Micro-organismos	Biossurfactantes
Glicolipídeos	*Pseudomonas, Torulopsis, Arthrobacter, Nocardia, Mycobacterium*	Ramnolipídeos, Soforolipídeos, Trealolipídeos
	Candida sphaerica	Lunasan
Peptídeos e lipopeptídeos	*Candida lipolytica*	Rufisan
	Bacillus	Surfactina
Ácidos graxos, Fosfolipídeos e Lipídeos neutros	*Rhodococcus erythropolis, Aspergillus, Arthobacter, Pseudomonas*	Fosfatidiletanolamina
Biossurfactantes polimérico	*Acinetobacter calcoaceticus, Candida lipolytica, Saccharomyces cerevisiae, Schizonella malanogramma*	Emulsan, liposan, Manoproteinas, Complexo de polissacarideo-proteina

Fonte: Singh (2012), modificada

3.2.1.2 Propriedades físico-químicas dos biossurfactantes

As aplicações dos surfactantes estão diretamente relacionadas com suas propriedades físico-químicas. Algumas das principais propriedades dos surfactantes como redução da tensão superficial e interfacial, Concentração Micelar Crítica (CMC) e emulsificação estão diretamente relacionadas com a sua importância em aplicações industriais nas mais diversas áreas (SAHARAN et al., 2011; JAMAL et al., 2012; SARAFIN et al., 2014).

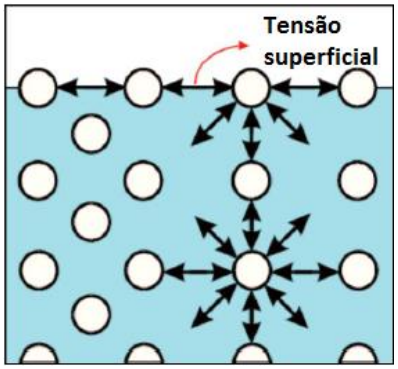
Tensão superficial

A tensão superficial é uma análise qualitativa podendo ser definida como o trabalho necessário para aumentar a área da superfície usualmente expressa em milinewtons por metro (mN/m), no Sistema Internacional de Unidades (SI) (DEL PINO e NETO, 1996; LUNA et al., 2011). As forças coesivas entre as moléculas no interior de um líquido são compartilhadas com os átomos vizinhos. As moléculas presentes na superfície não têm átomos vizinhos acima delas e exibem uma força atrativa mais forte sobre as moléculas vizinhas próximas a superfície. Este aumento das forças atrativas intermoleculares na superfície é chamado tensão superficial (HEWITT, 2002).

A tensão superficial permite avaliar a produção de surfactantes através da medida da redução da tensão superficial em relação à tensão da água (72 mN/m). De acordo com a literatura as tensões nas faixas de 35 mN/m a 40 mN/m, indicam que o micro-organismo é promissor na produção de biossurfactantes e abaixo de 35 mN/m, indica que o micro-organismo pode ser considerado um eficiente produtor (MULLIGAN, 2005; LUNA et al., 2011).

A tensão superficial tem sua efetividade dimensionada através da energia livre por unidade de área requerida para trazer a molécula da fase líquida para a superfície (MULLIGAN, 2005). Quanto menor trabalho é requerido para trazer a molécula para a superfície, mais a tensão superficial é reduzida. Quando um surfactante é adicionado à água, suas moléculas tendem a se arranjar de modo a minimizar a repulsão entre os grupos hidrofóbicos e a água. Os grupos polares ficam na solução aquosa, próximo à superfície, e os grupos apolares ficam na interface água-ar, minimizando o contato com a água (Figura 3). Esse fato gera uma diminuição na tensão superficial da água ao provocar um desarranjo em sua superfície (APARNA et al., 2011).

Figura 3 - Esquema das forças intermoleculares no interior e na superfície do líquido.



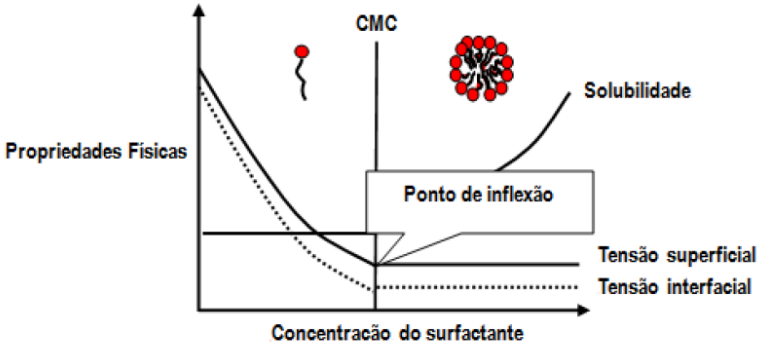
Fonte: Pirrôlo (2006)

Concentração Micelar Crítica (CMC)

A Concentração Micelar Crítica é uma propriedade que avalia a eficiência da atividade do surfactante conferindo propriedades de detergência e solubilização de compostos hidrofóbicos (MAKKAR et al., 2011). Está diretamente relacionada com a tensão superficial e é definida como a concentração mínima de surfactante utilizado para o processo de formação de micelas determinado pelo ponto de inflexão (SOBERÓN-CHÁVEZ e MAIER, 2011) (Figura 4).

As micelas são agregados de monômeros de surfactantes que a partir de uma determinada concentração (CMC) se associam. Abaixo da CMC, o surfactante está predominantemente na forma monomérica. Desta forma, a intensidade de adsorção do biossurfactante à superfície depende de sua concentração ocasionando uma variação na ordenação destas moléculas sobre a superfície. Em concentrações muito baixas de biossurfactante, o mesmo se distribui na superfície e tende a se orientar paralelamente a esta. Quando ocorre um aumento da concentração de surfactante, observa-se uma diminuição da área disponível para as moléculas iniciando o processo de ordenação das mesmas à superfície (CHEN et al., 2011; SAHARAN et al., 2011).

Figura 4 - Representação esquemática do comportamento de um surfactante em solução aquosa após atingir a CMC.



Fonte: http:// www.virtuallaboratory.ne

Emulsificação

Os biossurfactantes apresentam a propriedade de formar e estabilizar emulsões de hidrocarbonetos em água ou água em hidrocarbonetos. A emulsificação é a dispersão de um líquido em outro, e ela é possível porque o surfactante é capaz de reduzir as forças de repulsão entre líquidos com diferentes graus de polaridade permitindo que as duas fases se misturem (MUTHUSAMY et al., 2008).

As emulsões são muito instáveis e, portanto, não se formam espontaneamente, sendo necessário fornecer energia, tal como a agitação. Neste processo ocorre a formação de gotas microscópicas que variam em tamanho (0,1 e 100 nm de diâmentro). Quanto menor o diâmetro das gotículas, mais estável a emulsão formada (NITSCHKE e PASTORE, 2002; LIMA et al., 2007). Contudo, com o tempo, as emulsões tendem a retornar para o seu estado inicial.

Esta é, provavelmente, a propriedade mais versátil dos surfactantes para uso em aplicações industriais. Em indústrias de cosméticos e alimentos esta propriedade de formar e estabilizar emulsões é especialmente útil (MUTHUSAMY et al., 2008; RUFINO et al., 2014).

3.2.1.4 Vantagens da utilização dos biossurfactantes em relação aos sintéticos

Os biossurfactantes oferecem várias vantagens sobre os surfactantes sintéticos que, consequentemente, determinaram suas aplicações no setor industrial, podendo ser aplicados em condições ambientais desfavoráveis (NITSCHKE e COSTA, 2007; APARNA et al., 2012). Dentre essas vantagens podem-se citar:

- Tolerância à temperatura, pH e força iônica: muitos biossurfactantes podem ser utilizados sob condições extremas pois suas propriedades físico-químicas não são afetadas por mudanças extremas de temperatura, pH e força iônica. Alguns deles podem suportar temperaturas de até 90°C

e apresentam maior estabilidade térmica em condições extremas quando comparados aos sintéticos (MAX et al., 2012). De igual forma, possuem maior funcionalidade em valores extremos de pH, entre 5 e 12 por exemplo, e são potencialmente ativos quando submetidos a concentrações de 10% de NaCl, enquanto que 2-3% de sal são suficientes para inativar surfactantes convencionais (MAKKAR e CAMEOTRA, 2002; DAVISHI et al., 2011)

- Biodegradabilidade: são facilmente biodegradados na água e no solo, o que os torna adequados para aplicação na biorremediação e no tratamento de resíduos (COLIN et al., 2012).

-Baixa toxicidade: apresentam baixa toxicidade quando comparados com os surfactantes sintéticos. Por isso, têm recebido maior atenção devido à crescente preocupação da população com os efeitos alérgicos dos produtos artificiais, sendo permitido em alimentos, cosméticos e produtos farmacêuticos (NITSCHKE e PASTORE, 2002; ANYANWU et al., 2011; TIMMA et al., 2014).

- Facilidade de síntese a partir de material renovável e de baixo custo: vários estudos mostram a produção de biossurfactantes produzidos através de uma grande variedade de substratos orgânicos de baixo custo (MAKKAR et al., 2011; ROCHA e SILVA et al., 2014).

3.2.1.5 Principais aplicações dos biossurfactantes

A demanda mundial dos biossurfactantes é ainda crescente devido ao enorme potencial de aplicação que eles têm em diversos setores industriais. Segundo pesquisas realizadas pelo Transparency Market Research (2012) "Biosurfactants Market - Global Scenario", o volume do mercado global de biossurfactantes é esperado para ser 476,512.2 de toneladas até 2018. Desse total, 21% do consumo de volume virão de regiões como a Ásia, África e América Latina.

Na indústria de alimentos os biossurfactantes possuem uma variedade de aplicações, devido a sua habilidade de formar emulsões estáveis no processamento de matérias primas, no controle da aglomeração de glóbulos de gorduras em alimentos processados, tais como: creme de leite, manteiga, margarina, maionese, molhos para salada, entre outros (SILVA et al., 2009; SILVA, 2012). Estes bioprodutos podem ser usados também como agentes anti-adesivos durante processo de fabricação de alimentos, particularmente na redução de contaminação por patógenos ou na remoção de micro-organismos aderidos (COSTA, 2010).

Na área terapêutica os biossurfactantes também têm aplicação como agentes anti-adesivos e antimicrobianos, na formulação de produtos de limpeza e como agentes terapêuticos (COSTA, 2010; GUDIÑA et al., 2013). Além disso, tem sido demostrada a atividade antitumoral de vários biossurfactantes, o que destaca seu potencial como compostos a ser usados na terapia de diferentes tipos de câncer em humanos (BURGOS-DÍAZ et al., 2013; JANEK et al., 2013).

Alguns biossurfactantes possuem ação de detergência e propriedade espumante, o que os tornam aplicáveis na indústria de cosméticos, em sabonetes líquidos e xampus (CAROLEI e GUTZ, 2005). Dentre eles, os soforolipídeos, raminolipídeos e lipídeos manosileritirol têm apresentado excelentes propriedades e estão sendo produzidos para utilização em loções, hidratante de pele e produtos anti-rugas (LOURITH e KANLAYAVATTANAKULI, 2009).

Contudo, a área onde os biossurfactantes têm sido aplicados mais amplamente é o setor petroleiro, incluindo a limpeza de derramamento de óleos, a remoção de petroderivados de tanques de estocagem, a recuperação melhorada de petróleo e a biorremediação de ambientes terrestres e aquáticos contaminados com hidrocarbonetos (RON e ROSENBERG, 2002; SILVA et al., 2014).

3.3 Biorremediação de compostos derivados do petróleo

A contaminação causada pela exploração do petróleo e seus derivados causa grande impacto ambiental, pois essas substâncias apresentam propriedades tóxicas, mutagênicas e carcinogênicas aos seres humanos (MULLIGAN, 2009; SOUZA et al., 2014). Diante disso, diversos métodos, sejam eles: físicos, químicos e biológicos, vêm sendo desenvolvidos para a recuperação, remoção ou degradação *in-situ* ou *ex-situ* do petróleo derramado e consequentemente, para a minimização de seus efeitos sobre o ecossistema (APARNA et al., 2011). As técnicas convencionais apresentam problemas operacionais em razão do seu alto custo, necessidade de pessoal e de equipamentos (RIBEIRO, 2014). Portanto, os processos de biorremediação tornam-se uma alternativa não convencional para remediação de áreas contaminadas com petróleo e seus derivados, com um menor custo operacional e mínimos efeitos adversos ao ambiente (BENTO et al., 2008; RIBEIRO, 2014; SILVA et al., 2014).

O processo de biorremediação, também conhecido como remediação microbiana, pode ser definido como a utilização de micro-organismos para desintoxicar ou remover os poluentes, devido às suas diversas capacidades metabólicas (CHAILLAN et al., 2004). Portanto, a biorremediação é um método para a remoção e degradação de muitos poluentes ambientais, incluindo os produtos da indústria de petróleo (MEDINA-BELLYER et al., 2005). Além disso, esta tecnologia tem as vantagens de ser relativamente não invasiva e de baixo custo (APRIL et al., 2000).

Os estudos de degradação de compostos químicos têm mostrado vários micro-organismos extremamente versáteis em catabolizar moléculas recalcitrantes. Trabalhos atuais em biotecnologia indicam os fungos e as bactérias como principais micro-organismos eficientes na degradação de poluentes, possuindo alto potencial de ação na recuperação de ambientes contaminados (RAJASEKAR et al., 2012; ROY et al., 2014; SHANKAR et al., 2014). A eficiência de um ou

outro micro-organismo depende, em muitos casos, da estrutura da molécula e da presença de enzimas hábeis em degradar o produto, as quais apresentam especificidade para a maioria dos substratos (BALAJI et al., 2014; WACKETT, 2014). É por meio deste mecanismo que a biorremediação é efetivada. Este processo é mais provável quando a estrutura química do xenobiótico é semelhante à estrutura de moléculas naturais (GAYLARD et al., 2005).

O processo de biodegradação de petróleo é complexo, uma vez que depende da natureza e da quantidade dos hidrocarbonetos presentes (APARNA et al., 2011). Um dos fatores importantes que limitam a biodegradação destes poluentes no ambiente é a sua disponibilidade limitada para os micro-organismos. Compostos de hidrocarbonetos de petróleo se ligam aos componentes do solo, e que são difíceis de ser removidos ou degradados (BARATHI e VASUDEVAN, 2001; SOUZA et al., 2014). Além disso, os hidrocarbonetos diferem na sua susceptibilidade ao ataque microbiano, a que pode ser geralmente classificada da seguinte forma: n-alcanos> alcanos ramificados> aromáticos de baixa massa molecular> cicloalcanos> aromáticos de alta massa molecular (PERRY e GREEN, 1984; ULRICI, 2000; VIEIRA et al., 2006). Alguns compostos, como os hidrocarbonetos policíclicos aromáticos (HPAs), não podem ser totalmente degradados (GAYLARDE et al., 2005; PINHATIA et al., 2014).

