KARLA SILVA TEIXEIRA SOUZA

PRODUÇÃO DE BIOSSURFACTANTES POR LEVEDURAS

LAVRAS – MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, área de concentração em Microbiologia, para a obtenção do título de Doutor.

Orientador
Prof. Dr. Disney Ribeiro Dias

Coorientadores
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2016

Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Souza, Karla Silva Teixeira.
Produção de biossurfactantes por leveduras / Karla Silva Teixeira Souza. - 2016.
82 p.

Orientador(a): Disney Ribeiro Dias.

Tese (doutorado) - Universidade Federal de Lavras, 2016.
Bibliografia.

1. Glicolipídeo. 2. Fermentação. 3. Antimicrobiano. I. Dias, Disney Ribeiro. II. Título.

KARLA SILVA TEIXEIRA SOUZA

PRODUÇÃO DE BIOSSURFACTANTES POR LEVEDURAS

BIOSURFACTANT PRODUCTION BY YEAST

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, área de concentração em Microbiologia, para a obtenção do título de Doutor.

APROVADA em 19 de dezembro 2016.

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LAVRAS - MG

2016

A Deus pela maravilhosa oportunidade.

Aos meus pais João e Neuza, pelo grande exemplo de força de vontade, pelo amor incondicional e por me mostrarem que sempre valerá a pena.

Ao meu marido por me apoiar em todas as decisões.

A minha irmã e aos meus sobrinhos pela amizade e carinho.

Dedico

AGRADECIMENTOS

Inicio agradecendo a Deus. Ele esteve sempre ao meu lado durante esta caminhada, na maioria das vezes não foi fácil, no entanto, Deus me concedeuas características necessárias: persistência e determinação! E na sua infinita bondade e misericórdia me capacitou em todas as etapas. Contudo, não teria chegado até aqui sem a ajuda de alguns anjos que Ele colocou no meu caminho:

Agradeço aos meus pais, João e Neuza, que em todos os momentos, mesmo naqueles em que estive tão longe, não deixaram de me apoiar, amando-me incondicionalmente e acreditando em meu potencial. Eu amo vocês!

Sou muito grata ao meu marido Thiago por toda compreensão, pois se não bastasse os dias, noites e finais de semana, ainda me apoiou em uma das grandes decisões em minha vida e suportou minha ausência total por um ano.

Obrigada a minha irmã e sobrinhos que mesmo pela distância estiveram sempre presentes me dando todo o amor e carinho necessários, com certeza isso fez toda a diferença no meu dia-a-dia.

Aos meus amigos Jovens Consagrados, em especial ao Gustavo e Thaísa, Jessé e Debynha, Juninho e Elen, pelo apoio, compreensão e momentos tão especiais que me proporcionam, vocês também acreditaram em meu potencial e suas orações me mantiveram de pé! Aos também amigos Corredores JC, agradeço pelos momentos de lazer, descontração e por todo incentivo ao esporte.

Agradeço ao meu orientador Professor Disney Ribeiro Dias. Obrigada por acreditar em mim e aceitar-me como orientada, incentivar-me, apoiar-me sempre que precisei, pelas companhias em diversos Congressos, inclusive com minhas experiências internacionais. E muito obrigada por tornar meu sonho em ser doutora uma realidade. Também devo agradecer as professoras Rosane Freitas Schwan, Cristina Silva Ferreira e Batista e Carla Ávila, exemplos de mães e profissionais que sempre levarei comigo.

Não há como descrever a gratidão que sinto pelo Professor José Antônio Teixeira, que tão prontamente abriu as portas do laboratório, sob sua responsabilidade, na Universidade do Minho em Braga, Portugal para me receber, essa com certeza foi uma das melhores experiências de vida pessoal e profissional. Meus agradecimentos também não seriam suficientes a Professora Lígia Rodrigues e ao Dr. Eduardo Gudiña, sem vocês este trabalho não poderia ser concretizado e nenhum conhecimento poderia ser obtido, foi maravilhoso trabalhar com os autores em que mais citei nos meus trabalhos. Um agradecimento especial ao

Dr. Eduardo Gudiña (a quem eu respeitosamente chamava de chefe), muito obrigada pela paciência e disponibilidade em todo o tempo do meu trabalho em Portugal. Agradeço também aos grandes amigos que conquistei e que levarei sempre no meu coração, do LF, vocês tornaram meus dias frios e chuvosos em alegres e divertidos.

Ao Departamento de Microbiologia Agrícola e todas as minhas queridas amigas que foram essenciais em todos esses anos. Dentre elas não poderia deixar de citar a Angélica, Suzana,Maria Gabriela, Ana Luiza, Kelly e Luciana!!! A Deus minha maior gratidão por ter colocado vocês em minha vida!

As técnicas Ivani, Cidinha e Juliana, sei que não foi fácil trabalhar comigo e conviver com toda minha hiperatividade. Muito obrigada a Janúsia (Jajá) e D. Luzia, o que seria de nós se não fossem vocês neste prédio?

Não poderia de fazer um agradecimento especial a secretaria Rose, que por tantos anos me suportou com minha falta de atenção, esquecimentos, nervosismo e sempre me atendendo com tanto amor, carinho e dedição, você com certeza é um dos patrimônios da Microbiologia Agrícola.

Por fim, agradeço ao Programa de Pós-graduação em Microbiologia Agrícola e a todos os professores, que foram essenciais na minha formação. Muito Obrigada!

Deus abençoe a todos!

RESUMO GERAL

O objetivo deste trabalho foi avaliar a produção de biossurfactantes por cinco espécies de leveduras: *Yarrowia lipolytica* CCMA 0357 (solo da Amazônia), *Yarrowia lipolytica* CCMA 0242 (Kefir), *Wickerhamomyces anomalus* CCMA 0358 (fermentação do café), *Lindnera saturnus* CCMA 0243 (solo do Cerrado), and *Cryptococcus humicola* CCMA 0346 (solo do Cerrado), em oito meios de cultivo diferentes. *Wickerhamomyces anomalus* CCMA 0358 cultivada em azeite de Oliva, apresentou a menor tensão superficial (32.05 mN/m). O Delineamento Composto Central Rotacionado (DCCR) foi realizado para otimizar os parâmetros e aumentar a produção do biossurfactante. A inclusão do extrato de levedura apresentou efeito significativo de 89% e a tensão superficial foi reduzida para 31.5 mN/m. Fermentações em batelada foram realizadas com agitação de 500 rpm e a tensão superficial foi reduzida para 29.3 mN/m após 24 horas, e rendimento de 2.60 g/L. Ensaios antimicrobianos e para recuperação do petróleo foram realizados com o biossurfactante. As espécies *Streptococcus sanguis*, *Streptococcus oralis* e *Streptococcus agalactiae*, tiveram seu crescimento inibido em 100% pelo biossurfactante bruto, *Candida albicans* obteve 78% de inibição e *Staphylococcus epidermitis* 66%. Vinte por cento do petróleo foi recuperado utilizando apenas o sobrnadante da fermentação, livre de células, contendo o biossurfactante. O biossurfactante estudado neste trabalho, foi caracterizado como glicolipídeo, onde a fase lipídica compreende de ácido oléico. No entanto, a fase glicídica não pôde ser identificada, indicando se tratar de um novo composto. Estes resultados mostraram pela primeira vez a produção de um novo glicolipídeo produzido pela levedura *Wickerhamomyces anomalus* CCMA 0358, e que pode ser utilizado em diferentes aplicações biotecnológicas.

Palavras-chave: Biossurfactante. Glicolipídeo. *Wickerhamomyces anomalus*. Fermentação. Antimicrobiano. Recuperação de petróleo.

GENERAL ABSTRACT

The main aim of this work was to evaluate the biosufarctant production by five wild yeasts species *Yarrowia lipolytica* CCMA 0357 (from Amazon soil), *Yarrowia lipolytica* CCMA 0242 (Kefir), *Wickerhamomyces anomalus* CCMA 0358 (coffee fermentation), *Lindnera saturnus* CCMA 0243 (Cerrado soil), and *Cryptococcus humicola* CCMA 0346 (Cerrado soil), in eight different culture media. *Wickerhamomyces anomalus* CCMA 0358 cultivated in olive oil presented lowest value of surface tension (32.05 mN/m). A rotatable center composite design (RCCD) was performed for optimization of parameters in order to increase the production of the biosurfactant. The inclusion of yeast extract was 89% significant and the surface tension after optimization was 31.5 mN / m. Batch fermentations were carried out with stirring 500 rpm, and increasing the surface tension of 29.3 mN / m after 24 hours and yield of 2.60 g / L. Antimicrobials and oil recovery tests were performed with the biosurfactant. For the first species of *Streptococcus sanguis*, *Streptococcus oralis* and *Streptococcus agalactiae*, were growth inhibited by 100 % crude biosurfactant, *Candida albicans* has 78 % inhibition of *Staphylococcus epidermitis* and 66%. Twenty percent of the oil was recovered using only the supernatant of the fermentation, cell-free, containing the biosurfactant. The biosurfactant studied in this work was characterized as glycolipid, wherein the lipid phase comprises oleic acid. However, the glycidic phase could be identified, indicating that it is a novel compound. These results showed for the first time the production of biosurfactants by *Wickerhamomyces anomalus* CCMA 0358 which can be used in different biotechnological applications.

Keywords: Biosurfactant. Glycolipid. *Wickerhamomyces anomalus*. Fermentation. Antimicrobial. Oil removal.

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1 INTRODUÇÃO

Surfactantes são compostos químicos com atividade superficial extensivamente utilizado em várias indústrias, tais como, alimentícias, farmacêuticas, petróleo, entre outras (APARNA; SRINIKETHAN; SMITHA, 2012). Alguns surfactantes tradicionais são sintetizados a partir de fontes de petróleo, no entanto, estes apresentam riscos para o meio ambiente, devido a sua natureza recalcitrante (APARNA; SRINIKETHAN; SMITHA, 2012; REDDY et al., 2016). Nesse âmbito, biossurfactantes derivados de microrganismos exibem benefícios sobre os seus homólogos químicos, tais como menor toxicidade, biodegradabilidade, alta especificidade, estabilidade em extremos de temperatura, pH, salinidade e sua possível produção a partir de diferentes fontes renováveis (BORAH et al., 2015; REDDY et al., 2016).

A maioria dos biossurfactantes microbianos descritos na literatura é de origem bacteriana e os gêneros mais relatados como produtores são *Pseudomonas sp.*, *Acinetobacter sp.*, *Bacillus sp.* e *Arthrobacter sp.*, contudo, devido a natureza patogênica de alguns desses microrganismos, a aplicação destes compostos é limitada, não sendo adequada para utilização na indústria alimentar (SHEPERD; ROCKEY; SUTHERLAND, 1995). Diante desta temática, o estudo da produção de biossurfactantes por leveduras vem crescendo em importância, sendo relatados principalmente pelos gêneros, *Candida sp.*, *Pseudozyma sp.*, e *Yarrowia sp.*, a grande vantagem do uso de leveduras na produção de biossurfactantes é que a maioria das leveduras se apresentam como microrganismos seguros (GRAS), não sendo tóxicos ou patogênicos, permitindo suas aplicações nas indústrias alimentícias e farmacêuticas (BARTH; GAILLARD, 1997).

Biossurfactantes têm atraído muita atenção nos últimos anos, sendo estes compostos com atividade superficial sintetizados por microrganismos que exibem diversas estruturas químicas, incluindo glicolipídeos, lipopeptídeos, complexos de proteínas e polissacarídeos, fosfolipídeos, ácidos graxos e lipídeos neutros (GUDIÑA et al., 2013; SHEKHAR; SUNDARAMANICKAM; BALASUBRAMANIAN, 2015).

