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DAYLIN RUBIO RIBEAUX

PRODUÇÃO, OTIMIZAÇÃO, CARACTERIZAÇÃO E ATIVIDADE ANTIFÚNGICA DO BIOSSURFACTANTE PRODUZIDO POR *Candida tropicalis* UCP 1613

Recife

2020

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como parte dos requisitos para a obtenção do título de Doutora em Ciências Biológicas

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Orientadora: Profª. Dra. Galba Maria de Campos Takaki

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RESUMO

Candida tropicalis UCP 1613 é uma levedura isolada do Rio Formoso no Estado de Pernambuco, Brasil, e que foi investigada quanto à sua capacidade para produzir biossurfactante a partir de resíduos ago-industriais. Dessa forma foi avaliado um planejamento fatorial 2^4 utilizando como substratos: glicose, glicerol residual, glutamato, óleo pósfritura e uma solução de sais. A análise estatítica permitiu selecionar o glutamato e glicerol como variáveis para otimizar o meio de produção através de um Desenho Composto Central Rotacional. A excelente capacidade da levedura para produzir biossurfactante foi comprovada a partir redução da tensão superficial até 25,3 mN/m na condição 4 (8,31 % de glicerol residual e 3,35 g/L de glutamato). O rendimento do biosurfactante extraido foi de 3,6 g/L. A caraterização preliminar baseada na sua composição bioquímica e espectrometria FT-IR permitiu identificar a molécula como glicolipídio. A concentração micelar crítica da molécula foi de 350 mg/mL e a atividade da água de 0,41aw. O potencial antifúngico *in vitro* do biossurfactante contra *Alternaria alternata* foi comprovado a uma concentração de 750 μg/mL e corroborado através da microscopia de luz e eletrônica de varredura. No entanto, a inibição do crescimento *in vivo* foi avaliada em tomate cereja (*Solanum lycopersicum*) mostrando excelente resultado na inibição. Além disso, a levedura produz biossurfactante através da fermentação em estado sólido a partir de uma solução umedecedora e 6 resíduos: glicerol residual, óleo pós fritura, resíduo de miojo, casca de abacaxi, bagaço e borra de café. Os resultados obtidos mostraram que melhor associação foi a do resíduo de miojo e glicerol devido à redução da tensão superficial da água de 72 para 33,2 mN/m. O rendimento máximo do biossurfactante foi de 19.5 g/100 g e foi caracterizado como glicolipídeo. O efeito não fitotóxico e estimulante da germinação do biossurfactante foi detectado em sementes de cebola (*Alliun cepa*) com um índice de germinação máximo de 120 %. Alem disso, foi testada a capacidade da levedura para sintetizar biossurfactante utilizando resíduo de miojo e glicerol através de um planejamento fatorial 2^4, tendo como variáveis: tamanho do inóculo, temperatura, tamanho das partículas e volume do glicerol residual. Os resultados mostraram o valor mínimo da tensão superficial na condição 8 (10^{-6} células/mL; 31°C; mesh de 32 e 0 mL do glicerol residual) com 25,8 mN/m. O biossurfactante foi estável em diferentes faixas de temperatura, salinidade e pH. A levedura *C. tropicalis* UCP 1613 mostrou ser um micro-organismo versátil devido à sua habilidade para utilizar diversos substratos e gerar uma biomolécula de alto valor agregado.

Palavras chave: Resíduos agro-industriais. Biossurfactante. Glicerol. Atividade antifúngica. Germinação de sementes

ABSTRACT

Candida tropicalis UCP is a yeast isolated from the Rio Formoso in the State of Pernambuco, Brazil, and has been investigated for its ability to produce biosurfactant from agro-industrial waste. Thus, a factorial design 2^4 was evaluated using as substrates: glucose, residual glycerol, glutamate, post-frying oil and a salt solution. The statistical analysis allowed to select glutamate and glycerol as variables to optimize the production medium through a Central Rotational Composite Design. The excellent ability of the yeast to produce biosurfactant was proven from a reduction in surface tension to 25.3 mN/m in condition 4 (8.31% residual glycerol and 3.35 g/L glutamate). The yield of the extracted biosurfactant was 3.6 g/L. The preliminary characterization based on its biochemical composition and FT-IR spectrometry allowed to identify the molecule as glycolipid. The critical micellar concentration of the molecule was 350 mg/mL and the water activity was 0.41aw. The *in vitro* antifungal potential of the biosurfactant against Alternaria alternata was proven at a concentration of 750 μg/mL and corroborated through light and scanning electron microscopy. However, growth inhibition *in vivo* was evaluated in cherry tomatoes (*Solanum lycopersicum*) showing excellent results in inhibition. In addition, yeast produces biosurfactant through solid state fermentation from a moistening solution and 6 residues: residual glycerol, post-frying oil, noodle residue, pineapple peel, bagasse and coffee grounds. The results obtained showed that the best association was that of the noodle residue and glycerol due to the reduction in the surface tension of the water from 72 to 33.2 mN/m. The maximum yield of the biosurfactant was 19.5 g/100 g and was characterized as glycolipid. The non-phytotoxic and stimulating effect of the germination of the biosurfactant was detected in onion seeds (*Allium cepa*) with a maximum germination index of 120%. In addition, the ability of the yeast to synthesize biosurfactant was tested using noodle and glycerol residue through a factorial design 2^4, having as variables: inoculum size, temperature, particle size and residual glycerol volume. The results showed the minimum value of surface tension in condition 8 (10-6 cells / mL; 31 ° C; 32 and 0 mL mesh of residual glycerol) with 25.8 mN/m. The biosurfactant was stable in different temperature, salinity and pH ranges. The yeast *C. tropicalis* UCP 1613 proved to be a versatile microorganism due to its ability to use different substrates and generate a biomolecule with high added value.

Keywords: Agro-industrial residues. Biosurfactant. Glycerol. Antifungal activity. Seed germination

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1 INTRODUÇÃO

Os biossurfactantes vêm despertando grande interesse, devido a sua crescente aplicação na área ambiental e industrial, considerando ainda, a sua produção de forma sustentável a partir de fontes renováveis, o que permite valor agregado no processo fundamentado na economia circular (BANAT et al., 2014a; PERFUMO et al., 2018; JAHAN et al., 2020). O fato dos biossurfactantes serem moléculas de origem biológica e sua capacidade de cumprir a maioria dos requisitos de seus similares sintéticos (derivados do petróleo) justifica-se com crescente número de investigações (VAN RENTERGHEM et al., 2018; MARCELINO et al., 2020). Assim, a produção dessas biomoléculas é considerada uma tecnologia fundamental para a implementação de processos industriais sustentáveis que reduzem significativamente a dependência de recursos fósseis (SANTOS et al., 2016; SATPUTE; PŁAZA; BANPURKAR, 2017).

Biodegradabilidade, baixa toxicidade ambiental, elevada diversidade estrutural e atividade antimicrobiana, entre outras propriedades, torna os biossurfactantes viáveis nas áreas de produtos de limpeza, biorremediação ambiental, recuperação melhorada do petróleo (MEOR) e agentes antimicrobianos (GEETHA; BANAT; JOSHI, 2018; Liu et al., 2020). Porém, apesar do reconhecimento das suas vantagens, a produção em larga escala para a maioria destes compostos, ainda não compete economicamente com a produção de surfactantes químicos. Isto se deve ao elevado custo dos substratos utilizados para sua síntese, a baixa produtividade de linhagens microbianas e os métodos ineficientes de bioprocessamento (LIMA et al., 2020).

Considerando as limitações de uma população mundial crescente e das mudanças climáticas, há um interesse crescente em desenvolver práticas agrícolas para reduzir o uso de agroquímicos e simultaneamente garantir sustentabilidade agrícola, relação custo-benefício e segurança alimentar. Ao longo dos anos, os pesticidas químicos foram utilizados para enfrentar as pragas e doenças de plantas. No entanto, o uso extensivo de pesticidas químicos levou ao desenvolvimento de resistência a inseticidas, além de toxicidade para a saúde humana e danos ambientais. Por outro lado, para alcançar uma agricultura de precisão, um fator de sucesso crucial para o mercado de tratamento de sementes é o desenvolvimento de uma solução completa de proteção que seja econômica, ecologicamente correta e ambientalmente responsável (HAZRA E PATANJALI, 2016). Neste sentido, os biossurfactantes têm mostrado excelente atividade antimicrobiana contra fitopatógenos, além de servir como estimulantes da germinação de sementes.

Assim, apesar da produção microbiana dessas biomoléculas ser prioritariamente por bactérias, os fungos, como leveduras do gênero *Candida* demonstram elevado potencial biotecnológico, considerando seu elevado metabolismo e catabolismo de diferentes substratos provenientes das indústrias (alimentos, agricultura, entre outros). Desse modo, visando ampliar o conhecimento sobre biossurfactantes produzidos por leveduras não convencionais para sua utilização como agentes antifúngicos e protetores de sementes foram realizados estudos que demonstraram um caminho promissor, diferencial e inédito para *Candida tropicalis* UCP 1613, levedura isolada de sedimentos do manguezal do Rio Formoso, Pernambuco, Brasil.

1.1 OBJETIVOS

1.2 OBJETIVO GERAL

Produzir, otimizar e caracterizar o biossurfactante isolado de *Candida tropicalis* UCP 1613, empregando resíduos agro-industriais e investigar o potencial antimicrobiano e de estimulação da germinação do biossurfactante e aplicação na agricultura.

1.2.1 Objetivos específicos

- Selecionar as melhores condições de produção de biossurfactante por *Candida tropicalis* UCP 1613 através de um planejamento fatorial completo;

- Otimizar a produção de biossurfactante por *Candida tropicalis* UCP 1613 através de um Delineamento Composto Central Rotacional com diferentes concentrações dos resíduos industriais;

- Realizar o perfil de cinética de crescimento de *Candida tropicalis* UCP 1613 e da produção do biossurfactante utilizando como substratos: glicerol residual e glutamato;

- Realizar o isolamento e purificação do biossurfactante;

- Caracterizar o biossurfactante;

- Avaliar a estabilidade do biossurfactante produzido frente a diferentes pH, temperatura e salinidade;

- Avaliar o potencial de atividade antimicrobiana frente a *Alternaria alternata*;

- Avaliar a produção de biosurfactnate por *Candida tropicalis* UCP 1613 através da fermentação em estado sólido;

- Determinar o efeito na germinação de sementes de cebola de formulações de revestimento de biossurfactante obtido através de fermentação em estado sólido;

- Determinar a influência da temperatura, tamanho de partícula, concentração de inóculo e indutor, na produção de biosurfactante por *Candida tropicalis* UCP 1613 através da fermentação em estado sólido;

- Validar os resultados obtidos através de tratamento estatístico.

2 REVISÃO DA LITERATURA

2.1 SURFACTANTES

Os surfactantes constituem um grupo de compostos químicos que se distinguem por sua capacidade de misturar fases imiscíveis, tipicamente óleo e água. Eles constituem componentes indispensáveis em quase todos os setores da indústria moderna, e sua importância é evidenciada pelos enormes volumes usados e pela diversidade de aplicações que incluem, alimentos, saúde, agricultura, controle da poluição ambiental entre outros (SAŁEK; GUTIERREZ, 2016; LIANG et al., 2019).

Estes compostos são definidos quimicamente como moléculas anfifílicas com uma região hidrofílica, usualmente nomeada de cabeça, e a região hidrofóbica, chamada de cauda. No caso da parte hidrofílica ou polar, caracteriza-se pela presença de grupos iônicos (catiônico ou aniônico) e não-iônico ou anfotérico (GEETHA; BANAT; JOSHI, 2018a; XIE; LAI; SHI, 2020) (Figura 1).

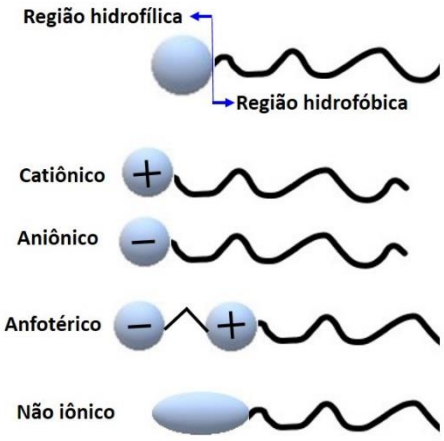

Figura 1- Classificação química dos surfactantes. Fonte: Autor

Podem-se mencionar como exemplos de grupos hidrofílicos catiônicos as sais de amônio, enquanto os aniônicos podem ser derivados de grupos sulfatos e sulfonatos (HOCINE et al., 2016) (Tabela 1). Assim, os surfactantes não iônicos contêm grupos sem carga e, os surfactantes anfotéricos, apresentam na mesma molécula grupos com uma carga negativa e uma positiva, dependendo do pH (FINIZIO et al., 2020; LV et al., 2020). A presença de ambos grupos (hidrofílicos e hidrofóbicos) na mesma molécula, faz com que eles se organizem nas

interfaces entre fases fluidas com diferentes graus de polaridade (óleo/água e água/óleo) (Figura 2).

Tabela 1- Tipos de surfactantes químicos e características bioquímicas

Surfactate	Nome	Fórmula química	Peso molecular (mg/mol)	Carga
Tween 20	Monolaurato de Sorbitan Etoxilado 20	$C_{58}H_{114}O_{26}$	1227.54	Não iônico
Tween 80	Polisorbato 80	$C_{17}H_{35}COOS_6(OCH_2CH2)20OH$	1309	Não iônico
Brij 35	Polietoxilato de alquila	$C_{12}H_{25}(OC_2CH_2)_{23}OH$	1200	Não iônico
CTAB		$CH_3(CH_2)_{15}NBr(CH_3)_3$	364.45	Catiônico
Goma de guar	Galactomanano	$C_{18}H_{32}O_{16}$	504.438	Catiônico
SDS	Dodecilsulfato de sódio	$C_{12}H_{25}OSO_3Na$	288.38	Aniônico
SDBS	Sulfonato de Dodecilbenzeno de Sódio	$C_{12}H_{25}C_6H_4\ SO_3Na$	348.48	Aniônico
Lecitina	Lecitina	$C_{35}H_{66}NO7P$	643.887	Zwitteriônicos
Cyclodextrina	β-ciclodextrina	$C_{42}H_{70}O_{35}$	1135	Zwitteriônicos

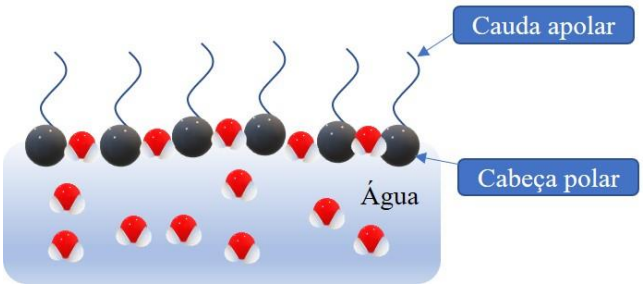

Figura 2- Desenho ilustrativo do comportamento do biossurfactante na superfície entre um líquido e o ar.
Fonte: Autor

Consequentemente, a formação de um filme molecular ordenado nas interfaces, reduz a tensão interfacial e superficial, sendo responsável pelas propriedades únicas dos surfactantes

como: detergência, emulsificação, lubrificação, capacidade espumante, capacidade molhante, solubilização e dispersão de fases (CHEVALLIER et al., 2020; SAGIR et al., 2020; WANG et al., 2020).

2.2 BIOSURFACTANTES

Dentre os seres vivos, as plantas, os micro-organismos e os humanos, possuem a capacidade de sintetizar compostos com propriedades tensoativas que são considerados surfactantes naturais. Entretanto, dessas biomoléculas as de maior importância são as de origem microbiana denominados bossurfactantes, que consistem em subprodutos metabólicos produzidos por bactérias, fungos filamentosos e leveduras (FENIBO et al., 2019; MARCELINO et al., 2020). Em geral, os biossurfactantes são sintetizados de forma intracelular, extracelular ou associados às membranas celulares. Dessa forma, entre as funções atribuidas a essas moléculas destacam-se a estimulação do crescimento microbiano, o aumento da disponibilidade de substratos e o transporte de nutrientes entre outras (KHAN; BUTT, 2016).

2.2.1 Classificação e micro-organismos produtores

Ao contrário dos surfactantes quimicamente sintetizados que são classificados de acordo com a sua dissociação na água, os biossurfactantes são categorizados pela sua composição química, peso molecular, propriedades físico-químicas e modo de ação e origem microbiana (PACWA-PŁOCINICZAK et al., 2011; ROY, 2017; VARJANI; UPASANI, 2016). Com base no peso molecular, são divididos em biossurfactantes de baixa massa molecular, incluindo: glicolipídios, fosfolipídios e lipopeptídeos e em biossurfactantes/bioemulsificantes de alta massa molecular: polissacarídeos anfipáticos, proteínas, lipopolissacarídeos, lipoproteínas ou misturas complexas desses biopolímeros (FENIBO et al., 2019).

Os biossurfactantes de baixa massa molecular são eficientes na redução das tensões superficiais e interfaciais, enquanto os biossurfactantes de massa molecular elevada são mais eficazes na estabilização das emulsões óleo-em-água (DAS, AMBUST, KUMAR et al., 2020) (Tabela 2).

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

Clase/tipo de biossurfactante	Micro-organismo
Glicolipídios	*Pseudomonas aeruginosa*
Ramnolipídios	*Torulopsis bombicola, T. apicola*
Trealolipídios	*Rhodococcus erythropolis, Mycobacterium* sp.
Soforolipídios	
Lipopeptídeos e lipoproteínas	
Viscosina	*Pseudomonas fluorescens*
Serrawetina	*Serratia marcescens*
Surfactina	*Bacillus subtilis*
Ácidos graxos, lipídeos neutros e fosfolipídeos	
Ácidos graxos	*Corynebacterium lepus*
Lipídios neutros	*Nocardia erythropolis*
Fosfolipídios	*Thiobacillus thiooxidans*
Surfactantes poliméricos	
Emulsan	*Acinetobacter calcoaceticus*
Biodispersan	*Acinetobacter calcoaceticus*
Liposan	*Candida lipolytica*
Carboidrato-Lipídeo-Proteína	*Pseudomonas fluorescens*
Manana-Lipídeo-Proteína	*Candida tropicalis*
Surfactantes particulados	
Vesículas	*Acinetobacter calcoaceticus*
Células	Várias bactérias

Fonte: SILVA et al., 2014.

A estrutura comum destas biomoléculas se baseia em uma porção lipofílica usualmente composta por cadeia hidrocarbônica de um ou mais ácidos graxos, que podem ser saturados, insaturados, hidroxilados ou ramificados, ligados à uma porção hidrofílica, que pode ser um éster, um grupo hidróxi, fosfato, carboxilato ou carboidrato. A maioria dos biossurfactantes são neutros, ou aniônicos variando desde pequenos ácidos graxos até grandes polímeros (DE ALMEIDA et al., 2016; SAŁEK; GUTIERREZ, 2016).

No entanto, a variedade estrutural desses metabólitos encontra-se relacionada à diversidade de produtores e ambientes dos quais eles podem ser isolados. Convencionalmente micro-organismos produtores têm sido mais frequentemente isolados de locais contaminados por hidrocarbonetos (PERFUMO et al., 2018). Exemplo de alguns dos principais consórcios

microbianos em solos e sedimentos incluem: *Pseudomonas, Burkholdeiria, Bacillus, Streptomyces, Sphingomonas* e *Actinobacter*. No entanto, *Pseudoalteromonas, Halomonas, Alcanivorax* e *Acinetobacter* são mais frequentes nos ecossistemas marinhos. Além disso, microorganismos produtores de biossurfactantes tem sido isolados em ambientes extremos com alta salinidade (WAGHMODE et al., 2020), temperatura (DHAGAT, ESWARI 2020; TAO et al., 2020) e ambientes fríos (BUENO et al., 2019; COLLINS, MARGESIN, 2019). .