Muitos micro-organismos têm sido utilizados para a degradação de gasolina, óleo diesel e resíduos de petróleo provenientes de derramamentos, nos oceanos ou no solo (PEREIRA e FREITAS, 2012). Algumas características das bactérias facilitam sua adaptação as mais diversas condições ambientais, como sua capacidade de crescimento rápido, flexibilidade metabólica, plasticidade genética e ampla habilidade de adaptação a variações do meio (MARTINS, 2004). Portanto, vários gêneros de bactérias têm sido descritos por sua potencialidade para degradar petróleo de ambientes contaminados, como *Acidovorans*, *Acinetobacter*, *Agrobacterium*, *Alcaligenes*, *Aeromonas*, *Arthrobacter*, *Beijemickia*, *Burkholderia*, *Bacillus*, *Comomonas*, *Corynebacterium*, *Cycloclasticus*, *Flavobacterium*, *Gordonia*, *Microbacterium*, *Moraxella*, *Mycobacterium*, *Micrococcus*, *Neptunomonas*, *Nocardia*, *Paracoccus*, *Pasteurella*, *Polaromonas*, *Pseudomonas*, *Ralstonia*, *Rhodococcus*, *Sphingomonas*, *Stenotrophomonas*, *Streptomyces* e *Vibrio* (CRAPEZ et al., 2002; JACQUES et al., 2007; MANDRI e LIN, 2007; TONINI et al., 2010).

Serratia marcescens também tem sido estudada na degradação de hidrocarbonetos e derivados do petróleo (IJAH, 1998; OKORO et al., 2012; IBRAHIM et al., 2013). Rajasekar et al. (2007) reportaram 58% de degradação de diesel por *S. marcescens* ACE2, enquanto Wongsa et al. (2004) isolaram uma cepa de *S. marcescens* com capacidade de degradação de 50–60% de gasolina, querosene, diesel e óleo lubrificante. Esses resultados indicam o potencial biotecnológico desta bactéria em processos de biorremediação de ambientes contaminados com petróleo e derivados.

3.3.1Aplicação de biossurfactantes na biorremediação de hidrocarbonetos

Um dos problemas associados à biodegradação de compostos hidrofóbicos, que incluem os hidrocarbonetos do petróleo, é sua ligação às partículas do solo e à pouca solubilidade de água que resulta em baixa disponibilidade para os micro-organismos, o que pode retardar ou mesmo paralisar esse processo (APARNA et al., 2011; COLIN et al., 2014). Um dos processos mais investigados para a resolução desse problema consiste na utilização de compostos surfactantes (VAN HAMME et al., 2006; SILVA et al., 2014).

Muitos micro-organismos liberam biossurfactantes na presença de hidrocarbonetos hidrofóbicos, promovendo o aumento da solubilidade desses compostos no meio (BANAT et al., 2010; AL-WAHAIBI et al., 2014; SOUZA et al., 2014). Os biossurfactantes aumentam a interação água/óleo, aceleram a degradação de vários hidrocarbonetos por micro-organismos e promovem a biorremediação de águas e solos contaminados (MULLIGAN, 2005; AYED et al., 2015). Diversas pesquisas com micro-organismos que produzem biossurfactantes demonstraram o potencial de biorremediação de hidrocarbonetos de petróleo em solos e areia (LUNA et al., 2011; NOPARAT et al., 2014; SILVA et al., 2014b; MONTAGNOLLI et al., 2015). A Tabela 2 apresenta uma lista de diferentes tipos de biossurfactantes e seus micro-organismos produtores com aplicações potenciais na biorremediação de ecossistemas contaminados com petróleo.

Tabela 2. Biossurfactantes, micro-organismos produtores e aplicações na biorremediação de ecossistemas contaminados com petróleo.

Micro-organismo	Tipo de biossurfactante	Aplicações
Rhodococcus erythropolis 3C-9	Glicolipídio e trehalolipídio	Operações de limpeza de derramamento de petróleo
Pseudomonas aeruginosa S2	Ramnolipídeo	Biorremediação de sítios contaminados com petróleo
Rhodococcus sp. TW53	Lipopeptídio	Biorremediação de poluição marinha por petróleo
R. wratislaviensis BN38	Glicolipídeo	Aplicações em biorremediação
Bacillus subtilis BS5	Lipopeptídio	Biorremediação de sítios contaminados com hidrocarbonetos
Azotobacter chroococcum	Lipopeptídio	Aplicações ambientais
Pseudomonas aeruginosa BS20	Ramnolipídeo	Biorremediação de sítios contaminados com hidrocarbonetos
Micrococcus luteus BN56	Trehalose tetraéster	Biorremediação de ambientes contaminados com petróleo

Nocardiopsis alba MSA10	Lipopeptídio	Biorremediação
Pseudoxanthomonas sp. PNK-04	Ramnolipídeo	Aplicações ambientais
Pseudomonas alcaligenes	Ramnolipídeo	Aplicações ambientais
Nocardiopsis lucentensis MSA04	Glicolipídeo	Biorremediação de ambientes marinhos
Calyptogena soyoae	Lipídeo manosileritirol	Biorremediação de ambientes marinhos
Pseudozyma hubeiensis	Glicolipídeo	Biorremediação de poluição marinha por petróleo
Pseudomonas cepacia CCT6659	Ramnolipídeo	Biorremediação de poluição marinha por petróleo
Candida bombicola	Soforolipídeo	Aplicações ambientais
C. glabrata UCP1002	Complexo proteína - carboidrato -lipídeo	Recuperação de óleo da areia
C. lipolytica UCP0988	Soforolipídeo	Recuperação de petróleo
C. sphaerica UCP0995	Complexo proteína - carboidrato -lipídeo	Remoção de óleo da areia
C. lipolytica UCP0988	Soforolipídeo	Controle da poluição ambiental por petróleo
C. sphaerica UCP0995	Complexo proteína - carboidrato -lipídeo	Processos de biorremediação
C. glabrata UCP1002	Complexo proteína - carboidrato -lipídeo	Remoção de petróleo
C. guilliermondii UCP0992	Glicolipídeo complexo	Remoção de óleo de motor da areia
C. tropicalis UCP0996	Complexo proteína - carboidrato -lipídeo	Remoção de petróleo e óleo de motor adsorbido em areia
C. lipolytica UCP0988	Soforolipídeo	Remoção de petróleo e óleo de motor adsorbido em areia
C. sphaerica UCP0995	Complexo proteína - carboidrato -lipídeo	Remoção de petróleo

Fonte: Silva et al. (2014c).

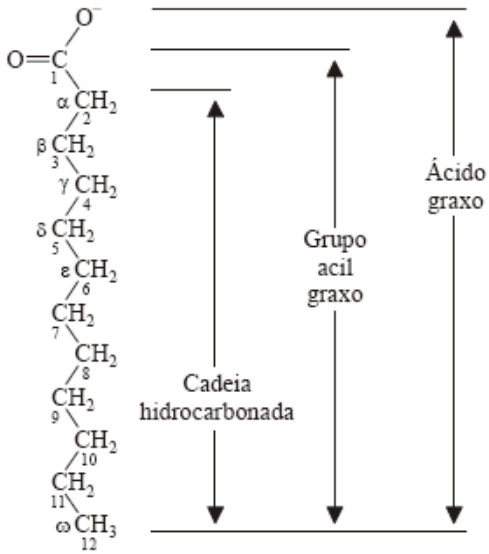
3.4 Lipídeos microbianos

Os lipídeos são substâncias hidrofóbicas, ou seja, insolúveis ou com reduzida solubilidade em água, devido a sua natureza apolar apresentando, no entanto, maior solubilidade em solventes orgânicos, como clorofórmio, metanol, hexano e benzeno. São basicamente constituídos por ácidos graxos que correspondem a uma cadeia carbônica apolar e um grupo carboxila polar, sendo representados pela fórmula geral RCOOH, podendo possuir de 4 a 36 átomos de carbonos e nenhuma ramificação**.** Os ácidos graxos se diferenciam entre si a partir de três características: o tamanho de sua cadeia hidrocarbonada, o número de insaturações e a presença de grupamentos químicos (NELSON et al., 2002; LEHNINGER et al., 2006).

Os exemplos mais conhecidos de lipídeos são os ácidos graxos e seus derivados, esteróis, ceras e carotenoides. Compostos que apresentam cadeias orgânicas com um elevado número de carbonos, o que lhes confere o caráter hidrofóbico, podendo apresentar apenas átomos de carbono e hidrogênio ou, ainda, grupos funcionais com heteroátomos, como álcoois, fenóis, ácidos carboxílicos, ésteres, entre outros (FAHY et al., 2005).

Os ácidos graxos podem ser saturados ou insaturados (contêm uma ou mais dupla ligações). Na nomenclatura IUPAC (*International Union of Pure and Applied Chemistry)* os carbonos são numerados a partir do carbono carboxílico. Em geral, apresentando número par de átomos de carbono. Na nomenclatura comum, o átomo de carbono adjacente ao carbono carboxílico é denominado α, e os carbonos seguintes são nomeados β, γ, δ, etc. O átomo de carbono mais distante do carbono carboxílico é chamado carbono ω, independentemente do tamanho da cadeia (Figura 5). Os mais abundantes contêm C_{16} e C_{18} átomos (MOTTA, 2004).

Figura 5. Estrutura e nomenclatura dos ácidos graxos: ácido graxo laureato (ou dodecanoato), tem 12 carbonos e não contêm duplas ligações (MOTTA, 2004).



A maioria dos ácidos graxos são sintetizados pelo homem, exceto o ácido linoleico e o ácido linolênico, denominados ácidos graxos essências e são obtidos da dieta. Os ácidos graxos essenciais são precursores para a biossíntese de vários metabólitos importantes. Dietas pobres em ácidos graxos podem ocasionar dermatites, demora na cura de ferimentos, redução na resistência a infecções, alopecia e trombocitopenia (MOTTA, 2004).

Os óleos microbianos, comumente denominados óleos de células simples (SCO, do inglês *single cell oils*), os quais são lipídeos produzidos por micro-organismos oleaginosos, têm sido de interesse potencial para vários pesquisadores nas últimas décadas devido às suas diversas

aplicações, como em aditivos alimentares, farmacêuticos, combustíveis e ingredientes de alimentos para a aquicultura (POLI et al., 2013). Tradicionalmente, os micro-organismos, que incluem bactérias, leveduras, fungos e microalgas, que podem acumular mais de 20% de lipídeos do seu peso seco, podendo chegar a capacidade de acumular até 70% durante o período de estresse metabólico, são considerados micro-organismos oleaginosos (SZCZESNA-ANTCZAK, 2006; ROSSI et al., 2011; ZHENG et al., 2012).

Dentre das espécies de micro-organismos oleaginosos, as representantes mais estudadas de bactérias são *Arthobacter sp.*, *Bacillus alcalophillus*, *Gordona sp.* e *Rhodococcus opacus*, e de micro-algas, *Chlorella vulgaris, C. emersonii, C. protothecoides, C. sorokiniana, Nannocloropsis sp.* e *Niczschia sp.* (ILLMAN et al., 2000; CHISTI, 2006; GOUDA et al., 2008; MENG et al., 2009). Além desses micro-organismos citados estão as leveduras e fungos filamentosos destacando-se os gêneros: *Yarrowia, Candida, Rhodotorula, Rhodosporodium, Cryptococcus* e *Lypomyces* como representantes das leveduras e *Cunninghamella echinulata, Umbelopsis isabelina* e *Mucor circinelloides* como representantes de fungos filamentosos mais investigado (LI et al., 2008; ZHAO et al. 2008; ROSSI et al., 2009; VICENTE et al., 2009; MACHADO JUNIOR, 2010; AGEITOS et al., 2011).

Os lipídeos produzidos por micro-organismos apresentam composição similar e valor energético aos óleos vegetais e animais, mas como produtores de lipídeos os micro-organismos não competem por recursos alimentares, especialmente se a fonte de carbono for de baixo custo, como matérias-primas, subprodutos e excedentes, apresenta grande rapidez de geração, e sua produção não é sujeito a variações climáticas e sazonais cíclico, requer menor área de produção e melhor controle da produção e do produto (MACHADO JUNIOR, 2010; ROSSI et al., 2011).

O uso de micro-organismos como fonte de lipídeos tem sido bastante estudado para aplicação biotecnológica, como sua aplicação em aditivos alimentares, farmacêuticos, combustíveis e ingredientes de alimentos para a aquicultura (RATLEDGE, 1991; RATLEDGE, 2004; SZCZESNA-ANTCZAK, 2006).

3.4.1 Aplicações dos lipídeos microbianos na obtenção de biodiesel

Atualmente, existe um aumento no interesse em compostos lipídicos de origem microbiana, devido ao seu grande potencial de aplicação biotecnológica, como a produção de biodiesel, suplementos alimentares, atividade microbiana, entre outros (POLI et al., 2013).

Nos últimos anos, o desenvolvimento das tecnologias sustentáveis tem ganhado espaço na política ambiental de diversos países bem como dos diferentes setores. Dentro do segmento de

energia os biocombustíveis vêm se consolidando como alternativa a utilização de combustíveis fósseis (CHERUBINI, 2010; LEIVA-CANDIA et al., 2014). A produção de biocombustíveis baseada na biomassa surgiu como uma abordagem importante para permitir a independência energética, reduzindo emissões de gases do efeito estufa, revitalizando comunidades rurais e melhorando o desenvolvimento sustentável (XIA et al., 2011). O biodiesel, tradicionalmente produzido a partir de óleos vegetais e gorduras animais, é uma alternativa atraente por ser biodegradável e atóxica apresentando características renováveis, bem como propriedades semelhantes ao diesel convencional (DEMIRBAS, 2008; LIANG e JIANG, 2013).