Glicolipídeos compreendem estruturalmente de ácidos graxos em combinação com um carboidrato e corresponde a um grupo de compostos que diferem com respeito da natureza da fração lipídica e de carboidrato. Com um resultado do tipo de carboidrato,

glicolipídeos podem ser subdivididos em lipídeos de ramnose, lipídeos de soforose, lipídeos de celobiose, lipídeos de manosileritritol, entre outros (MNIF; GHRIBI, 2016).

Além disso, os glicolipídeos apresentam várias funções, como, emulsificante, espumante, formação antiadesiva e antibiofilme, e ainda propriedades biológicas como atividade antimicrobiana, favorecendo sua utilização em indústrias alimentícias, como aditivos alimentares e conservantes (MNIF; GHRIBI, 2016). Na agricultura, apresenta atividade destrutiva contra algumas pragas, como fungos, ervas daninhas e insetos e o uso dos glicolipídeos tornou-se de grande interesse devido a sua baixa toxicidade, biodegradabilidade e sua natureza ambientalmente segura (CORTÉS-SÁNCHES; HERNÁNDES-SÁNCHEZ; JARAMILLO-FLORES, 2013).

Glicolipídeos podem ser produzidos a partir de materiais disponíveis em grandes quantidades tais como, resíduos e subrpodutos oleosos, incluindo hidrocarbonetos, resíduos de óleo de fritura e azeite (MNIF; GHRIBI, 2016). Além disso, a eficiência na produção de glicolipídeos por microrganismos foi melhorada juntamente com o avanço da biotecnologia como resultado do melhoramento das condições de fermentação, processos de fermentação em estado sólido, e a otimização da produção utilizando a metodologia da superfície de resposta (ABBASI et al., 2013; KIRAN; ANTO THOMAS; SELVIN, 2010; MANIVASAGAN et al., 2014; NALINI; PARTHASARATHI, 2014; VEDARAMAN; VENKATESH, 2010).

Dentro do contexto apresentado, objetivou-se neste estudo a avaliação da produção de biossurfactantes por cinco leveduras isoladas de diferentes ambientes, bem como a utilização de diferentes meios de cultivo visando maior produção do biossurfactante relacionado à maior redução da tensão superficial e atividade emulsionante, otimizaçãodo processo fermentativo para produção de biossurfactante em escala laboratorial com produção em biorreator, caracterização do biossurfactante e avaliaçãodas possíveis aplicações do biossurfactante como antimicrobiano e agente recuperador de petróleo.

2 REFERENCIAL TEÓRICO

2.1 Biossurfactantes

Biossurfactantes são compostos anfílicos, que podem reduzir a tensão superficial e interfacial, pelo acúmulo de fluídos imiscíveis que aumentam a solubilidade, mobilidade, e subsequente biodegradação de compostos orgânicos hidrofóbicos ou insolúveis, sendo produzidos por microrganismos (bactérias, fungos e leveduras) (SHEKHAR; SUNDARAMANICKAM; BALASUBRAMANIAN, 2015; SINGH; HAMME; WARD, 2007).

Os biossurfactantes microbianos constituem uma das principais classes de surfactantes naturais, podendo ser classificados de acordo com a origem microbiana e sua composição química. As principais classes conhecidas incluem glicolipídeos, lipopeptídeos, fosfolipídeos e ácidos graxos e ainda os surfactantes poliméricos e particulados (NITSCHKE; PASTORE, 2002).

Alguns microrganismos tem a habilidade de produzir moléculas com atividade superficial. Dois tipos de compostos de atividade superficial são produzidos por microrganismos: biossurfactantes e bioemulsionantes. Os biossurfactantes, reduzem significativamente a tensão superficial água-ar, enquanto os bioemulsionantes, não reduzem a tensão superficial mas estabilizam emulsões de óleo em água (ROSENBERBG; RON, 1999).

A produção do biossurfactantee pode ser espontânea ou induzida através da presença de compostos lipídicos, variações de pH, temperatura, aeração e agitação, ou ainda quando o crescimento celular é mantido sob condições de estresse como baixas concentrações de nitrogênio e alterações nas condições ótimas de pH e temperatura (BEZERRA, 2012).

2.2 Classificação dos biossurfactantes

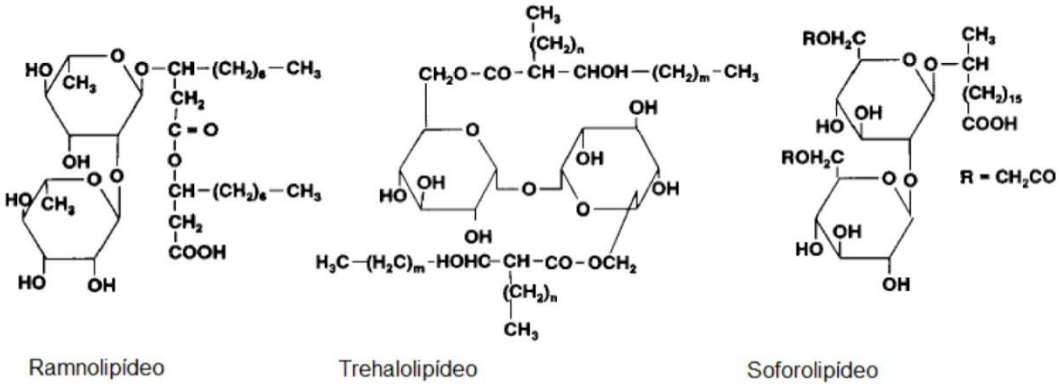
2.2.1 Glicolipídeos

Os biossurfactantes glicolipídeos compreendem uma seção de carboidratos hidrofílicos e uma cadeia de ácidos graxos hidrofóbica. O açúcar na parte hidrofílica final é denominado soforose em soforolipídeos, ramnose em ramnolipídeos e eritritol em lipídeos de manosileritritol (MARCHANT; BANAT, 2012).

Soforolipídeos são glicolipídeos extracelulares produzidos geralmente por espécies de *Candida*, quando cultivadas em carboidratos, presença de ácidos graxos ou a mistura de ambos. Os soforolipídeos consistem tipicamente de uma glicose dimérica (também chamada de soforose), ligados por uma ligação glicosídica a o penúltimo grupo hidroxil de um ácido graxo de 18 carbonos (GORIN; SPENCER; BHATTACHAFCJEE, 1969).

Os ramnolipídeos são os glicolipídeos mais bem estudados. A maioria das espécies de ramnolipídeos se apresenta aniônica quando em solução (ABALOS et al., 2001), possuem uma ou duas moléculas de ramnose ligadas a uma ou duas moléculas de ácido β-hidroxidecanóico. A primeira descrição da produção dos glicolipídeos contendo ramnose foi feita por Jarvis e Johnson (1949). A figura 1 representa a estrutura química de alguns exemplos de glicolipídeos.

Figura 1 - Estrutura química de três glicolipídeos, sendo eles: Ramnolipídeo, Trehalolipídeo e Soforolipídeo (FELSE et al., 2007).



Geralmente, os glicolipídeos são reconhecidos por sua boa estabilidade em condições extremas de pH, salinidade e temperatura (MNIF; GHRIBI, 2016). Trealolipídeo produzido por *Rhodococcus sp.* produz emulsões que são estáveis a ampla variedade de condições: pH 2-10, temperatura na faixa de 20 – 100 ^{o}C e concentrações de NaCl entre 5 - 25% (WHITE; HIRD; ALI, 2013).

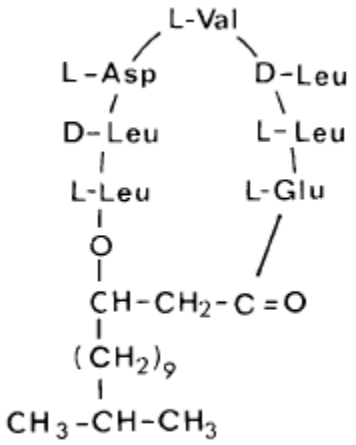
2.2.2 Lipopeptídeos

Os lipopeptídeossão comumente produzidos por espécies de *Bacillus subtilis* e recebeu o nome usual de surfactina, sendo conhecido como um dos biossurfactantes mais potentes (FIECHTER, 1992). Este biossurfactante é aniônico, possui capacidade de reduzir a tensão superficial de 72 para 27,9 mN/m em concentrações tão baixas quanto 0,005%. Sete

aminoácidos (Glu – Leu – Leu – Val – Asp – Leu – Leu) neste composto formam um anel e a porção lipídica é composta por um ácido graxo beta-hidroxilado contendo de 13 a 15 carbonos (ARAUJO; FREIRE, 2013).

Alguns lipopeptídeos apresentam-se como potentes antibióticos com propriedades de tensão superficial. *Bacillus subtilis* produz um lipopeptídeo ciclico chamado surfactina (Figura 2) (ARIMA; KAHINUMA; TAMURA, 1968), que tem sido relatado por ser um dos biossurfactantes mais ativos (ROSENBERG; RON, 1999).

Figura 2 - Estrutura química da surfactina.



Geralmente os lipopeptídeos são produzidos por microrganismos aeróbicos como: bactérias, fungos, leveduras e actinobactérias. Os surfactantes lipopeptídeos são produzidos naturalmente como mistura de várias macromoléculas pertencentes à mesma família ou classe, por exemplo, *Bacillus subtilis* JKK328 e *B. subtilis* S499, podem produzir três tipos de homólogos (surfactina, iturina e fegicina) (INÈS; DHOUHA, 2015).

2.2.3 Ácidos graxos, Fosfolipídeos e Lipídeos Neutros

Algumas espécies de bactérias e leveduras sintetizam grandes quantidades de surfactantes, ácidos graxos e fosfolipídeos durante seu crescimento em n-alcanos (CIRIGLIANO; CARMAN, 1985). O balanço hidrofílico e lipofílico é diretamente proporcional ao tamanho da cadeia de hidrocarbonetos e sua estrutura. Fungos, leveduras e bactérias possuem a capacidade de crescer em substratos hidrofóbicos como alcanos, e secretar grandes quantidades de fosfolipídeos, ácidos graxos ou lipídeos neutros para facilitar

a absorção da fonte de carbono (SHEKHAR; SUNDARAMANICKAM; BALASUBRAMANIAN, 2015).

2.2.4 Biossurfactantes poliméricos

Os mais bem estudados biossurfactantes poliméricos são alasan, liposan, lipomanan, emulsan e alguns outros complexos de polissacarídeos-proteínas. Mesmo em concentrações baixas (0,001% a 0,01%), emulsan é um efetivo agente emulsionante para hidrocarbonetos em água. Liposan é um emulsionante solúvel em água, sintetizado por *Candida lipolytica*, composto por 83% de carboidratos e 17% de proteínas, um complexo de carboidratos-proteínas sintetizado pela levedura *Yarrowia lipolytica* (SHEKHAR; SUNDARAMANICKAM; BALASUBRAMANIAN, 2015).

2.3 Aplicações Biotecnológicas dos biossurfactantes

Na natureza, biossurfactantes desempenham papel fisiológico aumentando a disponibilidade de moléculas hidrofóbicas, estes estão envolvidos na promoção da motilidade de microrganismos e participam de processos celulares fisiológicos de sinalização e diferenciação, além de estarem envolvidos no processo de formação de biofilme. Os surfactantes apresentam a capacidade de interagir com proteínas microbianas e podem ser manipulados para modificar a conformação da enzima, alterando sua atividade, estabilidade e/ou especificidade (SINGH; HAMME; WARD, 2007).