2.3 LEVEDURAS DO GÊNERO *Candida*

O gênero de leveduras conhecido como *Candida* foi identificado em 1923 pela microbiologista dinamarquesa Christine Berkhout, que atribuiu nove espécies anteriormente incluídas no gênero *Monilia* à nova unidade taxonômica (BARNETT, 2004). O nome do gênero tem origem na palavra latina *candidus* (branca), relacionada à ausência de corantes carotenóides nas células desses fungos. O gênero *Candida* foi identificado na época de acordo com características morfológicas, bioquímicas e fisiológicas selecionadas. Porém, nas últimas décadas o desenvolvimento de métodos avançados de biologia molecular resultou em mudanças significativas na nomenclatura de *Candida*. Atualmente, o gênero inclui mais de 200 espécies, que são membros do reino fúngico, família *Saccharomycetaceae,* ordem *Saccharomycetales*, classe *Hemiascomycetes*, filo *Ascomycota* (Fungi) (MELO et al., 2019; WICKES, ROMANELLI, 2020).

As leveduras do gênero *Candida* são unicelulares e a forma e tamanho das células depende principalmente da espécie, da fase de crescimento, da condição fisiológica e das condições ambientais da cultura. Elas são geralmente ovais, elipsoidais ou fortemente alongados, e seu tamanho está na faixa de (1 x 8) e (1 x 6,0) µm (KURTZMAN; FELL, 2000). As leveduras do gênero *Candida* são tipicamente micro-organismos aeróbicos. Durante a aeração, elas produzem pequenas quantidades de álcool ou não o produzem, enquanto aumentam rapidamente sua biomassa. A temperatura ótima e pH varia entre, 25 e 30°C e 4 – 6 respectivamente. As leveduras deste gênero podem formar pseudomicélio, micélio rudimentar, e micélio verdadeiro bem desenvolvido ou não formar de forma alguma. Isso depende principalmente de suas espécies e das condições ambientais (KIELISZEK et al., 2017).

2.3.1 Potencial biotecnológico de leveduras do Gênero *Candida*

Durante a evolução, as leveduras têm sido um componente essencial das sociedades humanas na fabricação de pão, vinho, cerveja ou outras bebidas destiladas. Além disso, algumas espécies servem como organismos modelo para elucidar os mecanismos moleculares por trás

dos processos celulares relevantes para a biotecnologia (ANDERSEN, WINTER, 2019). Na era da bioeconomia, acredita-se que os processos biotecnológicos associados às leveduras são um fator-chave para o estabelecimento da economia circular. Isso se deve ao seu potencial para gerar compostos industrialmente relevantes a partir de fontes naturais e fluxos de resíduos de uma forma rentável e ambientalmente amigável (SABU, MUFEEDHA, PRAMOD, 2019).

Tradicionalmente “levedura” denota a *Saccharomyces cerevisiae* e grupos próximos. No entanto, os biotecnologistas agruparam todas as leveduras não *Saccharomyces cerevisiae c*omo leveduras “não convencionais (ROZPĘDOWSKA et al., 2011; SOUZA et al., 2019). Uma das características mais notáveis de *S. cerevisiae* é a sua habilidade para crescer em concentrações extremamente altas de açúcar. Porém, a maioria dos habitats naturais, não fornece tais condições extremas de substrato. Dai que a maioria das leveduras não convencionais fornecem rotas metabólicas alternativas para utilização de substrato e formação de produtos e padrões regulatórios diferentes (MATTANOVICH; SAUER; GASSER, 2014; WENDLAND, 2020).

No caso das leveduras do gênero *Candida* o número de pesquisas relacionadas ao seu alto potencial biotecnológico devido a sua grande versatilidade metabólica. KATHIRESAN SARAVANAKUMAR (2011) e SENTHILRAJA et al. (2011) mostraram que espécies de *Candida* não são apenas agentes patogênicos, senão que podem ser ferramentas úteis para a produção de bioetanol. Desse modo, estes investigadores, obtiveram etanol a partir de *C. tropicalis* e *C. albicans* isoladas de ambiente marinho. Por outo lado, representantes deste gênero tem sido utilizados com sucesso na produção de biossurfactantes, por exemplo: *C. glabrata* (LUNA; SARUBBO; CAMPOS-TAKAKI, 2009), *C. bombicola* (VAUGHN et al., 2014), *C. sphaerica* (LUNA et al., 2011) *C. lipolytica* (RUFINO et al., 2014) e *C. tropicalis* (RUBIO et al., 2017; VERMA et al., 2015).

2.4 PROPRIEDADES FÍSICO-QUIMICAS DOS BIOSSURACTANTES

A propriedade mais importante destes compostos se encontra relacionada a sua capacidade de diminuir a tensão superficial da água. Assim, este parâmetro pode ser definido como a força por unidade de comprimento ou de energia por unidade de área, que se origina a partir da diferença entre as forças intermoleculares que atuam na interface (fluido-fluido ou fluido- gás) (HANTAL et al., 2019). Esta propriedade se manifesta em um líquido ao manter as moléculas unidas na sua superfície sendo uma consequência das forças intermoleculares.

Dessa forma, cada molécula é atraída por outras moléculas em todas as direções do espaço no interior do líquido, enquanto que as moléculas superficiais só estão submetidas à tensão das moléculas que têm por baixo (HAYAKAWA et al., 2019; OLAYIWOLA, DEJAM, 2019) (Figura 3).

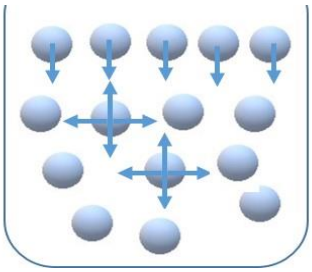

Figura 3- Efeito do biossurfactante na tensão superficial. Fonte: Autor

De acordo com a literatura a tensão superficial (TS) da água destilada é de 72 mN/m, e quando se adiciona um biossurfactante reduz-se esse valor. Sendo assim, a diminuição das tensões nas faixas de 35 mN/m a 40 mN/m, indica que o micro-organismo é promissor na produção destes compostos e abaixo de 35 mN/m, que o micro-organismo pode ser considerado um eficiente produtor. No entanto, a tensão interfacial (TI) entre a água e n-hexadecano pode ser diminuída de 40 a 1 mN/m (DE ALMEIDA et al., 2016; FRACCHIA et al., 2015). Portanto, a medição da TS e TI entre fases líquidas são medidas úteis para determinar se uma cultura microbiana está produzindo biossurfactante, mas não pode ser usada de maneira quantitativa, porque uma vez que o mínimo TS ou TI é alcançado, a produção adicional de biossurfactante não leva a qualquer alteração no valor (DE ALMEIDA et al., 2016; SATPUTE; PŁAZA; BANPURKAR, 2017).

Por outro lado, a atividade dos biossurfactantes depende de sua concentração micelar crítica (CMC) que se define como a concentração mínima requerida para atingir a menor tensão superficial. Em concentrações acima da CMC, moléculas de biossurfactante se associam para formar micelas, bicamadas e vesículas. No entanto, abaixo da CMC, o surfactante está predominantemente na forma monomérica (Figura 4) (FENIBO ET AL., 2019; JAHAN ET AL., 2019).

A intensidade de adsorção do biossurfactante à superfície depende de sua concentração. Quando ocorre um aumento da concentração de biosssurfactante, observa-se uma diminuição da área disponível para as moléculas iniciando o processo de ordenação das mesmas para formar micelas. Assim, estas estruturas permitem que os biossurfactantes reduzam a tensão superficial

e interfacial e aumentem a solubilidade e a biodisponibilidade de compostos orgânicos hidrofóbicos. Deste modo, biossurfactantes eficientes possuem um valor de CMC baixo, o que significa que menos biossurfactante é necessário para diminuir a tensão superficial (GUAN et al., 2017; PERFUMO et al., 2018).

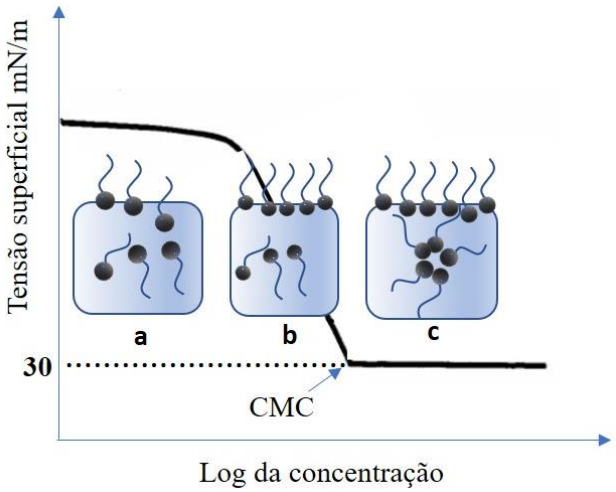

Figura 4- Gráfico representativo do efeito da concentração do biossurfactante na tensão superficial: a, b, c posição dos surfactantes na interfase e na formação das micelas na medida que aumenta do surfactante. Fonte: RAIGER e LOPÉZ, 2009

Os valores de CMC são importantes em praticamente todas as aplicações de biossurfactantes na indústria, desde o processamento de minerais até a formulação de produtos e alimentos para cuidados pessoais, sistemas de liberação de medicamentos e novas tecnologias de remediação de surfactantes (PERFUMO et al., 2018; SATPUTE; PŁAZA; BANPURKAR, 2017). Nestes processos, o surfactante geralmente deve estar presente em uma concentração maior que a CMC pois o maior efeito seja na redução da tensão interfacial, emulsificação, estabilização da suspensão, como um veículo de liberação, ou na promoção da estabilidade da espuma, é alcançado quando uma concentração significativa de micelas está presente (DE et al., 2015a).

Outra importante propriedade dos surfactantes microbianos é a emulsificação que se define como dispersão de um líquido em outro. Sendo assim, os biossurfactantes agem como moléculas que facilitam a formação de uma emulsão devido à capacidade de diminuir a tensão interfacial entre fases com diferente grau de polaridade. Desse modo aumenta a solubilidade de substratos insolúveis ou com pouca solubilidade (KARINA, STEPHEN, 2019; ESWARI, DHAGAT, SEN, 2019).

Emulsões são sistemas dispersos compostos por duas fases imiscíveis (geralmente água e óleo) com um (a fase dispersa) dispersos no segundo (a fase contínua) na forma de pequenas gotículas (WEBBER et al., 2019). Existem dois tipos principais de emulsões: água-em-óleo (A/O) e óleo-água (O/A), com exemplos notáveis sendo leite (O/A) ou margarina (A/O). Este tipo de emulsões duplas são de particular interesse para o indústrias médicas, cosméticas e alimentícias, especialmente quando usadas para encapsulamento de produtos farmacêuticos (sistemas de liberação de medicamentos, hormônios, esteroides), cremes (componentes ativos encapsulados ou vitaminas) e alimentos funcionais enriquecidos com produtos nutricionais e à base de vitaminas e também cores, sabores, minerais e conservantes (MICHELON et al., 2019; MORELLI et al., 2019; ZHUN et al., 2019).

As emulsões são sistemas instáveis e podem ser formadas de forma espontânea, mecánica ou manual, por ultrasom ou altas pressões de momogenização. Assim, o tamanho varia entre 0.5-50 (μm), 0.01-0.10 (μm) e 0.05-50 (nm) para as emulsões, microemulsões e nanoemulsões respectivamente. Quanto menor o diâmetro das gotículas, mais estável a emulsão formada (SOUZA et al., 2016). Esta propriedade faz dos surfactantes compostos atrativos para seu uso nas indústrias de cosméticos e alimentos. (NITSCHE; SILVA, 2016; PARTHASARATHI, R. AND SUBHA, 2018; SANTOS et al., 2016).

De igual forma o valor de balanço hidrófilo-lipófilo é uma medida para indicar se um biossurfactante está relacionado à emulsão de água em óleo ou óleo em água. Este fator pode ser usado para determinar a aplicabilidade adequada dos biossurfactantes. Essa propriedade é representada por uma escala arbitrária de 0 a 20, em que os materiais mais hidrofílicos têm o maior número. A escala HLB indica a capacidade do agente tensoactivo para formar emulsões de água em óleo ou óleo em água, comparando com tensoactivos de valores e propriedades conhecidos de HLB. Por exemplo, a escala HLB pode ser construída atribuindo-se um valor de 1 para o ácido oleico e um valor de 20 para o oleato de sódio. Além disso, utilizando uma gama de misturas destes dois componentes em diferentes proporções, podem ser obtidos valores intermédios. Os emulsionantes com valores de HLB inferiores a 6 favorecem a estabilização da emulsificação de água em óleo, enquanto os emulsificadores com valores de HLB entre 10 e 18 têm efeito oposto e favorecem a emulsificação de óleo em água (MANIGLIA ET AL., 2019; THAVASI, BANAT; 2019; HALECKÝ, KOZLIAK, 2020).

2.5 VANTAGENS DOS BIOSSURFACTANTES EM RELAÇÃO AOS SURFACTANTES SINTÉTICOS

A demanda industrial por surfactantes químicos na indústria é alta. Contudo, com o aumento da preocupação ambiental entre os consumidores como consequência de seu deterioro, juntamente com as legislações de controle do meio ambiente a procura de alternativas se torna maior. Assim, com essa finalidade, os biossurfactantes tem sido objeto de pesquisas intensas (DE ARAUJO; FREIRE; NITSCHKE, 2013; GUDIÑA et al., 2015). Nesse contexto podem-se mencionar como principais vantagens:

- Baixa toxicidade: os biossurfactantes exibem baixa toxicidade quando comparados com os surfactantes sintéticos. Assim, estes compostos têm recebido maior atenção devido à crescente preocupação em relação aos efeitos alérgicos dos produtos artificiais, sendo permitidos em alimentos, cosméticos e produtos farmacêuticos (FEI et al., 2020; REBELLO et al., 2020).

- Biodegradabilidade: o fato de serem produtos naturais faz com que possam ser facilmente biodegradados em ambientes aquáticos e terrestres, tornando-os adequados para aplicação na biorremediação e no tratamento de resíduos MARCELINO et al., 2020; REN et al., 2020).

- Disponibilidade a partir de substratos de baixo custo: Os biossurfactantes podem ser produzidos utilizando matérias-primas muito baratas e o que tem sido demonstrado em vários estudos (JIMOH, LIN, 2020; LIMA et al., 2020).

- Tolerância à temperatura, pH e força iônica: muitos dos biossurfactantes não perdem as suas propriedades quando submetidos a condições extremas de temperatura, pH e força iônica. Neste sentido, alguns deles podem suportar temperaturas de até 90°C e apresentam maior estabilidade térmica em condições extremas quando comparados aos sintéticos. 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 (SOMOZA-COUTIÑO et al., 2020; JAVEE, SUBRAMANI, 2020).

2.6 APLICAÇÕES INDUSTRIAIS DOS BIOSSURFACTANTES

Os biossurfactantes possuem ampla gama de aplicações biotecnológicas em áreas como: petróleo, alimentos, bebidas, cosméticos, detergentes, têxteis, tintas, mineração, celulose,

farmacêutica e nanotecnologia (SANTOS et al., 2016). Ainda assim, a tendência no mercado global para biossurfactantes foi estimada em 344 quilotoneladas em 2013, enquanto se espera que atinja 462 quilotoneladas até 2020, com uma taxa de crescimento anual de 4,3% de 2013 a 2020 (DE ALMEIDA et al., 2016).

2.6.1 Aplicação na indústria dos cosméticos

Atualmente, percebe-se o significativo nível de interesse em produtos naturais pela indústria cosmética, principalmente devido às crescentes preocupações dos consumidores com a sustentabilidade ambiental e com a saúde. Portanto, a esta indústria procura produtos mais sustentáveis e saudáveis (FERNÁNDEZ-PEÑA et al., 2020). Os biossurfactantes são usados da mesma maneira que os surfactantes quimicamente sintetizados. Eles podem ser empregados na detergência, emulsificação, de-emulsificação, umedecimento, formação de espuma, dispersão, solubilização de substâncias hidrofóbicas ou para modificar superfícies, entre outras atividades ou funções possíveis (MARCHANT; BANAT, 2012; VECINO et al., 2017).

A função emulsionante é provavelmente a propriedade mais importante dos biossurfactantes na formulação de cosméticos, porque as emulsões apresentam vantagens consideráveis em relação a outros tipos de preparações. Portanto, eles são fáceis de aplicar, relativamente menos onerosos (devido à sua alta proporção de água) e permitem simultaneamente o uso de substâncias lipossolúveis e hidrossolúveis. Os biossurfactantes de baixo peso molecular reduzem a tensão superficial nas interfaces ar/água e a tensão interfacial nas interfaces óleo/água (O/W), enquanto os biossurfactantes de alto peso molecular são mais eficazes na estabilização de emulsões O/W (VECINO et al., 2017).

Vários autores (BUJAK; WASILEWSKI; NIZIOŁ-ŁUKASZEWSKA, 2015; LU; MOORE, 2012) têm discutido que um dos problemas mais desafiadores relacionados ao uso de surfactantes químicos em formulações cosméticas se baseia no seu potencial para causar irritações na pele e reações alérgicas. Surfactantes não naturais podem interagir com proteínas, remover lipídios da superfície epidérmica desorganizando a estrutura intercelular desses lipídios e também afetar as células vivas da pele. Esses efeitos prejudiciais podem ser potencialmente evitados ou reduzidos pelo uso de biossurfactantes, como, por exemplo, aqueles produzidos por bactérias do ácido láctico (IWANAGA et al., 2020; SONG et al., 2020). Alguns destes biossurfactantes também podem ser encontrados em subprodutos agroindustriais, como

licor de maceração de milho, devido à fermentação espontânea que ocorre durante o processo de refino do milho (VECINO et al., 2014, 2015).

2.6.2 Aplicação na indústria dos alimentos

O principal objetivo do processamento de alimentos é fornecer produtos seguros que mantenham as características organolépticas. Qualquer material que modifique a propriedade distintiva do alimento, uma vez adicionado, é caracterizado como um aditivo alimentar. O objetivo de incorporar aditivos é melhorar e manter o valor nutricional, a textura, a segurança, o sabor e a aparência do alimento. A mudança na tendência dos consumidores ao natural, a redução ou eliminação de aditivos sintéticos e também as crescentes preocupações com a saúde e o meio ambiente, tem criado alta demanda por novos aditivos "verdes" nos alimentos (COLLEY et al., 2020; MEIJER et al., 2020). Neste contexto, as características demonstradas pelos biossurfactantes como: antiadesividade, atividade antimicrobiana e propriedades de emulsificação, abrem uma nova oportunidade para sua incorporação como aditivos versáteis ou ingredientes para processamento de alimentos (GUDIÑA, RODRIGUES, 2019).

As indústrias de processamento de alimentos podem utilizar os biossurfactantes de duas maneiras distintas: na utilização indireta para o tratamento/limpeza de superfícies de contato com alimentos; no uso direto como aditivo/ingrediente alimentar. Alternativamente, as indústrias de alimentos podem tirar proveito de seus resíduos como substratos para a produção de biossurfactantes, contribuindo, assim, com a valorização de resíduos e redução dos custos de tratamento de resíduos (ALKAN et al., 2019; RIVERA, URBINA, LÓPEZ, 2019).

2.6.3 Aplicação na medicina

Vários biosurfactantes têm forte atividade antibacteriana, antifúngica e antiviral e podem desempenhar o papel de agentes anti-adesivos contra patógenos o que os torna úteis para o tratamento de muitas doenças, bem como a sua utilização como agentes terapêuticos e probióticos (SAIMMAI et al., 2020). Vários destes compostos têm sido investigados como alternativas adequadas para medicamentos sintéticos e antimicrobianos. Dessa forma, possíveis aplicações incluem a transfecção de genes, a utilização como adjuvantes para os antigênicos e o uso como inibidores da formação de coágulos de fibrina. Além de que têm sido empregados como anti-adesivos para revestimentos de biomateriais e de igual forma, tem-se incorporado

como probióticos para combater infecções do trato urogenital e para imunoterapia pulmonar (DÍAZ DE RIENZO et al., 2015; FRACCHIA et al., 2015).