Contudo, o alto custo do biodiesel tornou-se um dos maiores obstáculos para seu desenvolvimento e aplicação em larga escala. Além disso, os óleos vegetais utilizados como matéria-prima para a produção de biodiesel poderiam competir com óleos comestíveis (DEMIRBAS, 2008). Assim, a procura de fontes mais eficientes e baratas de processos de produção não tradicional de triglicerídeos, especialmente aquelas que podem ser operados de forma contínua e sem qualquer condição de extensas terras aráveis, é essencial para uma indústria de biodiesel sustentável (RATLEDGE, 2004; CERTIK, 2006). A este respeito, muita atenção tem sido dada para o desenvolvimento de óleos microbianos, e tem sido encontrado que muitos micro-organismos como microalgas, fungos e bactérias, têm a capacidade de acumular lipídeos sob algumas condições de cultivo especial (YI e ZHENG, 2006; LIANG e JIANG, 2013).

Em comparação com outros óleos vegetais, os óleos microbianos têm muitas vantagens, tais como o ciclo de vida curto dos micro-organismos, menos trabalho requerido, são menos afetados pelo local, estação e clima (MA, 2006; MONTONERI et al., 2009). Com a rápida expansão do biodiesel, os óleos microbianos podem se tornar uma das matérias primas lipídicas com potencial para produção de biodiesel no futuro, segundo os resultados obtidos por muitos pesquisadores (VICENTE et al., 2009; SAENGE et al., 2011; POLI et al., 2013).

3.5 Utilizações de substratos alternativos na produção de biossurfactantes e lipídeos microbianos

Apesar das vantagens apresentadas, os biossurfactantes e lipídeos microbianos não são amplamente utilizados pelas indústrias devido ao alto custo de produção, associado à baixa produtividade e ao uso de substratos caros (CAMPOS-TAKAKI et al., 2010; CHEIRSILP et al., 2013; LUNA et al., 2013). Uma possível estratégia para reduzir os custos da produção seria o uso de substratos alternativos, como os resíduos agroindustriais ou de indústrias alimentícias, que geralmente contêm altos níveis de carboidratos ou lipídeos necessários para a biossíntese destas

biomoléculas (BANAT et al., 2000; VICENTE et al., 2009; BANAT et al., 2014). Além disto, o uso de resíduos contribui para a redução da poluição ambiental e para a valorização econômica destes produtos (MAKKAR et al., 2011).

Os sistemas industriais brasileiros abrangem uma vasta gama de atividades agrícolas ou agropecuárias, pois o país é considerado um grande fornecedor de alimentos para o mundo (ARAÚJO, 2010). Contudo, os resíduos líquidos gerados pela indústria alimentícia apresentam grande complexidade física e química, o que dificulta seu tratamento, podendo causar risco ao meio ambiente onde são descartados (CERQUEIRA e COSTA, 2009; LUNA et al., 2013).

Manipueira

A manipueira é um líquido extraído da mandioca durante o processo de fabricação da farinha (BARROS et al., 2008). De acordo com Cordeiro (2006), este efluente de cor amarelada possui uma composição química variada que está associada à variedade da mandioca utilizada, ao período da safra, à fertilidade do solo, entre outros fatores. Merece uma especial atenção devido a seu efeito agressivo ao ambiente, tanto pelo teor de cianeto total quanto pela alta carga orgânica (CARVALHO et al., 2006; ANDRADE, 2010).

O potencial tóxico da manipueira é associado à presença da linamarina, um glicosídeo cianogênico que é enzimaticamente hidrolisado a cianeto, o qual possui afinidade com o ferro promovendo a combinação da hemoglobina para formar a cianohemoglobina e inibe o transporte de oxigênio no sangue e consequentemente, a cadeia respiratória (CARVALHO et al., 2006; BARROS, 2008; ARAÚJO, 2010). A alta carga poluidora deste resíduo é associada à presença de carboidratos, nitrogênio e diversos sais minerais que o tornam passível de ser aproveitado para o cultivo de micro-organismos (BEZERRA, 2012; SALGADO, 2013). A Tabela 3 apresenta a composição química da manipueira segundo alguns trabalhos publicados (NITSCHKE e PASTORE, 2006; ROSSMAN, 2008; COSTA et al., 2009).

Tabela 3- Composição química média da manipueira.

Componentes	**Nitschke e Pastore (2006)**	**Rossman (2008)**	**Costa et al. (2009)**
Sólidos Totais (g/L)	*	45,02	*
Açúcares Totais (g/L)	35,3	47,07	56,4
Açúcares redutores (g/L)	12,8	0,35	*
Açúcares não-redutores (g/L)	22,2	47,03	*

Nitrogênio total (g/L)	2,5	0,21	*
Fosforo (mg/L)	225,9	643	900
Potássio (mg/L)	2665,1	49	3600,00
Cálcio (mg/L)	272,5	352,00	*
Magnésio (mg/L)	519,0	8,12	500,00
Enxofre (mg/L)	104,0	*	*
Ferro (mg/L)	7,8	ND	6,1
Zinco (mg/L)	7,3	*	11,1
Manganês (mg/L)	1,8	0,16	4,1
Cobre (mg/L)	0,6	*	14,1
pH	5,9	4,56	*

* Análises não realizada

Óleos pós-fritura

Os óleos e gorduras são, por definição, substâncias hidrofóbicas que podem ser de origem animal ou vegetal (LIMA, 2007). Sua constituição química é composta por triglicerídeos, que são formados da condensação entre glicerol e ácidos graxos. A diferença entre gordura e óleo é baseado no seu estado físico, em que a gordura é sólida e o óleo é líquido, ambos a uma temperatura de até 20ºC (DABDOUB et al., 2006). O óleo vegetal, que é o que dá origem aos óleos de cozinha, pode ser obtido de várias plantas, ou sementes como o buriti, mamona, soja, canola, girassol, milho, etc. (SALGADO, 2013).

Os óleos vegetais são larga e universalmente consumidos para a preparação de alimentos nos domicílios, estabelecimentos industriais e comerciais de produção de alimentos (MORÁS e SILVA, 2009). Esses óleos, após serem degradados termicamente, se descartados de maneira imprópria podem causar prejuízos ao meio ambiente, como por exemplo: quando em contato com a água de rios e lagos, o óleo se concentra na superfície, criando uma barreira sobrenadante que dificulta a entrada de luz e oxigênio na água, sendo comprometida a base da cadeia alimentar aquática. Também, quando em contato com o solo impermeabiliza-o impedindo que a água se infiltre, agravando o problema das enchentes de higiene, mau cheiro e entupimentos nas redes de esgoto, e ainda uma pequena quantidade de óleo polui uma grande quantidade de água, como por exemplo, um litro de óleo de cozinha pode poluir cerca de 10000 litros de água (DABDOUB et al., 2006; GHESTI et al., 2012; SALGADO, 2013).

O alto custo da produção de biossurfactantes ou biodiesel a partir de lipídeos microbianos, deve-se principalmente aos custos associados aos meios de cultura, o qual é estimado em aproximadamente 80% do custo total do processo de produção (LUNA et al, 2011; VICENTE et al., 2009). Assim, consideráveis esforços têm sido direcionados para minimizar os custos da fonte de carbono e encontrar novas fontes alternativas (TSIGIE et al., 2011; BANAT et al., 2014). Em particular, a exploração de resíduos agroindustriais e subprodutos como matérias-primas podem reduzir muito o custo, além de possibilitar um novo destino mais sustentável a estes resíduos (MAKKAR et al., 2011; LEIVA-CANDIA et al., 2014).

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CAPÍTULO 2

ARTIGO I

Ability of *Serratia marcescens* UCP/WFCC 1549 for biosurfactant production using industrial wastes and fuels biodegradation

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Ability of *Serratia marcescens* UCP/WFCC 1549 for biosurfactant production using industrial wastes and fuels biodegradation

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Biosurfactants are microbial molecules which acting on the liquid surface and interface, providing the formation of stable emulsions. Their production is closely related to the ability of microorganism to grow in hydrocarbon contaminated environments and degrade fuels. Besides, they can be produced from renewable and low-cost substrates such as agro-industrial residues. This work was aimed for biosurfactant production by *Serratia marcescens* UCP/WFCC 1549 using wastes and its potential for fuels biodegradation after its acclimatization in diesel. The best results were obtained in medium containing 6% of cassava wastewater and 7.5% of corn post frying oil, with reduction in surface tension of the water from 70 to 26.2 mN/m. The emulsification index obtained in this condition was 64.0% to diesel, 57.69% to corn post frying oil and 54.17% to engine burned oil. *S. marcescens* pre-incubated on 15% of diesel showed the higher values of fuels degradation: 79.63% (cotton biodiesel), 65.57% (sunflower biodiesel), 60.50% (diesel), 57.20% (gasoline) and 39.26% (kerosene).

Keywords *Serratia marcescens*; biosurfactants; agro-industrial wastes; biodegradation; fuels.

1. Introduction

Biosurfactants are microbial molecules with hydrophobic and hydrophilic portions that are distributed preferentially at the interface between fluid phases. These properties give these compounds the ability to reduce surface tension and promote the formation of microemulsions for the solubilization of hydrocarbons [1].

Although biosurfactants exhibit several important advantages, they have not been yet employed extensively in industry because of relatively high production costs. One possible strategy for reducing costs is the utilization of alternative substrates such agro-industrial wastes, in order to contribute to environmental pollution reduction and allow them aggregate market value [2, 3]. In addition, their production is closely related to the ability of microorganism to grow and degrade hydrocarbons and fuels [4].

This work was aimed to verify the ability of *Serratia marcescens* UCP/WFCC 1549 for biosurfactant production using agro-industrial residues as substrates. Also, it was evaluated the microbial potential for fuels biodegradation after its acclimatization in diesel.

2. Materials and Methods

Serratia marcescens UCP/WFCC 1549 was kindly supplied from the Culture Collection of the Nucleus of Research in Environmental Sciences, Catholic University of Pernambuco, Recife-PE, Brazil. The microorganism was maintained in Nutrient Agar at 5° C.

The pure culture was transferred to Luria Bertani (LB) solid medium (trytone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L and agar 15 g/L) and incubated for 24 h at 28^{o}C. Then, a loopful of the red-coloured culture was transferred to a 250 mL Erlenmeyer flask containing 100 mL of LB broth and maintained under orbital shaker at 150 rpm during 18 h at 28^{o}C to obtain the pre-inoculum.

The biosurfactant production was evaluated using a 2^3 full factorial design to analyze the effects and interactions of the independent variables concentration of cassava wastewater, corn post frying oil and lactose about the response variable surface tension. In this design, a set of 12 experiments, with four replicates at the central points, was performed. The analysis of the results was accomplished using the Statistical 7.0 software package (StatSoft, Inc. 2005). The fermentation experiments were carried out using 250 mL Erlenmeyer flasks containing 100 mL of production medium, in agreement with the experimental factorial design (Table 1), inoculated with 1 mL of pre-inoculum (10^7 cells/mL). The flasks were maintained for 72 h, under orbital agitation (150 rpm) at 28 °C. After this period, aliquots were used to measure surface tension and emulsification index on the metabolic cell-free liquid obtained by centrifugation (10 000g for 15 min) and subsequent filtration of samples. The surface tension was determined using a Tensiometer model Sigma 70

(KSV Instruments LTD - Finland) using the Du Nouy ring method at room temperature [5]. The emulsification index was analysed according to Cooper and Goldenberg [6] for the better condition determined by measuring of surface tension. The test was done using 2 mL of the metabolic cell-free liquid and 2 mL of hydrophobic compounds (soybean oil, corn oil, soybean post frying oil, corn post frying oil, diesel, kerosene, engine oil and engine burned oil) in a graduated screw cap test tube and vortexed at high speed for 2 min. After 24 h, the emulsification index (E24) was calculated by dividing the measured height of the emulsion layer by the mixture's total height and multiplying by 100 to expressing in percentage.

For the fuels biodegradation assay, *S. marcescens* was pre-incubated in LB medium solid supplemented with different concentrations of diesel (2, 4, 6, 8, 10, 12 and 15%) for 24 h. Then, were prepared cell suspensions of 10^7 UFC/mL and 1 mL of each suspension was inoculated in test tubes containing 5 mL Bushnell-Hass medium (BHM) ($MgSO_4$ 0.2 g/L, K_2HPO_4 1.0 g/L, KH_2PO_4 1.0 g/L, $CaCl_2$ 0.02 g/L, $FeCl_3$ 0.05 g/L and NH_4NO_3 1.0 g/L), 5 μg/mL of the redox indicator 2,6 dichlorophenol-indophenol (DCIP) and 50 μL of fuels (diesel, sunflower biodiesel, cotton biodiesel, gasoline or kerosene). The biodegradation potential was evaluated after 30 days by visualization of decolourization of the medium and determination of degradation percentage by optical density at 610 nm.

3. Results and Discussion

3.1 Biosurfactant production

The use of alternative low-cost substrates, such as agro-industrial wastes, is an important strategy to improve the economics and to facilitate industrial development of biosurfactant production. Cassava wastewater and frying vegetable oils are industrial residues which disposable causes environmental problems; however, they are very attractive substrates for biotechnological processes. Previous works demonstrated that these wastes were suitable as feedstock for biosurfactant production [7, 8]. In the present study, it was investigated the ability of *S. marcescens* UCP/WFCC 1549 to produce biosurfactant using variables concentrations of cassava wastewater, corn post frying oil and lactose. The results are showed in Table 1, where numbers one (1) to eight (8) are the runs corresponding to the experimental conditions obtained from the combination of the variables tested and numbers nine (9) to twelve (12) are the runs corresponding to the experimental conditions of the central point.

Table 1 Surface tension values obtained in the 2^3 full factorial design used for biosurfactant production by *S. marcescens* UCP/WFCC 1549 at 28 °C and 150 rpm during 72 hours.