Algumas funções têm sido atribuídas aos biossurfactantes, como (NITSCHKE; PASTORE, 2002):

a) emulsificação e solubilização de hidrocarbonetos ou compostos insolúveis em água, facilitando o crescimento de microrganismos nesses substratos;
b) transporte de hidrocarbonetos: função atribuída aos biossurfactantes ligados à parede celular de *Candidatropicalis;*
c) aderência-liberação da célula a superfícies. Os microrganismos podem utilizar surfactantes ligados à parede para regular as propriedades da superfície celular, visando aderir ou se desligar de um local;
d) atividade Antibimicrobiana: demonstrada por vários biossurfactantes, principalmente da classe dos lipopeptídeos e glicopeptídeos. Os ramnolipídeos, por exemplo, funcionam como antibióticos, solubilizando os principais componentes

das membranas celulares microbianas. A inturina, um lipopeptídeo, apresenta atividade antifúngica, afetando a morfologia e a estrutura da membrana celular de leveduras.

O potencial de aplicação de compostos tensoativos produzidos a partir de microrganismos é baseado em suas propriedades funcionais, que incluem: emulsificação, separação, umedecimento, solubilização, desemulsificação, inibição de corrosão, redução de viscosidades de líquidos e redução da tensão superficial. Essas propriedades são aplicadas em campos diversos da agricultura, construção, nas indústrias alimentícias, de bebidas, papel, metal, têxtil, farmacêuticas e de cosméticos, bem como em pesquisas com biorremediação de poluentes (MUKHERJEE; DAS; SEN, 2006).

Tabela 1 - Aplicações industriais de surfactantes químicos e biossurfactantes (SINGH; HAMME; WARD, 2007)

Indústria	Aplicação	Papel dos surfactantes
Petróleo	Recuperação do petróleo	Melhorar a drenagem de óleo no solo; simultaneamente liberação do óleo retido nos capilares; humedecimento de superfícies sólidas; redução da viscosidade e ponto de fluidez do óleo.
Meio Ambiente	Biorremediação	Emulsificação de hidrocarbonetos, redução da tensão interfacial; sequestro de metais
Alimentos	Emulsificação e De-emulsificação	Agente de emulsificação, solubilização, desemulsificação, espumação
Alimentos	Ingredientes funcionais	Interação com lipídeos, proteínas e carboidratos, agente protetor
Biológicas	Microbiologia	Comportamente fisiológicos bem como a atividade celular, comunicação celular, competição entre célula-célula.
Agricultura	Biocontrole	Facilitação dos mecanismos de biocontrole dos microrganismos como parasitismo, antibióticos, competição, indução sistemática de resistência
Bioprocessos	Processos de Downstream	Biocatálises em sistemas aquosos de duas fases e microemulsões, biotransformação, recuperação de produtos intracelulares, aumentar a produção de enzimas extracelulares e produtos de fermentação
Cosméticos	Produtos de saúde e beleza	Emulsificantes, agentes espumantes, solubilizantes, agentes microbianos, mediadores de ação enzimática

Em todas as potenciais aplicações comerciais, o custo da produção é um fator crítico para determinar se um novo composto pode ser incorporado na formulação de produtos (cosméticos, produtos de higiene pessoal e limpeza, alimentos, entre outros). Neste caso, o passo inicial é determinar se o biossurfactante tem as características necessárias para substituir parcialmente ou na totalidade o surfactante químico já empregado (MARCHANT; BANAT, 2012).

2.3.1 Biorremedição

O uso de surfactantes sintéticos em sistemas de tratamentos de solo contaminado com hidrocarbonetos melhora a recuperação das áreas, aumentando a superfície de ação dos microrganismos (CUNHA et al., 2004).

Assim os biossurfactantes têm se destacado uma vez que podem ser utilizados em remediações de numerosos tipos de hidrocarbonetos contaminantes. Exemplo dessa atuação é dada pela surfactina, raminolipídeos e soforolipídeos, quesão capazes de remover cobre e zinco de solos contaminados com hidrocarbonetos (MULLIGAN; YONG; GIBBS, 2001).

Propriedades físico-químicas dos biossurfactantes estão envolvidas na interação entre células microbianas e os hidrocarbonetos imiscíveis pelos seguintes mecanismos: (i) emulsificação, (ii) micelarização, (iii) adesão de microrganismos para e a partir de hidrocarbonetos e (iv) dessorção de contaminantes. Estes fenômenos que ocorrem naturalmente podem ser explorados para melhorar tratamentos de biorremediação adicionando biossurfactantes e surfactantes químicos (RON; ROSENBERG, 2001; SINGH; HAMME; WARD, 2007).

2.3.3 Emulsionantes e Indústria de alimentos

Biossurfactantes são de grande interesse por suas propriedades físico-químicas e biológicas que podem ser exploradas nas indústrias farmacêuticas, de óleos minerais e de alimentos. A propriedade de formação e estabilização de emulsões é a principal característica a ser influenciada pela adição de biossurfactantes em alimentos. De forma geral, a função dos emulsificantes em alimentos é promover a estabilidade da emulsão, controlando a aglomeração de glóbulos de gordura e estabilizando sistemas aerados (BARROS et al., 2007).

Bioemulsionantes podem ser produzidos a partir de fontes renováveis, são biodegradáveis e apresentam atividade em variadas condições, como condições extremas de pH, salinidade e temperatura. As espécies de interesse para produção de emulsionantes são aquelas que produzem biossurfactantes termofílicos e halofílicos (ROSENBERG; RON, 1999).

A emulsificação tem papel importante na formação da consistência e textura, bem como na dispersão de fase e na solubilização de aromas (BANAT; MAKKAR; CAMEOTRA, 2000). Os biossurfactantes são utilizados como emulsionantes no processamento de matérias-primas. Os agentes tensoativos encontram aplicação em panificação e produtos derivados de

carne, onde influenciam as características reológicas da farinha e a emulsificação de gorduras. Um exemplo é o bioemulsinante produzido por *Candida utilis* que tem sido utilizado em molhos prontos para salada (NITSCHKE; PASTORE, 2002).

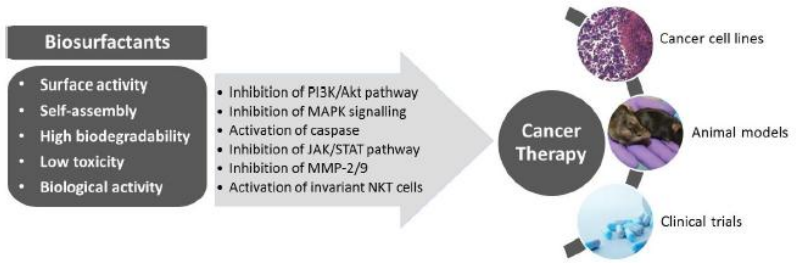
A elaboração de testes e avaliação de qualquer novo ingrediente é requerida de acordo com o regulamento da "U. S. Food and Drug Administration" e este processo pode ser longo. Os fatores a serem considerados nessa avaliação estão relacionados às questões ambientais e toxicológicas do novo aditivo, além disso, outros fatores devem ser considerados, tais como fatores econômicos, quando comparados aos surfactantes sintéticos para o mesmo uso, aceitação pelo consumidor, regulação legal e hábitos alimentares dos consumidores (BARROS et al., 2007).

2.3.4 Indústria farmacêutica

A surfactina possui várias aplicações farmacêuticas como a inibição da formação de coágulos, formação de canais iônicos em membranas, atividade antibacteriana e antifúngica, atividade antiviral e antitumoral (PEYPOUX; BONMATIN; WALLACH, 1999).

Biossurfactantes, em particular lipopeptídeos e glicolipídeos, tem sido destaque por seu potencial para serem utilizados como agentes anti-câncer interferindo no processo da formação do câncer (Figura 3). Esses compostos têm sido implicados em vários processos de reconhecimento molecular intracelular, compreendendo o sinal de transdução, diferenciação celular e resposta imune da célula, entre outros (RODRIGUES et al., 2006). Além disso, eles exibem baixa toxicidade, alta eficácia e fácil biodegradabilidade, que são características relevantes em qualquer tratamento anti-câner (GUDIÑA; TEIXEIRA; RODRIGUES, 2016).

Figura 3 - Propriedades e efeitos anti-câncer de biossurfactantes em direção a um novo desenho de terapias contra o câncer (GUDIÑA; TEIXEIRA; RODRIGUES, 2016).



Foram propostos diferentes mecanismos subjacentes à atividade anticancerígena de biossurfactantes, incluindo o atraso da progressão do ciclo celular; inibição de vias de sinalização cruciais; redução da angiogênese; ativação das células T assassinas e indução da apoptose através de receptores de morte em células cancerígenas. Além disso, os biossurfactantes possuem a capacidade de romper a membrana celular, levando a uma consequência de eventos que incluem a lise, aumento da permeabilidade da membrana e extravasamento de metabólitos, que também foram apontados como mecanismo de atividade anti-câncer (GUDIÑA; TEIXEIRA; RODRIGUES, 2016; JANEK et al., 2013).

Devido à versatilidade bioquímica, bem como excelente propriedade interfacial, o biossurfactante lipídeo Manosileritritol, apresenta extensivas aplicações farmacêuticas como na atividade de indução da diferenciação contra células de leucemia em humanos, células de feocromocitoma em ratos e células de melanoma em ratos, além disso, também inibem a secreção inflamatória mediada por mastócitos, sendo também utilizados no tratamento da esquisofrenia ou doenças causadas pela disfunção da dopamina, e ainda infecções microbianas (YU et al., 2015).

Dentre as diversas aplicações dos biossurfactantes, a mais expressiva, é a sua capacidade de controlar uma variedade de funções celulares em mamíferos e, por conseguinte, o seu potencial para atual como agentes anti-tumorais (Tabela 2) que interferem com alguma progressão no processo do câncer (GUDIÑA et al., 2013).

Tabela 2 - Biossurfactantes com atividade antitumoral contra células de câncer humanas.

Biossurfactantes	Descrição	Atividade
Lipídeos de Manosileritritol (MELs)	Leucemia mielóide	Inibição do crescimento/diferenciação
Soforolipídeos	Leucemia promielocítica/ Câncer no fígado/ Câncer no pulmão/ Câncer no pâncreas	Interação com a membrana plasmática/ inibição do crescimento/ indução da apoptose/ necrose
Surfactina	Carcinoma hepatocelular/ Leucemia mielóide/ Adenocarcinoma do cólon/ Câncer de mama/ Câncer cólonretal/ Câncer no cólon	Inibição do crescimento/ indução da apoptose/ parada do ciclo celular/
Glicoproteínas de *Lactobacillus paracasei*	Câncer de mama	Inibição do crescimento/ Parada do ciclo celular

Fonte: Gudiña et al. (2013)

2.3.5 Agricultura

Os biossurfactantes de origem microbiana podem ser utilizados com diversas funções na agricultura, como mostrado na Figura 4. Os compostos ativos são geralmente hidrofóbicos, sendo necessários agentes emulsificantes para dispersá-los em soluções aquosas.

Ramnolipídeos que possuem potencial para o controle biológico de fitopatógenos produtores de zoósporos (NITSCHKE; PASTORE, 2002), e os biossurfactantes produzidos por rizobactérias são conhecidos por possuírem atividade antagonista (NIHORIMBERE et al., 2011).

Alguns biossurfactantes microbianos possuem atividade antagônica contra patógenos de plantas. Assim, são considerados como moléculas promissoras de biocontrole para alcançar a agricultura sustentável (SACHDEV; CAMEOTRA, 2013).

Soforolipídeos, lipídeos de celobiose e lipídeos de manosileritritol, estão envolvidos na proteção de plantas pela inibição do crescimento de fungos patogênicos. Os soforolipídeos, por exemplo, tem atividade contra numerosos fungos, incluindo *Cladosporium, Aspergillus, Fusarium, Penicillium, Gloeophyllum* e *Schizophyllu* (MNIF; GHRIBI, 2016).