2.6.4 Aplicação de biossurfactantes na biorremediação de hidrocarbonetos

A maioria dos informes na literatura para estes compostos referem-se a sua aplicação na indústria petroleira. Neste sentido, a sua natureza não toxica e biodegradável tem tornado eles alternativas sustentáveis e compatíveis com o meio ambiente (GEETHA; BANAT; JOSHI, 2018b; PERFUMO et al., 2018). A biorremediação consiste na decomposição biológica de hidrocarbonetos por micro-organismos que utilizam estes poluentes como uma fonte de carbono para obter energia, degradando-os até dióxido de carbono, água, sais minerais e gases (BANAT et al., 2010; HAZEN, 2019).

Quanto maior for a população de micro-organismos degradadores mais rápido e eficiente será o processo de bioremediação. Estudos realizados 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 (DONATI, 2019; YANG et al., 2019). Assim, a eficiência de um micro-organismo degradador depende, em muitos casos, da estrutura da molécula do contaminante e da presença de enzimas específicas capazes de degradar o produto (BALAJI et al., 2014; WACKETT, 2014). Adicionalmente a ocorrência deste mecanismo é mais provável quando a estrutura química do xenobiótico é semelhante à estrutura de moléculas naturais (ANGELUCCI et al., 2019; BAKER, IROUMALECHETTY, MOHAN, 2019).

Porém, dentre as dificuldades associados à biodegradação de compostos hidrofóbicos, que incluem os hidrocarbonetos do petróleo, podem-se mencionar sua baixa solubilidade e alta hidrofobicidade, o que diminui a disponibilidade para os micro-organismos e pode retardar ou paralisar o processo (COLIN et al., 2014). Considerando esta situação, uma alternativa tem sido o uso de compostos surfactantes (SILVA et al., 2014a).

Assim, surfactantes naturais ou biossurfactantes são sintetizados por micro-organismos em presença de compostos hidrofóbicos, promovendo o aumento da solubilidade deles no meio (SAJNA, GOTTUMUKKALA, 2019). Estes compostos aumentam a interação água/óleo diante a diminuição da tensão superficial, aceleram a degradação de vários hidrocarbonetos por micro-organismos e promovem a biorremediação de águas e solos contaminados (NIMRAT et al., 2019;

JOE et al., 2019). Estudos realizados com micro-organismos produtores de biossurfactantes evidenciaram o potencial de biorremediação de hidrocarbonetos de petróleo em solos e areia (LUNA et al., 2011; SILVA et al., 2014b).

2.6.5 Aplicação na agricultura

Os biossurfactantes são componentes integrais de muitos produtos comerciais para diversas aplicações agrícolas, tanto em sistemas de cultivo de plantas quanto alimentação de animais de fazenda (GALLO et al., 2017). Na criação de animais de fazenda, a manipulação nutricional/dietética é uma das principais direções das aplicações de biossurfactantes. Biossurfactantes naturais, como alquil poliglucósidos derivados de plantas (PDP), demonstraram ser eficazes na nutrição de ruminantes devido aos seus efeitos positivos nos parâmetros fisiológicos e de produção em, por exemplo, ruminantes. A digestibilidade ruminal e intestinal da matéria orgânica aumenta junto com a síntese protéica microbiana ruminal, resultando no aumento do fluxo microbiano duodenal de nitrogênio (NAUGHTON et al., 2019). Além disso, o PDP pode ter efeitos indiretos positivos em termos de sua capacidade de modificar a comunidade microbiana do rúmen, pois aumenta a produção volátil total de ácidos graxos no rúmen in vivo.

Pesquisas disponíveis indicariam que os biossurfactantes microbianos podem ter efeitos semelhantes aos atribuídos aos PDP na nutrição de ruminantes, por exemplo, um ramnolipídeo produzido por *P. aeruginosa* mostrou influência a enzima xilanase e taxas gerais de degradação da matéria orgânica *in vitro* (LIU et al. 2011). Pesquisas anteriores também reconheceram que a incorporação de culturas de leveduras com glicoproteína emulsificada em dietas para ruminantes pode melhorar a digestibilidade da matéria orgânica, incluindo a digestibilidade da celulose e hemicelulose (NAUGHTON et al., 2019). Além de melhorar a atividade das enzimas fibrolíticas na nutrição de ruminantes, biossurfactantes microbianos com suas propriedades emulsificantes têm sido sugeridos para melhorar a digestibilidade de gorduras/óleos em dietas de animais. As gorduras/óleos são normalmente adicionadas às dietas animais como uma fonte de energia mais econômica. No entanto, seu uso é limitado pela capacidade fisiológica do animal de digerir altos níveis de gorduras/óleos alimentares (WILLIAMS et al., 2017; PLASCENCIA, ZINN, 2019).

Mais recentemente, o potencial dos biossurfactantes na proteção de sementes e na estimulação do crescimento foi investigado, mostrando a eficácia de lipopeptídeos (TORAL et

al. 2018) contra fitopatógenos, incluindo *Botrytis cinerea* e de ramnolipídeos (BORAH et al. 2016) contra *Fusarium verticillioides*, um dos principais patógenos. de milho. Além disso, os ramnolipídeos têm mostrado potencial como biopesticidas (SOLTANI et al. 2016) e fungicidas (SHAH et al. 2005). SHA et al. (2012) atribuíram o efeito antifúngico do caldo de cultura livre de células de ramnolipídeos à atividade superficial e à ruptura das membranas plasmáticas.

Os revestimentos de sementes são classificados de acordo com suas características físicas. Embora a nomenclatura usada na literatura não seja consistente, a terminologia mais utilizada e reconhecida entre a indústria e a academia se baseia no peso, tamanho e propriedades de classificação das sementes revestidas. O tratamento básico de revestimento é o revestimento de filme, onde uma camada fina de material externo (geralmente <10% do peso da semente) é aplicado. O peso da semente é aumentado em 100-500% (dependendo da morfologia da semente), o procedimento é descrito como 'incrustante' e é definido como tal desde que a forma original da semente ainda seja evidente. A quantidade de material externo torna impossível discriminar a forma inicial da semente (o resultado geralmente é uma forma esférica) (PEDRINI et al., 2017).

Os materiais estruturais utilizados no revestimento de sementes são classificados em aglutinantes e cargas. Os aglutinantes são polímeros de origem natural e sintética que fornecem aderência e coesão de material à semente e retenção de ingredientes ativos. Eles geralmente são aplicados na forma líquida (em água ou solventes) e, quando secos, os monômeros dissolvidos são reunidos em longas cadeias poliméricas, formando um filme contínuo ao redor da semente, partículas de ligação e produtos químicos (AMIRKHANI et al., 2016; PAWLICKI et al., 2019).

Diferentes camadas de polímero podem ser aplicadas em diferentes estágios do processo de revestimento, algumas carregando tratamentos e outras fornecendo um amortecedor para evitar o contato direto entre as camadas ativas e a semente, o ambiente externo ou outras camadas ativas (PEDRINI et al., 2017). O revestimento de sementes tem sido realizado com aglutinantes comerciais de composição não revelada. No entanto, os aglutinantes mais comumente relatados são metilcelulose, polietileno glicol, quitosana, polivinil álcool, etilcelulose, acetato de polivinil e goma arábica (HONG et al., 2016; YELWA et al., 2017).

As cargas normalmente são pós inertes, como bentonita, carbonato de cálcio, talco, terra de diatomáceas, areia e pó de madeira. As propriedades físicas e químicas dos diferentes pós, em combinação com os aglutinantes, fornecem uma variedade de possíveis resultados

mecânicos e biológicos para os revestimentos. A distribuição do tamanho de partícula, por exemplo, afeta fortemente o comportamento do pellet; partículas pequenas fornecem resistência física mais alta, mas troca limitada de gás e água, enquanto partículas maiores aumentam a porosidade, mas reduzem a integridade mecânica e a resiliência do revestimento (PEDRINI et al., 2017; PAWLICKI et al., 2019).

2.7 ESTRATÉGIAS PARA A PRODUÇÃO DE BIOSSURFACTANTES

O custo de fabricação dos biossurfactantes é 3 a 10 vezes maior que a produção dos surfactantes químicos. Assim, diversas estratégias têm sido adotadas para reduzir os custos de produção. Os pesquisadores têm se concentrado em aumentar o rendimento do produto usando substratos renováveis e facilmente disponíveis, desenvolver processos usando mutantes hiperprodutores e linhagens recombinantes, otimizar as condições de fermentação (temperatura, pH, agitação, aeração) bem como selecionar o tipo de fermentação (fermentação submersa ou em estado sólido). De igual forma outro fator importante é a purificação dos produtos com bom custo-benefício (KANDASAMY et al., 2019).

2.7.1 Utilização de substratos alternativos na produção de biossurfactantes

A sociedade moderna atravessa um processo de mudança durante o qual o desenvolvimento sustentável deve ser alcançado para evitar esgotar seus recursos naturais (BRUMANO; SOLER; DA SILVA, 2016). Um forte setor industrial de base biológica desenvolvido reduzirá significativamente a dependência de recursos fósseis, ajudará os países a cumprir as metas de mudança climática e levará a um crescimento mais ecológico e mais favorável ao meio ambiente. Neste sentido, a bioeconomia engloba a produção de recursos biológicos renováveis e a sua conversão em alimentos, rações e produtos biológicos (químicos, materiais e combustíveis) através de tecnologias inovadoras e eficientes fornecidas pela biotecnologia industrial (MOORE et al., 2017; SATPUTE; PŁAZA; BANPURKAR, 2017).

O fato dos biossurfactantes serem moléculas de origem biológica e sua capacidade de cumprir a maioria dos requisitos de seus similares sintéticos (derivados do petróleo) justifica-se com crescente número de investigações (SATPUTE; PŁAZA; BANPURKAR, 2017; VAN RENTERGHEM et al., 2018). Contudo, apesar do crescente reconhecimento das vantagens dos biossurfactantes, sua produção ainda não é capaz de competir economicamente com a produção

de surfactantes químicos (LOUHASAKUL et al., 2020). Os biossurfactantes possuem altos custos de produção, principalmente devido ao uso de substratos caros, métodos ineficientes de bioprocessamento, baixa produtividade de cepas microbianas e o alto custo das técnicas de jusante.

Essa situação reforça a necessidade de novas estratégias que permitam escala de produção de biossurfactante através de processos biotecnológicos (SANTOS et al., 2016). As estratégias para reduzir o custo de produção de biossurfactantes baseiam-se principalmente na seleção e engenharia de novos micro-organismos capazes de aumentar a produção; o uso de substratos de baixo custo para a formulação de meios de fermentação que diminuem os custos iniciais de matéria-prima; e o desenvolvimento de bioprocessos eficientes, incluindo otimização das condições de cultura e processos de recuperação de baixo custo para o máximo de biossurfactante (BANAT et al., 2014a; LOUHASAKUL et al., 2020). Neste sentido, as fontes de carbono e nitrogênio dos meios de cultura, especialmente para fins industriais, devem satisfazer, tanto quanto possível, os seguintes parâmetros: provêm de substratos econômicos, (b) estejam disponíveis durante todo o ano, (c) possibilitem o máximo rendimento de biomassa e formação do produto, (e) ser compatíveis com diferentes modos de cultivo (batelada, batelada alimentada ou contínuo), (f) não gerar resíduos perigosos e em quantidade maior que o resíduo inicial e (g) ser fáceis de manipular em todas as fases da produção (cultivo, extração, purificação e tratamento de resíduos) (KANDASAMY et al., 2019).

2.7.2 Glicerol residual

Na atualidade, o biodiesel ganhou amplo interesse como combustível alternativo e o Brasil se encontra entre os 5 grandes produtores de biodiesel do mundo. O biodiesel pode ser definido como um conjunto de ésteres monoalquílicos de ácidos graxos de cadeia longa derivados de triglicerídeos, ou fontes lipídicas renováveis por transesterificação ou esterificação com álcoois de cadeia curta (DING et al., 2016; ANDRADE et al., 2016; VELUTURLA et al., 2018). O biodiesel pode ser usado diretamente em motores a diesel sem (ou com poucas) modificações. Os mercados de biodiesel globalmente estão entrando em um período de crescimento rápido e transitório (AVHAD; MARCHETTI, 2015; VELUTURLA et al., 2018).

Por outro lado, o glicerol se obtém como subproduto fabricação de biodiesel e bioetanol. Também pode ser obtido a partir de processos de saponificação em indústrias oleo- químicas. Assim, o aumento na produção de biodiesel resultou em um aumento concomitante na

quantidade de glicerol bruto produzido (BASKAR; AISWARYA, 2016; SAMUL; LEJA; GRAJEK, 2014). Ele representa aproximadamente 10% do volume de uma reação para sintetizar biodiesel e como consequência, as empresas produtoras de biodiesel enfrentam sérios problemas em como resolver o excesso de glicerol, já que sua eliminação implica um alto custo. Assim, soluções para o gerenciamento dessa matéria-prima são extremamente necessárias (JOSÉ DE ANDRADE et al., 2016; SAMUL; LEJA; GRAJEK, 2014).

Este subproduto apresenta um baixo valor agregado devido à presença de impurezas. Além disso, dependendo da fonte de triglicéridos usada na produção de biodiesel, o glicerol bruto pode conter elementos nutricionais como fósforo, enxofre, magnésio, cálcio, nitrogênio e sódio, que podem ser usados por micro-organismos (SALAZAR-BRYAM; LOVAGLIO; CONTIERO, 2017). Daí que uma possível abordagem como alternativa para sua disposição seria seu reaproveitamento como substrato de baixo custo para a bioprodução de produtos de alto valor agregado, como biossurfactantes (GARLAPATI; SHANKAR; BUDHIRAJA, 2016).

2.7.3 Óleo pós fritura

Os óleos residuais de cozinha ou óleos pós fritura são gerados a partir de óleos vegetais (coco, girassol, soja, palmeira, semente de algodão, azeitona, entre outros) empregados para fritar alimentos (RINCON, CADAVID, ORJUELA, 2019). Durante o processo de fritura, que ocorre em altas temperaturas (160 °C a 200 °C), os óleos vegetais (compostos por triacilgliceróis, TAGs) sofrem muitas modificações físicas e químicas e compostos tóxicos são formados por meio de reações de oxidação, hidrólise e polimerização de TAGs (TSOUTSOS et al., 2016). Após um processo de fritura ao ar livre, a estrutura dos óleos de cozinha é modificada por reação de oxidação e é produzido hidroperóxido, que pode ser oxidado ainda mais em produtos tóxicos, como 4-hidroxi-2-alcenais (PANADARE, RATHOD, 2015).

Todos esses compostos gerados pela degradação dos óleos de cozinha têm efeitos prejudiciais à saúde humana. Eese efeitos podem ser mutagênicos, carcinogênicos, neurotóxicos e hepatoxicos, entre outros (TSOUTSOS et al., 2016; SCHLÖRMANN et al., 2020). Parâmetros físicos dos óleos vegetais, como cor, viscosidade, densidade e a tensão superficial também é afetada pelos processos de fritura. Algumas mudanças físicas podem ser avaliadas rapidamente por inspeção visual. O aumento da escuridão do óleo de cozinha é atribuído ao desenvolvimento de pigmentos durante a oxidação de ácidos graxos, reações de Maillard e oxidação de compostos fenólicos de óleos vegetais (SHENG et al., 2020). A

viscosidade aumenta com o número de ciclos de fritura devido a dímeros não polares e compostos poliméricos de alto peso molecular produzidos durante a polimerização de TAGs (TARMIZI, NIRANJAN, GORDON, 2013; KANDASAMY et al., 2019).

Estima-se que aproximadamente 0,9 milhões de toneladas de óleos pós fritura sejam produzidas por ano na União Europeia. Em países altamente populosos, grandes quantidades de resíduos são produzidas: China - 5,6 milhões de toneladas, Estados Unidos da América - 1,2 milhões de toneladas, Índia - 1,1 milhões de toneladas, Japão - 570 mil toneladas, Alemanha - 493 mil toneladas, República da Coréia - 411 mil toneladas, Espanha - 300 mil toneladas e Canadá - 148 mil toneladas (TEIXEIRA, NOGUEIRA, NUNES, 2018).

O descarte final do óleo pós fritura representa um problema devido ao seu alto volume, e a descarga incorreta em esgotos ou drenos causa bloqueios e problemas de odor ou vermes. Esse resíduo possui compostos que permanecem no ambiente por muitos anos, aumentam a carga orgânica nas fontes de água e formam uma fina camada sobre a superfície da água, reduzindo a concentração de oxigênio dissolvido necessária para espécies subaquáticas, alterando o ecossistema (GUERRERO-ROMERO, SIERRA, 2011; KANDASAMY et al., 2019). Para reduzir os impactos negativos nos ecossistemas, torna-se urgente a gestão, reciclagem e valorização do óleo pós fritura.

Neste sentido a produção de biossurfactantes a partir deste resíduo tem sido investigada em várias bactérias como *Pseudomonas aeruginosa* (CHEN et al., 2018; WADEKAR et al., 2012), *Bacillus* sp. (DURVAL et al., 2019; HENTATI et al., 2019; VEDARAMAN & VENKATESH, 2011) e *Streptomyces* sp. (SANTOS et al., 2019), entre outros. Em relação às linhagens de leveduras, as espécies de *Candida*, particularmente *Candida tropicalis* (ALMEIDA et al., 2017; BATISTA et al., 2010; JUNIOR et al., 2018; RUBIO-RIBEAUX et al., 2017) são as mais estudadas para a produção de biossurfactantes. Outras espececies de fungos filamentosos que tem sido utilizado para este fim são *Cunninghamella echinulata* (SOUZA et al., 2018) e *Rhizopus* spp. (PELE et al., 2019).

2.7.4 Resíduo de macarrão instantâneo

O macarrão é um dos alimentos diários mais populares do mundo. A indústria de fabricação de macarrão instantâneo é uma das indústrias mais importantes e depende completamente do suprimento de amido (CHA, WANG, 2020). A maioria dos relatos sobre o

resíduo de macarrão estão relacionandos à sua conversão como matéria prima para a produção de bioenergia (YANG et al., 2014 a, b).

Durante o processo de fabricação do macarrão instantâneo, uma enorme quantidade de fluxo de resíduos é drenada como um substrato naturalmente abundante do açúcar de fermentação com componentes constantes (LI et al., 2015; KUMAR, RABHASANKAR, 2017). Embora o desperdício de macarrão instantâneo tenha sido reconhecido como prejudicial à alimentação animal, devido ao óleo de palma adsorvente nos fragmentos residuais (SIKANDER, MALIK, KHAN, 2017).

O desperdício de macarrão é geralmente descartado sem nenhum tratamento adicional. O resíduo é gerado durante a produção, processamento, cozimento ou consumo de macarrão. Existem dois tipos principais de resíduos de macarrão: resíduos líquidos, que contêm principalmente amido formado durante a fase de produção industrial, e resíduos sólidos, que contêm amido, lignocelulose e óleo (TANNADY et al., 2019). No entanto, o resíduo de macarrão contém outros nutrientes como proteínas, vitaminas e minerais (TANG et al., 2019). Dessa forma, hidrolisado de resíduos de macarrão tem sido utilizado para o cultivo de microorganismos na produção de enzimas, pigmentos e lipídios. Wang et al. relataram a produção de glucoamilase usando resíduos de alimentos misturados como meio para o cultivo de *Aspergillus niger* sob condições de fermentação submersa. YANG et al., 2014 informaram uma conversão máxima teórica de bioetanol de 98,5% do por *Saccharomyces cerevisiae*. ANDRADE et al., 2018 investigaram a produção de biossurfactante por *Cunninghamella echinulata* em meio contendo resíduo de macarrão instantâneo (2%), licor de maceração de milho (2%) e óleo de pós-fritura (0,5%) utilizando fermentação submersa. Contudo, a produção de biossurfactante a partir deste resíduo atavés de fermentação em estado sólido não tem sido informada na literatura.

2.7.5 Borra de café

O café é uma das bebidas mais populares do mundo (JUNG et al. 2012). O consumo de café tem aumentado constantemente e o processamento do café produz grandes quantidades de sólidos resíduos na forma de polpa de café, casca de café prateado, borra de café gasto e casca de café. Esses resíduos têm aplicações limitadas, como fertilizantes, alimentos para animais, entre outros. Vários processos como fermentação, digestão anaeróbica, transesterificação e

extração foram adotados na valorização da borra de café para a obtenção de bioprodutos (KAVITHA et al., 2020).