Conditions	Lactose (%)	Cassava wastewater (%)	Corn post frying oil (%)	Surface tension (mN/m)
1	0.0	1.0	5.0	30.1
2	1.0	1.0	5.0	36.0
3	0.0	6.0	5.0	34.0
4	1.0	6.0	5.0	30.8
5	0.0	1.0	7.5	31.3
6	1.0	1.0	7.5	35.0
7	0.0	6.0	7.5	26.2
8	1.0	6.0	7.5	29.9
9	0.5	3.5	6.25	31.8
10	0.5	3.5	6.25	30.8
11	0.5	3.5	6.25	30.9
12	0.5	3.5	6.25	31.9

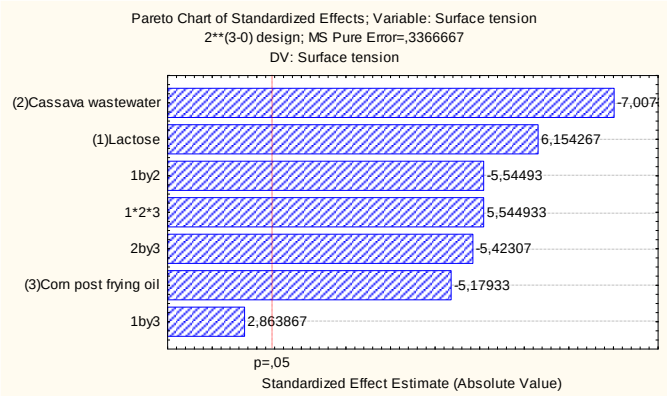
After 72h of cultivation the higher reduction in surface tension of the water was observed from 70 to 26.2 mN/m in condition 7 of factorial design, in medium constituted by 6% of cassava wastewater, 7.5% of corn post frying oil and without lactose. Araújo et al. [9] obtained similar results (26.78 mN/m) with this strain using 6% of cassava wastewater, 7.5% of corn oil and 0.2% of lactose, in similar conditions of cultivation. However, in the present work was achieved dispense with lactose and change a valuated product (corn oil) by a waste cooking oil.

3.2 Effect of variables used on the surface tension

Figure 1 illustrates the Pareto Chart, with 95 % confidence level, for effect estimates, in absolute values. From the chart, it can be seen that all independent variables were statistically significant. The cassava wastewater was the independent variable that most influenced in reducing the surface tension. Thus, the association of cassava with lactose as manipueira with the corn post frying oil

influenced also in reducing the surface tension. On the other hand, the three associated factors were significant from the statistical point of view, of lactose with cassava wastewater contributed to decreasing the response variable after 72 hours of cultivation.

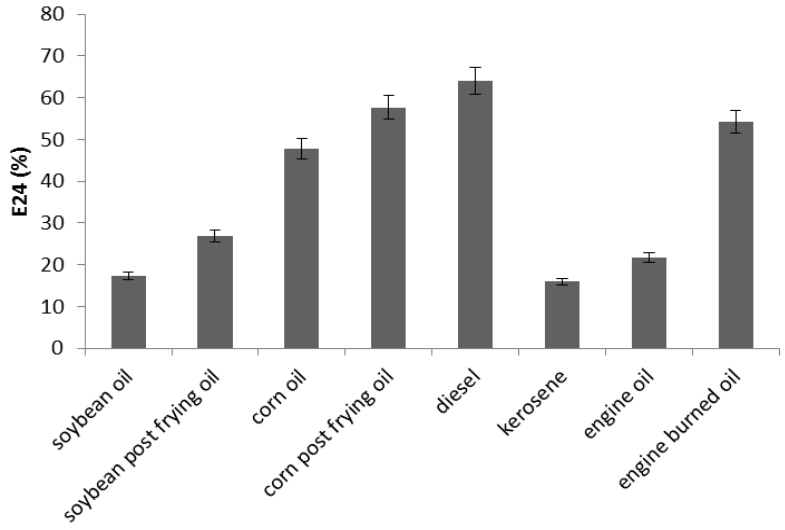
Fig 1. Pareto Chart of standardized effects for surface tension of the cell-free broth from *S. marcescens* after 72 hours of cultivation for the 2^3 full factorial design. The point at which the effect estimates were statistically significant (at p = 0.05) is indicated by the broken vertical line.



3.3 Emulsification index (E_{24})

The ability to form and stabilize emulsions is one of the most important features to be considered for the practical application of a surfactant [10]. The emulsifying activity against different hydrophobic substrates of the biosurfactant produced by *S. marcescens* in the condition 7 of the 2^3 full factorial design is presented in Fig. 2. The results showed that the significative values were 64.0% to diesel, 57.69% to corn post frying oil and 54.17% to engine burned oil. The property of biosurfactants to form stable emulsions with hydrocarbon-water mixtures has been demonstrated to increase hydrocarbon degradation suggests its potential application in oil spill management and enhanced oil recovery [11].

Fig. 2 Emulsification index (E_{24}) of the biosurfactant produced by *S. marcescens* UCP 1549 in medium consisting in 6% of cassava wastewater and 7.5% of corn post frying oil.



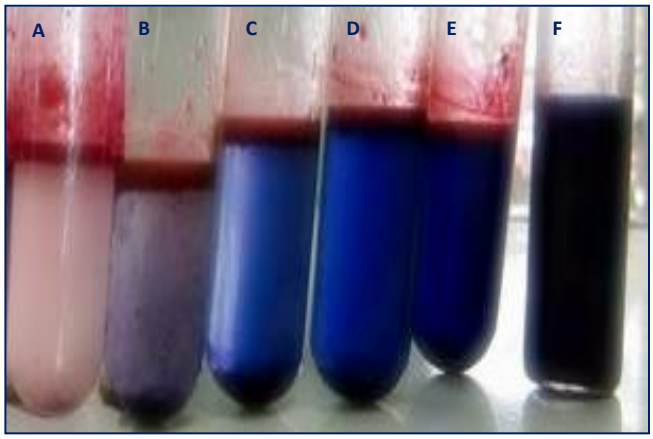
3.4 Fuels biodegradation assay

Biosurfactants are directly involved in the process of hydrocarbon removal from the environment through increased bioavailability and subsequent biodegradation of the hydrocarbons by direct cell contact [12]. For this reason, is considered that biosurfactant production is closely related to the ability of microorganism to grow and degrade hydrocarbons and fuels.

In this study, a biosurfactant-producing bacteria, *S. marcescens* UCP/WFCC 1549 was tested for its potential in fuels biodegradation after its acclimatization on diesel oil. This strain was pre-incubated on different concentrations of diesel (2, 4, 6, 8, 10, 12 and 15%) in order to analyze the influence of this acclimatization on its potential for biodegradation of fuels: diesel, sunflower biodiesel, cotton biodiesel, gasoline and kerosene.

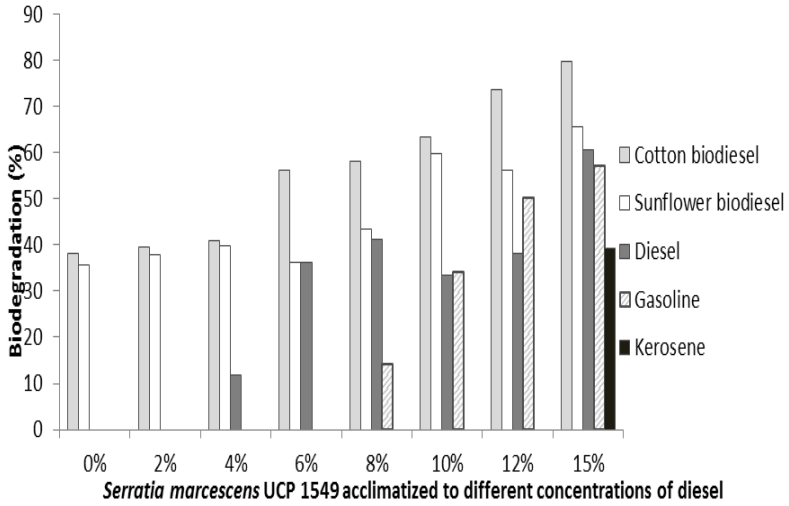
The qualitative determination (visualization) showed that an increase in diesel concentration during the acclimatization step corresponded to an increase of fuels degraded and in the percentage of degradation. *S. marcescens* acclimatized in 15% diesel was the one with the greatest potential to biodegrade the fuels tested, with colour changes of BHM from dark blue to light blue or colourless (Figure 3).

Fig. 3 Test of fuels biodegradation by *S. marcescens* UCP/WFCC 1549 acclimatized to 15% diesel, using the redox indicator 2,6-dichlorophenol-indophenol: (A) degradation of cotton biodiesel, (B) degradation of sunflower biodiesel, (C) degradation of diesel, (D) degradation of gasoline, (E) degradation of kerosene and (F) control.



While the strain acclimatized to lower concentrations of diesel, it degraded the two types of biodiesel in all conditions tested. However, it did not occur with other substrates in which only the degradation occurred when was used the strain acclimatized to higher concentrations of diesel. These results were confirmed by determination of percentage of biodegradation, which are shown in Figure 4. It was shown that the strain acclimated to 15% diesel showed the highest values of degradation: 79.63% (biodiesel cotton), 65.57% (sunflower biodiesel), 60.50% (diesel), 57.20% (gasoline) and 39.26% (kerosene).

Fig. 4 Potential of fuels biodegradation (%) of *S. marcescens* UCP/WFCC 1549 acclimatized on different concentrations of diesel after incubation for 30 days.



The results obtained in this study were considered satisfactory due to in the literature it is showed limited reports describing the involvement of *Serratia* species in biodegradation of hydrocarbons [13, 14], and they are mostly degraders of aromatic compounds. However, experiments carried out by Wongsa *et al.* [15], showed a *S. marcescens* strain (HokM) with a relatively high capacity to degrade hydrocarbons in gasoline, kerosene and diesel. These strain showed percentages of degradation similar to the obtained in present study. In this work was demonstrated the ability of a *S. marcescens* UCP/WFCC 1549 to fuels biodegradation, which would have great application in bioremediation of hydrocarbon-contaminated sites.

S. marcescens strains have also been implicated by many investigators in petroleum hydrocarbon biodegradation [15-17] and biosurfactant production [18, 19] but there are few works involving *Serratia marcescens* strains in both biodegradation and biosurfactant production [20].

4. Conclusions

In conclusion, this study demonstrated the ability of *S. marcescens* UCP/WFCC 1549 to produce biosurfactant using wastes as substrates, what allows a reduction in costs of the process. Also, it showed its potential for fuels biodegradation that suggests its likely biotechnological applications on the bioremediation of polluted environments.

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CAPÍTULO 3
ARTIGO II

Conversion of Agro-industrial Wastes by *Serratia marcescens* UCP/WFCC 1549 into Lipids Suitable for Biodiesel Production

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Conversion of Agro-industrial Wastes by *Serratia marcescens* UCP 1549 into Lipids Suitable for Biodiesel Production

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Abstract

Microorganisms that can accumulate lipids at more than 20% of their dry biomass are defined as oleaginous species. The majority of these lipids are triacylglycerol containing long-chain fatty acids, which are comparable to conventional vegetable oils. This paper shows the potential of *Serratia marcescens* UCP/WFCC 1549 to produce lipids using agro-industrial wastes, indicating that these wastes are a renewable resource and can be used as additives of Luria Bertani medium. *S.marcescens* has been proved to have good ability for growth and can store large amounts of lipids (higher than 40%) in media consisting only of wastes. Its potential application as a source of biodiesel was analyzed by identifying fatty acid methyl esters (FAMEs) using gas chromatography. In media comprising only residues, more balanced profiles of FAMEs were found in terms of the proportion of saturated, mono-unsaturated and poly-unsaturated fatty acids (SFAs, MUFAs and PUFAs, respectively). The best result was obtained in lipids produced in medium containing cassava wastewater (CW) and soybean oil waste (SOW), which had the highest percentage of MUFAs (48.09%), in accordance with the standards for biodiesel quality. Also, a high content of oleic acid (46.82%) was achieved in this medium, thus showing *S. marcescens* as a potential feedstock for producing good quality biodiesel, and the potential of oleaginous bacterium was demonstrated by the favorable composition of the MUFAs.

Keywords: *Serratia marcescens*; lipids; fatty acids; biodiesel; agro-industrial wastes.

1. Introduction

In recent years, energy high prices, energy security, protecting the environment and concerns about petroleum supplies have attracted great attention and led to strong efforts to find a renewable biofuel [1]. However, crude fossil oil has a limited and finite supply, which could run out during this century depending upon the extent to which this energy source continues to be used to meet the increasing demands for energy. Therefore, if plant biomass can be increasingly exploited, this will increase energy security, thereby reducing the dependency on crude oil (a non-renewable). There are at least three distinct advantages of a biorefinery using renewable feedstocks for production of bioenergy, biofuels and biochemicals, compared to chemical refining of petrochemical feedstocks, namely: energy security, prevention of climate change and rural development [2].

One of the most promising renewable biofuels is biodiesel, which is a mixture of fatty acid methyl esters (FAMEs) obtained by the transesterification of triglycerides (in most cases, vegetable oils and animal fats) with methanol or ethanol [3]. Biodiesel is biodegradable, nontoxic and has properties similar to conventional diesel [4,5].

However, the cost of biodiesel is high due to the high cost of the raw materials (about 70–75% of the total cost). A cheaper raw material for biodiesel production would help to reduce the total cost [6,7]. Much attention has been paid to the development of microbial oils and it has been found that many microorganisms, such as microalgae, yeasts, bacteria, and fungi, have the ability to accumulate oils under some special cultivation conditions [8]. Researchers exploit oleaginous microorganisms for biodiesel production due to their short life cycle, less labor being required, and their being less affected by location and easier to scale up, compared with other biodiesel sources [9,10]. Therefore, microbial lipids are now of interest as promising potential feedstock for biodiesel production [11].

On the other hand, modern society produces a high quantity of waste materials through activity related to industries, forestry, agriculture and in municipal environments [12]. The enormous costs associated with treating these residues using conventional treatment methods have been a major concern for those who generate wastes and responsible municipal authorities. The high content of fats, oils and other nutrients in these wastes make them interesting and cheap raw materials for industries involved in useful metabolite production [13]. The use of such residual materials serves a dual role, namely that of generating a usable product and that of reducing waste disposal [1,13].

Cassava wastewater (CW) is a yellowish liquid obtained from cassava during the cassava flour manufacturing process, and is rich in many nutrients such as potassium, nitrogen, magnesium, phosphorus, calcium and sulfur [14]. Currently, CW is discharged into rivers or released on soil

without any kind of treatment, thus causing damage to the environment and human health [14,15]. Similarly, large amounts of waste cooking oil are produced from restaurants, catering establishments and food industries every year and discarding them has serious consequences including that of contaminating natural reserves of water [16]. These residues have a high nutrient content and have been used as alternative substrates for several biotechnological processes [14,17,18].

Industrial wastes have been used as substrates to cultivate oleaginous microorganisms so as to produce lipids [1,7,11]. Thus, this study sets out to investigate the potential of *Serratia marcescens* UCP 1549 for biosurfactant and lipid production using cassava wastewater (CW) and corn oil waste (COW) or soybean oil waste (SOW) as substrates.