2.4 Microrganismos potencialmente produtores de biossurfactantes

2.4.1 Bactérias

O principal grupo de microrganismos produtores de biossurfactantes são as bactérias, apesar de também serem produzidos por espécies de fungos filamentosos e leveduras. Esses microrganismos são capazes de sintetizar biossurfactantes a partir de hidrocarbonetos, compostos solúveis em água como glicose, sacarose, glicerol ou etanol e podem tanto serem excretados ou ligados a parede celular (DESAI; BANAT, 1997).

As bactérias desempenham papel importante na produção de biossurfactante, sendo *Pseudomonas* o gênero predominante (SHEKHAR; SUNDARAMANICKAM; BALASUBRAMANIAN, 2015). Contudo, *Bacillus sp.* é o mais conhecido na produção de lipopeptídeos (JENNEMAN et al., 1983).

Espécies de *Lactobacillus* sintetizam menor quantidade de biossurfactantes quando comparado a *Bacillus subitilis* e *Pseudomonas aeruginosa*, no entanto, constituem promissora fonte de biossurfactantes por serem considerados microrganismos seguros, já sendo utilizados na fabricação de alguns alimentos. Além disso, com a otimização das condições de cultivo, pode-se aumentar o rendimento da produção de biossurfactantes por essas espécies (GUDIÑA; TEIXEIRA; RODRIGUES, 2011).

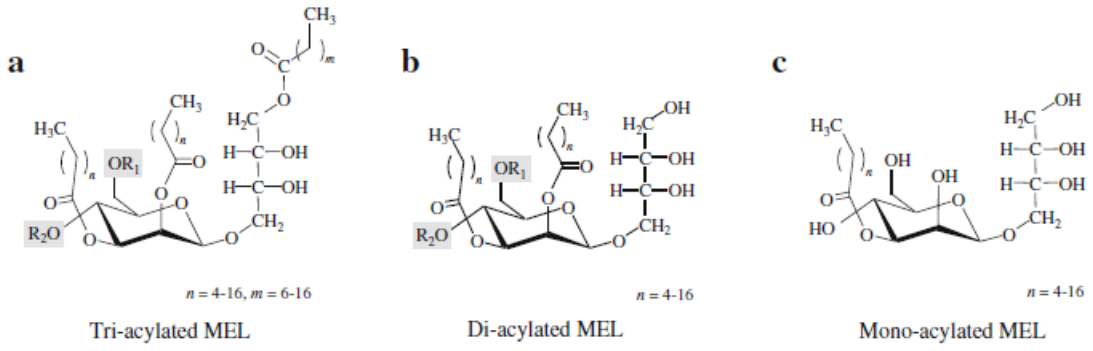
2.4.2 Leveduras

Assim como as bactérias, as leveduras também são potenciais produtoras de biossurfactantes e têm sido de grande importância, sendo relatados principalmente pelos gêneros *Candida* sp. *Pseudozyma* sp. e *Yarrowia* sp. A grande vantagem do uso de leveduras na produção de biotensoativo é que a maior parte dessas espécies são reconhecidas como GRAS (generally regarded as safe), como por exemplo, *Yarrowia lipolytica, Saccharomyces cerevisiae e Kluyveromyces lactis*,sendo permitido a aplicação de seus produtos na indústria farmacêutica e de alimentos.

Algumas leveduras como *Pseudozyma antarctica* e *Rhodococcus erythropolis*, são capazes de produzir grandes quantidades de glicolipídeos quando a fermentação é realizada em meio contendo glicerol bruto (subproduto da indústria de biodiesel) (FONTES et al., 2012). Outros estudos também relataram a produção de compostos de interesse como biossurfactantes, ácidos orgânicos, lipídeos e ácido cítrico pela levedura *Yarrowia lipolytica*a partir de glicerol puro e bruto (FONTES et al., 2012; PAPANIKOLAOU et al., 2003; SOUZA; SCHWAN; DIAS, 2014).

Um exemplo de biossurfactante produzido abundantemente por leveduras é o lipídeo manosileritritol (MEL) (Figura 4), um glicolipídeo que apresenta não apenas excelente propriedade interfacial como tambémexibe alta atividade antimicrobiana contra bactérias Gram-positivas. Este glicolipídeo apresenta excelentes propriedades cosméticas, como hidratação de pele seca e reparação de danos nos cabelos (MORITA et al., 2013).

Figura 4 - Estrutura química do lipídeo manosileritritol. **a:** um tipo de MEL triacilado; **b:** MEL diacilado; **c:** MEL monoacilado.



Algumas espécies de leveduras e os seus respectivos biossurfactantes produzidos, estão descritos na Tabela 3.

Tabela 3 - Biossurfactantes produzidos por espécies de leveduras (AMARAL et al., 2008)

Biossurfactante	Microrganismos produtores
Soforolipídeos	*Candida bombicola*
	Torulopsis petrophilum
	Candida (torulopsis) apícola
	Torulopsis apícola
	Candida bogorienses
Lipídeos de Manosileritritol	*Candida antártica*
	Pseudozyma rugulosa
	Candida sp. SY16
	Pseudozyma aphidis
	Kurtzmanomyces sp. I-11
	Pseudozyma fusifornata, P. parantarctica
	P. tsukubabaensis
Complexos de lipídeos-Carboidratos-proteínas	*Candida lipolytica UCP0988*
	Candida lipolytica IA 1055
	Yarrowia lipolytica NCIM 3589
	Debaromyces polymorphus
	Candida tropicalis
Complexos de proteínas-carboidratos	*Candida lipolytica ATCC 8662*
	Yarrowia lipolytica IMUFRJ 50682
Mananoproteína	*Saccharomyces cerevisiae*
	Kluyveromyces marxianus
Ácidos graxos	*Candida ingens*
Lipídeos	*Rhodotorula glutinis*

2.5 Fontes utilizadas para produção de biossurfactantes

O fator chave que indica o sucesso da produção de biossurfactantes, é o desenvolvimento de processos econômicos que utilizam materiais de baixo custo, obtendo-se alto rendimento e produtividade (SANTOS et al., 2013). Diferentes elementos como nitrogênio, ferro e manganês são descritos por afetar o rendimento do biossurfactante (MUKHERJEE; DAS; SEN, 2006). A limitação de nitrogênio é relatada por aumentar a produção do biossurfactante por *Pseudomonas aeruginosa* BS-2 (DUBEY; JUWARKAR, 2004) e *Ustilago maydis* (HEWALD; JOSEPHS; BOLKER, 2005).

Na literatura, diferentes fontes renováveis e resíduos agroindustriais têm sido utilizados para produção de biossurfactantes por microrganismos, tais como: melaço (MAKKAR; CAMEOTRA, 1997), manipueira (NITSCHKE; PASTORE, 2002), águas ruças (MERCADE; MANSERA, 1994), gordura animal (DESPHANDE; DANIELS, 1995), glicerol da produção de biodiesel (FONTES et al., 2012), sendo utilizados como substratos de baixo custo.

Meios de cultivo de baixo custo, baseado em gordura animal e água residual de maceração, combinados com glicose, extrato de levedura, uréia e outras fontes de nitrogênio inorgânicas também podem ser utilizadas na produção de biossurfactantes por leveduras (SANTOS et al., 2013).

O aproveitamento de resíduos ou subprodutos é um dos maiores problemas enfrentados pelas indústrias, que buscam a exploração total das matérias-primas e o desenvolvimento de tecnologias limpas. Neste contexto, algumas leveduras são capazes de crescer em resíduos industriais como gorduras, óleos vegetais, glicerol bruto, soro de queijo desproteinizado e melaços, podendo produzir agentes ativos de superfície durante o crescimento (SANTOS et al., 2013).

A Tabela 4 descreve a cepa microbiana e o respectivo biossurfactante produzido a partir de fontes de baixo custo.

Tabela 4 - Uso de materiais brutos para a produção de biossurfactantes por diferentes microrganismos (MUKHERJEE; DAS; SEN, 2006).

Material bruto de baixo custo	**Tipo do biossurfactante**	**Microrganismo produtor**
Óleo de Colza	Ramnolipídeos	*Pseudomonas* species
Óleo de Babaçu	Soforolipídeos	*Candida lipolytica*
Óleo de milho turco	Soforolipídeos	*Candida bombicola*
Óleo de girassol e soja	Ramnolipídeos	*Pseudomonas aeruginosa*
Óleo de girassol	Lipopeptídeo	*Serratia marcescens*
Óleo de soja	Lipídeo de manosileritritol	*Candida sp.*
Resíduo de óleo de fritura (óleo de girassol ou soja)	Ramnolipídeos	*Pseudomonas aeruginosa*
Resíduo de borra de soja	Ramnolipídeos	*Pseudomonas aeruginosa*
Resíduo de óleo de girassol	Ramnolipídeos	*Pseudomonas aeruginosa*
Resíduos de refinaria de petróleo	Glicolipídeos	*Candida antarctica/ apícola*
Resíduos de soja de refinaria de petróleo	Ramnolipídeos	*Pseudomonas aeruginosa*
Soro de leite e resíduos de destilaria	Ramnolipídeos	*Bacilus subtilis*
Efluentes do processamento da batata	Lipopeptídeo	*Bacilus subtilis*
Farinha de Manipueira	Lipopeptídeo	*Bacilus subtilis*

3 CONSIDERAÇÕES FINAIS

Os biossurfactantes apresentam diversas vantagens em relação aos surfactantes sintéticos, podendo ser utilizados em uma gama de aplicações industriais, tais como, antimicrobianos, biorremediação de água e solos, indústria alimentícia, farmacêutica, entre outras.

Em virtude do aumento dos esforços no desenvolvimento de novas tecnologias de aplicação, na busca por microrganismos potencialmente produtores e dos processos de produção, e devido à sua versatilidade, biodegradabilidade e baixa toxicidade, os biossurfactantes poderão se tornar compostos de uso comum nas indústrias.

O sucecsso na ampliação de escala da produção de biossurfactantes por leveduras, capazes de torná-los economicamente competitivos em relação aos surfactantes químicos, são necessários desenvolvimentos acerca dos tópicos abordados nesta revisão, abrindo novas perspectivas para aumentar a eficiência da produção, tornando possível a aplicação industrial desses compostos.

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SEGUNDA PARTE

ARTIGO 1

New glycolipid biosurfactants produced by the yeast strain *Wickerhamomyces anomalus* CCMA 0358

Submetido a Colloids and Surfaces B: Biointerfaces

Abstract

In this work, biosurfactant production by several yeast strains was evaluated using different culture media. The best results were obtained with the strain *Wickerhamomyces anomalus* CCMA 0358 growing in a culture medium containing glucose (1 g/L) and olive oil (20 g/L) as carbon sources. This strain produced 2.6 g of biosurfactant per liter after 24 hours of growth. The crude biosurfactant reduced the surface tension of water to values around 31 mN/m, and its critical micelle concentration was 0.9 mg/mL. This biosurfactant was characterized through mass spectrometry (MS), and nuclear magnetic resonance (NMR) as a mixture of two different glycolipids, comprising a sugar moiety linked to one or three molecules of oleic acid. To the best of our knowledge, these biosurfactants are structurally different from those previously reported. Furthermore, the crude biosurfactant exhibited antimicrobial activity against several microorganisms, including the pathogens *Candida albicans*, *Escherichia coli*, *Staphylococcus epidermidis* and*Streptococcus agalactiae*, which opens the possibility for its use in several biomedical applications.

Keywords: biosurfactant; glycolipid; antimicrobial activity; *Wickerhamomyces anomalus*.