A toxicidade inerente de vários constituintes no café também apresenta uma preocupação de contaminação ambiental (FERNANDES et al., 2017). Portanto, os esforços de pesquisa concentram-se na valorização dos resíduos gerados dentro do paradigma da economia circular, reduzindo a tonelagem enviada para aterro explorando a biomassa como matéria-prima em potencial (MATA et al., 2018). Assim, a utilização deste resíduo para a produção de metabólitos microbianos como biossurfactantes

2.7.6 Casca de abacaxi

O abacaxi (*Ananas comosus* (L.) Merrill), ocupa o terceiro lugar na colheita de frutas tropicais. A área global de colheita de abacaxi representa cerca de 35% da área global de colheita de frutas tropicais. A produção massiva de abacaxi e subprodutos relacionados, gera uma grande quantidade de resíduos. No caso da casca, o subproduto do processamento do abacaxi representa 40% a 50% do peso total de toda a fruta (OKPANACHI, AGBAJI, YARO, 2020). Além disso, o teor de proteína bruta e cinzas na casca é superior ao da polpa. A casca de abacaxi é rica em açúcares, proteínas, pectinas, vitaminas, minerais, fibras alimentares e pigmentos (BRITO et al., 2020).

As cascas de abacaxi contêm açúcares que podem apoiar o crescimento de microrganismos como *Saccharomyces cerevisiae, Candida utilis* e *Trichoderma viride*, converter esses resíduos lignocelulósicos por fermentação em produtos de valor acessível, nutritivo e seguro para o meio ambiente (ARUNA, 2019). No caso da produção de biossurfactante esse resíduo tem sido utilizado em diversas pesquisas como forma de minimizar a poluição ambiental.

Por exemplo, ALMEIDA et al., (2015) otimizaram e avaliaram a produção de biossurfactante por *Pantoea* sp. usando casca de abacaxi, gordura vegetal e licor de maceração de milho. AL-KASHEF et al., (2018) obtiveram glicolipídios fúngicos derivados da conversão microbiana de torta de óleo de girassol e mistura de casca de abacaxi como substratos econômicos para avaliar sua eficácia como inibidores de corrosão. MAUREEN, KAYODE, PEACE (2020) estudaram a produção de biossurfactante a partir de diferentes isolados

bacterianos. Os autores selecionaram *Pseudomonas fluorescens* PC20 a partir da sua capacidade de utilizar casca de mandioca e abacaxi como substratos não convencionais.

2.7.7 Bagaço de cana de açúcar

O suco de açúcar é extraído da cana e a parte fibrosa residual é chamada de bagaço. Inicialmente, o bagaço era usado principalmente na produção de papel. O descarte inadequado deste material pode criar problemas ambientais em torno das usinas de açúcar. Dessa forma, dentro das estratégias para enfrentar essa situação, esse resíduo matéria-prima de combustível na caldeira de cogeração da indústria açucareira para produzir eletricidade, devido ao seu valor calorífico, o bagaço também é usado como matéria-prima de combustível na caldeira de cogeração da indústria açucareira para produzir eletricidade (STAI 2015). Contudo, várias pesquisas têm abordado seu uso como fonte nutricional para a produção de diferentes metabólitos microbianos. Assim, CAMILIOS et al. (2011) investigaram a produção de ramnolipídios usando um método de cultivo em estado sólido com diferentes fontes de carbono e obtiveram a melhor produção de ramnolipídeos, 45 g/L da solução de impregnação utilizada, com uma mistura 50:50 (m / m) de bagaço de cana e milho farelo, suplementado com uma solução contendo 6% (v/v) de glicerol e óleo de soja. LOPES et al., (2017) investigaram a co-fermentação para a produção simultânea de ramnolipídios e etanol a partir do bagaço usando *Saccharomyces cerevisiae*, *Pseudomonas aeruginosa* e concluiram que o bagaço é uma excelente fonte nuticional para a síntese deses bioprodutos. MARTINS e MARTINS (2018) informaram que *Corynebacterium aquaticum* apresentou produção eficiente de biossurfactante ao usar resíduos de peixe e bagaço como fonte de carbono. A tensão superficial obtida para esses tratamentos foi de 27,8 mN/m e uma capacidade de emulsificação foi de 87,6%. O biossurfactante obtido potencial para a solubilização e remoção de tintas.

2.8 FERMENTAÇÃO EM ESTADO SÓLIDO (FES)

A fermentação em estato sólido é um processo realizado por micro-organismos que crescem em substratos sólidos e úmidos que atuam como fontes de nutrientes e suportam o crescimento microbiano na ausência ou quase ausência de água (MARCELINO et al., 2020). A busca por processos sustentáveis e ecológicos para transformar os processos químicos tradicionais destaca o potencial deste processo. Assim, a bioconversão de resíduos orgânicos em valiosos produtos biológicos poderia substituir materiais não renováveis e transformar processos químicos em práticas mais limpas no setor industrial (SINGH, PATIL, RALE, 2019).

O interesse particular na fermentação em estado sólido se deve a que esta tecnologia utiliza substratos de baixo custo e equipamentos simples, baixos volumes de água, baixa demanda de energia e maior concentração de produtos obtidos, em comparação à fermentação submersa (BANERJEE, BASAK, GHOSH, 2019).

Desde os primeiros estudos de produção de biossurfactantes, a fermentação submersa tem sido um campo de pesquisa dominante.

2.8.1 Substratos e micro-organismos utilizados em FES

Vários resíduos sólidos orgânicos podem ser utilizados na FES para produzir diversos biossurfactantes (Tabela 3).

Tabela 3- Micro-organismos produtores de biossurfactantes em fermentação em estado sólido

Micro-organismos	Substrato	Biossurfactante
Bacillus amyloliquefaciens	Palha de arroz e soja farinha	Surfactina
Bacillus pumilus UFPEDA 448	Okara e bagaço de cana de açúcar	Surfactina
Bacillus subtilis SPB1	Farinha de resíduo de folha de oliveira e farinha de azeitona	Lipopeptídeo
Brevibacterium aureum MSA13	Melaço pré-tratado	Lipopeptídeos
Pleurotus djamor *Pleurotus eryngii* *Pleurotus*	Casca de semente de girassol	Complexo polissacarídeo proteico-lipídeo
Bacillus subtilis DM-03 e DM-04	Cascas de batata	Lipopeptídeo
Pleurotus ostreatus	Casca de semente de girassol	Complexo de carboidrato-peptídeo-lipídeo
Starmerella bombicola NRRL Y-17069	Farelo de trigo, casca de isabgol	Soforolipídio

Adapatado de COSTA et al. (2018)

Entretanto, além de com baixos rendimentos e fluxos de produtos diluídos, pode ocorrer espuma severa e um aumento gradual na viscosidade (KANDASAMY et al., 2019). A adição de antiespumantes químicos para superar o problema implica um efeito negativo na produção de biossurfactantes (DOLMAN, WANG, WINTERBURN, 2019).

Por exemplo, resíduos de processamento de soja cultivados mostraram que *Candida guilliermondii* produziu um complexo glicolipídico, enquanto *B. subtilis* produziu lipoproteína e glicolipídios (SITOHY et al., 2010). Também foi relatado que *B. subtilis* produziu biossurfactantes lipopeptídicos quando cultivados em casca de batata a partir de resíduos orgânicos de cozinha (DAS, MUKHERJEE, 2007). O rendimento máximo de soporolipídeos (18 g/mg de substrato seco) foi obtido pelo cultivo de *Starmerella bombicola* em uma mistura de farelo de trigo, glicose e ácido oleico. Os autores informaram que empregando uma mistura a produção de soporolipídios aumentou 31% (JIMÉMENZ-PEÑALVER et al., 2016). Além disso, o cultivo de *Pseudomonas aeruginosa* em uma mistura de bagaço de cana e farelo de milho suplementado com glicerol e óleo de soja na FES produziu 45 g/L de ramnolipídeos (CAMILIOS et al., 2011). Por outro lado, uma grande variedad dessas moléculas tem sido obtida através do uso dessa tecnologia.

3 ARTIGO 1- GLYCOLIPID PRODUCTION BY *CANDIDA TROPICALIS* UCP 1613 AS SUSTAINABLE ALTERNATIVE FOR APPLICATION IN AGRICULTURE

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Abstract

In this study was evaluated the production of biosurfactant by *Candida tropicalis* UCP 1613. The lowest surface tension (25.3 mN m^{-1}) was obtained when the yeast was grown in an optimized medium which consisted in minimal salt medium supplemented with 8.41 % (w/w) of residual glycerol and 3.35 g L^{-1} of glutamate. The biosurfactant exhibited a critical micelle concentration of 350 mg L^{-1} and the maximum yield was detected in the stationary phase with 3.6 g L^{-1}. The chemical structure of the molecule was confirmed as glycolipid through the biochemical tests and the Fourier transform infrared spectroscopy and the water activity assessed was 0.41 aw. The biosurfactant showed an inhibitory effect *in vitro* on *Alternaria alternata* and *in vivo* using cherry tomatoes. The experimental evidence showed that the compound obtained is a suitable antimicrobial agent with potential application in the management disease caused by the phytopathogenic fungi.

Keywords: biosurfactant, raw glycerol, water activity, cherry tomato, phytopathogenic fungi

1 Introduction

Glycolipids biosurfactants include a heterogeneous family of amphiphilic compounds produced by microorganisms, plants, animals and humans. These molecules comprise structurally a fatty acid moiety bound by glycosidic linkage to a carbohydrate moiety (Jahan et al., 2019; Santos et al., 2016). The type of carbohydrate moiety allows mainly subdivided these molecules into: rhamnose lipids, sophorose lipids, cellobiose lipids, trehalose lipids, mannosylerythritol lipids, lipomannans monoacylglycerol, and galactosyl-diglyceride (Mnif and Ghribi, 2016). Among

the diverse group of biosurfactants, glycolipids are the most studies and commercialized (Lodens et al., 2019). Compared to their synthetic homologues or plant-derived, microbially-produced glycolipids biosurfactants exhibit higher surface activity, lower critical micelle concentrations, higher emulsifying power and biodegradability as well as lower ecotoxicity. In addition, they display selectivity and specific activity at extreme conditions of pH, salinity and temperature (Pacwa-Plociniczak et al., 2011; Makkar et al., 2011; Fenibo et al., 2019). In addition, they display interesting properties as emulsifiers, solubilizers and antimicrobial agents which allow them its application in different areas such as cleaning, food industry, bioremediation, medicine and agriculture (Mulligan 2005; Andrade et al., 2018, Araújo et al., 2019). However, the low quantities produced by microorganisms along with the high cost of the downstream processing (~60-80%) make most of them not competitive in the market (Banat et al., 2014; Santos et al., 2016). To overcome these drawbacks alternatives such as, the use of inexpensive raw materials and the optimization of production by means of response surface methodology have been the main subject of several studies (Banat et al., 2014).

Cherry tomato (*Lycopersicon esculentum*) is a crop widely consumed that stands out for presenting an excellent taste and higher nutritional quality (Sun et al, 2015). However, significant postharvest losses are reported due to the susceptibility of the fruit to several phytopathogens. Among them, *Alternaria alternata* have been described as one of the most destructive pathogens (Black–Solis et al., 2019; Moghaddam et al., 2019). In this sense, chemical synthetic fungicides are the principal mean to manage the fungal proliferation. However, the high risk of these compounds to the environment and the development resistant phytopathogens encourages the search for novel eco-friendly molecules with effective antifungal activity (Hammami et al., 2011; Mnif et al., 2015).

Previous studies have highlighted the important role of glycolipids biosurfactants in plant defense through the inhibition the growth of fungal phytopathogens. For instance, in vivo development of Botrytis cinerea was inhibited by a derived cellobiose lipids from *Ustilago maydis* which was inoculated with fungi sporidia (Teichmann et al., 2007). Studies conducted to evaluate the antifungal efficacy of a biosurfactant produced by *Pseudomonas aeruginosa* JS29 on *Colletotrichum capsica* revealed the inhibition of both spore and mycelia of the phytopathogen, while in vivo tests confirmed the significant reduction the fungal growth. Similarly, the severity of the disease caused by *Fusarium oxysporum* in tomato was reduced by 95 % under the action of a rhamnolipid obtained *Pseudomonas aeruginosa* KVD-HM52. However, the reports are scarce regarding the evaluation of glycolipids biosurfactants obtained from *Candida* sp. as inhibition agents for the control of phytopathogen fungi. Bearing this in

mind, the objectives of this study were to optimize a culture medium for biosurfactant production from *Candida tropicalis* UCP 1613 and to investigate the effect of the molecule on *Alternaria alternata*, pathogen of cherry tomato.

2 Material and Methods

Substrates and culture preparation of microorganisms

Raw glycerol of cotton biodiesel (RG) and post fry soybean oil (PSO) were kindly provided by obtained from Cetene (Centro de Tecnologias Estratégicas do Nordeste, Recife, Pernambuco; Brazil) and a local restaurant (Recife, Pernambuco; Brazil) respectively. The yeast *C. tropicalis* UCP 1613 was received from the culture collection of Nucleus of Resources in Environmental Sciences, Catholic University of Pernambuco, Recife, Brazil. The microorganism was kept at 4 °C in Yeast Mold Agar (YMA) slant with the following composition (g/L): yeast extract, 3; malt extract, 3; peptone, 5; glucose, 10; agar, 20 at final pH 6.5. The Erlenmeyer flask (150 mL) containing 50 mL Yeast Mold Broth (YMB) (the same composition above without agar) were inoculated with a loop full of the microorganism freshly grown on YMA slant (Luna et al., 2011). Subsequently the culture was incubated at 48 h at 28 °C; 150 rpm in a rotary incubator shaker (TECNAL, TE-421) and after that period the concentration of cells was adjusted to 1×10^7 cells/mL. *Alternata alternata* was provided by the Plant Physiology Department of the Federal Rural University of Pernambuco and maintained on potato dextrose agar medium (PDA) which contained: extract of 200 mL of boiled potatoes, 20 g of dextrose, 20 g of agar and 800 mL of distilled water (He et al., 2011).

Optimization of carbon and nitrogen sources

For the preliminary studies, a 2^4 full factorial design was developed to determine which component significantly affected the biosurfactant production. In this study was used a mineral salt medium (MSM) for the screening stage as follows (g/L): 0.2 Mg_2SO_4, 1.0 K_2HPO_4, 1.0 KH_2PO_4, 0.02 CaCl2, 0.05 $FeCl_3$, supplemented with different concentrations of Soybean waste frying oil (SWO) (X_1), Glycerol (X_2) and Glucose (X_3) as carbon sources, and Glutamate (X_4) as nitrogen source as shown the Table 1.

All media were adjusted to pH 6.5 using 2 M HCl before autoclaving and Glucose and Soybean waste frying oil were autoclaved apart and added to pre-sterilized medium conditions. The flasks were incubated for 96 h at 28°C; 150 rpm in a rotary incubator shaker and the biosurfactant production assessed through the reduction of the surface tension in the cell-free culture was considered as the dependent variable.

Table 1- Values of the input variables used in the full factorial design

Variables	Levels		
	-1	0	1
X_1	2	4	6
X_2	2	4	6
X_3	1	2	3
X_4	1	0.5	1.5

Subsequently, with the variables indicated as significant from the previous design, a further central composite rotated design (CCRD) of 22 was established to study the performance in the optimum region of surface tension reduction (Z1). Two independent variables (Raw glycerol: Y1, Glutamate: Y2) were established as input variables with the reduction of surface tension as output variable (Table 2). A set of 11 experiments with three replicates at the central points was considered in this new approach.

Table 2- Values of the variables used in the Central Composite Rotational Design (CCRD)

Variables	Levels				
	-1.41	-1	0	1	1.41
Y_1	5.59	6	7	8	8.41
Y_2	2.29	2.5	3	3.5	3.71

The response surface regression procedure allowed to fit the experimental results through the following second-order polynomial equation:

Moreover, the coordinates of optimum point were calculated through the equation to estimate the maximum response which is higher surface tension reduction. The coefficient of determination R^2 was used to evaluate the fitting quality of the quadratic model. The effects of variables on response and the validity of the chosen quadratic model, were estimated using the p-value and f-value respectively.

Surface activity measurements

The measurements of surface tension were performed on cell-free broth after cell removal by centrifugation at 5000 rpm for 20 min in a centrifuge (Hermle Z513K) at 4°C. The Du–Nuoy ring method was employed for this purpose using a Tensiometer Sigma 70 (KSV Instruments LTD, Finland) at room temperature (25°C) (Kuyukina et al., 2005).

Time course of biosurfactant production

The growth profile was obtained from the optimized condition prepared in 500 mL Erlenmeyer flasks each containing 200 mL of production media. The growth was initiated with 10^4 cells/mL and the flasks were incubated at 28°C in an orbital shaker at 150 rpm for 96 h. Samples were withdrawn at regular intervals, every 24 h samples to determinate surface tension, biomass, pH and yield of biosurfactant. The measurements were carried out in triplicate. The crude biosurfactant and the biomass were determined by the gravimetric method and their yields were reported in g/L (Rufino et al., 2014).

Extraction of the Biosurfactant

The cell-free supernatants were used after 96 h to recover the biosurfactant. The samples were adjusted to pH 2 with HCl and precipitated with two volumes of methanol and maintained for 24 h at 4 °C. Subsequently, the obtained precipitates were obtained by centrifugation (10,000g, 20 min, 4 °C) and washed twice with cold methanol. The crude biosurfactants were dried for 48 h at 37°C, and then placed in a desiccator to reach a constant weight. The biosurfactant yields were expressed in g/L all were measured in triplicate (Luna et al., 2009).

Biochemical analysis of biosurfactant

Protein was estimated using the method of Lowry et al. (1951). Carbohydrate was assessed by phenol–sulphuric acid method (Dubois et al. 1951). Lipid component was determined according to the methods described by Manocha et al. (1980) through gravimetric estimation. The results were reported as the average of measurements in triplicate. The water activity (aw) of the biosurfactant was measured using (Novasina AG CH 8853 Lanchen, Switzerland) at room temperature. Approximately, 0.5 g of the compound was placed in a plastic container to the a_w meter and the registered value of the aw was showed directly. The crude biosurfactant was analyzed using Fourier transform infrared spectroscopy (FTIR) to identify some component from the unknown mixture. The FTIR spectrum with the main functional groups was obtained through a FTIR spectrophotometer (Bruker IFS 66) at a frequency range of 4000-400 cm^{-1} using KBr pellet as the background reference (Ghojavand et al., 2008).

Critical micelle concentration (CMC)

The Critical Micelle Concentration (CMC) was determined from known biosurfactant amounts prepared in distilled water. The surface tension of the crude biosurfactant samples were measured in triplicate at room temperature (25°C). The CMC was determined when the surface tension showed a constant value (Manivasagan et al., 2013).

Application of the glycolipid produced by *Candida tropicalis* UCP 1613

Antifungal action of glycolipid against the phytopathogen *Alternaria alternata*

The in vitro antifungal assay of the biosurfactant was examined by using different concentrations of biosurfactant (175, 350, 500, 750 μg/mL) supplemented in PDA medium. Petri dishes of 90 mm containing the medium were inoculated at the center with mycelial plugs (5 mm diameter) obtained from the perimeter of growing active colonies. The negative controls were established as the plates with culture medium without biosurfactant. The mycelial growth (diameters) was verified after 7-10 days of incubation at 24°C and was compared with the controls. The fungal growth inhibition (FGI) was calculated according to the following formula:

$$FGI\ (\%) = ((A-B)/A) \times 100$$

Where: A and B denote the diameter of fungal mycelium growing on control and treated Petri dishes, respectively. All experiments were informed as the average of standard deviation in triplicates (Lutz et al., 2017).