2. Materials and Methods

2.1 Microorganism

The bacteria *Serratia marcescens* UCP/WFCC 1549 was kindly supplied from the Culture Collection of the Nucleus of Research in Environmental Sciences and Biotechnology, Catholic University of Pernambuco, Recife, State of Pernambuco, Brazil. The strain was registered in the World Federation for Culture Collection (WFCC). The microorganism was maintained in Luria Bertani (LB) solid medium (trytone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L and agar 15 g/L) at 5°C. For pre-culture, the strain from the 24-h culture on LB medium was transferred to 50 mL of LB broth and maintained in an orbital shaker at 150 rpm during 18 h at 28ºC, to obtain the seed culture.

2.2 Materials

All chemicals were of reagent grade. Cassava wastewater (CW) was obtained from an industry food in the municipal district of Carnaíba, state of Pernambuco, Brazil. Corn oil waste (COW) and soybean oil waste (SOW) were kindly supplied from a local restaurant in the city of Recife, state of Pernambuco, Brazil. The residues were stored according to the suppliers' recommendations and used without any further processing.

2.3 Culture conditions

This study tested the following different culture media for lipid production: LB medium, LB medium supplemented with 7.5% SOW and LB medium supplemented with 7.5% COW. Also tested were two alternative media consisting of distilled water supplemented with 6.0% CW and 7.5% SOW and distilled water supplemented with 6.0% CWW and 7.5% COW. The pH of the media was adjusted to ±7.0 and they were sterilized by autoclaving. One percent aliquots (v/v) of the seed culture (0.8 optical density at 600 nm, corresponding to 10^7 cells/mL), was used to

inoculate 250 mL Erlenmeyer flasks containing 100 mL of sterile production medium. Cultivations were carried out in triplicate in an orbital shaker at 150 rpm for 72 h at 28°C.

2.4 Biomass determination

To determine the biomass, the culture samples were centrifuged at 10 000 g for 15 min. The cell pellets were washed three times with distilled water to remove residue from the cultivation medium and were centrifuged again. Then, they were frozen and lyophilized to constant weight. Cell dry weight was determined gravimetrically.

2.5 Extraction and determination of total lipids of biomass

The lipid content of biomass was determined according to Manocha et al. [19]: 1.0 g of the lyophilized biomass was extracted with chloroform: methanol in different proportions (2:1, 1:1, 1:2, v/v). The organic extracts were washed with sodium chloride 0.9%, then evaporated in a vacuum and the lipid content was determined by gravimetric estimation.

2.6 Extraction and transesterification of fatty acids

Cellular lipids were converted to their fatty acid methyl esters (FAMEs) according to Dunlap and Perry [20]. Briefly, ten milligrams of lyophilized biomass were transferred to tubes containing a solution of boron trifluoride-methane (2 mL) at 14% and benzene (2 mL) and incubated at 60ºC overnight. Then, distilled water (2 mL) was added and samples were shaken in vortex for 5 minutes and centrifuged (1700 rpm, 10 minutes at 4ºC). After centrifugation, the benzene was removed and evaporated in a nitrogen atmosphere. The FAMEs were re-suspended in n-hexane and subjected to reading in gas chromatography (GC).

2.7 Gas chromatography (GC)

The FAMEs were analyzed by gas chromatography (GC) using a chromatograph model Agilent Technologies 7890A with automatic injector (Analytical Central - CETENE). It was equipped with a flame ionization detector (FID), fused silica capillary column HP - 5 (5% diphenyl and 95% dimethylpolysiloxane), 30 m x 0.25 mm. The temperature of the column oven was as follows: heating ramp - initial temperature 150°C for 4 min; increasing at a rate of 4°C min^{-1} until 250°C, and remained so for 20 min. The temperature of the injector and detector was 280 °C, and helium ($1cm^3.min^{-1}$) was used as carrier gas.

The microbial FAMEs were identified by comparison of retention times with standard FAMEs (Sigma Aldrich) and were quantified by area normalization using the software supplied with the equipment.

3. Results and Discussion

3.1 Production of biomass

World production of oils and fats is about 2.5-3 million tons, 75% of which is derived from plants and oil seeds [21]. The vegetable industries generate great amounts of wastes and disposing of them is a serious problem [22]. Brazil is also among the main producers of vegetable oils, such as soybean oil, babassu oil and palm oil [23].

Figure 1 shows the yield of biomass produced by *S. marcescens* in the different culture media tested. The strain was able to grow in all media, with best results in Luria Bertani (LB) medium supplemented with 7.5% COW (3.07 g/L) and LB supplemented with 7.5% SOW (2.20 g/L). Probably, the considerably higher carbon and nitrogen contents in these media compared with the others tested, favored this result.

<PLEASE INSERT FIGURE 1>

In this study, the main strategy to achieve excellent biomass yields was to assess the substrates of COW or SOW as product output, focusing on the appropriate use of *S. marcescens*, the nutritional balance and the use of a cheap alternative to lower the costs involved in the process of the transesterification reaction.

S. marcescens showed a good ability to grow in media comprising only wastes (CW and SOW or COW), and its performance in these media was better than in LB medium. These agro-industrial residues are rich in many nutrients which are useful for microbial growth and producing metabolites with high added value, such as biosurfactant and chitin, as suggested in several studies [14,24,25].

3.2 Production of Microbial Lipids

Oleaginous microorganisms are defined as such because the content of microbial lipids in this species is in excess of 20% biomass weight [26]. Microbial oils might become one of the potential feedstocks for biodiesel production in the future. To reduce their cost, exploring other carbon sources instead of glucose is very important especially when using such oils for biodiesel production. It was reported that xylose, glycerol, corn straw, and other agricultural and industrial wastes could be used as the carbon sources for accumulating microbial oils [1,6].

This study assessed the use of CW and vegetable waste frying oils both alone and as LB medium additives for the production of lipids by *S. marcescens.* The percentages of total lipids obtained in each culture media tested are shown in Figure 2. The best results were obtained in media comprising only wastes, with percentages higher than 40%, thus showing that this strain is an oleaginous microorganism. Recently, Barthi et al. [27] reported that *Serratia* sp. produced 0.647 g of lipid/g dry cell weight in a minimal salt medium supplemented with sodium bicarbonate. In the present study, *S. marcescens* was able to produce 0.417 and 0.531 g lipid/g dry biomass, related to

biomass cultured in medium containing only CW+COW and CW+SOW, respectively. The results indicate that these agro-industrial residues are promising renewable substrates for producing lipids which would help to reduce the total costs of the process. Also, the possible conversion of organic compounds present in CW and vegetable oil wastes into lipids is important since this gives wastes an added value.

<PLEASE INSERT FIGURE 2>

3.3 Biodiesel Production from Microbial Oil

Demand for fatty acid methyl esters (FAMEs) as diesel fuel has increased significantly in recent years due to the decrease in global petroleum and the instability of oil prices [5,27]. Microbial lipids can potentially be used as raw material for biodiesel production using the commonest way to produce FAMEs in the biodiesel industry, namely base-catalyzed transesterification [28]. However, this process has disadvantageous and undesirable reactions that can be avoided by using acid catalysts, which also produce FAMEs and increase the biodiesel yield [7,29]. In this study, biodiesel from lipids of *S. marcescens* was produced by acid-catalysed transesterification in the presence of BF_3 and methane. The results obtained are showed in Table 1.

<PLEASE INSERT TABLE 1>

Considerable variations in the fatty acid composition of lipids of *S. marcescens* were observed when the medium composition was modified (Table 1). Several authors state that the substrate composition influences the FAME profile of microbial oils and hence biodiesel properties and quality [1,3,30,31].

Saturated fatty acid (SFA) content was predominant (90.59%), in relation to unsaturated fatty acids (UFAs), in FAMEs from *S. marcescens* cultured in LB medium. In contrast, the profiles of FAMEs from microorganisms cultured in media with wastes presented a higher content of UFAs than of SFAs (Figure 3), as suggested by the standards for biodiesel quality [32-34].

According to a report from the US Department of Energy, an ideal biodiesel should consist of more monounsaturated fatty acids (MUFAs) and less saturated and polyunsaturated fatty acids (PUFAs) [35]. High levels of PUFAs would negatively impact the oxidative stability and increase nitrogen oxide exhaust emissions, which do not suit diesel engines [36-38]. On the contrary, biodiesel derived from SFAs would have good oxidative stability, but poor fuel properties at low temperatures, which is a disadvantage in cold weather conditions [1,5,6]. Consequently, it has been

predicted that high levels of oleic acid (C18:1) and a low content of linoleic, linolenic and other PUFAs are best suited for biodiesel production [24,39].

In the present study, the FAMEs obtained from *S. marcescens* UCP 1549 cultured in media containing wastes, presented a good proportion of MUFAs:PUFAs:SFAs as is shown in Figure 3. However, FAMEs produced in LB medium supplemented with SOW or COW showed high levels of linolenic acid (18:3), very similar to that of the oleic acid content (Table 1). The best result was achieved in medium containing CW which would simulate a condition of limiting nitrogen, as suggest Sousa et al. [40], and SOW, with a high content in MUFAs (48.09%), predominantly oleic acid and a minor content of linolenic and linoleic acids, compared to medium containing CW and COW.

<PLEASE INSERT FIGURE 3>

4. Conclusions

In conclusion this study demonstrated the potential of oleaginous bacterium *S. marcescens* UCP/WFCC 1549 as a sustainable candidate to be used in the production of biodiesel. In principle, biofuels offer a huge advantage over fossil fuels. This strain was shown to be an oleaginous microorganism with the ability to accumulate lipids using agro-industrial wastes. The use of inexpensive raw materials such as agroindustrial wastes is an attractive strategy to reduce the production costs associated with lipids production and, at same time, contribute to the reduction of environmental impact generated by the discard of residues, and the treatment costs. Also, it presented balanced profiles of FAMEs, with the best results in lipids produced in medium containing cassava wastewater and soybean waste frying oil, with the highest percentage of MUFAs (48.09%), mainly oleic acid. These are desirable properties for a good quality biodiesel, according to international standards. The results obtained indicate that cassava wastewater and vegetable waste oils are appropriate alternative substrates for producing microbial lipids.

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FIGURE LEGENDS

Figure 1. Biomass production on different culture media by *Serratia marcescens* UCP/WFCC 1549. (LB), Luria Bertani; (SOW) Soybean oil waste; (COW) Corn oil waste; (CW), cassava wastewater**.**

Figure 2. Total lipid yields of *Serratia marcescens* UCP/WFCC 1549 cultured in different culture media for 72 h at 150 rpm and 28°C. (LB) Luria Bertani; (SOW) Soybean oil waste; (COW) Corn oil waste and (CW) cassava wastewater.

Figure 3. Composition of saturated and unsaturated fatty acid methyl esters produced by *Serratia marcescens* UCP/WFCC 1549 cultured in different culture media. (LB), Luria Bertani; (SOW) Soybean oil waste; (COW) Corn oil waste; (CW), cassava wastewater; (SFAs) Saturated fatty acids; (MUFAs) mono-unsaturated fatty acids; (PUFAs), poly-unsaturated fatty acids.

FIGURES

Figure 1.

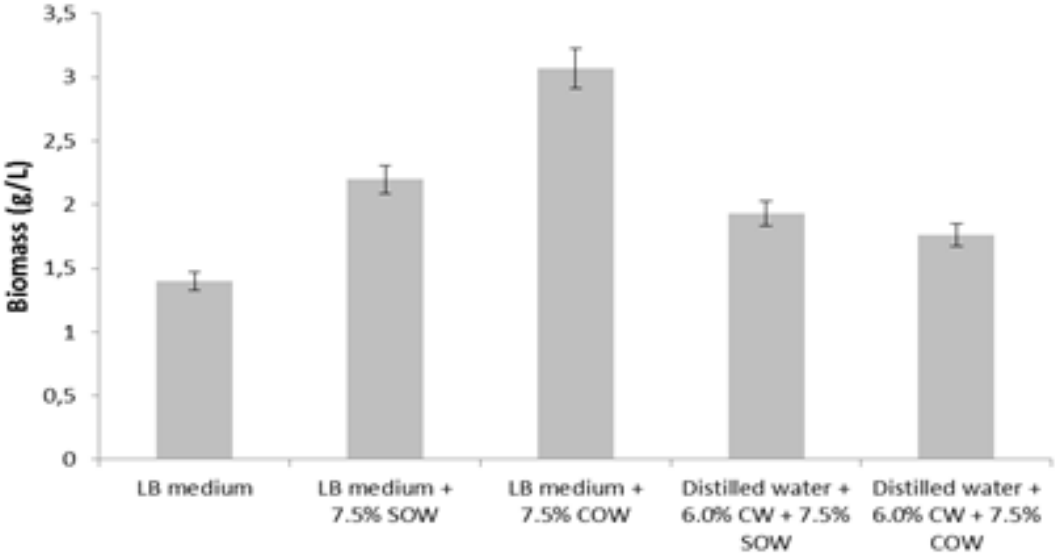


Figure 2.