1. Introduction

Microbial surfactants or biosurfactants are a natural class of surface-active molecules with diverse structures that are produced by different microorganisms, including bacteria, yeasts and filamentous fungi. They are amphiphiliccompounds, comprisinga hydrophilic head and a hydrophobic tail, that are usually classified according to their chemical structure, being glycolipids and lipopeptides the most important classes [1, 2]. Biosurfactants can be synthesized by microorganisms growing on water-immiscible substrates (e.g. vegetable oils, hydrocarbons) as well as on water-soluble compounds (e.g. carbohydrates, glycerol), and their production and composition are usually conditioned by the culture conditions and the composition of the culture medium [3-5]. These compounds play a critical role in the survival of their producing microorganisms, as they enhance the solubility of water-insoluble compounds (facilitating their transport into the cell), and participate in processes such as cell adhesion and aggregation, quorum sensing, biofilm formation and defense against other microorganisms [1, 2].

Besides reducing surface and interfacial tensions in multi-phase systems, these moleculesexhibit some interesting properties as compared to chemical surfactants, such aslow toxicity and high biodegradability, improved environmental compatibility, ability to form foams and emulsions, as well as tolerance to extreme temperature, pH and salinity conditions [6-8]. For that reason, biosurfactants constitute an environmentally friendly alternative to traditional synthetic surfactants in several fields.

In addition, several biosurfactantsexhibit antibacterial, antifungal and antiviral activities, which makes them relevant molecules for applications envisaging fighting diseases and infections [9-12]. Given the current efforts in searching and developing new agents that can replace traditional antibiotics, biosurfactants represent an extremely promising approach that ought to be further explored. Indeed, some biosurfactants have been described as viable alternatives to synthetic medicines and can be used as safe therapeutic agents, as anticoagulants and as anti-adhesive coatings on biomaterials [10, 12, 13, 14].Moreover, several biosurfactants exhibit anti-tumor activity and have shown potential to interfere with some cancer progression processes [15, 16, 1].

Most of the microbial surfactants reported are produced by bacteria such as *Bacillus* and*Pseudomonas*species [17, 18]. However, due to the pathogenic nature of some of these microorganisms, the use of these compounds has been limited to few applications, often not being suitable for use in the food industry, among others.The evaluation of biosurfactant production by yeasts has increased in the last years, and several strains have been reported as promising biosurfactant producers due to their high production yields and their high substrate conversion rates; among them stand out species belonging to the genera *Candida*, *Starmerella*, *Pseudozyma* or *Yarrowia* [19, 20, 3, 4].Another great advantage of using yeasts for the production of biosurfactants is the GRAS (Generally Regarded as Safe) status that most of these strainsexhibit, thus allowing the use of their products in the food and pharmaceutical industries.In addition, the microbial surfactants produced by yeasts are expected to present distinct structures and performances as compared to the commonly reported ones, which hold a great promise regarding the possibility of finding new bioactive agents.

The aim of this study was to evaluatethe production ofbiosurfactants by five wild type yeast strainsisolated from differentenvironments, as well as to characterize potentially novel biosurfactants regarding their physicochemical and biological features.

2. Material and Methods

2.1 Strains and culture conditions

The yeast strains used in this study were obtained from the Culture Collection of Agricultural Microbiology, CCMA (Department of Biology, Federal University of Lavras, Brazil), and included: *Yarrowia lipolytica* CCMA 0357 (isolated from Amazon soil); *Yarrowia lipolytica* CCMA 0242 (isolated from Kefir water); *Wickerhamomyces anomalus* CCMA 0358 (isolated from coffee processing by-products); *Lindnera saturnus* CCMA 0243 and *Cryptococcus humicola* CCMA 0346 (both isolated from Cerrado soil). All strains were grown in YEPG medium (10 g/L yeast extract; 20 g/L peptone; 20 g/L glucose, pH 6.5) at 28ºC.The following strains used in the antimicrobial assays were kindly provided by the Faculty of Pharmacy, University of Porto (Portugal): *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Streptococcus oralis* and *Streptococcus sanguis*. These strains were grown at 37ºC. *C. albicans*, *E. coli*, *P.*

aeruginosa, *S. aureus* and *S. epidermidis* were grown in Luria-Bertani (LB) medium, whereas *S. agalactiae*, *S. oralis* and *S. sanguis* were grown in Todd-Hewitt Broth (THB). Both media were purchased from OXOID (Basingstoke, England).

2.2 Screening biosurfactant-producing yeast strains

Eight culture media were used to evaluate the production of biosurfactants by the different yeast strains under study (Table 1). Olive oil and soybean oil were purchased from local markets. Olive oil mill wastewater (OMW), a residue generated during olive oil production, was obtained from an olive oil mill located in the north of Portugal. The assays were performed in flasks (500 mL capacity) containing 200 mL of the different media. Each flask was inoculated with a pre-culture of the corresponding yeast strain (grown overnight in YEPG medium at 28ºC and 200 rpm) to attain an initial cell concentration of 10^7 cells/mL. Subsequently, the flasks were incubated at 28ºC and 200 rpm up to 144 h. Samples (4 mL) were taken every 24 h to evaluate cell growth and biosurfactant production. The cell growth was determined according to the number of cells counted using a Neubauer improved cell counter (Marienfeld GmbH, Germany). Afterwards, the samples were centrifuged (2700 × *g*, 15 min) and the cell-free supernatants were used to assess the biosurfactant production through surface tension measurement and determination of the emulsifying activity, as described below.

Table 1. Composition (g/L)and surface tension values (ST, mN/m) ofculture media evaluated for the production of biosurfactants by the yeast strains under study.

	Culture medium							
Composition (g/L)	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**
Glucose	5.0	25.0	25.0	25.0	1.0	1.0	1.0	1.0
Yeast extract	0.5	1.0	1.0	1.0	4.0	4.0	-	-
$(NH_4)_2SO_4$	0.5	-	-	-	-	-	4.0	4.0
NaCl	-	0.1	0.1	0.1	-	-	-	-
Glycerol	30.0	30.0	-	-	-	-	-	-
Olive oil	-	-	-	-	20.0	-	20.0	20.0
OMW	-	-	-	30.0	-	20.0	-	-
Soybean oil	-	-	30.0	-	-	-	-	-
Tween 80	-	-	-	-	-	4.0	4.0	-
KH_2PO_4	7.0	-	-	-	-	-	-	-
Na_2HPO_4	2.5	-	-	-	-	-	-	-
$MgSO_4.7H_2O$	1.5	-	-	-	-	-	-	-
$CaCl_2.2H_2O$	0.15	-	-	-	-	-	-	-
$ZnSO_4.7H_2O$	0.02	-	-	-	-	-	-	-
$MnSO_4.H_2O$	0.06	-	-	-	-	-	-	-
ST (mN/m)	53.4 ± 0.1	50.6 ± 0.1	48.6 ± 0.1	44.2 ± 0.1	52.4 ± 0.1	41.7 ± 0.1	37.5 ± 0.1	50.5 ± 0.1

2.3 Surface-activity measurement

The surface tension of the cell-free supernatants was measured according to the Ring method as described elsewhere [17]. A KRÜSS K6 Tensiometer (KRÜSS GmbH, Germany) equipped with a 1.9cm De Noüy platinum ring was used. All the measurements were performed in triplicate at room temperature (25ºC).

2.4 Emulsifying activity determination

The emulsifying activity was determined adding 2 mL of *n*-hexadecane to the same volume of cell-free supernatants in glass test tubes. The tubes were mixed with a vortex at high speed for 2 min and subsequently incubated at 25ºC for 24 h. The emulsification indexes (E_{24}, %) were calculated as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm) [7]. All the emulsification indexes were determined in triplicate.

2.5 Biosurfactant recovery

The yeast strain that offered the best results regarding biosurfactant production was selected to perform the following studies. The biosurfactant produced was recovered from the cell-free supernatant by adsorption chromatography, using a glass column (430 mL) filled with the polystyrene resin Amberlite XAD-2 (Sigma-Aldrich, USA), as described by Gudiña et al. [18]. Briefly, 250 mL of the cell-free supernatant obtained at the end of the fermentation were passed through the column until the surface tension of the effluent was equal or higher than 50 mN/m. Subsequently, the column was washed with three volumes of demineralized water to remove the non-adsorbed compounds. Finally, the biosurfactant adsorbed to the resin was eluted with three volumes of methanol; the methanol was removed using a rotary evaporator at 40ºC and the product obtained was dissolved in a minimal amount of demineralized water and freeze-dried. The crude biosurfactant obtained was weighed and stored at -20ºC for further studies.

2.6 Critical micelle concentration (cmc) calculation

Critical micelle concentration (*cmc*) is defined as the concentration of an amphiphilic compound in solution at which the formation of micelles is initiated. Different concentrations of the freeze-dried crude biosurfactant were prepared in demineralized water, and the surface tension of each sample was measured as described above. The *cmc* was determined by plotting the surface tension as a function of the logarithm of biosurfactant concentration, and it was found at the point of intersection between the two lines that best fit the pre- and post-*cmc* data, as described elsewhere [17]. All the measurements were performed in triplicate.

2.7 Biosurfactant chemical characterization

Mass spectrometry (MS)

A sample of crude biosurfactant was analyzed by direct injection of a methanolic solution into an Electrospray ionization source, ion-trap quadrupole mass spectrometer (LCQ DECA XP MAX, ThermoFinnigan) in positive and negative mode. Tandem mass spectra were obtained for the most intense ions to charge mass ratios.

Liquid Chromatography–Mass Spectrometry (LC-MS)

A sample of crude biosurfactant was injected into a HPLC-DAD-UV (LCQ Thermo-Finnigan Surveyor) detection system using a previously reported method [21] with some modifications. The column used was a LiChroCART C18 column (4.6 x 150 mm; 5 µm), operating at 0.5 mL/min and 210 nm. The mobile phase was a gradient of acetonitrile and a solution of 1% of formic acid with an elution profile of 10% of acetonitrile for 5 min to 100% acetonitrile for an additional 10 min and 100% acetonitrile for more 30 min. The HPLC was connected to the mass spectrometer with an ESI probe at a spray voltage of 5 KV, capillary voltage of 4.5 V and capillary temperature of 325^{o}C, in positive and negative mode and data dependent analysis scan mode.

Transesterification/Silylation

An aliquot of 2 mg of crude biosurfactant sample was dissolved in methanol in the presence of catalytic amounts of sulfuric acid. The mixture was then extracted with *n*-hexane and the FAME (fatty acid methyl ester) recovered were analyzed by GC-MS. An aliquot of 2 mg of crude biosurfactant sample was mixed with hexamethyldisilazane (1 mL) in dimethylformamide (DMF) solvent (1mL). The mixture was heated in oil bathat 100ºC during 1 h and the tetramethylsilane (TMS) fatty acids derivatives were analyzed by GC-MS. An aliquot of 1 mg of the crude biosurfactant sample was hydrolyzed with pectinase in sodium acetate buffer at pH of 4.8 at 40ºC. The enzyme was precipitated with acetonitrile and the resulting solution, previously adjusted to pH 2 with glacial acetic acid, was evaporated to dryness in a rotary evaporator. The resulting fraction was silylated to the TMS derivatives and analyzed by GC-MS.

Gas Chromatography-Mass spectrometry (GC-MS)

FAME was analyzed by gas chromatography (GC) coupled to a single quadrupole mass detector. Separation was achieved using a TG-5MS capillary column (60 m long, 0.25 mm i.d., 1 µm film thickness). Mass spectrometry (MS) transfer line, ion source and injector temperatures were 300ºC, 280ºC and 300ºC, respectively. The mass detector was operating in the electron impact ionization (EIMS) with an electron energy of 70 eV. The initial column temperature was 150ºC, which was kept for 5 min, and then raised to 300ºC at a rate equal to 6ºC/min, where it was maintained for more 15 min. The injector was used in the splitless mode during 1 min. Helium was used as carrier gas at a constant flow rate of 1.2 mL/min. Mass ranges was from 40 to 1000 with scan times every 0.2 sec.