Optical and scanning electron microscopy of mycelia treated with glycolipid

The detect the morphological alterations the hyphae treated were stained with lactophenol cotton blue and observed using an optic microscopy (Zeiss Standard 20) at 100X of magnification. The samples for scanning electron microscopy (SEM), were washed with phosphate buffered saline (PBS) at pH 7.2. After that, they were placed in a solution of 2.5% of glutaraldehyde in phosphate buffer (0.1 M), pH 7.4 for 1 h at room temperature. Then, the dehydration of the cells was carried out with increasing concentrations of ethanol: water according to Zeraik and Nitschke (2010). The mycelia were treated with 0.5 mL 1,1,1,3,3,3-Hexamethyl-disilazane and the samples were mounted on stubs prepared with sticky carbon tapes placed and coated with gold to analyze in scanning electron microscopy (LSM JEOL 5600 LV) operating at 20 Kv.

Effect of glycolipid in postharvest *in vivo* assay

Sets of 20 Cherry tomatoes (Lycopersicon esculentum) were selected taking into account the following criteria: equal size and absence of any disease of wound. A sodium hypochlorite 2% was used to disinfect the surface of the fruits for 10 min, followed by rinsing 3 times by sterilized distilled water (Sharma et al., 2018). After this process, the tomatoes were dried with a towel paper and placed at room temperature. A sterile syringe was used to make a wound in each fruit. Subsequently, the negative control group was treated with 30 μL of sterile distilled water and the treated group was inoculated with 30 μL of the biosurfactant at 750 μg/mL. 10

μL of a spore suspension of A. alternata (10^4 spores mL^{-1}) was inoculated into each wound in the positive control group and in the treated group. Afterwards, the cherry tomatoes were stored in enclosed in Petri dishes with a high relative humidity at 25 °C. The experiments were repeated in triplicate twice and the after 10 days the incidence was evaluated as follow:

The incidence percentage (I%) was calculated as = 100 × [(number of inoculated wounds − number of disease wounds)/number of inoculated wounds]

Efficiency of glycolipid in the cell membrane permeability of the phytopathogen

For this assay the phytopathogen was grown in PDB at 28°C for 14 days with (750 μg/mL) and without biosurfactant and after that period the biomass was centrifuged and washed with deionized water. Subsequently, for each treatment 1 g was weighted and placed into beakers containing 20 mL deionized water. In order to disrupt the plasma membrane of the second group, the beakers were heated in the boiling water bath for 30 min. The conductivity of both treatments was determined with a conductivity meter (Novasina AG CH–8853 Lanchen), and the electrolyte leakage was established as the ratio of the conductivities of the measurements. The data were expressed as the average of three independents results (Yan et al., 2014).

Statistical analysis

STATISTICA software 7 allowed the graphical analysis of the data and the experimental designs. All the experiments were expressed as the arithmetic averages of measurements repeated three times with standard deviation (±).

3 Results and discussion

Production of biosurfactant

Table 3 shows a set of 16 experiments with four repetitions at the central point considered for this study as well as the experimental and predicted results. As the presence of microbial surfactants reduced the surface tension, *C. tropicalis* UCP 1613 was considered a biosurfactant producer from the tested substrates (Table 3). The excellent ability of this yeast to produce biosurfactant was confirmed from the data obtained with values of the surface tension which varied from 40 mN/m to 26.2 mN/m. Similar results were reported by other authors when investigated the culture medium composition from alternative substrates (Sobrinho et al. 2009; Santos et al., 2013).

Table 3- Full factorial design (2^4) matrix with the observed and predicted values with surface tension as response

Run	X_1	X_2	X_3	X_4	Surface Tension (mN/m)	
					Experimental	Predicted
1	-1	-1	-1	-1	39.4	34.7
2	1	-1	-1	-1	40.0	35.4
3	-1	1	-1	-1	32.1	31.5
4	1	1	-1	-1	27.0	32.2
5	-1	-1	1	-1	34.7	34.1
6	1	-1	1	-1	27.4	34.7
7	-1	1	1	-1	36.8	30.9
8	1	1	1	-1	31.9	31.5
9	-1	-1	-1	1	28.5	30.4
10	1	-1	-1	1	34.2	31.1
11	-1	1	-1	1	26.2	27.2
12	1	1	-1	1	30.4	27.9
13	-1	-1	1	1	27.1	29.8
14	1	-1	1	1	36.7	30.4
15	-1	1	1	1	27.7	26.6
16	1	1	1	1	27.2	27.2
17	0	0	0	0	27.5	31
18	0	0	0	0	28.1	31
19	0	0	0	0	26.8	31
20	0	0	0	0	27.1	31

Figure 1 illustrates the Pareto chart with the main effects of the substrates evaluated on the surface tension. Since the factorial design allows to select the variables and it is not for optimization, it was established 90 % as the confidence interval. Among the variables screened the Raw Glycerol showed the most significant contribution on the reduction of the surface tension, followed by Glutamate. As a result, an increase in the concentrations of these two factors caused a negative effect on the surface tension and consequently, this variable decreased, which was desirable. Moreover, the influence of the Glucose was no significant

statistically and the PFO concentration had an adverse effect despite its effect was no significant statistically. On the other hand, the lack of adjustment of the linear model was determined through the curvature effect that indicated the proximity of the optimal region, based on its negative and statistically significant effect on surface tension.

Interestingly, the current study showed that *C. tropicalis* UCP 1613 preferred the raw glycerol as the main carbon source for biosurfactant production. In this sense, once that Glycerol is in the intracellular environment a glycerol kinase acts quickly to convert it to glycerol–3–phosphate which is oxidized to dihydroxyacetone phosphate (DHAP) by a glycerol phosphate dehydrogenase. Subsequently, DHAP is transformed into its isomer glyceraldehyde–3–phosphate (G3P) and both can be converted to glucose by gluconeogenesis. This process is an alternative to provide the sugars present in the hydrophilic moiety of the biosurfactant structure or also a path to guarantee the cellular growth (Fontes et al., 2012). By other side, the lipidic moiety could be synthetized by partial β–oxidation of oil fatty acids, which are abundant in this substrate. Hence, the synthesis de novo it is not necessary in the process. In addition, an advantage of this finding is that glycerol is not a component of food and feed, which is the case of glucose (Nakamura et al., 2003). Since raw materials account for 10 to 30 % of the production of biosurfactant, an economical solution for this drawback is the use of low–cost raw substrates (Cameotra and Makkar, 1998; Olasanmi and Thring, 2018).

Regarding the use of monosodium glutamate as nitrogen source, several microorganisms have used this compound for biosurfactant production. For instance, *Selenomonas ruminantium* CT2, showed the minimum value of surface tension and yield of biosurfactant (33.49 mN/m and 1.60 g/L, respectively) when monosodium glutamate was tested in the culture medium. In addition, the yield improved nearly three folds when compared with $(NH_4)_2SO_4$, along with the biomass and the emulsification index (Saimmai et al., 2013). *Ochrobactrum anthropi* 2/3 produced a biosurfactant that reduced the surface tension to 25.0 mN/m in an optimized medium containing a minimal salt medium supplemented with 25% (v/v) palm oil decanter cake and 1% (w/v) commercial monosodium glutamate. Moreover, under these conditions the highest biosurfactant production and the critical micelle concentration were 4.52 g/L and 8.0 mg/L respectively (Noparat et al., 2013). By other side, *Rhizopus arrhizus* UCP 1607 synthetized a biosurfactant/bioemulsifier when the main components in the culture medium were soybean post-frying oil (5% v/v) and sodium glutamate (1% w/v) medium. The fungi decreased the surface tension to 31 mN/m and formed stable emulsions in crude oil with a maximum value of 94.8 % (Pele et al., 2017).

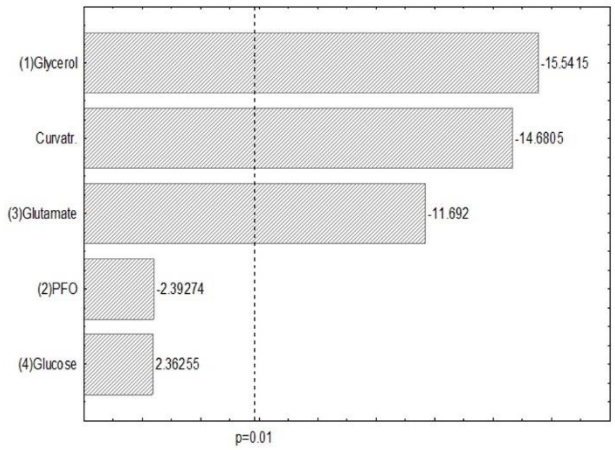

Figure 1- Pareto diagram of standardized effects of Raw Glycerol, PFO, Glucose and Glutamate used by *C. tropicalis* UCP 1613 on surface tension as response variable. The dashed line indicates the statistically significant (p = 0.050)

Based on the data from the factorial design, the glycerol and glutamate concentrations were selected as the factors to determine the response surface in the proximity of the optimum. The Table 4 shows the combination of possibilities between the two proposed variables with three repetitions at the central point and 4 axial points for the central composite design (CCRD).

The performance of the surface tension reduction in function of the independent variables was obtained through the second-order model equation which consisted in six terms were Y_1 and Y_2 were associated to Raw Glycerol and Glutamate concentrations, respectively.

The regression analysis on the experimental results fitted with a second-order polynomial equation (Equation 2):

Surface tension (mN/m) = $84.7 - 44{,}8\ Y_1 + 6.54\ Y_1^2 + 4{,}51\ Y_2 - 0.36\ Y_2^2 + 0.15\ Y_1Y_2$

The statistical significance of the second-order model equation was tested through the ANOVA. In addition, multiple correlation coefficient R and the determination coefficient R^2 were determined from ANOVA. The data showed that only the linear and quadratic terms of Glycerol were statistically significant ($p < 0.05$).

Moreover, the value of R (0.93985) which was close to 1, indicated the high correlation between the observed and predicted values. By other side, the value of R^2 was 0.96992, which means that when the computed F–value is greater than the tabular F–value the proposed predicts experimental data well.

Table 4- Central composite design (CCD) with surface tension as response for the optimization of biosurfactant production by *C. tropicalis* UCP 1613

Run	Y_1	Y_2	Surface Tension	
			Experimental	Predicted
1	-1	-1	29.6	30,0
2	1	-1	25.5	25,3
2	-1	1	29.1	29,9
4	1	1	25.3	25,5
5	-1.41	0	33.6	32,9
6	1.41	0	26.4	26,5
7	0	-1.41	25.8	25,7
8	0	1.41	26.2	25,7
9 (C)	0	0	26.7	26,4
10 (C)	0	0	26.5	26,4
11 (C)	0	0	26.0	26,4

In this study the computed F–value (31.97) was nine times higher than the tabular F–value (3.32) at the 5% level. Consequently, the regression model was suitable which indicates could be used to describe biosurfactant production.

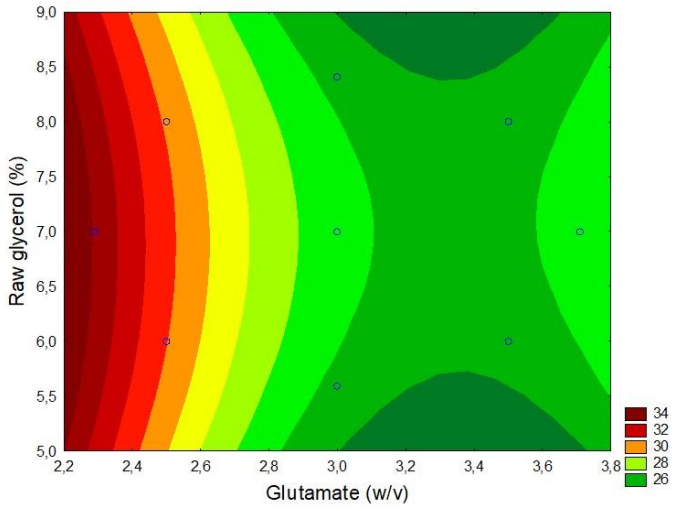

Figure 2- Contour plot for biosurfactant production by *C. tropicalis* UCP 1613 in mineral salt medium supplemented with Glycerol (%) and Glutamate (w/v).

In this sense the validation of the statistical model was performed through the repetition in triplicate at the maximum biosurfactant production, which corresponded to 8.41 % of Raw glycerol and 3.35 g L^{-1} of Glutamate with a surface tension of 25.3 mN/m.

Time course of biosurfactant production

After the analysis of the statistical model though RSM, the variations of the biomass, pH, surface tension and synthesis of biosurfactant from *Candida tropicalis* UCP 1613 were studied during 96 h in the condition selected (Glycerol: 8.41% (w/w) and Glutamate: 3.35 g L^{-1}). As can be seen from the figure 3, the exponential growth was observed from 48 h of the experiment. In addition, the lowest value of surface tension was recorded in the stationary phase with a decreased from 65 to 24.8 mN/m. However, the highest yield of biosurfactant was detected at the end of the stationary phase with 3.6 g/L as the maximum quantity of the metabolite.

Concerning the yield of biosurfactants obtained from agroindustrial wastes, the literature has showed the potential of *Candida* spp. to produce these metabolites. Almeida et al. (2018) when evaluated the production of biosurfactant by *C. tropicalis* obtained a maximum biosurfactant yield of 4.11 g/L in a culture medium composted by 2.5% waste frying oil, 2.5% corn steep liquor and 2.5% molasses.

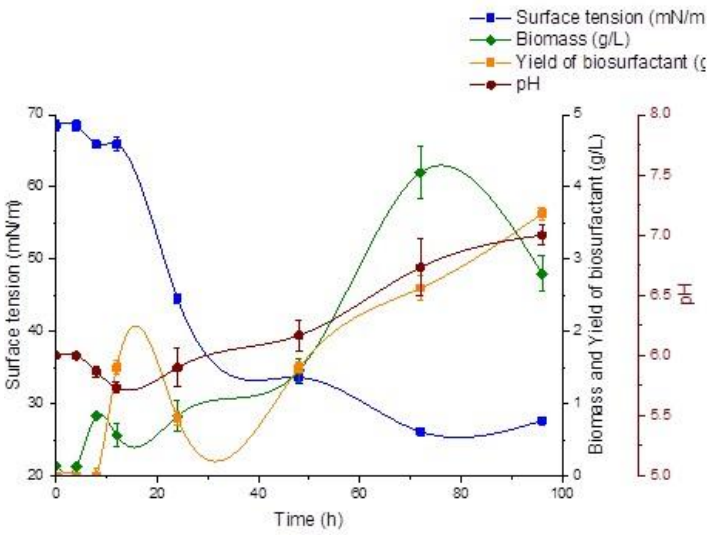

Figure 3- Profile of biosurfactant production with growth, pH, surface tension and yield of *C. tropicalis* UCP 1613. The data represents three independent measurements with standard deviations.

However, the experimental evidence obtained from studies conducted with some yeasts values results comparable with those informed for bacteria (Luna et al., 2013; Rufino et al., 2014).

Sobrinho et al. (2008) reported a yield of biosurfactant of 4.5 g/L from *C. sphaerica* grown on industrial refinery residue of soybean oil and corn steep liquor as precursors of the microbial

synthesis. A vast number of reports in the literature have shown the excellent ability of bacteria such to decrease the surface tension to values around the 25 mN/m (Varjani et al., 2017).

In this study, the pH practically did not change until the 24 h and after this period began to rise until approximately 7 at 72 h. Some authors have shown that the lack of control of the pH during the process of fermentation along with the slight variation during the process could contribute to high yields of biosurfactant (Luna et al., 2013, Rufino et al., 2008). Alternatively, Cunha et al. (2004) investigated the production of biosurfactant from gasoline as substrate and observed that the pH of the medium was around 6.0 at the end of the experiment. These results suggest that the adequate pH required for the synthesis of biosurfactants is specific for each microorganism, depending on the type of biosurfactant.

Preliminary characterization of biosurfactant

Preliminary chemical analysis of the biosurfactant produced by *C. tropicalis* UCP 1613 indicated that the molecule was a glycolipid which consisted of 74 % of carbohydrates and 21% of lipids. Similar composition was observed in the glycolipid produced by *C. sphaerica* which contained of 70% lipids and 15% carbohydrates (Luna et al., 2013). However, 83% carbohydrate and 17 % were detected the Liposan synthetized by lipolytica when grown in hexadecane as substrate (Cirigliano and Carman, 1985).

The biosurfactant exhibited 0.41 aw, which is consider a low water activity. This result agrees to the value reported by Ghazala et al., (2018), with 0.437 aw for a biosurfactant obtained from Bacillus mojavensis I4. Interestingly, Bouassida et al. (2017) noticed values of water activity between 0.2 and 0.32 in formulations containing a lipopeptide obtained from Bacillus subtilis SPB1. According to Barbosa et al. (2007) an acceptable aw for toothpaste should be between 0.585 and 0.984. On the contrary, fungi require above 0.80 aw to initiate the metabolic activity (Gizachew et al., 2019). For instance, Pose et al. (2009) detected the shortest germination time of Alternaria alternate at 0.982 aw, both at 21°C and 35°C. Likewise, Vaquera et al. (2014) informed a similar value of water activity (0.995 aw), measured at both 25°C and 30°C for *Alternaria arborescens*. The result obtained in the current study for the biosurfactant produced by *C. tropicalis* UCP 1613 suggests that the molecule could have an optimal performance above the value registered, which is below the minimum established for the fungal growth.

Figure 4 illustrates the IR spectral analysis of the biosurfactant produced by *C. tropicalis* UCP 1613. The weak bands at 3337.05 cm^{-1} and 1406.65 $cm^{-}1$ are representative of O-H groups suggesting that the molecule contains alcohol groups. In addition, the excitation bands at

2921.13 cm^{-1} and 2845.89 cm^{-1} correspond to C–H stretch may include contributions of alkane groups. The band at approximately 1735.42 cm^{-1} was consistent with a C=O which could be related to lactone, ester or acid group. By other side, the absorption peaks at 1104.95 cm^{-1} and 1029 cm^{-1} confirmed the presence of C–N and C–O bonds vibrations, respectively. In general, the structural patterns of the molecule were previously reported in the literature to others biosurfactants produced by *Candida* sp. (Hu et al., 2001; Chandran et al., 2011; El-Sheshtawy et al., 2013).

In addition, among the properties of microbial surfactants, critical micelle concentration (CMC) is one of the most important due to determine their efficiency (Satpute et al., 2017; Veshareh et al., 2018). The minimum concentration of biosurfactant at which micelles are formed and the surface tension reaches its lowest and steady value is known as the critical micelle concentration.

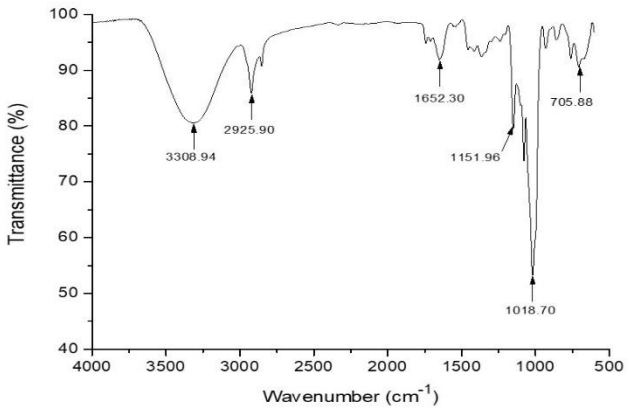

Figure 4- FT-IR absorption spectrum of the biosurfactant produced by *C. tropicalis* UCP 1613

on culture medium containing

In addition, among the properties of microbial surfactants, critical micelle concentration (CMC) is one of the most important due to determine their efficiency (Satpute et al., 2017; Veshareh et al., 2018). The minimum concentration of biosurfactant at which micelles are formed and the surface tension reaches its lowest and steady value is known as the critical micelle concentration. The crude biosurfactant was dissolved in pure water at concentrations ranging from 50 to 500 mg/L. As shown in Fig. 5, CMC of the biosurfactant produced by *C. tropicalis* UCP 1613 was 350 mg/L at a surface tension of 25,9 mN/m.