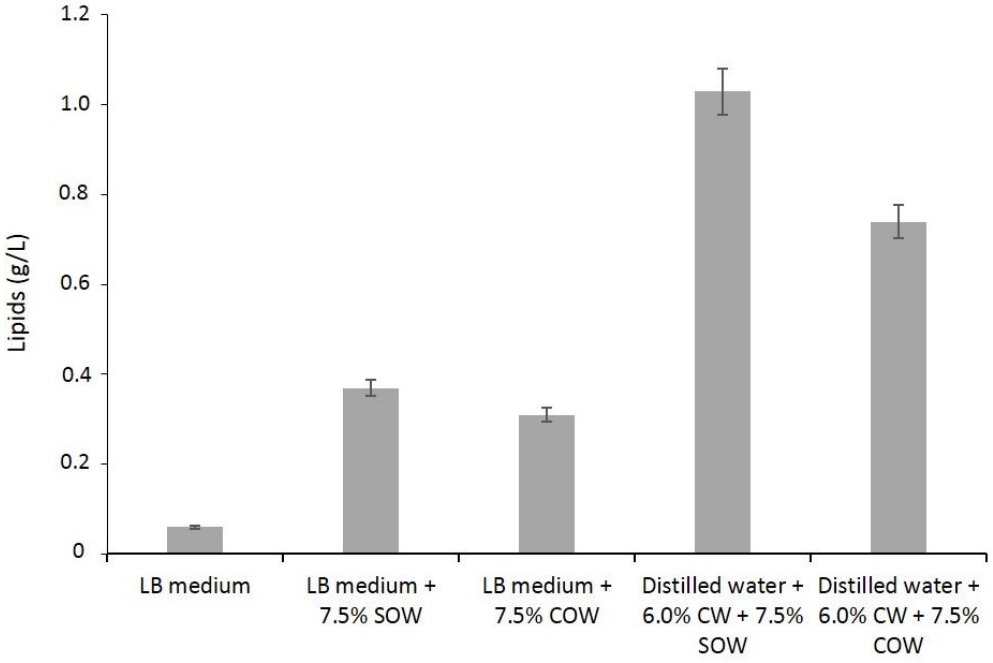
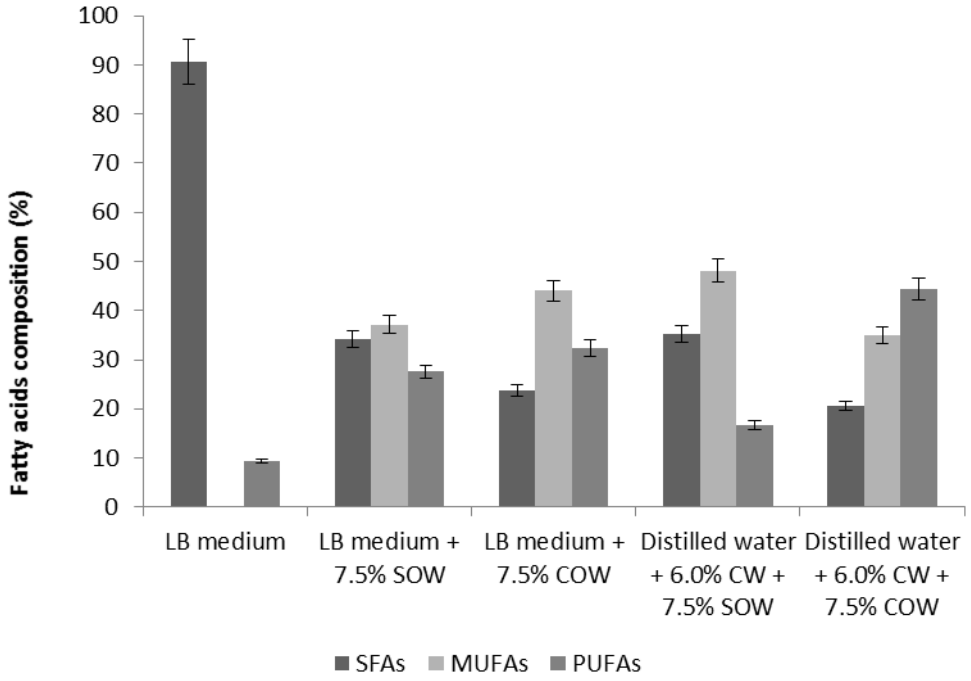


Figure 3.


Fatty acids composition (%)
100
90
80
70
60
50
40
30
20
10
0
LB medium
LB medium + 7.5% SOW
LB medium + 7.5% COW
Distilled water + 6.0% CW + 7.5% SOW
Distilled water + 6.0% CW + 7.5% COW
SFAs
MUFAs
PUFAs

TABLES

Table 1. Percent composition of fatty acids methyl esters obtained after transesterification of lipids produced by *Serratia marcescens* UCP/WFCC 1549 cultured in different culture media.

FAMEs		Production media (% FAMEs)				
Name	Formule	LB	LB + SOW	LB + COW	CW + SOW	CW + COW
Caprylic acid	(C8:0)	-	-	-	1.11	0.21
Capric acid	C10:0)	-	-	-	0.43	0.18
Lauric acid	(C12:0)	-	0.53	-	-	-
Myristic acid	(C14:0)	13.23	3.52	1.78	0.70	0.32
Pentadecanoic acid	(C15:0)	8.56	2.04	-	-	-
Palmitic acid	(C16:0)	68.80	22.05	18.64	22.98	15.66
Margaric acid	(C17:0)	-	0.99	-	-	0.12
Linoleic acid	(C18:2)	-	-	-	14.39	43.36
Oleic acid	(C18:1)	-	37.12	43.99	46.82	34.46
Linolenic acid	(C18:3)	9.38	27.57	32.33	2.30	1.08
Stearic acid	C18:0)	-	5.14	2.69	7.17	3.07
Gadoleic acid	(C20:1)	-	0.51	-	0.75	0.47
Arachidic acid	(C20:0)	-	-	0.56	0.97	0.73
Erucic acid	(C22:1)	-	0.54	-	0.52	-
Behenic acid	(C22:0)	-	-	-	1.87	0.31

CAPÍTULO 4

ARTIGO III

Bioremediation of hydrophobic pollutants using biosurfactant produced by *Serratia marcescens* UCP/WFCC 1549 in low-cost medium

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Article

Bioremediation of hydrophobic pollutants using biosurfactant produced by *Serratia marcescens* UCP/WFCC 1549 in low-cost medium

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Abstract: Environmental pollution by petroleum and petrochemical products has attracted much attention in recent decades. Biosurfactants are microbial compounds capable of reducing surface

and interfacial tension at interfaces between liquids, solids and gases, thereby allowing them to mix or disperse readily emulsions in water or other liquids. Microbial biosurfactants are found to have widely applications in environmental protection, which includes enhancing of oil recovery, oil spills control, biodegradation and detoxification of oil-contaminated industrial effluents and soils. Moreover, they can be obtained with the use of agro-industrial wastes as substrates, which helps reduce overall production costs. This work was aimed for biosurfactant production by *Serratia marcescens* UCP/WFCC 1549 through bioconversion of agro-industrial residues (cassava wastewater and corn waste oil) using a 2^2 factorial design. The best production was obtained in medium containing 6% cassava wastewater and 7.5% corn waste oil, with reduction of water surface tension to 27.8 mN/m. The crude biosurfactant showed ability to emulsify hydrophobic compounds (EI_{24} > 60% of soybean oil, diesel, engine oil and burned engine oil), as well as stability in a wide range of pH, temperature and salinity. Also, the crude biosurfactant exhibited excellent properties to dispersing engine oil in water (78%) and to removing engine burned oil in beach sand and mangrove sediment (88.27% and 73.70%, respectively). These results demonstrated the high potential of biosurfactant produced by *S. marcescens* as sustainable for application in bioremediation processes of hydrophobic pollutants derivated of petroleum.

Keywords: *Serratia marcescens*; biosurfactant; agro-industrial wastes; bioremediation; hydrophobic pollutants.

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1. Introduction

Petroleum is one of the most important energy resources around the world and its use as fuel has contributed to intensive economic development [1,2]. However, petrochemical industry produces considerable amounts of hazardous wastes that contaminate waters and soils as a consequence of leaks and spills from petroleum refinery process, oil transportation and storage tanks [3-5]. Therefore, the environmental contamination by petroleum derivatives has been increasing over the last years, due to its use in several industrial segments [2,6].

Hydrocarbons are described as extremely pollutant, toxic, with carcinogenic and mutagenic potential for humans [4,6]. The concern with these compounds increases due to difficulties in removing them from the environment [7]. The remediation of contaminated sites can be achieved by physicochemical or biological methods [3,8]. Conventional physicochemical methods can rapidly remove the majority of spilled pollutants but cannot remove them completely [2,9]. Thus, with the advance of sustainable technologies, the search for natural method for removal and/or degradation of soil and water contaminated has increased [3].

Biological treatment, or bioremediation, is a desirable alternative due to its cost-effectiveness and safety [8,10,11]. Low solubility and high hydrophobicity of hydrocarbons make them highly

unavailable to microorganisms. Several microorganisms produce biosurfactants in order to degrade hydrocarbons and use them as carbon source [12-16].

Biosurfactants are surface-active compounds produced by microorganisms, mainly aerobic ones, such as bacteria, yeasts and filamentous fungi [17,18]. There are many types of biosurfactants, based on their molecular weight and chemical structures [19,20]. Low-molecular weight biosurfactants (glycolipids, phospholipids and lipopeptides) lower the surface tension at the air/water interfaces and the interfacial tension at the oil/water interfaces. High-molecular weight biosurfactants (proteins, polysaccharides, lipopolysaccharides, lipoproteins, or complex mixtures of these biopolymers), also called bioemulsifiers, are more effective in stabilizing oil in water or water-in-oil emulsions [9,21]. These microbial products have received considerable attention in the field of environmental remediation because they influence such processes due to their efficacy as dispersion and remediation agents [8,22]. Also, their environmentally friendly characteristics, such as low toxicity and high biodegradability make them suitable for application in environments [2,23].

Despite these important advantages exhibited by biosurfactants, they have not yet been employed extensively in the industry due to their relatively high production costs [24,25]. Currently, their prices range between 2 and 3 USD/kg and are 20-30% more expensive than their synthetic equivalents [20,26]. The reduction of production costs of biosurfactants requires enhancement of biosynthesis efficiency and the selection of inexpensive medium components since they constitute 50% of the total production costs [27,28]. One possible strategy for reducing these costs is the utilization of alternative substrates, such as waste or by-products from the agro-industry [29-31].

In addition, modern society produces high quantity of waste materials through activity related to industries, forestry, agriculture and municipalities [32]. It was reported that millions of tons of hazardous and non-hazardous wastes are generated each year throughout the world and therefore there is a global concern for its management and utilization [29]. One of the possibilities explored extensively is the use of organic matter rich but cheap agro-based raw materials or industrial wastes as substrates for microbial production. This approach will help to achieve double benefits of reducing pollution while producing useful products [5,29,30]. A variety of these cheap raw materials have been employed for biosurfactant production including vegetable oils, waste frying oils, distillery and dairy wastes, cassava wastewater and corn steep liquor [5,22,29,33,34].

Thus, the aim of this work was to produce biosurfactants by the bacterium *Serratia marcescens* UCP/WFCC 1549 cultivated in low-cost medium in order to reduce the cost of process and evaluate its application in the removal of hydrophobic pollutants from petroleum industry.

2. Results and Discussion

2.1 Production of biosurfactant by Serratia marcescens

One of main factors governing the success of biosurfactants production is the development of an economical process that uses low-cost materials [28]. Cassava wastewater and vegetable waste oil are agro-industrial wastes which disposal causes environmental problems; however, their high content of nutrients make them attractive substrates for several biotechnological processes [29;34-38]. Then, this paper describes the use of two residues - cassava wastewater (CW) and corn waste oil (CWO)- as low-cost medium components for biosurfactant production by *S. marcescens* UCP/WFCC 1549.

The microorganism was cultivated during 72 h, in different concentrations of CW and CWO, according to a 2^2 full factorial design (FFD). The results shown in Table 1 demonstrated that *S. marcescens* had the ability to reduce the surface tension in presence of these agro-industrial wastes, with the higher reduction in condition 4, corresponding to medium composed by 6% CW and 7.5% CWO. These results confirm those obtained by Montero-Rodríguez et al. [35], with the same residues, ratifying the suitability of both wastes as alternative substrates for biosurfactant production [35,39].

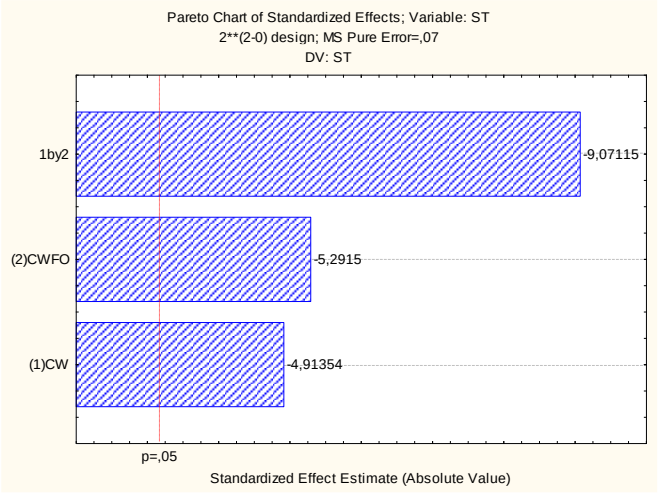
Table 1. Surface tension values obtained in the 2^2 full factorial design used for biosurfactant production by *S. marcescens* UCP/WFCC 1549 at 28 °C and 150 rpm during 72 hours.

Conditions	Cassava wastewater	Corn waste oil	Surface tension (mN/m)
1	-1	-1	30.5
2	+1	-1	31.6
3	-1	+1	31.5
4	+1	+1	27.8
5	0	0	32.8
6	0	0	32.2
7	0	0	32.7
8	0	0	32.5

The estimated effects of CW and CWO on surface tension, as well as the interaction between them, are shown in Pareto chart illustrated in Figure 1. As can be seen, the interaction of both substrates had a significant influence on reducing the surface tension in the culture medium. Also,

both substrates by separate were statistically significant for biosurfactant production by *S. marcescens*. These results are in accordance with the literature that affirms that physiological biosurfactant production is associated with the assimilatory mechanism in response to exposure to hydrophobic substrates, isolated or combined with a soluble substrate [28,40-42].

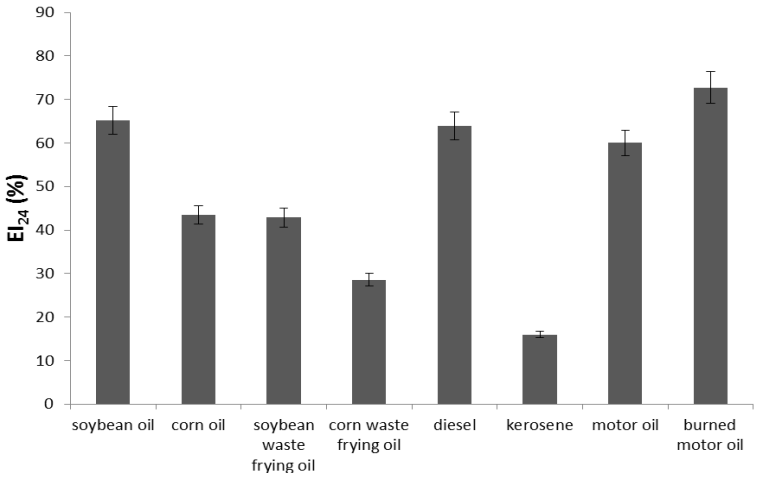
Figure 1. Pareto chart of standardized effects of (1) cassava wastewater and (2) corn waste oil on the surface tension of culture media by *S. marcescens* UCP/WFCC 1549. The point at which the effect estimates were statistically significant (p = 0.05) is indicated by the broken vertical line.