Partial Purification of the biosurfactant sample

The biosurfactant sample was purified by solvent extraction in two septs. Firstly, the crude biosurfactant sample was extracted with *n*-hexane and centrifuged. Secondly the resulting solid was extracted with ethyl acetate, and centrifuged again. The residue was kept for further analysis.The solvents were vacuum-dried removed and the two fractions, hexane fraction and ethyl acetate fraction, were analyzed by NMR and MS.

Nuclear Magnetic Resonance Spectroscopy (NMR)

For the NMR analysis aliquots of each fraction were dissolved in CDCl3 in a 5 mm NMR tube.

^{1}H NMR (500.13 MHz) and ^{13}C NMR (125.77 MHz) spectra were recorded in CD_3OD/ TFA (98:2) on a Bruker-Avance 500 spectrometer at 303 K and with TMS as an internal standard (chemical shifts (δ) in parts per million, coupling constants (J) in hertz). Multiplicities are recorded as singlets (s), doublets (d), triplets (t), doublets of doublets (dd), multiplets (m) and unresolved (*).^{1}H chemical shifts were assigned using 2D NMR (COSY) experiment while ^{13}C resonances were assigned using 2D NMR techniques (*g*HMBC and *g*HSQC). The delay for the long range C/H coupling constant was optimized to 7 Hz.

2.8 Antimicrobial assays

The antimicrobialactivity of the crude biosurfactant against several microorganisms was determinedusing the microdilutionmethodin96-well plastic tissue culture plates (Orange Scientific, Belgium), as described by Gudiña et al. [22]. Briefly, the crude freeze-dried biosurfactant was dissolved in demineralized water at a concentration of 5.2 g/L, and sterilized by filtration through a 0.2 µm pore-size filter. Subsequently, 125 µL of double strength culture medium (LB or THB, depending on the microorganism) were placed into the 1st column of the 96-well microplate, and 125 µL of single strength culture medium in the remaining wells. After that, 125 µL of the crude biosurfactant solution were added to the 1st column of the microplate and gently mixed with the medium. Subsequently, 125 µL from the 1st column were transferred to the 2nd column and mixed; serially, 125 µL were transferred to the subsequent wells, discarding 125 µL of the mixture in the 10th column, so that the final volume in each well was 125 µL. This process resulted in two-fold serial dilutions of the biosurfactant in the first 10 columns. Columns 11 and 12 did not contain biosurfactant and

served as negative and growth controls, respectively. All the wells (except for the 11th column) were inoculated with 2.5 µL of a pre-culture of the corresponding microorganism grown overnight in the appropriate culture medium (LB or THB) at 37ºC. The microplates were covered and incubated for 24 or 48 h (depending on the microorganism) at 37ºC. After the incubation time, the optical density at 600 nm was determined for each well. The growth inhibition percentages at different biosurfactant concentrations for each microorganism were calculated as:

$$\% \text{ growth inhibition}_c = [1 - (OD_c/OD_0)] \times 100, \quad \text{(Equation 1)}$$

where OD_c represents the optical density of the well with a biosurfactant concentration c, and OD_0 is the optical density of the control well (without biosurfactant). Triplicate assays were performed for each microorganism and biosurfactant concentration.

3.Results and Discussion

3.1 Screening biosurfactant-producing yeast strains

The yeast strains under study were grown indifferent culture media (Table 1) in order to evaluate their potential as biosurfactant producers. Since biosurfactants reduce the surface tension of the medium, this potential was assessed as the difference between the surface tension at time zero (i.e. no biosurfactant produced) and the lowest surface tension value obtained along the fermentation (Table 2). The yeast strains were considered biosurfactant producers when this difference was equal or higher than 8 mN/m, as reported elsewhere [22].
The carbon sources used to evaluate biosurfactant production included glycerol (media I and II), olive oil (media V, VII and VIII) and soybean oil (medium III), which have been used previously as substrates for biosurfactant production by yeasts [4, 19,21,23, 24] The agro-industrial waste OMW was also evaluated as a potential low-cost carbon source (media IV and VI). The culture media were formulated according to previous reports [4]. In all the cases, the different lipophilic substrates (olive oil, OMW and soybean oil) were used in combination with glucose, as it has been reported that biosurfactant production by yeasts is higher when hydrophobic and a hydrophilic carbon sources are supplied together in the culture medium [5, 19, 20,23, 24].

Table 2. Surface tension reductions obtained at the optimum time (h) with the yeast strains grown in different culture media. Culture media composition is given in Table 1. Only those media where surface tension reductions were observed are presented. Results represent the average of triplicate experiments ± standard deviation.

Yeasts	Culture medium					
	I	**II**	**III**	**V**	**VI**	**VIII**
Yarrowia lipolytica CCMA 0357	10.8± 0.4 (120)	6.0±0.1 (96)	1.5 ± 0.3 (120)	12.4 ± 0.3 (24)	4.1±0.8 (120)	10.5±0.1 (72)
Yarrowia lipolytica CCMA 0242	1.9±0.2 (24)	2.6±0.8 (24)	1.0± 0.7 (72)	12.3± 0.1 (96)	2.1±0.3 (24)	10.3± 0.1 (72)
Cryptococcus humicola CCMA 0346	4.1±0.6 (120)	4.4± 1.4 (96)	5.6 ± 1.1 (72)	8.9 ± 1.1 (24)	1.3±0.9 (24)	8.0±0.3 (48)
Lindnera saturnus CCMA 0243	0.4 ± 0.1 (96)	4.6 ± 1.3 (96)	0.7 ± 0.4 (120)	9.5 ± 0.1 (72)	0.7± 0.0 (24)	9.9± 0.1 (24)
Wickeramomyces anomalus CCMA 0358	10.9±1.0 (72)	5.2 ± 0.1 (24)	3.5 ± 0.4 (24)	16.0 ± 0.1 (24)	1.7±0.1 (72)	10.1± 0.7 (24)

Biosurfactant production was observed with three of the culture media assayed. Using medium I, which contains glycerol and glucose as carbon sources, *Y. lipolytica* CCMA 0357 and *W. anomalus* CCMA 0358 were identified as biosurfactant producers. In both cases, the surface tension of the culture medium was reduced to values around 43 mN/m, being this reduction achieved earlier (72 h) in the case of *W. anomalus* CCMA 0358 (Table 2). Glycerol is a readily available substrate, and large amounts of raw glycerol are expected to be available as by-products of biodiesel industries at low cost, making it an inexpensive carbon source for the production of different metabolites, including biosurfactants. Several studies reported the ability of *Y. lipolytica* of producingadded-value compounds (includingbiosurfactants) using wasteglycerol as carbon source[4, 25]; however, higher surface tension reductions than the obtained in this work (up to 22 mN/m) have been reported for other *Y. lipolytica* strains using similar culture media [4].

Biosurfactant production was also observed using the media V and VIII (both of them containing olive oil). With these media, the surface tension reductions achieved with all the yeast strains studied were enough to consider them biosurfactant producers. The best results were obtained with *W. anomalus* CCMA 0358 growing in medium V, which reduced the surface tension to values around 36 mN/m after 24 h, followed by the *Y. lipolytica* strains (Table 2). Olive oil and oleic acid (the main fatty acid present in olive oil) have been successfully used, in combination with glucose, for the production of biosurfactants by different yeasts, including *Starmerella bombicola* and species belonging to the genera *Candida* and *Wickerhamiella*, among others [8, 19, 23,24].

OMW, an agro-industrial waste generated during olive oil production, was also used as a substrate to evaluate biosurfactant production by these yeasts. Although biosurfactant production by yeasts using OMW has not been previously reported, it was selected as it contains long-chain fatty acids similar to those present in olive oil (including palmitic, stearic, oleic and linoleic acid) [26]; furthermore, OMW has been successfully used as an inducer of biosurfactant production by *Pseudomonas aeruginosa*[26]. OMW is a significant pollutant that represents a waste disposal issue in countries that produce large amounts of olive oil, such as Portugal. Consequently, its use as substrate to produce biosurfactants is interesting from an economical and environmental point of view. However, using the culture media containing OMW (IV and VI), none of the yeaststrains under study was able to produce biosurfactants.

Soybean oil has also been used as carbon source to produce biosurfactants by different yeasts [19, 21]. However, in this case, none of the isolates exhibited biosurfactant production using the culture medium containing soybean oil (medium III).

Besides the ability of reducing the surface tension, also the emulsifying activity was evaluated for all the yeast strains grown in the different culture media; however none of them was found to produce surface active compounds able to form stable emulsions under the studied conditions. Other authors reported previously that some biosurfactants produced by yeasts are not particularly effective emulsifiers, which excludes them from a number of applications, but makes them suitable for non-foam requiring purposes [2, 6]. However, in other cases, good emulsifying activity with different hydrophobic substrates has been reported for biosurfactants produced by yeasts [8, 19, 20].

Y. lipolytica and *C. humicola* strains have been previously reported as biosurfactant producers [4, 27]. Regarding *W. anomalus* (formerly known as *Pichia anomala*), only the strain PY1 has been reported to produce biosurfactants [21]. However, to the best of our knowledge, this is the first report of biosurfactant production by a *L. saturnus* strain.

Taking into account the surface tension reductions obtained, *W. anomalus* CCMA 0358 growing in medium V was selected to further characterize its growth and biosurfactant production kinetics (Figure 1). This yeast strain achieved the lowest surface tension value (36.4 ± 0.7 mN/m) after 24 h of fermentation; at this point, the number of cells was 2.4×10^8 cells/mL. After that, an increase in the surface tension values was observed, which can be due to the partial degradation of the biosurfactant previously produced, whereas the number of cells remained almost constant up to 96 h (Figure 1). These results suggest that biosurfactant production by *W. anomalus* CCMA 0358 in medium V is growth-associated. Similar biosurfactant production profiles were also reported by other authors for several yeast strains [19, 21].

3.2 Critical micelle concentration (cmc)

The *cmc* is the minimum concentration of a surface-active compound necessary to reduce the surface tension to the maximum extent; once achieved the *cmc*, further increases in its concentration do not have effect in the surface tension, which remains stable. The *cmc* is a

property characteristic of each surface-active compound, and it is commonly used to define its efficiency.The lowest surface tension value that can be achieved with a surface-active compound is also a characteristic property, and it is used to define its effectiveness. The *cmc* calculated for the crude biosurfactant produced by *W.anomalus*CCMA0358 in medium V was 0.9 mg/mL, and the minimum surface tension value was 31.2 ± 0.4 mN/m (Figure 2).

The lowest surface tension value obtained for the crude biosurfactant produced by *W.anomalus*CCMA0358 was similar to those reported for other biosurfactants produced by yeasts (between 30.3 and 35.9 mN/m) [5, 6, 8,19, 20, 27]. However, the *cmc* herein obtained was higher when compared with some values reported by other authors (0.02-0.366 mg/mL) [6, 8, 10, 19, 23], which may be due to differences in the purification processes.

Nevertheless, it is important to notice that the productivity reported in the current study is higher when compared with other examples from the literature. Different authors reported productivities between 1.2 and 62.5 mg/L/h for *Candida sphaerica*, *P. anomala* or *S. bombicola* strains [10, 20, 21]. In the present work, 2.6 g/L of biosurfactant were produced by *W. anomalus* CCMA 0358 after 24 h of growth, which results in a productivity of 108.3 mg/L/h.

3.3 Biosurfactant chemical characterization

GC-MSanalysis

Thepartially purified biosurfactant produced by *W.anomalus*CCMA0358 in medium Vwas transesterified/silylated to the corresponding FAME or TMS fatty acids in order to determine its fatty acid composition. Oleic acid appears to be the principal fatty acid present in the biosurfactant sample. The identification was based in other FAME samples analysis and on the MS NIST Library. Moreover, it was not observed any peak of methylated or silylated sugar or other significant compound on the GC-MS chromatogram obtained (Figure 3). The silylated hydrolyzed sample showed the presence of two major TMS sugar by its mass spectra; however it was not possible to clearly identify its structure (data not shown).