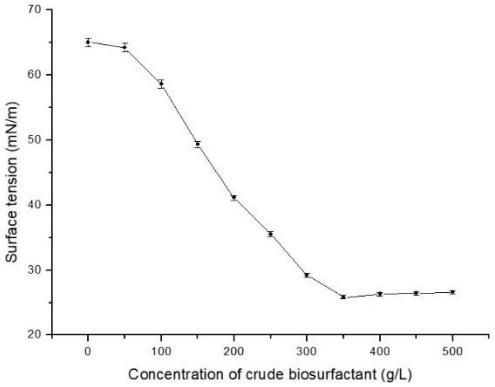

Figure 5- Critical micelle concentration of the biosurfactant produced by *C. tropicalis* UCP 1613 (results expressed as the average of three independent experiments ± standard deviation)

Senthil et al. (2019) observed a CMC of 30 mg/L at 28 mN/m from a glycolipid produced by *Cyberlindnera sa*turnus SBPN-27 in glucose mineral salts medium, which diverges greatly from the value informed in this study. In contrast, Luna et al. (2011) reported a reduction up to 25 mN/m at a CMC of 250 mg/L for a biosurfactant synthetized by *Candida sphaerica* UCP 0995 from 9% refinery residue of soybean oil and 9% corn steep liquor. In addition, Galdino et al., (2019) found that a biosurfactant obtained from *Candida utilis* UFPEDA 1009 in mineral medium containing 6% canola frying oil and 6% glucose was able to reach the CMC at 600 mg/L. According to Oliveira et al., (2013), variations in the CMC may be attributed to the level of purity of the biosurfactant as well as the solvent used. Alternatively, conventional surfactants such as sodium dodecyl sulfate (SDS) and tetra decyl trimethyl ammonium bromide (TTAB) possesses CMC values between 1000 mg/L and 2000 mg/L (Meneses et al., 2017). From these findings, the CMC of the biosurfactant produced by *C. tropicalis* UCP 1613 show a better activity than those reported for synthetic surfactants.

Antifungal activity of the glycolipid against the phytopathogen *Alternaria alternata*

The antifungal effect of four different concentrations of the biosurfactant (0, 350, 500, 750 μg/mL) on the growth of *A. alternata* was also verified. The data revealed that the increasing concentrations of the biomolecule had a negative influence on the growth rate of the phytopathogen. At the concentration of 350 μg/mL the biosurfactant showed a low inhibitory effect on mycelial growth of A. alternata with 29,63%, while at 500 μg/mL the inhibition ratio recorded was 49,53%. In this sense, Yan et al., (2014) tested the fungicidal activity of a glycolipid produced from *P. aerugi*nosa ZJU-211 against *A. alternata* and they observed that

around 50% of the fungal growth was inhibited at 1000 µg/mL. However, the percentage of inhibition in the current study was similar with less concentration of the biosurfactant, indicating its effectiveness.

Several studies have focused on the use of glycolipids as antifungal agent against phytopathogenic fungi (Kim et al., 2002). In general, direct effect on the cell surface structure is described as the main mechanism of action of glycolipids as fungicidal compounds. The physical assembly of the membrane bilayer is modified by these molecules when disrupted the protein conformation. This structural state affects directly the transport and energy production in the cell of the phytopathogen and consequently cause the cellular lysis (Lakhar et al., 2015).

Morphological observations of mycelia treated with glycolipid

By other side, in order to observe the direct inhibitory effect of the biosurfactant on the mycelial growth, the microorganism was cultured with different concentrations of biosurfactant in potato dextrose broth (PDB) at 28°C for 10 days. Microscopic examinations of *A. alternata* treated with 350, 500 and 750 µg/mL of the glycolipid produced by *C. tropicalis* UCP 1613 exhibited alterations of the hyphae morphology. As shown in the figure 6, with the increasing concentrations of the biosurfactant the hyphae treated depicted modifications in the structure such as: higher septation and vegetative spore formation (6B), disrupted patterns of growth and branching along with atypical bending (6C) and cellular lysis (6D). However, a normal growth with a smooth surface was detected in the hyphae from the control group (6A).

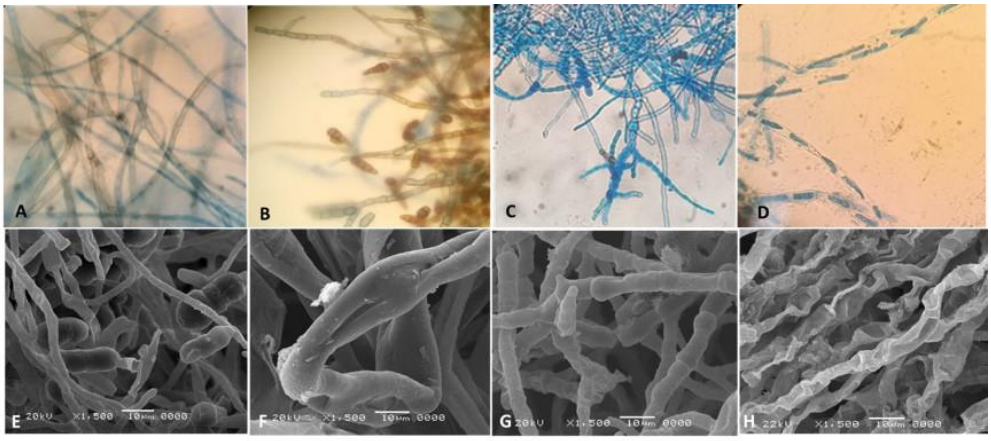

Figure 6- Optical and scanning electron microscopy of the hyphae of *A. alternata* treated with glycolipid obtained from *C. tropicalis* UCP 1613. Hyphae cultured without biosurfactant, hyphae cultured without biosurfactant (A, E); hyphae cultured with glycolipid: 350 µg mL^{-1} (B, F), 500 µg mL−1 (C, G), 750 µg mL^{-1} (D, H).

Regarding SEM observations, the images showed a normal growth with a straight appearance in the hyphae without biosurfactant (6E), while a swollen and irregular morphology was observed in the hyphae treated (Figure 6F, 6G). In addition, it was evident the blended of the hyphae with each other, causing distortion in the mycelial structure (6H). The effect was more accentuated as the concentrations of biosurfactant were increasing from the lowest to the highest. Since mycelium is a key structure due to its function on asexual reproduction in pathogen infection, the finding of biomolecules with effective action is imperative to control the phytopathogen development.

Effect of glycolipid in postharvest *in vivo* assay

For this study, cherry tomatoes were selected due to is an important commercial crop in Brazil. The infection post–harvest with *A. alternata* cause severe yield which lead a negative impact on the production (Adhikari et al., 2017; Moghaddam et al., 2019). As the biosurfactant produced by *C. tropicalis* UCP 1613 showed remarkable in vitro effect against *A. alternata*, its efficacy was tested on cherry tomatoes. Figure 7 displayed that fungal growth starts from the wounded spot and expands to the rest of the fruit in the control group, while when treated with biosurfactant the lesions are much when compared to those in the control group. In addition, the fungal development is reduced in the surrounding areas which indicates that the biosurfactant had and effective action on *A. alternata.*

Figure 7- Effects of glycolipid produced by *C. tropicalis* UCP 1613 on cherry tomatoes:

(A) Control, (B): Treatment with *A. alternata*, (C): Treatment with biosurfactant.

In terms of agricultural production, the use of synthetic pesticides is undeniable, but their effect to human health and environment overcome the benefits they offer. Thus, advanced approaches with greater reliability are essential to minimize the volume of pesticides and their side impacts. In this sense, the biological control constitutes an interesting method that might be integrated in the management of plant diseases (Yoshida et al., 2016; Mnif et al., 2016; Ayed et al., 2019). Traditionally, biological control has focused on antagonistic microorganisms to control phytopathogens. However, the number of studies on microorganisms producing biosurfactants with fungicidal activities has increased in the last decade (Shadev et al., 2015).

Lahkar et al. (2018) pointed that like other agrochemicals, biosurfactants can be easily handled which makes their distribution uniform in the system to ensure the inhibition of the pathogen. However, Yan et al. (2014) outlined that in the case of rhamnolipids, as they are only slightly soluble in water, the system formed will be unstable. Hence, to solve this drawback the selection of some proper solvents leads this kind of biosurfactants to commercial application. Thus, more studies are required to find formulations with these molecules as additives to chemical fungicides. Consequently, lower doses of synthetic compounds will be required which becomes more cost–effective the crop cultivation, with the additional advantage of less damage to the environment.

Leakage membrane fungi

At the concentration of 750 μg/mL the efficacy of the electrolyte leakage was 40.5%. This result was higher that reported by Yan et al. (2014) who informed an efficiency of 35.9% when treated *A. alternata* with a rhamnolipids obtained from *P. aeruginosa* ZJU-211. They highlighted that the cell permeability is destabilized with increased concentrations of rhamnolipid and consequently lead to electrolytic leakage. This evidence suggests that the biosurfactant type glycolipid produced by *C. tropicalis* UCP 1613 follows the same mechanism. In this sense, the asymmetric insertion of these molecules into the bilayer may cause a curvature strain and lead the mechanical failure. Therefore, the consequences of these physical changes are evidenced through the relaxation followed by annealing of the membrane. Another mechanism that could explain the leakage is the monolayer curvature strain which occurs when the membrane is partially relaxed due to the surfactants form highly curved rims that cover hydrophobic edges of toroidal pores (Heerklotz, 2008).

4 Conclusion

Raw glycerol and glutamate are adequate precursors for the synthesis of biosurfactant by *C. tropicalis* UCP 1613. The microorganism showed excellent for biosurfactant production, confirmed by the reduction of the surface tension in an optimized medium. The molecule was characterized preliminarly as a glycolipid and had an antagonistic *in vitro* activity against *A. alternate*. Moreover, the biosurfactant exhibited promising results when inhibited fungal growth of the phytopatogen on cherry tomatoes which suggest that the compound may be used to manage the incidence of the phytopathogen on this crop. The results of the current study demonstrated that the glycolipid produced has a potential as fungicide and could be used in post-harvest applications.

5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 Author Contributions

GM conceived the study. GM and DR designed the experiments. DR, DM, RA and AD performed the experiments. IR interpreted the results and performed the data analysis. MB performed the microscopy analyzes and GM reviewed the manuscript.

RS and DD contributed reagents, materials, and analysis tools. SM wrote the manuscript. All authors read, reviewed, and approved the final manuscript.

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4 ARTIGO 2- VALUE-ADDED BIOCONVERSION OF AGROINDUSTRIAL WASTES BY SOLID-STATE FERMENTATION FOR BIOSURFACTANT PRODUCTION BY *CANDIDA TROPICALIS* AND COATING APPLICATION ON SEEDS GERMINATION

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Abstract

Solid state fermentation (SSF) is currently used in a range of applications including classical applications, such as enzyme or antibiotic production, recently developed products, such as tensoactive compound such as biosurfactant production looking for economic advantage to reduce the effective cost with substrates. The present study explored the potential of *Candida tropicalis* UCP 1613 to synthesize biosurfactant from four solid substrates: coffee grounds, sugarcane bagasse, pineapple peels, and instant noodle waste, two inductors: raw glycerol and post frying soybean oil. The yield of the biosurfactant was determined from two different solvents (methanol and ethyl acetate). The preliminary structure of the biosurfactant was identified through attenuated total reflection (ATR) technique in FTIR (ATR-FTIR). The potential toxicity of biosurfactant and two synthetic surfactants (sodium dodecyl sulfate (SDS) and Tween 80) on onion (*Allium cepa*) seeds was evaluated. Therefore, seeds coating solution

were prepared using polyvinyl alcohol (PVA), Arabic gum, as binders, calcium carbonate as filler, and the biosurfactant and synthetic surfactant (SDS) as active agents. The results showed that instant noodle waste in combination raw glycerol as inductor was the most suitable systems for solid state fermentation. In addition, the higher yield detect was 19.5 g/100 g with ethyl acetate as solvent. The preliminary identification of the compound indicated a biosurfactant type glycolipid. Alternatively, the biosurfactant displayed no toxicity against the onion seeds neither the other surfactants. Moreover, seeds coating containing biosurfactant displayed a positive effect on seeds germination and the fresh and dry total weight. The results obtained suggested the potential of the biomolecule for the replacement anionic surfactant in seeds coating formulation.

Keywords: glycolipid, instant noodle waste, *Allium cepa*, seeds coating,

Introduction

The environmental impact of synthetic surfactants has led to the search for similar compounds in the different areas of the industry (Rebello et al., 2020). It is expected that the large growing market of surfactants reaches $36.1 billion by 2020 (Jiménez-Peñalver et al., 2016). Biosurfactants are surface-active molecules synthesized mainly by microorganisms. These metabolites represent an alternative to conventional surfactants due to their biodegradability, low toxicity and the possibility of production from renewable feedstocks (Marcelino et al., 2020).

In this sense, the expenses related to raw materials are classically a significant part of biosurfactant production costs. However, the use of industrial wastes as sources of nutrients may significantly reduce the cost making the process viable (Al-Bahry et al., 2013). Agro-industrial wastes are attractive to be used as substrates due to the high content of carbohydrates, lipids, and proteins. However, the correct selection of nutritional sources involves the difficulty of finding a waste with the right balance to promote microbial growth and the production of metabolites (Costa et al., 2018).

Solid-state fermentation (SSF) is an alternative technology that has allowed the successful production of several compounds, such as enzymes, at high yields that are sometimes higher than those obtained in submerged fermentation. For instance, solid-state fermentations are already developed at the commercial scale in the food industry, waste management and bioremediation processes (Costa et al., 2018). Among the advantages of SSF, it can be

mentioned the requirement of simple equipment, low volumes of water, low energy consumption, and the solution for disposal problems (Chen et al. 2012; Mussatto et al., 2012).

On the other hand, seed coating technologies research are focused on prolonging or enhance the viability and health of successful germination. Several methods have been developed to protect crops from biotic (e.g. phytopathogens) and abiotic stress (e.g. drought and salinity) (Rocha et al., 2019). The formulation often contains aqueous suspension preparations with the active agent (e.g., pesticide, bactericide, microorganism, and fertilizer), dispersants, thickeners, membrane formers and other components (Ma, 2019). However, the presence of chemical compounds in seeds coating is the most common trend and it is not efficient due to the cumulative effect of these substances in the environment (Ziani et al., 2010). With this in mind, the present study evaluated the capacity of *Candida tropicalis* UCP 1613 to produce biosurfactant through solid state fermentation and also, it determined the effect of the biosurfactant obtained as an active agent in seeds coating formulations.

Materials and methods

Yeast strain and inoculum preparation

Candida tropicalis UCP 1613 was obtained from the culture collection of Nucleus of Resources in Environmental Sciences, Catholic University of Pernambuco, Recife, Brazil. The yeast was kept at 4 °C in Yeast Mold Agar (YMA) slant containing (g/L): yeast extract, 3; malt extract, 3; peptone, 5; glucose, 10; agar, 20 at final pH 6. The culture seed was prepared from a loopful of yeast freshly grown on an agar slant which was added to a 250 mL Erlenmeyer flask containing 50 mL Yeast Mold Broth (YMB) (the same composition above without agar). The microorganism was incubated at 48 h at 28 °C; 150 rpm in a rotary incubator shaker (TECNAL, TE–421). The concentration was adjusted to 1×10^4 cells/mL after that period (El-Sheshtawy et al., 2013; Santos et al., 2013).

Screening of substrates for biosurfactant production by SSF

Sugarcane bagasse, coffee grounds, pineapple peels, and post frying soybean oil (PFSO) were kindly donated by a local restaurant. Raw glycerol (RG) was provided by Cetene (Centro de Tecnologias Estratégicas do Nordeste, Recife, Pernambuco; Brazil) and the instant noddle waste (INW) was obtained from the food industry. Coffee grounds, bagasse, and pineapple peels were dried at 60 °C for 48 h. After grinding all the solid substrates, they were passed through a 32-mesh sieve. Erlenmeyer flasks contained 10 g of the dry solid substrate along with

the impregnating solution containing (g/L): 3.0 g KH_2PO_4, 7.0 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 1 g $(NH_4)_2SO_4$ were autoclaved at 121 °C for 15 min. The volume of impregnating solution was established according to the liquid absorption capacity of each substrate: sugarcane bagasse (40 mL), coffee grounds (15 mL), pineapple peels (10 mL), instant noodle waste (10 mL).

The amount of medium (mL) that was added to 10 g of the dry substrate without the appearance of free liquid was determined as the liquid absorption capacity. The seed culture, the amount of glycerol, soybean or the mixture (1:1) was quoted as a percentage (v/v; volume of glycerol or soybean oil per total volume of impregnating solution). After the addition of each aliquot, the substrates were mixed and then incubated at 28 °C for 96 h. Erlenmeyer flasks received 100 ml of distilled water and the mixture was homogenized for 1 h at 200 rpm at 30 °C on an orbital shaker. The content was filtered through cheesecloth and this procedure was done twice times. The metabolic liquids were centrifuged at 5 000 rpm and 4 °C for 15 min and used for further analysis.

Surface Tension

The surface tension of the cell-free broth was assessed with a tensiometer (Sigma 700, KSV Instruments LTD, Finland), based on the principle of the Du Nuoy ring method (Rubio-Ribeaux et al., 2017).

Biosurfactant extraction and yield

The extract obtained from the SSF with INW as support and RG as inductor was extracted different solvent system methods. In the first case, the metabolic liquid was acidified with HCl (6 M) to pH 2 and precipitated with two volumes of methanol. Samples were kept for 24 h at 4 °C and centrifuged at 5000 g for 20 min. Then, they were washed twice with cold methanol and dried at 37 °C for 24 – 48 h (Pareilleux, 1979).

The second method involved a solvent extraction with ethyl acetate. The cell-free broth as placed into a 250-mL separatory funnel and 100 mL ethyl acetate were added. The mixture formed two phases and the upper phase was transferred to a round-bottom flask. The ethyl acetate in equal volume was used to extract the lower phase three times, for the complete recovery of biosurfactant. The round-bottom flask was coupled to a roto-evaporator to evaporate ethyl acetate under vacuum at 80°C. To remove fatty acids, the semi-crystalline product, ambar colored and honey-like was washed with hexane, two times and dried in the oven to a constant weight (Sarubbo et al., 2007). The yield was defined as grams of

biosurfactant per g of the total dry mass of fermentation. Moreover, to compare the results with the data reported in the literature the yield was also reported as grams of biosurfactant per 100 g of substrates (Jiménez-Peñalver et al., 2016).

Preliminary characterization of the biosurfactant

Fourier transform infra-red (FTIR) analysis

The structural identification of functional groups in the biosurfactant was confirmed by attenuated total reflection (ATR) technique in FTIR (ATR-FTIR). The spectrum was measured on a Varian 640 IR FTIR spectrometer (Varian, Australia) with a wavelength range of 4000 to 400 cm^{-1} (Santos et al., 2018).

Ionic charge

The electrokinetic of potential zeta was analyzed in the Zeta potentiometer (ZM3-D-G, Zeta Meter System 3.0+). The biosurfactant (100 mg) was dissolved in 5 mL of an aqueous solution of KCl, with the corresponding ionic strength at 0.001 M (Silva et al., 2014).

The ionic charge of the biosurfactant was measured in a Zeta potentiometer (ZM3-D-G, Zeta Meter System 3.0+). The top row was filled with sodium dodecyl sulfate (SDS) as anionic surfactant (0.02 M) and barium chloride (0.05 M)) as cationic surfactant. Petri dish was incubated at room temperature for 48 h and the result was calculated when the precipitation lines appeared (Meylheuc et al., 2001).

Phytotoxicity assay

The toxicity of the biosurfactant was assessed on seeds of onion (*Allium cepa*). Sodium dodecyl sulfate (Scharlau) and Tween 80 (Sigma–Aldrich) were used as anionic and non-ionic surfactants, respectively. The concentrations of the compounds tested were 10, 50 and 90 mg/L. The surface of the seeds was disinfected with Na–hypochlorite (10%) for 20 min to avoid the fungal growth and then subsequently washed with sterile distilled water. Three replicates of ten seeds were placed on sterilized Petri dishes (100 mm diameter) previously sterilized containing Whatman N°1 filter paper. Afterward, 8 mL of distilled water (control) or aqueous solutions of the surfactant tested were dissolved in distilled water and added to Petri dishes, which were covered with aluminum foil and incubated in the dark at 20 ± 1°C for 5 days (Galvés et al., 2018). After that period, the germination index was determined as follows:

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

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

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

Process of coating seed

Different solutions for coating seeds were prepared and homogeneously dissolved using binders (polyvinyl alcohol (PVA), Arabic gum), fillers (calcium carbonate) and active agents (biosurfactant and synthetic surfactant) (Table 1). The solutions were homogenized in a constant speed mixer for several minutes. The composition of each solution is listed in Table 1. The treatments were performed in triplicate.