2.2 Emulsifier property

The emulsifying property determines the strength of biosurfactant in retaining the emulsion of hydrocarbons or oils in water [43]. The emulsification index (EI_{24}) was determined to biosurfactant produced by *S. marcescens* in condition 4 of the 2^2 FFD, using various vegetable oils, vegetable waste oils and hydrophobic compounds derivatives from petroleum (Figure 2). The results showed that stable emulsions were formed using soybean oil and derivatives from petroleum (diesel, motor oil and burned motor oil) reflected by good emulsification indices up to 60% after 24 h. The maximum EI_{24} was observed with burned motor oil (75%). Previously, few studies had reported biosurfactant produced by *S. marcescens* strains, with good properties emulsifying kerosene as hydrocarbon [44,45], but this is the first study showing the ability of emulsify efficiently burned motor oil. One of the most important characteristic of hydrocarbon degrading bacteria is the ability to emulsify hydrocarbons in solution by producing surface active agents that cause dispersion of hydrocarbons in water emulsion, microdroplets or micelles which are subsequently transported into the cell [46,47].

Figure 2. Emulsification index of the biosurfactant produced by *S. marcescens* UCP/WFCC 1549 in medium containing 6% cassava wastewater and 7.5% corn waste oil.



2.3 Stability studies

The diverse application of biosurfactants in different fields depends on its stability at different temperatures, pH and salinity [30]. For example, many hydrocarbon-contaminated environments are characterized by extreme environmental conditions such as very low or elevated temperatures, highly acidic or alkaline pH, high saline concentrations and or high pressures [48].

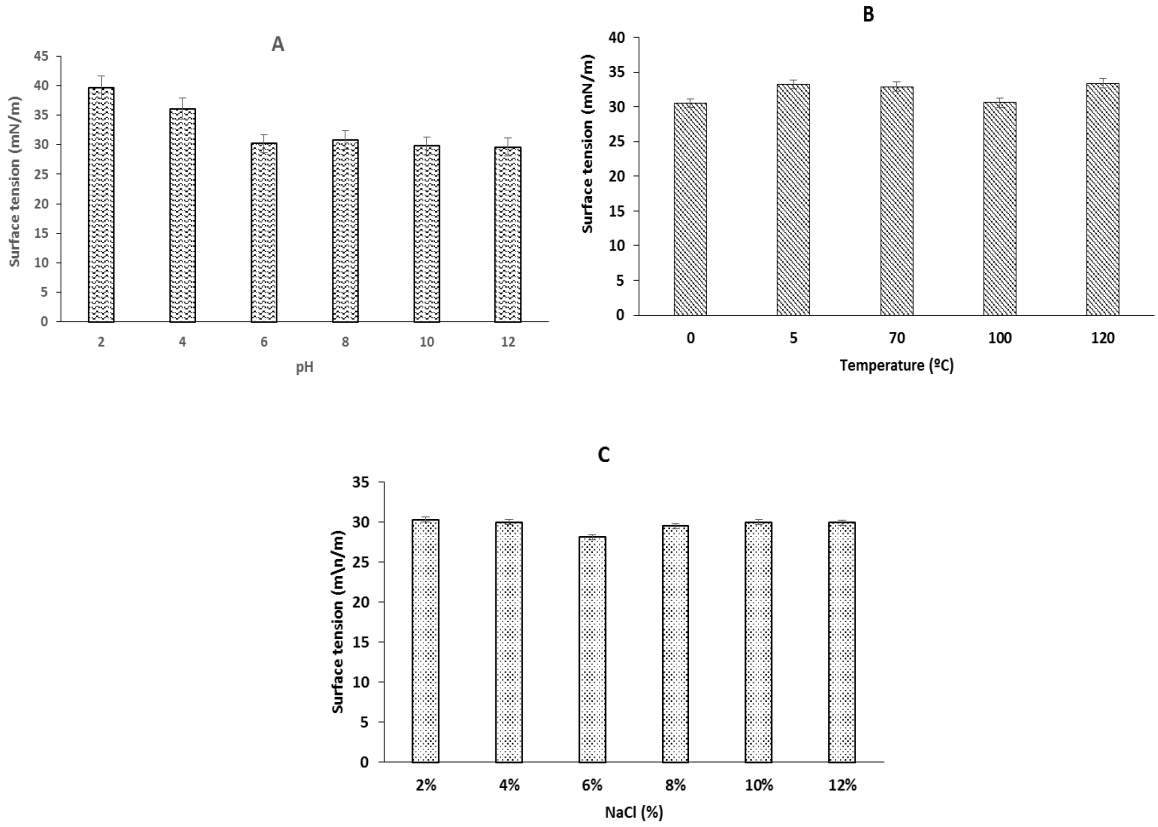
Figure 3 shows the effects of temperature, pH and salinity concentration in stability of surface tension of the biosurfactant produced by *S. marcescens.* The results showed that crude biosurfactant was stable to the neutral to basic pH range, with an increase in surface tension in acid pH (Figure 3A). In case of temperature, the surface tensions remained practically uniform at different values (0-120ºC) (Figure 3B), indicating that variation in temperature had no appreciable effect. Similarly, the addition up to 12% (w/v) sodium chloride to the cell-free liquid showed no noticeable effect in the surface tension. Biosurfactant produced by other *S. marcescens* strains have indicated stability in a wide range of pH, temperature and salinity as was demonstrated in present study, make them potential candidates for microbial enhanced oil recovery or bioremediation of environment in extreme conditions [45,49].

2.4 Oil displacement test

The oil displacement test is an indirect measurement of surface activity of surfactant sample tested against oil; a larger diameter indicates a higher surface activity of the testing solution [45]. Figure 4 illustrates the dispersant activities of Triton X-100 and crude biosurfactant produced by *S. marcescens*. The dispersion rate obtained with the use of synthetic surfactant was 89%, whereas the

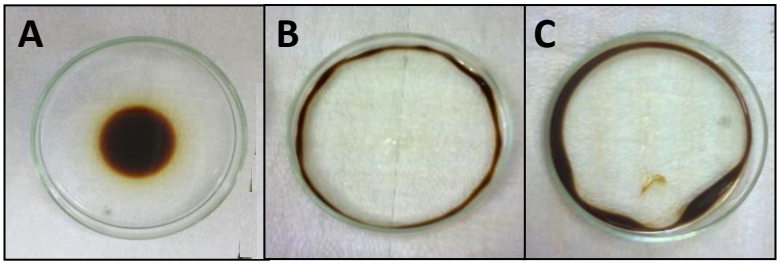
cell-free metabolic liquid (crude biosurfactant) achieved a dispersion rate of 78%, showing its potential in the dispersion of oil spills.

Figure 3. Stability of surface tension of biosurfactant produced by *S.marcescens* using cassava wastewater and corn waste oil. Influence of pH (**A**), temperature (**B**), and sodium chloride concentrations (**C**) on surface tension stability.



Sitohy et al. [50] reported an oil displacement rate of 57% using a biosurfactant produced by *Bacillus subtilis* whereas Rocha e Silva et al. [22] achieved an 80% oil displacement rate using crude biosurfactant of *Pseudomonas cepacia*. The present study shows excellent property of biosurfactant produced by *S. marcescens* UCP/WFCC 1549 which was better than those showed by others *S. marcescens* strains previously studied [44,45].

Figure 4. Oil displacement activity of crude biosurfactant produced by *S. marcescens* UCP/WFCC 1549. Motor oil droplet without application of surfactant (A), after dispersion with synthetic surfactant (B) and after dispersion with crude biosurfactant produced by *S. marcescens*.



2.5 Bioremediation test of burned motor oil from contaminated sand and mangrove sediment

The release of contaminants, such as petroleum and petroleum byproducts, into the environment is one of the main causes of global pollution and has become a focus of great concern both in developing countries due to the broad environmental distribution [2,5,8]. The marine environment has suffered with constant oil spills, making oil one of the most abundant organic contaminants in the sea [4]. Half of world's oil production (around three billion tones/year) is transported by ships through the oceans, increasing hydrocarbon contamination levels in various marine ecosystems, such as beaches and mangroves due to possible accidents [2,27,51-53].

Although conventional physicochemical methods can rapidly remove the majority of spilled oil, in most cases, these methods cannot clean up completely crude oil or the removal simply transfers contaminants from one environmental medium to another. Thus, more attention is being given to biological alternatives [54,55]. Biosurfactants play an important role in remediation processes due to their efficacy as dispersion and remediation agents as well as their environmentally friendly characteristics, such as low toxicity and high biodegradability [5,27].

Figure 5 and Table 2 show the results obtained for the removal of burned motor oil adsorbed in samples of beach sand and mangrove sediments by the crude biosurfactant from *S. marcescens*. In comparison with distilled water (control), promising results were obtained by crude biosurfactant from *S. marcescens* with removal of 88.27% and 73.70%, respectively.

Results described in the literature show that the biosurfactants produced by strains of *Pseudomonas aeruginosa* removed 49-54% of crude oil adsorbed in sand [56], whereas Silva et al. [57] demonstrated high removal rates (above 85%) of diesel oil from sand samples, but lower (less than 20%) when petroleum was tested. Nalini and Parthasarathi [58] showed the recovery of 92% of used engine oil adsorbed in sand by biosurfactant produced by *Serratia rubidae*. The results obtained in present study demonstrated considerable potential of crude biosurfactant of *S. marcescens* UCP/WFCC 1549 for use in bioremediation processes of oil-polluted environments.

Figure 5. Removal capacity of burned motor oil adsorbed in beach sand and mangrove sediment using crude biosurfactant produced by *S. marcescens* UCP/WFCC 1549. (A) Sand adsorbed with burned motor oil without treatment (B) Sand with remaining motor oil after treatment with distilled water (control) (C) Sand with remaining motor oil after treatment with the crude biosurfactant (D) Mangrove sediment adsorbed with burned motor oil without treatment (E) Mangrove sediment with remaining motor oil after treatment with distilled water (control) (F) Mangrove sediment with remaining motor oil after treatment with the crude biosurfactant.

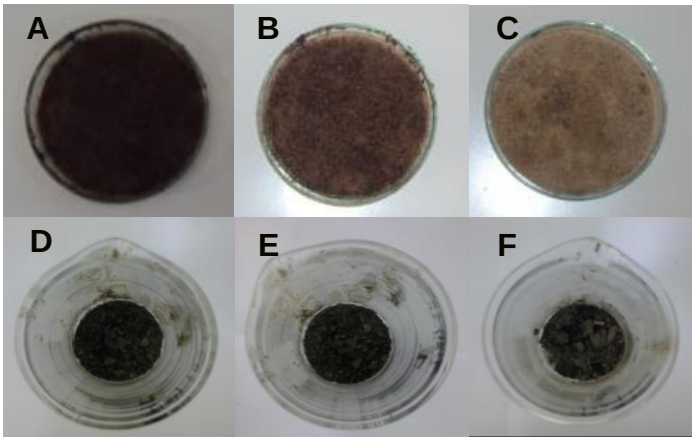


Table 2. Removal of burned motor oil adsorbed in beach sand and mangrove sediment by the cell-free culture medium containing the crude biosurfactant produced by *S. marcescens* UCP/WFCC 1549 and by distilled water (control).

Treatments	Removal (%)	
	Beach sand	Mangrove sediment
Distilled water (control)	51.80	65.47
Cell-free culture medium (crude biosurfactant)	88.27	73.70

3. Experimental Section

3.1 Microorganism

The bacterium *Serratia marcescens* UCP/WFCC 1549 was kindly supplied from the Culture Collection of the Nucleus of Research in Environmental Sciences and Biotechnology, Catholic University of Pernambuco, Recife, state of Pernambuco, Brazil. The microorganism was maintained in Luria Bertani (LB) solid medium (trytone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L and agar 15 g/L) at 5° C. For pre-culture, the strain from 24-h culture on LB medium was transferred to 50 mL of LB broth and maintained under orbital shaker at 150 rpm during 18 h at 28ºC, to obtain the seed culture.

3.2 Agro-industrial wastes

The production medium was composed by cassava wastewater (CW) obtained from an industry food at municipal district of Carnaíba, state of Pernambuco, Brazil and corn waste oil (CWO), kindly supplied from a local restaurants in the city of Recife, state of Pernambuco, Brazil. The

substrates were stored according to the suppliers' recommendations and used without any further processing.

3.3 Culture conditions and biosurfactant production

The experiments were carried out in 250 mL Erlenmeyer flasks containing 100 mL of production medium, with varying concentrations of CW and CWO, in agreement with the experimental factorial design. After to adjust pH of the media to 7.0 and sterilization by autoclaving, each flask was inoculated with 1% (v/v) of the seed culture (0.8 optical density at 600 nm, corresponding to 10^7 cells/mL). Cultivations were carried out on a rotatory shaker at 150 rpm, for 72 h at 28 °C.

A 2^2 full factorial design (FFD) was carried out to analyze the effects and interactions of CW and CWO on the surface tension as response variable, using a set of eight experiments, with four replicates at the central points (Table 1). The effects and significance of the variables were graphically illustrated using a Pareto chart. The data obtained from the experiments were subjected to statistical analysis by STATISTICA software version 7.0 (StatSoft Inc., USA) and the significance of the results was tested at $p < 0.05$ level.

Table 3. Design matrix for the factorial experiments used to evaluate the influence of two factors (cassava wastewater (CW) and corn waste oil (CWO)) on biosurfactant production by *S. marcescens* UCP/WFCC 1549, with experimental conditions set at the mean of two extreme levels (−1 and +1) and a central point (0).

Independent Variables	**Factor levels**		
	-1	**0**	**+1**
Cassava wastewater (CW) % (v/v)	3.0	4.5	6.0
Corn waste oil (CWO) % (v/v)	6.5	7.0	7.5

3.4 Determination of surface tension

The surface tension was determined on metabolic cell-free liquid obtained by centrifugation (10,000× g for 15 min) and subsequent filtration of cultures, using a tensiometer model Sigma 70 (KSV Instruments Ltd., Finland) by the Du Nouy ring method at room temperature (±28 °C). Measurements of surface tension from distilled water and from the conventional medium were used as control [59].

3.5 Emulsification index

The emulsification index was analysed according to Cooper and Goldenberg [43] for the better condition of the FFD, determined by measuring of surface tension. The test was done using 2 mL of

the metabolic cell-free liquid and 2 mL of hydrophobic compounds (soybean oil, corn oil, soybean waste oil, corn waste oil, diesel, kerosene, motor oil and burned motor oil) in a graduated screw cap test tube and vortexed at high speed for 2 min. After 24 h, the emulsification index (EI_{24}) was calculated by dividing the measured height of the emulsion layer by the mixture's total height and multiplying by 100 to expressing in percentage.