NMR analysis

To further understand the biosurfactant molecule structure, the sample was purified by extractions with increasing polarity of organic solvents, hexane and ethyl acetate, leading to

two main fractions. These fractions were analyzed by ^{1}H and ^{13}C NMR (Figure 4A and 4B, respectively). The NMR spectrum of each fraction confirmed the presence of a fatty acid, similar to the oleic acid ^{1}H and ^{13}C NMR. Cosy NMR spectrum indicated that this fatty acid moiety is linked to a C-O symmetric template (Figure 5). ^{13}C NMR spectrum showed the presence of a double bond with signals at 130 ppm. In addition, several CH_2 groups resonated at 22.7-34 ppm and a signal at 14.1, corresponding to a primary methyl group, confirmed that the fatty acid could not be a hydroxyl fatty acid. The spectrum also revealed signals of C-O bond at 62 and 69 ppm which could be from glycerol chain or C6 sugar carbon. No signals were resonated near 112 ppm (C1 sugar carbon). ^{13}C NMR spectrum of hexane fraction reveals a peak at 173.4 ppm and the one of ethyl acetate fraction shows an additional peak at 158 ppm, possibly due to the carbonyl of the acetyl group. However, the NMR spectrum of the hexane fraction was very similar to the one of a triglyceride sample [28].

MS analysis

ESI-MS analysis by direct infusion of the biosurfactant sample dissolved in methanol, in positive mode, showed a major peak at m/z=903. Tandem MS/MS of this pseudomolecular ion revealed a minor and a more abundant ion with masses of 885 and 603, respectively. No ions were detected in the negative mode analysis. This behavior is very similar to the one of triglycerides. To elucidate the composition of the biosurfactant sample, it was further analyzed by LC-MS, with UV detection at 210 nm, in positive and negative mode. Ion extracted chromatogram of masses m/z=608/652/696 and m/z=904/948/992 in positive mode showed two peaks at 15 and 26 min (Figure 6) corresponding to the very small peaks in the UV chromatogram (*data not shown*).

The mass spectra of these peaks is presented in Figures7A and 7B, which indicates the presence of multiple mass peaks with a Gaussian distribution that differs from each other by differences of m/z=18 or m/z=44. This mass spectra behavior has already been reported byDaverey and Pakshirajam [29].

According to the ESI-MS direct infusion, no important peaks were detected in LC-MS analysis in negative mode, which means that no important free acidic group is present in the biosurfactant molecule. Tandem MS fragmentation of each mass peak present in the mass spectrum showed a very similar fragmentation pattern, with the presence of new fragment

masses by the addition of 18 Da (MS^2 data dependent analysis) and 44 Da (MS^3data dependent analysis).

The LC-MS analysis of the hexane fraction revealed the presence of a peak that elutes at 26 min, with a mass spectrum similar to the one shown in fig. 4B, but it was contaminated with some triglycerides.So ethyl acetate fraction was used to further NMR characterization.

Therefore, the proposed structure of the biosurfactant is presented in Figure 7A and 7B, with a mass to charge ratio (m/z) of 696 (at 14.7 min) and 1168 (at 26 min).

According to the results obtained, the biosurfactant produced by *W.anomalus*CCMA0358 is a glycolipid, being the lipophilic fraction composed by oleic acid. Two different types of glycolipid biosurfactant structures were detected, containing one (Figure 7A) or three (Figure 7B) molecules of oleic acid. Additionally, although the structure of the sugar moiety could not be completely resolved, it was concluded that it was not sophorose neither trehalose, as reported for other glycolipid biosurfactants produced by different yeasts [6, 8, 19, 23].

P. anomala PY1 was previously reported to produce different sophorolipids in culture media containing glucose or soybean oil as carbon sources, with molecular weights of 675/691/707 Da and 658/675/691 Da, respectively [21]. The glycolipid biosurfactants produced by *W.anomalus*CCMA0358 are different from those produced by *P. anomala* PY1, as they are not sophorolipids and their molecular weights are 696 and 1168 Da.

To the best of our knowledge, the chemical structure and the molecular weight of the biosurfactants produced by *W.anomalus*CCMA0358 have not been previously described in the literature, and therefore represent new surface-active agents.

3.4 Antimicrobial activity

Microbial surfactants can interfere in the adhesion of microorganisms to different surfaces [11, 13, 14, 30, 31]; some of them have been reported to exhibit antibacterial and antifungal activity [9, 22, 32, 33], and in some cases anti-tumor activity, interfering with some cancer progression processes [1, 16]. These properties contribute to their potential use as alternatives to conventional therapeutic agents in many biomedical applications.

The antimicrobial activity of the crude biosurfactant produced by *W. anomalus* CCMA 0358 was determined by measuring the growth inhibition percentages obtained for different microorganisms at different biosurfactant concentrations. The results obtained at the highest biosurfactant concentration tested (2.6 g/L) are shown in Table 3. This biosurfactant exhibited high growth inhibition percentages (95-100%) against *S. oralis*, *S. sanguis* and *S. agalactiae*. A considerable inhibitory activity was also observed against *C. albicans* (78%) and *S. epidermidis* (66%). The lowest antimicrobial activity was observed against *E. coli* (16%), whereas no inhibitory activity was observed against *S. aureus* and *P. aeruginosa* (Table 3).

Table 3. Growth inhibition percentages obtained for different microorganisms exposed to the crude biosurfactant (2.6 g/L) produced by *W. anomalus* CCMA 0358. Results are expressed as means ± standard deviations of values obtained from triplicate experiments.

Microorganism	% Growth Inhibition
*Candida albicans**	78 ± 1
*Escherichia coli**	16 ± 4
*Pseudomonas aeruginosa**	-
*Staphylococcus aureus**	-
*Staphylococcus epidermidis**	66 ± 5
*Streptococcus agalactiae**	100 ± 1
Streptococcus oralis	100 ± 2
Streptococcus sanguis	95 ± 5

*: pathogenic microorganisms

Similarly to the results herein obtained, the biosurfactant produced by *Candida lipolytica* UCP 0988 also exhibited lower antimicrobial activity against *P. aeruginosa*, *S. aureus* and *E. coli* when compared with *S. oralis*, *S. sanguis* and *S. agalactiae* (Rufino et al., 2011). Furthermore, the crude biosurfactant produced by *W. anomalus* CCMA 0358 exhibited higher antimicrobial activities against all the microorganisms tested (with the exception of *S. aureus* (27.3%) and *P. aeruginosa* (12.5%)) when compared with the biosurfactant produced by *C. sphaerica* UCP 0995 at a similar concentration (2.5 g/L), although the *cmc* of this biosurfactant (0.25 mg/mL) was considerably lower when compared with the one produced by *W. anomalus* CCMA 0358 [10].

Also biosurfactants produced by the lactic acid bacteria *Lactococcus lactis* 53, *Streptococcus thermophilus* A, *Lactobacillus paracasei* sbsp. *paracasei* A20 or *Lactobacillus agilis* CCUG31450 exhibited antimicrobial activity against the same bacterial strains herein studied, although at higher concentrations (3-100 g/L), and in most of the cases, the antimicrobial activities observed were lower when compared with the ones herein reported [9, 13, 22].

Conclusions

The yeast strain *W. anomalus* CCMA 0358 produced a mixture of glycolipid biosurfactants in a culture medium containing olive oil and glucose as carbon sources. The crude biosurfactant reduced the surface tension to values around 31 mN/m and exhibited antimicrobial activity against several pathogenic microorganisms. Furthermore, these biosurfactants are structurally different from those previously reported. The properties exhibited by these biosurfactants, together with their relatively high productivity (108.3 mg/L/h), make them promising candidates for application in several fields, including biomedical applications.

Acknowledgements

This study was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UID/BIO/04469/2013 unit and COMPETE 2020 (POCI-01-0145-FEDER-006684);and by BioTecNorte operation (NORTE-01-0145-FEDER-000004) funded by the European Regional Development Fund under the scope of Norte2020 - Programa Operacional Regional do Norte. The authors also thank the FCT for the financial support under the scope of the Project RECI/BBB-EBI/0179/2012 (FCOMP-01-0124-FEDER-027462).

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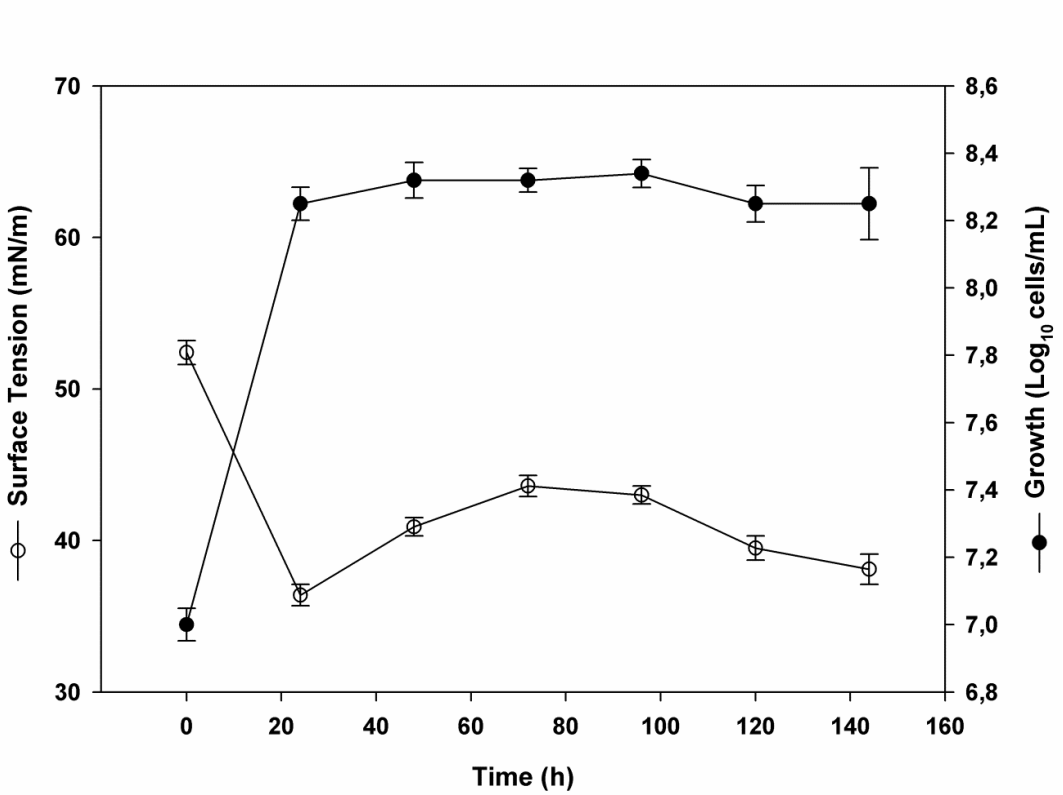

Figure 1. Growth and biosurfactant production kinetics of *Wickerhamomyces anomalus* CCMA 0358 in culture medium V. The composition of this culture medium is shown in Table 1. Results represent the average of three independent experiments ± standard deviation.