Table 1- Coating formulations used on *Allium cepa* seeds.

Coating solutions	PVA (%)	Arabic gum (%)	Calcium carbonate (%)	SDS (mg)	Biosurfactant (mg)
Solution 1	1	-	0.3		-
Solution 2	1	-	0.3	150	-
Solution 3		1	0.3	150	-
Solution 4	1	-	0.3	-	-
Solution 5	1	-	0.3	-	150
Solution 6	-	1	0.3	-	150

Coating appearance

The appearance of the seed coating was observed through a stereomicroscope (Zeiss, Stemi 1000). Besides, seed samples were dried at 50°C for 48 h, placed on stubs attached with sticky tape and sputter-coated with gold-palladium before being analyzed by using an SEM (JSM-6700F, Jeol, Tokyo, Japan) (Tu et al., 2016).

Growth Parameters

The analysis of the growth parameters was recorded after 10 days. For each treatment, 40 seeds were placed on Petri dishes (150 mm diameter) containing Whatman N°1 filter paper and covered with the same paper moistened with 15 mL of distilled water. All the treatments were

incubated at 20°C and performed in triplicate including controls (Ziani et al., 2010). Germination percentage was calculated according to the formula developed by Carley and Watson (1968).

Germination percentage (%) = Number of seeds germinated/ Total number of seeds placed × 100

The total plant fresh weight and total plant dry weight determinations were carried out by selecting 3 plants from each replicate randomly. The dry weight determination was performed at 105°C for 10 min and then at 75°C to obtain a constant weight (Tu et al., 2016).

Statistical analysis

The results were reported as Mean ± Standard Error (SE) for experiments repeated at least three times. The one–way ANOVA test followed by the Tukey test was used to analyze statistical differences, which were significant at $p<0.05$.

Results and discussion

Figure 1 shows the effects of the four substrates tested (sugarcane bagasse, coffee grounds, pineapple, and instant noddle waste) and the inductors on the surface tension for the biosurfactant production by *C. tropicalis* in SSF. Several studies have shown the suitability of sugarcane bagasse, pineapple peels and coffee grounds for biosurfactant production in SSF (Martínez-Ávila et al., 2018; Al-Kashef et al., 2018). However, in this study, these substrates were not efficient when compared with INW in combination with the inductors, which was corroborated from the statistical analysis.

The performance of an SSF depends on the feature of the strain, the culture conditions and the substrates tested which varies according to the process. The previous study with *Candida tropicalis* UCP 1613 showed the ability of this yeast as a biosurfactant producer from agro-industrial wastes in submerged fermentation (Rubio-Ribeaux et al., 2017). In the current study, the yeast was also able to reduce the surface tension using INW under the influence of RG, PFSO and the mixture of both inductors to 34.8, 33.6 and 37.6 mN/m respectively. In this sense, INW is generated during production, processing, cooking or consumption. Also, the residues do not receive any further treatment before disposal (Karmee, 2018). Significant amounts of starch, lignocellulosic material and oil are found in this food industry waste. Thus, the microbial conversion of noddle waste has been reported in the literature to produce lipid, enzyme, and

pigment under submerged fermentation conditions (Pimpa, 2004; Puangbut et al., 2018; Getha et al., 2016).

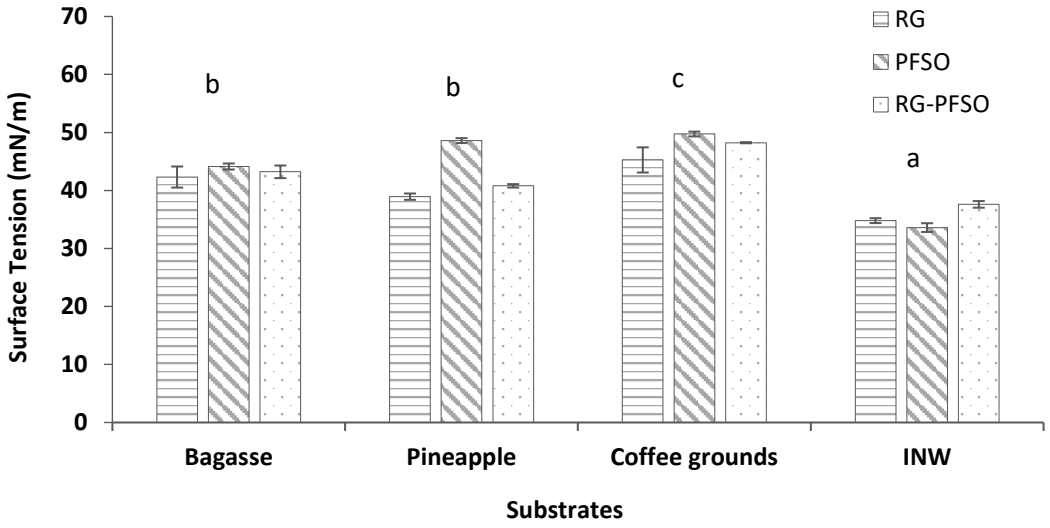

Fig. 1- Surface tension versus screened substrates for biosurfactant produced by *C. tropicalis* UCP 1613 in solid stated fermentation

The production of biosurfactant by *Cunninghamella echinulata* in a medium containing instant noodle waste (2%), corn steep liquor (2%), and post frying oil (0.5%) with a surface tension of 32.4 mN/m was informed by Andrade et al. (2018). However, to our knowledge, this is the first report on yeast using INW, under the influence of PFSO and RG for the synthesis of biosurfactants through SSF. Although, the contribution of the Glycerol-SWO was significant statistically, the value of surface tension was higher than the effect of the two separated substrates. As the statistic difference of Glycerol and SWO was similar, the Glycerol was selected as the inductor for further studies.

Yield and preliminary characterization of the Biosurfactant

The yield of biosurfactant was determined through two protocols. Regarding the extraction with methanol, the yield was 14.3 g/100 g. However, the mixture of methanol and ethyl acetate (1:1) gave a yield of 19.5 g/100 g. This last result was similar to the obtained by Jiménez-Peñalver et al. (2016), who detected a yield of 19.1 g/100 g of wet substrates synthesized by *Starmerella bombicola* from winterization oil cake by SSF. They increased the amount of biosurfactant recovered to 25.1 g/100 g of wet substrates after when applied intermittent mixing. Besides, Parekh et al. (2012) informed that other strain of the same yeast produced 17.48 g/100 g, which were extracted with ethyl acetate from the SSF of mango kernel. Rashad et al. (2014) obtained a yield of 15 g/100 g substrate when using methanol and ethyl acetate in the extraction of

biosurfactant from the SSF of sunflower oil cake and soybean oil by *Candida bombicola*. The use of the same method in the current study showed a higher yield. On the other hand, these authors reported 17 g/100 g substrate when using methanol as solvent extraction, and 49.5 g/100 g substrates when they applied a new concept based on two consecutive extractions with methanol and ethyl acetate. These facts evidence that the quantification of biosurfactants is strongly related to the extraction method (Jiménez-Peñalver et al., 2016).

The most significant bands of the FTIR spectrum are shown in Figure 2. The peak detected at 3308 cm^{-1} can be attributed to the O–H stretching vibrations of hydroxyl groups. The spectrum also showed a peak at 2926 cm^{-1} associated with aliphatic hydrocarbons, constituent fatty acids, and lipids. The peak detected at 1652 was from the stretching of unsaturated C=C bonds while the band at 1151.96 cm^{-1} suggested the presence of lactones in the molecule. Moreover, sugar C–H groups were found to be at 1018.70 and 705.88 cm^{-1} respectively. Since these structural details were found similar to those reported in the literature (Chandran e Das, 2010; Santos et al., 2017) it might be concluded that the fermentation product belongs to the sophorolipids group, which is typical biosurfactant produced by yeast species.

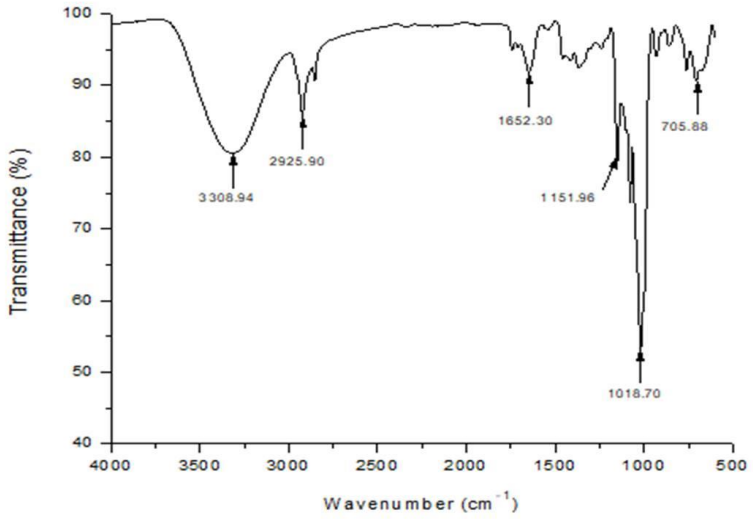

Fig. 2- FTIR spectrum of the biosurfactant obtained through SSF by *C. tropicalis* UCP 1613

The surface charge of the obtained biosurfactant was also determined through zeta potential. This kind of measurement offers information on the prediction and control of colloidal suspensions and emulsions. Thus, higher values are related to the good stability of the suspension due to the repulsion between hydrophilic particles (Silva et al., 2014; Araújo et al., 2019).

The biosurfactant produced by *Candida tropicalis* UCP 1613 under the experimental conditions of this work showed an anionic character −54.3 ZPmv, 3.10 μS/cm at 25.9 °C. This result agrees with other studies which previously reported the anionic character of biosurfactants produced by members of the genus *Candida* (Sobrinho et al., 2008; Andrade et al., 2015).

Germination assay

The concentration effects of synthetic and microbial surfactants are shown in Figure 3. was selected for this study due to be recommended as one of the species to test the ecological effect of toxic substances (Galvés et al., 2018). The results displayed that the different concentrations of the surfactant tested did not show inhibitory effects on seed germination and root elongation after 10 days since values of GI from 80% indicates the disappearance of toxicity (Araújo et al., 2019).

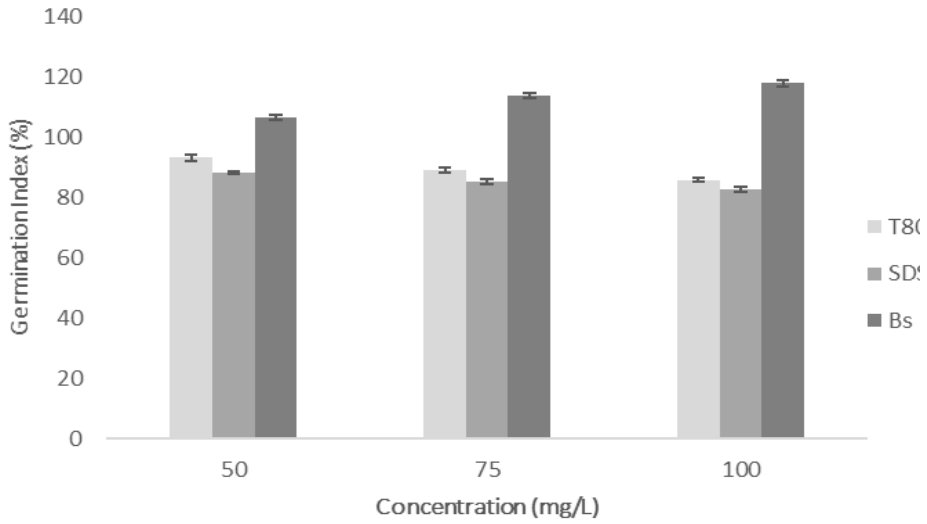

Fig. 3- Phytotoxicity of biosurfactant produced by *C. tropicalis* UCP 1613 and synthetic surfactants on seeds of onion (*Allium cepa*)

The germination index was encouraged with the increased of the biosurfactant concentration. However, the opposite effect was observed in the synthetic surfactants. These findings are probably due to the concentrations evaluated. Gálvez et al. (2018) determined the effect of synthetic surfactants on germination and root elongation of two horticultural crops. They observed that a high concentration of the compounds used inhibited seed germination. Moreover, sodium dodecyl sulfate was the most hazardous. However, Tween 80 and Brij35 were the least phototoxic compounds for the seeds tested.

They suggested that the high doses of detergents led to induced oxidative stress, which resulted in lipid peroxidation and increased permeability of the cell membrane to toxic ions (Heidari, 2013). Interestingly, leaves and secondary roots were observed for all the conditions tested of the biosurfactant when compared with synthetic surfactants. Similar results were informed by Luna et al. (2013) and Rufino et al. (2014) who verified the toxicity of biosurfactants produced by *Candida* spp. from renewable substrates but in submerged fermentation.

Microscopy analysis of seeds coating

Figure 4 shows the observations under the stereoscope microscope and SEM of *Allium cepa* seeds. The surface appearance revealed that homogeneous films with good dispersion were formed on the seeds with PVA-calcium carbonate-SDS and PVA-calcium carbonate-Bs treatments (Fig. 4A, 3C). Also, a bright coating was observed in these treatments. By other side, the seeds treated with Arabic gum-calcium carbonate-SDS and Arabic gum-calcium carbonate-Bs in the formulation displayed an opaque aspect with an irregular coating which altered the surface patterns.

Moreover, SEM micrographs showed that the combination of PVA-calcium carbonate-SDS was able to form a firm, smooth and round coating (Figure 3E). A similar observation was informed by Tu et al. (2016) who noted a regular film on cotton seeds with PVA-alginate-bentonite coating. They related this pattern with intermolecular hydrogen bonding between silanol groups (Si−OH) on the bentonite surface and the hydroxyl or carboxyl groups of alginates and PVA. In the current study, the interaction of the Na+ group of SDS and carbonate or carboxyl groups of calcium carbonate and PVA could be favored by the homogeneous distribution on the surface.

On the other hand, PVA-calcium carbonate- Bs, Arabic gum-calcium carbonate-SDS and Arabic gum-calcium carbonate- Bs films were irregular and presented cracks and faults, as shown in Figure 3 (F, G, H). In this sense, an important aspect related to seed coating is the right selection of adhesives which dissolves easily on contact with moisture and binds the stuffing material firmly on the seed surface. A desirable feature is that they are effective at very low concentrations, which could reduce the cost of the adhesives (Ma, 2019).

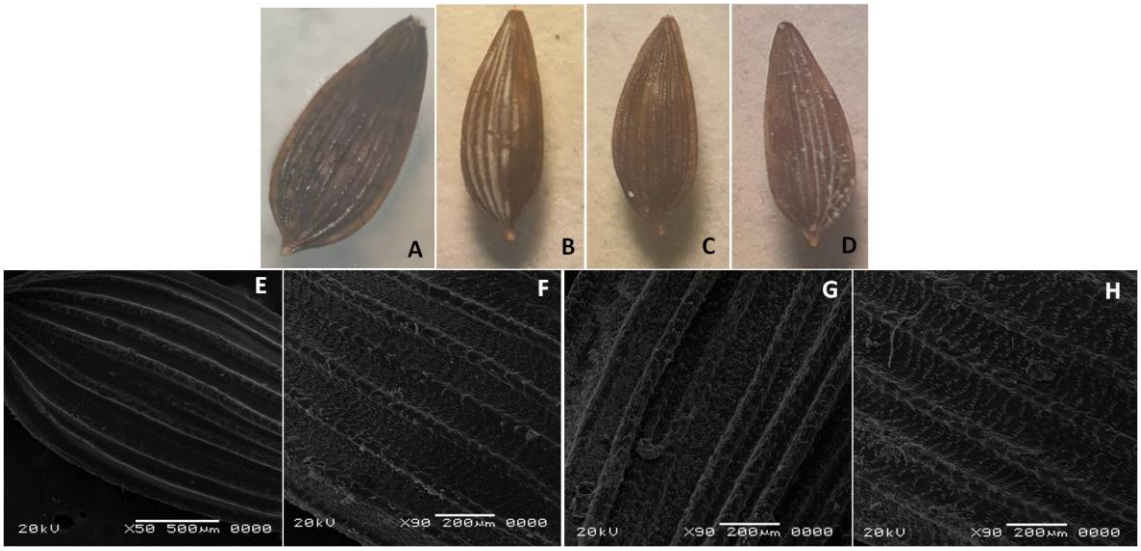

Fig. 4- Stereoscope microscope and SEM observation of onion seeds coating: PVA - calcium carbonate-SDS (A, E), PVA-calcium carbonate-Bs (B, F), Arabic gum-calcium carbonate-SDS (C, G), Arabic gum-calcium carbonate-SDS (D, H)

Growth parameters

The effects of applying different coating formulations on the germination rate of onion and the fresh and dry total weight are shown in Table 2. In the case of the treatments with PVA-calcium carbonate, regarding the control, there was an increase of 5% and 15% for the PVA-calcium carbonate-SDS and PVA-calcium carbonate-Bs coating, respectively. However, in the second the germination growth improved by 7.5% and 17% for Arabic gum-calcium carbonate-SDS and Arabic gum-calcium carbonate-Bs respectively in comparison to the control. The treatments containing biosurfactant showed germination rates higher than the treatments with SDS in the formulation. Thus, these results demonstrate this biosurfactant could be considered when used seed coating technologies to replace synthetic surfactants.

Sharifzadeh et al. (2006) suggested that the significant reduction in germination trait when evaluated the effect of synthetic surfactants is associated with the increased dose around the seeds. This is due to the water uptake is reduced leading to high osmotic potential, as a result, seed germination and metabolic activities are delayed, and production of oxygen free radical could cause the damage of the cell membrane. In this sense, further studies are required to evaluate the effect of increased concentrations in seeds coating of both surfactant (SDS) and biosurfactant (glycolipid type). However, the present studied showed that the presence of the biosurfactant significantly encouraged germination when compared to their respective controls.

Table 2- Influence of film coating formulations on *Allium cepa* seeds

Treatments	Germination rate (%)	Total plant fresh weight (g)	Total plant dry weight (g)
PVA-calcium carbonate-C	77.5 ± 1.24	2.20 ± 0.05	1.25 ± 0.06
PVA-calcium carbonate-SDS	82.5 ± 1.34	2.05 ± 0.04	1.15 ± 0.01
PVA-calcium carbonate-Bs	92.5 ± 1.15	2.35 ± 0.05	1.32 ± 0.03
Arabic gum-calcium carbonate-C	80.0 ± 0.00	2.70 ± 0.03	1.40 ± 0.05
Arabic gum-calcium carbonate-SDS	87.5 ±1.25	2.25 ± 0.08	1.32 ± 0.03
Arabic gum-calcium carbonate-Bs	97 ± 1.15	2.98 ± 0.05	1.57 ± 0.02

In the case of total plant fresh weight and total plant fresh weight, they showed a similar evolution. The treatments containing SDS in the formulation exhibited the lower weights. In this sense, the application of organic pollutants, such as petroleum-derived contaminants can induce changes in the root development, but also a reduction of water and nutrient availability to the plants. Consequently, the reduction in fresh and dry matter yield of the plant may be attributed to both the inherent pollutant toxicity and induced (Besalatpour et al. 2008). In contrast, Silva et al. (2015) evaluated the influence of different concentrations of biosurfactants on seed germination and development. These authors outlined that the biomolecules may have penetrated the tested seeds, helping to mobilize the reserve tissue, which supported the seedling development.