3.6 Stability of the biosurfactant

Stability studies were undertaken using the cell-free broth obtained after centrifugation of the cultures at 10,000× g for 15 min. Twenty millilitres of this broth were maintained at a constant temperature (0, 5, 70, 100 and 120 °C) for 1 h, and cooled to room temperature, after which the surface tension was measured. The effect of pH on surface tension was evaluated after adjustment of the broth pH to 2, 4, 6, 8, 10, 12 and 14 with 6.0 M NaOH or HCl. The effect of NaCl concentrations (2–12%, w/v) on the activity of the biosurfactant was also determined. The tests were performed in triplicate [23,25,34].

3.7 Oil displacement test

The oil displacement test was carried out by slowly dropping 1 mL of burned motor oil onto the surface of 30 mL of distillated water in a Petri dish (9 cm in diameter). This was followed by the addition of 500 μL of the cell-free metabolic liquid (crude biosurfactant) on the center of the oil layer. The mean diameter of the clear zones of triplicates experiments was measured and calculated as the rate of the Petri dish diameter [34,60]. The chemical surfactant Triton X-100 was used as control.

3.8 Bioremediation test of burned motor oil from contaminated sand and mangrove sediment

Testing the suitability of biosurfactant for bioremediation process was conducted by using 60 g of beach sand and mangrove sediment impregnated with 5 mL of burned motor oil. Biosurfactant produced by *S. marcescens* cultivated in the better condition of factorial design, was used in the removal tests. Fractions of 20 g of the contaminated sand or mangrove sediment were transferred to 250 mL Erlenmeyer flasks, which were submitted to the following treatments: addition of 40 mL of the cell-free metabolic liquid and addition of 40 mL distilled water (control). The tests were carried out in triplicate and the samples were incubated on a rotary shaker (150 rpm) for 48 h at 28 °C. Then, they were centrifuged at 5000 g for 10 min for separation of the wash solution and the sand or mangrove sediment. The amount of oil residing after the impact of biosurfactant was gravimetrically determined using hexane [27].

4. Conclusions

The extensive production and use of petroleum and petrochemical derivatives has resulted in widespread environmental contamination by these chemicals. Hydrocarbons, as the hydrophobic organic chemicals, exhibit limited solubility in groundwater and tend to partition to the soil matrix. A promising method that can improve bioremediation of hydrocarbon contaminated environments is the use of biosurfactants. In the present study, *S. marcescens* UCP/WFCC 1549 demonstrated their ability to produce biosurfactant using a low-cost fermentative medium based on cassava wastewater and corn waste oil as substrates. The crude biosurfactant was stable in a wide range of pH, temperature and salinity. Also, it exhibited excellent properties to emulsify hydrophobic compounds, dispersing and remove burned motor oil in water, beach sand and mangrove sediment. These results suggesting their feasibility to be applicated in bioremediation processes in extreme conditions.

Acknowledgements

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4. CONCLUSÕES GERAIS

- *Serratia marcescens* UCP/WFCC 1549 demonstra potencial de biodegradação de combustíveis derivados do petróleo e biocombustíveis, apresentando-se como promissora para os processos de descontaminação ambiental de poluentes hidrofóbicos.

- *S. marcescens* demonstra habilidade para utilizar resíduos agro-industriais (manipueira e óleos vegetais pós-fritura) como substratos para a produção de lipídeos e biossurfactante.

- Os lipídeos produzidos mostram perfis de ácidos graxos similares ao biodiesel, demonstrando ser um micro-organismo promissor na produção de biodiesel de boa qualidade.

- A melhor condição para a produção de biossurfactante é no meio contendo 6% de manipueira e 7,5% de óleo de milho pós-fritura.

- O biossurfactante produzido apresenta excelentes propriedades de redução da tensão superficial e na emulsificação de compostos hidrofóbicos.

- O biossurfactante produzido demonstra estabilidade na redução da tensão superficial frente a diferentes valores de pH, temperatura e NaCl.

- O biossurfactante produzido apresenta excelentes propriedades de dispersão e remoção de óleo motor em água, areia de praia e sedimento de mangue, demonstrando potencial de aplicação na biorremediação de ecossistemas contaminados por derivados do petróleo.

5. ANEXOS

TRABALHOS PUBLICADOS DURANTE O MESTRADO

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3. Doroshenko V, Airich L, Vitushkina M, Kolokolova A, Livshits V, et al. (2007) YddG from Escherichia coli promotes export of aromatic amino acids. FEMS Microbiol Lett 275: 312-318.

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Editorial Procedure and Peer-Review

Initial Checks

All submitted manuscripts received by the Editorial Office will be checked by a professional in-house *Managing Editor* to determine whether it is properly prepared and whether the manuscript follows the ethical policies of the journal, including those for human and animal experimentation. Manuscripts that do not fit the journal or are not in line with our ethical policy may be rejected before peer-review. Manuscripts that are not properly prepared will be returned to the authors for revision and resubmission. The *Managing Editor* will consult the journals' *Editor-in-Chief*, the *Guest Editor* or an *Editorial Board member* to determine whether the manuscript fits the scope of the journal and whether it is scientifically sound. No judgment on the significance or potential impact of the work will be made at this stage.

Peer-Review

Once a manuscript passes the initial checks, it will be assigned to at least two independent experts for peer-review. A single-blind peer-review process is applied, where authors' names are revealed to reviewers. In-house assistant editors generally invite experts recommended by the *Editor-in-Chief* or identified by literature searches. These experts may also include Editorial Board members and Guest Editors of the journal. Potential reviewers suggested by the authors may also be considered. Reviewers should not have published with any of the co-authors during the past five years and should not currently work or collaborate with one of the institutes of the co-authors of the submitted manuscript.

Editorial Decision and Revision

Based on the comments and advices of the peer-reviewers, an external editor – usually the *Editor-in-Chief* or a *Guest Editor* – will make a decision to accept, reject, or to ask authors to revise the manuscript.

For *Minor Revisions* the authors will have one week to resubmit their revised manuscript. For *Major Revisions* the authors will have two weeks to resubmit their revised manuscript. However, authors should contact the editorial office if extended revision time is anticipated.

Author Appeals

Authors may appeal a rejection by sending an e-mail to the Editorial Office of the journal. The appeal must provide a detailed justification, including point-by-point responses to the reviewers' and/or Editor's comments. The *Managing Editor* of the journal will forward the manuscript and relating information (including the identities of the referees) to an Editorial Board member who was not involved in the initial decision-making process. If no appropriate Editorial Board member is available, the editor will identify a suitable external scientist. The Editorial Board member will be asked to give an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. A reject decision at this stage will be final and cannot be revoked.

Production and Publication

Once accepted, the manuscript will undergo professional copy-editing, English editing, proofreading by the authors, final corrections, pagination, and, publication on the www.mdpi.com website.

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Suggestion of Reviewers

During the submission process, authors have the possibility to suggest potential reviewers with the appropriate expertise to review the manuscript. The editors will not necessarily approach these referees. Please provide detailed contact information (address, homepage, phone, e-mail address). The proposed referees should neither be current collaborators of the co-authors nor have published with any of the co-authors of the manuscript within the last five years. Proposed reviewers should be from different institutions to the authors. You may identify appropriate Editorial Board members of the journal as potential reviewers. You may also suggest reviewers from among the authors that you frequently cite in your paper.

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English Corrections

This journal is published in English. To facilitate proper peer-reviewing of your manuscript, it is essential that it is submitted in grammatically correct English. If you are not a native English speaker, we strongly recommend that you have your manuscript professionally edited before submission or read by a native English-speaking colleague. Professional editing will mean that reviewers and future readers are better able to read and assess the content of your manuscript. For additional information see the English Editing Guidelines for Authors.

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Publication Ethics Statement

IJMS is a member of the Committee on Publication Ethics (COPE). We fully adhere to its Code of Conduct and to its Best Practice Guidelines.

The editors of this journal take the responsibility to enforce a rigorous peer-review process together with strict ethical policies and standards to ensure to add high quality scientific works to the field of scholarly publication. Unfortunately, cases of plagiarism, data falsification, image manipulation, inappropriate authorship credit, and the like, do arise. The editors of *IJMS* take such publishing ethics issues very seriously and are trained to proceed in such cases with a zero tolerance policy.

Authors wishing to publish their papers in *IJMS* are asked to abide to the following rules:

- Any facts that might be perceived as a possible conflict of interest of the author(s) must be disclosed in the paper prior to submission.
- Authors should accurately present their research findings and include an objective discussion of the significance of their findings.
- Data and methods used in the research need to be presented in sufficient detail in the paper, so that other researchers can replicate the work.
- Raw data should preferably be publicly deposited by the authors before submission of their manuscript. Authors need to at least have the raw data readily available for presentation to the referees and the editors of the journal, if requested. Authors need to ensure appropriate measures are taken so that raw data is retained in full for a reasonable time after publication.
- Simultaneous submission of manuscripts to more than one journal is not tolerated.
- Republishing content that is not novel is not tolerated (for example, an English translation of a paper that is already published in another language will not be accepted).
- If errors and inaccuracies are found by the authors after publication of their paper, they need to be promptly communicated to the

editors of this journal so that appropriate actions can be taken. Please refer to our policy regarding publication of publishing addenda and corrections.

- Your manuscript should not contain any information that has already been published. If you include already published figures or images, please obtain the necessary permission from the copyright holder to publish under the CC-BY license.
- Plagiarism, data fabrication and image manipulation are not tolerated.
  - **Plagiarism is not acceptable** in *IJMS* submissions.

    Plagiarism includes copying text, ideas, images, or data from another source, even from your own publications, without giving any credit to the original source.

    Reuse of text that is copied from another source must be between quotes and the original source must be cited. If a study's design or the manuscript's structure or language has been inspired by previous works, these works must be explicitly cited.

    If plagiarism is detected during the peer review process, the manuscript may be rejected. If plagiarism is detected after publication, we may publish a correction or retract the paper.

  - **Image files must not be manipulated or adjusted in any way** that could lead to misinterpretation of the information provided by the original image.

    Irregular manipulation includes: 1) introduction, enhancement, moving, or removing features from the original image; 2) grouping of images that should obviously be presented separately (e.g., from different parts of the same gel, or from different gels); or 3) modifying the contrast, brightness or color balance to obscure, eliminate or enhance some information.

    If irregular image manipulation is identified and confirmed during the peer review process, we may reject the manuscript. If irregular image manipulation is identified and confirmed after publication, we may correct or retract the paper.

Our in-house editors will investigate any allegations of publication misconduct and may contact the authors' institutions or funders if necessary. If evidence of misconduct is found, appropriate action will be taken to correct or retract the publication. Authors are expected to comply with the best ethical publication practices when publishing with MDPI.

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Supplementary Materials and Data Deposit

In order to maintain the integrity, transparency and reproducibility of research records, and to retain important chemical and structural information, authors are strongly encouraged to make their experimental and research data openly available either by depositing into data repositories or by publishing the data and files as supplementary information in this journal. Additional data and files can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be available to the referees as part of the peer-review process, although referees are not specifically asked to review these files. Accepted file formats include (but are not limited to):

- spectral data (NMR, IR, Raman, ESR, etc.) in JCAMP (JDX) format
- 3D coordinate structures (in PDB, MOL, XYZ or other common format)
- crystallographic information (in CIF format)
- data tables and spreadsheets (text files, MS Excel, OpenOffice, CSV, XML, *etc.*)
- text documents (text files, PDF, MS Word, OpenOffice, *etc.*; text documents will usually be converted to PDF files for publication)
- images (JPEG, PNG, GIF, TIFF, BMP, *etc.*)
- videos (AVI, MPG, QuickTime, *etc.*)
- executables (EXE, Java, *etc.*)
- software source code

Citations and References in Supplementary files are permitted provided that they also appear in the main text and in the reference list.

Large data sets and files should be deposited to specialized service providers (such as Figshare) or institutional/subject repositories, preferably those that use the DataCite mechanism. For a list of specialized repositories for the deposit of scientific and experimental data, please consult databib.org or re3data.org. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal *Data* (ISSN 2306-5729) also accepts submissions of data set papers, and the publication of small data sets along with the paper, and/or software source codes is encouraged.

Guidelines for Deposition of Sequences and of Expression Data

New sequence information must be deposited to the appropriate database prior to submission of the manuscript. Accession numbers provided by the database should be included in the submitted manuscript. Manuscripts will not be published until the accession number is provided.

- **New nucleic acid sequences** must be deposited in one of the following databases: GenBank, EMBL, or DDBJ. Sequences should be submitted to only one database.
- **New high throughput sequencing (HTS) datasets**(RNA-seq, ChIP-Seq, degradome analysis, …) must be deposited either in the GEO database or in the NCBI's Sequence Read Archive.
- **New microarray data** must be deposited either in GEO or ArrayExpress databases. The "Minimal Information About a Microarray Experiment" (MIAME) guidelines published by the Microarray Gene Expression Data Society must be followed.
- **New protein sequences** obtained by protein sequencing must be submitted to UniProt (submission tool SPIN).
  All sequence names and the accession numbers provided by the databases should be provided in the Materials and Methods section of the article.

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Ethical Guidelines for the Use of Animals in Research

The editors will require that the benefits potentially derived from any research causing harm to animals are significant in relation to any cost endured by animals, and that procedures followed are unlikely to cause offense to the majority of readers. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs':

- Replacement of animals by alternatives wherever possible,
- Reduction in number of animals used, and
- Refinement of experimental conditions and procedures to minimize the harm to animals.

Any experimental work must also have been conducted in accordance with relevant national legislation on the use of animals for research. For further guidance authors should refer to the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures [1].

Manuscripts containing original descriptions of research conducted in experimental animals must contain details of approval by a properly constituted research ethics committee. As a minimum, the project identification code, date of approval and name of the ethics committee or institutional review board should be cited in the Methods section. During the electronic submission process, authors will be asked to inform

the Editor-in-Chief of any potential conflicts of interest.

1. Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care of Animals Used in Scientific Procedures. Available online: http://www.official-documents.gov.uk/document/hc8889/hc01/0107/0107.pdf.

International Journal of Molecular Sciences endorses the ARRIVE guidelines (www.nc3rs.org.uk/ARRIVE) for reporting experiments using live animals. Authors and reviewers can use the ARRIVE guidelines as a checklist, which can be found at www.nc3rs.org.uk/ARRIVEchecklist.

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