In general, it is well informed in the literature the use of synthetic surfactants, mainly anionic and non-ionic, as active agents in seeds coating formulations (Tu et al., 2015; Galvés et al., 2018; Ren et al., 2019). For instance, previous studies were developed with non-ionic surfactants on seed to treat water repellency of soils (Moore et al., 2010; Madsen et al., 2012) which consequently improved the seeds germination. Madsen et al. (2016) evaluated the application of a nonionic surfactant applied directly to turfgrass seed to enhance germination and subsequent plant establishment. They concluded that the presence of nonionic surfactants may create transient increases in membrane fluidity which increase the permeability of the

plasma membrane to growth regulators and/or nutrients. Kerbauy (2008) highlighted that it is necessary that wrapping embryonic tissues be permeable to water to begin the germination process. Thus, the amphipathic structure of biosurfactants probably allows them to act on external wrapping tissue, increasing the permeability to water to facilitate the germination.

Conclusions

The present study demonstrated the suitability of instant noodle waste and glycerol as substrates for biosurfactant production through SSF. Furthermore, the biosurfactant produced by *C. tropicalis* UCP 1613 showed to be an excellent candidate for seed coating formulation to improve seed germination. However, further studies are necessary to the optimization of the production and the evaluation of different concentrations of the biosurfactant on growth seeds.

Author Contributions

GM: Conceptualization, GM and DR: Design of experiments, DR, DM and RF: Methodology, MB: Microscopy analyzes, DR: Writing, GM: Reviewing and Editing.

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5 CONCLUSÕES GERAIS

- A levedura *Candida tropicalis* UCP 1613 demonstrou habilidade para utiliza glicerol residual e o glutamato como substratos para a produção de biossurfactante;

- O biossurfactente produzido por *Candida tropicalis* UCP 1613 reduziu a tensão superficial para 25,3 mN/m em meio mineral suplementado com 8% de glicerol residual e 3,5 g de glutamato;

- O biossurfactante produzido foi caracterizado como um glicolipídio;

- O metabólito produzido foi detectado na fase estacionária com um rendimento máximo de 3,6 g/L;

-O biossurfactante produzido mostrou excelente estabilidade frente a condições variáveis de temperatura, pH e salinidade;

- O efeito antifúngico do biossurfactante contra o fungo fitopatógeno *Alternaria alternata* foi avaliado *in vitro* e o efeito inibitório comprovado em tomate cereja com uma concentração de 750 μg/mL;

- A levedura foi capaz de produzir biosurfactante através da fermentação em estado sólido utilizando miojo como substratos e glicerol residual como indutor;

- O biossurfactante obtido através da fermentação em estado sólido mostrou potencial para estimular a germinação de sementes de cebola, em formulações contendo goma arábica, álcool polivinílico e carbonato de calcio, quando comparado com o surfactante sintético dodecil sulfato de sódio;

- A *Candida tropicalis* UCP 1613 apresentou excelente habilidade para produzir biossurfactante a partir de miojo na ausência de glicerol residual, com um valor de 25 mN/m, uma concentração de inóculo de 10-6 cells/mL; a uma temperatura de 31°C; e um tamanho de partícula de 32 mesh.

- O biossurfactnte produzido por *Candida tropicalis* UCP 1613 gera perspectivas futuras de aplicação em vários seguimentos industriais, utilizando substratos alternativos na obtenção de tensoativo que permite a utilização nas indústrias farmacêuticas, melhoramento nos bioprocessos de alimentos e recuperação de áreas contaminadas por poluentes hidrofóbicos e metais pesados

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Innovative production of biosurfactant by *Candida tropicalis* UCP 1613 through Solid-State Fermentation

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Abstract

Microbial surfactants are amphipathic molecules mainly produced by submerged fermentation, and capable of decreasing the surface and interfacial tension between two immiscible phases. However, their obtention by solid state fermentation (SSF) has gained attention due to the less complex equipment involved, less energy demand, low volumes of water and requirement of less solvent for extraction. Hence, the aim of this study was to evaluate the ability of *Candida tropicalis* UCP 1613 to produce biosurfactant through solid-state fermentation and determine its stability under different environmental conditions. A factorial design 2^4 was performed to determine the influence of four variables, which were inoculum size, temperature, and particle size and inductor volume on the surface tension. In addition, the stability of the biomolecule was analysed in different pH, temperature and salinity ranges. The results showed that yeast synthesized biosurfactant under all conditions tested. From the statistical analysis, it was bserved that all variables except the inductor concentration had a significant influence on the surface tension decrease. The lowest value 25.8 mN/m was detected at condition 8 (10^{-6} cells/mL; 31 °C; 32 mesh and 0 mL of the inductor). The biosurfactant displayed a good performance when analysing its activity in different temperature, salinity and pH ranges. The study showed the feasibility of the solid-state fermentation to obtain a biosurfactant with excellent activity produced by a yeast. At the same time this is the first report of its kind in the literature.

1. Introduction

Microbial surfactants are an important group of molecules synthetized by bacteria, filamentous fungi and yeast.

They are amphiphilic molecules that decrease the surface and interfacial tension by accumulating at immiscible interfaces (Souza et al., 2016). In addition, these compounds are produced in stationary phase of microbial growth. According to their chemical structure they are classified as glycolipids, lipopeptides, phospholipids, and polymeric or particulate compounds (Varjani and Upasani, 2017; Karlapudi et al., 2018). The hydrophobic moiety is usually made up of long-chain fatty acids, hydroxyl fatty acids or a-alkyl-b-hydroxyl fatty acids. In the case of the hydrophilic portion can be a carbohydrate, amino acid, cyclic peptide, phosphate, carbox- ylic acid or alcohol.

The interest in biosurfactants is based on their low toxicity, high biodegradability and effectiveness at extreme temperatures, pH and salinity. In addition, their functional properties such as emulsification/de-emulsification, dispersion, foaming and wetting determine its application in bioremediation, agriculture, food production, cosmetic and other industrial sectors. (Banat et al., 2014; Fenibo et al., 2019).
However, the low yields, the carbon source and high cost input required for downstream processing are among the main challenges to make most of them marketable. Hence, to overcome this scenario one of the strategies have been the use of inexpensive and easily available raw materials (Banat et al., 2014; Satpute et al., 2017). In this context, the increasing amounts of organic solid waste because of the human activity is a challenge that needs to be handled at every level. Thus, solid-state fermentation (SSF) is a technology that allows the biological transformation of this kind of residues without a previous pre-treatment to obtain products with added value (Lourenço et al., 2018; Sadh et al., 2018).
Moreover, the adequate selection of residual substrates is essential in the process to ensure the right balance of necessary nutrients for the microbial growth and the production of biosurfactants. Renewable substrates from different industrial activities such as, food processing and biodiesel production usually possess a high content of carbohydrates, lipids, and proteins that are attractive to be used as precursors for synthesis of microbial surfactants (Konishi et al., 2017; Satpute et al., 2017). Bearing this in mind, this study reports for the first time, the use of instant noddle waste as substrate for biosurfactant production from *Candida tropicalis* UCP 1613 through solid-state fermentation as well as the assessment of its stability in different conditions of pH, temperature and salinity.

2. Materials and methods

Microorganism

Candida tropicalis UCP 1613 was kindly provided by the Nucleus of Resources in Environmental Sciences, Catholic University of Pernambuco, Recife, Brazil. The yeast was kept at 4 °C in Yeast Mold Agar (YMA) slant containing (g/L): yeast extract, 3; malt extract, 3; peptone, 5; glucose, 10; agar, 20 at final pH 6.5.

Inoculum preparation

To obtain the inoculum a loop full of biomass was inoculated into Erlenmeyer flask of 250 mL containing 50 mL of YM broth and was incubated at for 24 h, at 28 °C and 150 rpm. After that period different concentrations (10^{-4}, 10^{-5}, 10^{-6}) were prepared to use as inoculum for the experimental design.

Culture medium for solid-state cultivation (SSF)

The raw glycerol (RG) was kindly provided by Cetene (Centro de Tecnologias Estratégicas do Nordeste, Recife, Pernambuco; Brazil) and its elementary composition was determined using a Carlo-Erba NA-111 Elemental Analyzer. The instant noddle waste (INW) was obtained from a food industry and the Table 1 shown the chemical composition of the substrates: raw glycerol and instant noddle waste (Yang et al., 2014, Andrade et a., 2018). The assays were performed in 250 mL Erlenmeyer flasks containing 5 g of instant noddle waste (which was used as support). Then 25 mL of impregnating solution contained the following composition (g/L): 3.0 g KH_2PO_4, 7.0 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 1 g $(NH_4)_2SO_4$ were added. The volume of impregnating solution used was defined as the amount of medium (mL) added to the instant noddle waste without the appearance of free liquid. The aliquots raw glycerol and inoculum were determined as 10 % of the volume of the impregnating solution.

Table 1. Chemical compositions of raw glycerol and instant noodle waste used in SSF for biosurfactant production by *C. tropicalis* UCP 1613

Raw glycerol	Quantity (%)
Carbon	65.18
Hydrogen	10.69
Nitrogen	0
Instant noddle waste	Quantity (mg)
Carbohydrates	51000
Proteins	9.400
Total fat	16.000
Others	1370.66

Experimental strategy to biosurfactant production by SSF

The effects of the temperature (25, 28 and 31 °C), particle size (16, 32 and 60 mesh), inoculum (10^{-4},10^{-5} and 10^{-6}) and concentration of raw glycerol (0,2 and 4 %) on the production of biosurfactant were assessed by a full experimental design consisting of 20 runs (16 experiments plus 4 replicates at the central point) (Table 1). The flasks were previously sterilized at 121 °C for 15 min, and subsequently, incubated for 96 h. After that period, the samples were collected by adding 50 mL of deionized water per flask and incubated in an incubator shaker (Tecnal, TE 421) 150 rpm at 28 °C for 1 h. The liquid obtained was filtered and centrifuged at 5000 rpm for 20 min to separate the cells.

Table 2. Full factorial design to biosurfactant production by *C. tropicalis* UCP 1613 through SSF

Runs	Temperature (°C)	Size particle	Inoculum (cell/mL^{-1})	Raw glycerol (%)	Surface tension (mN/m)
1	25	3	10^{-5}	0	43.6
2	31	3	10^{-5}	0	27.6
3	25	1	10^{-5}	0	37.0
4	31	1	10^{-5}	0	27.1
5	25	3	10^{-6}	0	45.7
6	31	3	10^{-6}	0	38.9
7	25	1	10^{-6}	0	36.7
8	31	1	10^{-6}	0	25.8
9	25	3	10^{-5}	4	45.0
10	31	3	10^{-5}	4	27.3
11	25	1	10^{-5}	4	41.7
12	31	1	10^{-5}	4	26.9
13	25	3	10^{-6}	4	36.9
14	31	3	10^{-6}	4	27.3
15	28	1	10^{-6}	4	53.3
16	28	1	10^{-6}	4	26.3
17	28	2	10^{-4}	2	28.9
18	28	2	10^{-4}	2	29.5
19	28	2	10^{-4}	2	28.3
20	28	2	10^{-4}	2	28.5

Measurement of surface tension

Surface tension was estimated using the Du-Nuoy ring method performed in a Tensiometer Sigma 70 (KSV Instruments LTD, Finland) at room temperature (25 °C) (Kuyukina et al., 2005).

Stability assays

The thermal stability of the biosurfactant was determined from the cell-free broth submitted at temperature range from 20 to 75 °C. To evaluate the effect of the pH the samples were adjusted to different values of pH (2-12) by adding 1 M HCL or 1 M NaOH. The influence of the salinity was investigated from different concentrations of NaCl (2-12 %) (Pinto et al., 2018).

3. Results and Discussion

The overall statistical approach consisted in the assessment of four variables on the biosurfactant production by *C. tropicalis* UCP 1613 through solid state fermentation. The results obtained showed that the yeast was able to produce biosurfactant under all conditions tested, with surface tension variations between 45 and 25.8 mN/m (Table 1). Even though surface tensions around 25 mN/m are more frequent for bacteria, this result is in good agreement with previous studies which demonstrate the potential of *Candida* spp. for biosurfactant production (Rubio et al., 2017; Almeida 2018). By other side, the use of SSF as fermentative process confirmed the feasibility of this technology for the bioconversion of organic substrates used as either nutrient or inert support for the biosurfactant production. In addition, another point that highlights the potential of SSF is the search for sustainable processes to transform traditional chemical processes (Abu et al., 2017).

Figure 1 shows the Pareto chart with the main effects of the input variables on the surface tension. As can be seen only temperature, inoculum and particle size had a significant from the statistic point of

view. In the case of the temperature and particle size, their increased produced an adverse effect on the reduction of surface tension. Similar effect was observed for inoculum but with positive influence. In the case of glycerol, even when exhibited a positive influence as inductor for the biosurfactant production, its influence was no significant. In this sense, the lower value of surface tension was detected in the condition 8 with 31 °C, 10^{-6} cells/mL, 32 mesh and 0 mL of glycerol. Regarding the use of instant noddle waste for the biosynthesis of biosurfactant, a few studies have displayed its potential as precursor for this purpose. However, this substrate was not explored until now for SSF applications. The present study showed that its influence was relevant to trigger the production of a microbial surfactant from the yeast. This fact is probably due to its rich nutritional composition.

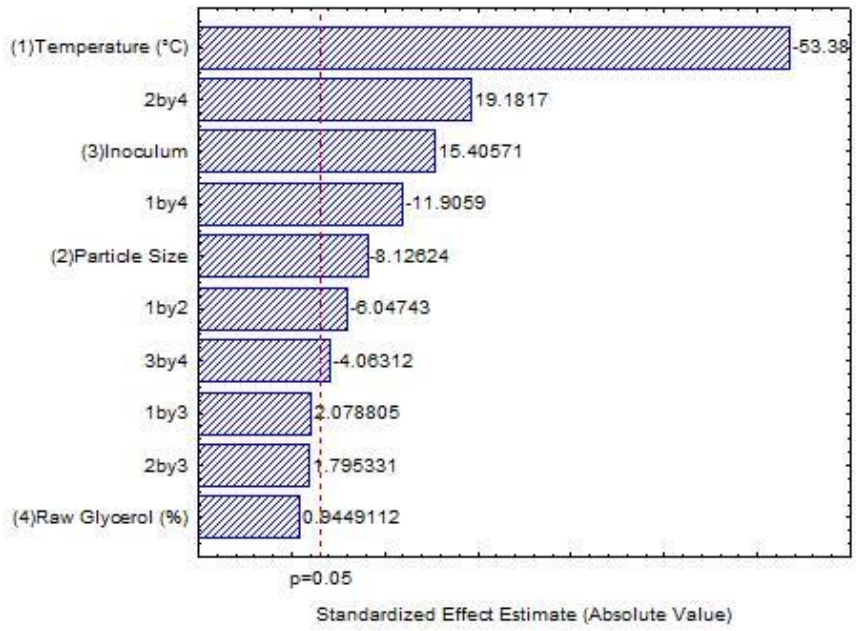

Figure 1. Pareto diagram of standardized effects of temperature, particle size, inoculum, and concentration of raw glycerol using surface tension as output variable. The dashed line specifies the statistically significant (p = 0.050

By other side, majority of research on biosurfactant production by *Candida* spp. have been based on utilize submerged fermentation (Elshafie et al., 2015; Almeida et al., 2017). In this sense, several reports have informed the synthesis of surface-active compound from yeast using inexpensive raw material which are residues various industries (Santos, et al., 2013, Luna et al., 2013; Chaprão et al., 2015). These wastes are rich in nutrients and its reuse allows the decreasing of the production cost and as well as the reduction of environmental pollution. In addition, the bioconversion of these substrates has demonstrated to be feasible to obtain efficient biosurfactants. Likewise, the current study confirmed the potential of *C. tropicalis* UCP 1613 to use a renewable substrate from food industry by SSF as beneficial approach (Table 2).

Table 3. Production of biosurfactant by Candida spp.

Yeast	Renewable substrate	Fermentation process	Surface tension (mN/m)	Reference
C. sphaerica UCP 995	Refinery residue and corn steep liquor	SmF	27	Luna et al., 2008
Candida lipolytica UCP0988	Animal fat and corn steep liquor	SmF	28	Santos et al., 2013
Candida glabrata UCP/WFCC1556	Cassava wastewater, whey and corn steep liquor	SmF	25	Andrade et al., 2015
C. tropicalis UCP 1613	Instant noddle waste	SSF	25.8	This study

The assessment of environmental factors it is essential, since they affect the characteristics of the produced biosurfactant and consequently determine its future applications. In general, when the biosurfactant was submitted to different environmental variations the values of surface tension were around 30 mN/m which indicates a good performance.

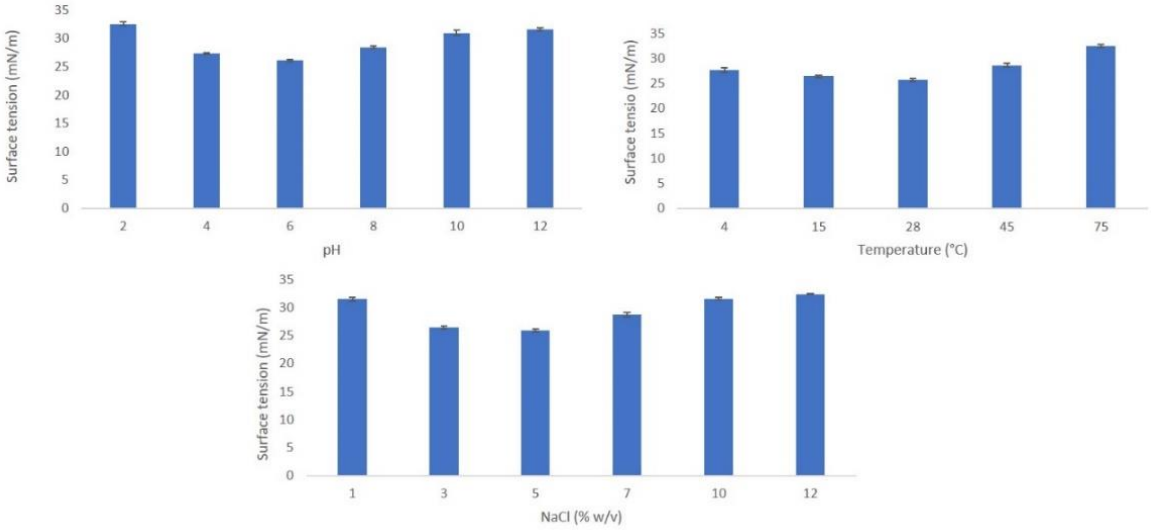

Figure 2. Stability of the biosurfactant produced by *C. tropicalis* UCP 1613

4. Conclusions

The current study demonstrated the ability of *C. tropicalis* UCP 1613 to produce biosurfactant by SSF. It is important to highlight the statistical analysis showed the potential of instant noddle waste as substrate to initiate the synthesis of the biomolecule, which represents an economical advantage for the process. The biosurfactant maintained a good activity when analyzing its performance at variable conditions of pH, temperatures and salinity which suggest its application in different industrial sectors.

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ANEXO A- CARTA SUBMISSÃO ARTIGO 2

Daylin Rubio Ribeaux <drubioribeaux@gmail.com>

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12 feb. a las 14:14

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Journal: Microbiological Research

Title: Value-added bioconversion of agroindustrial wastes by solid-state fermentation for biosurfactant production by *Candida tropicalis* and coating application on seeds germination

Corresponding Author: Galba Campos-Takaki

Co-Authors: Daylin Rubio-Ribeaux, Rosileide Fontenele, Dayana Montero, Alexandre D´Lamare M. Medeiros, Marcos Antonio Barbosa de Lima

Galba Campos-Takaki submitted this manuscript via Elsevier's online submission system, EVISE®. If you are not already registered in EVISE®, please take a moment to set up an author account by navigating
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ANEXO B- PRODUÇÃO ACADÊMICA GERADA DURANTE A TESE

Publicaciones/Conferências

Daylin R. Ribeaux, Carlos V. Jackes, Alexandre D. M. Medeiros, Jaqueline Marinho, Uiara Lins, Ilka Nascimento, Gilvanete Barreto, Galba M. C. Takaki. - Innovative Production of biosurfactant by *Candida tropicalis* UCP 1613 through Solid-State Fermentation. Chemical Engineering Transactions (2020) (Artigo aceite para publicação).

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