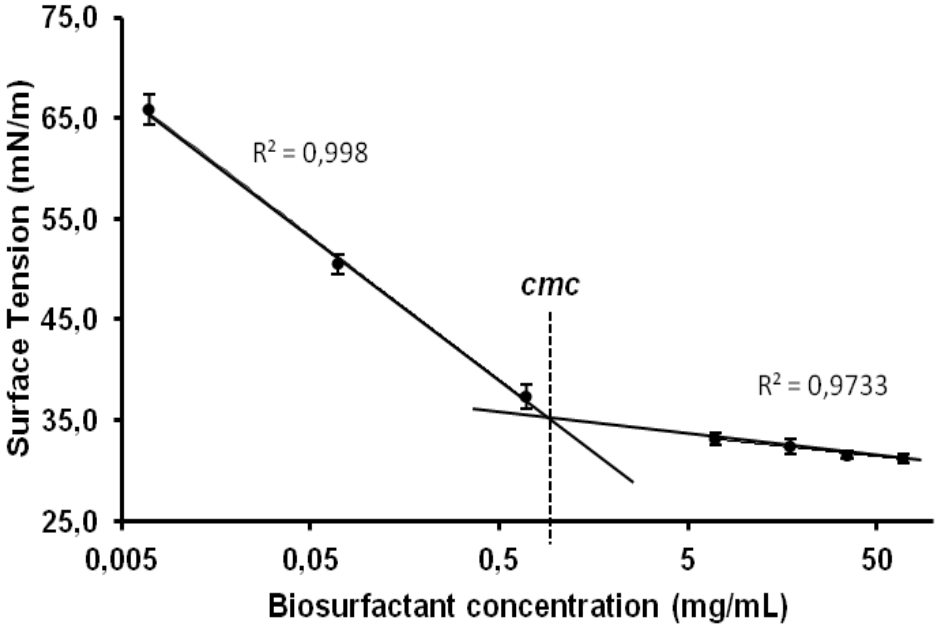

Figure 2. Surface tension values (mN/m)*versus* logarithm of biosurfactant concentration (mg/mL) obtained with the crude biosurfactant produced by *Wickerhamomyces anomalus* CCMA 0358 dissolved in demineralized water. Results represent the average of three independent measurements ± standard deviation.

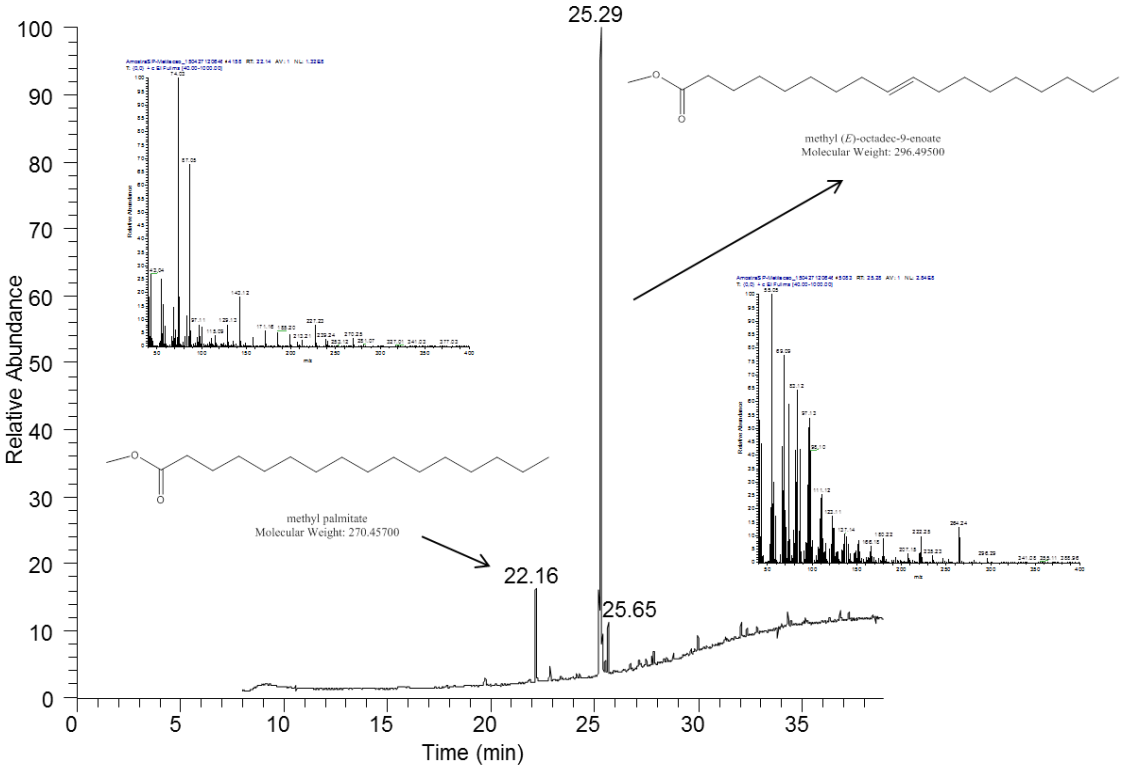

Figure 3. GC-MS Full scan chromatogram obtained from the analysis of methyl esters of fatty acids present in the crude biosurfactant produced by *Wickerhamomyces anomalus* CCMA 0358.

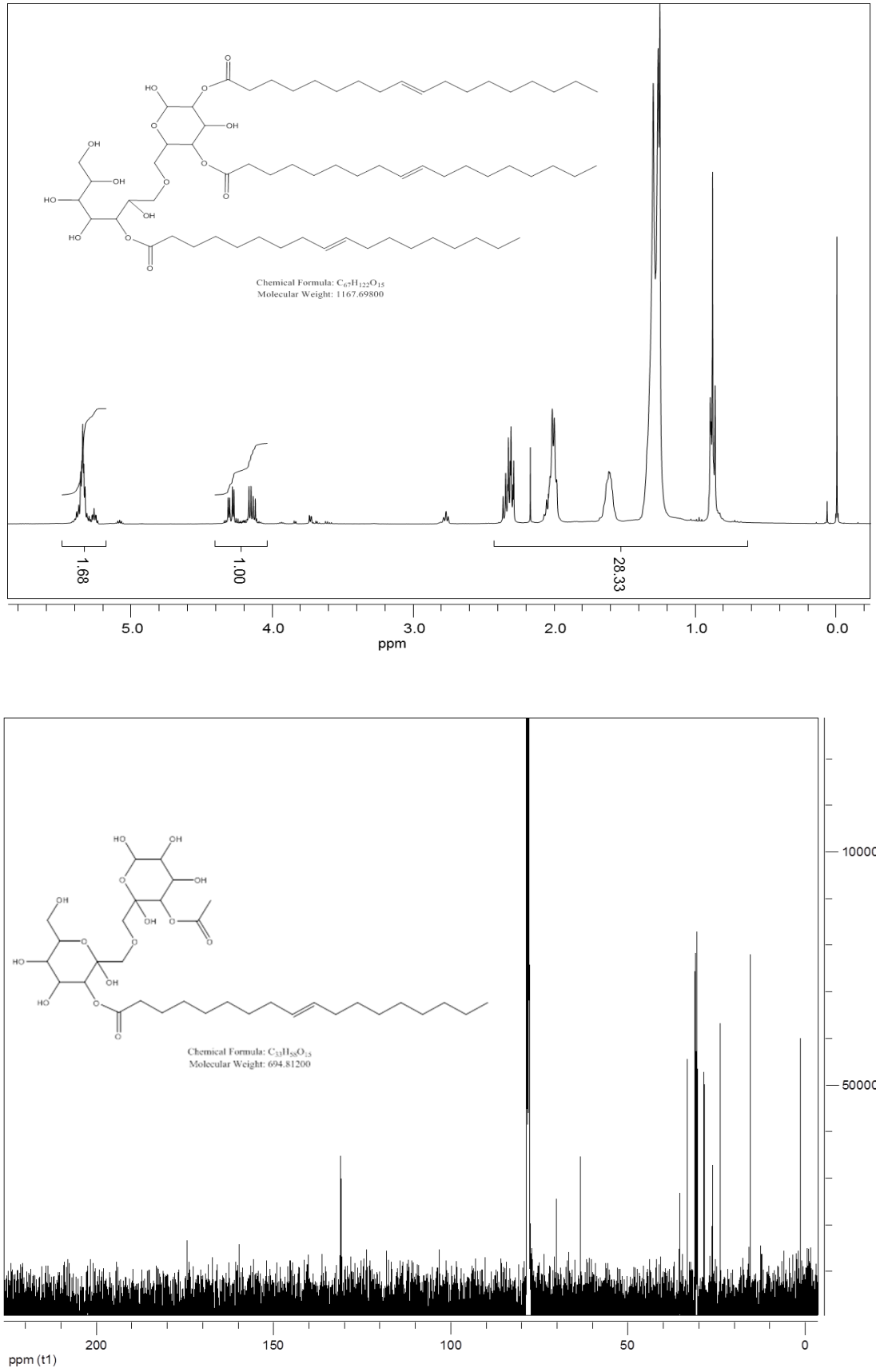

Figure 4. ^{1}H **(A)**and ^{13}C **(B)**NMR spectra obtained fromthe ethyl acetate fraction of the biosurfactant produced by *Wickerhamomyces anomalus* CCMA 0358.

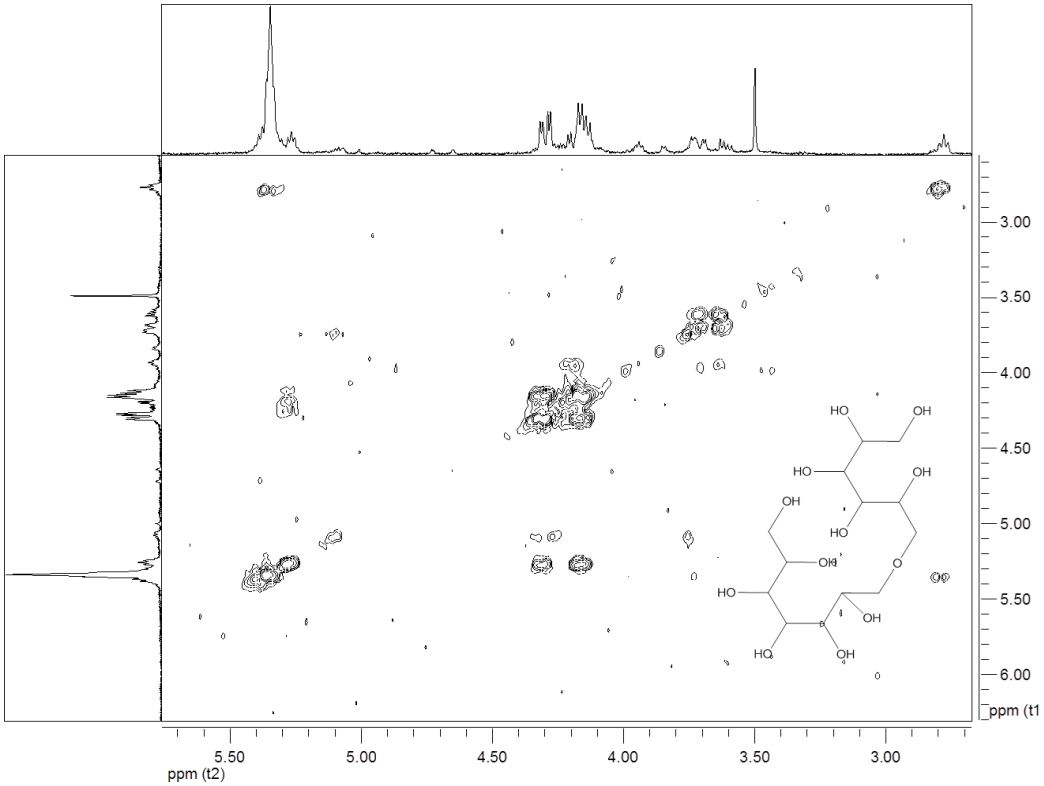

Figure 5. Cosy NMR spectrum(amplified in the range of 3 to 6 ppm) obtained fromthe ethyl acetate fraction of the biosurfactant produced by *Wickerhamomyces anomalus* CCMA 0358.

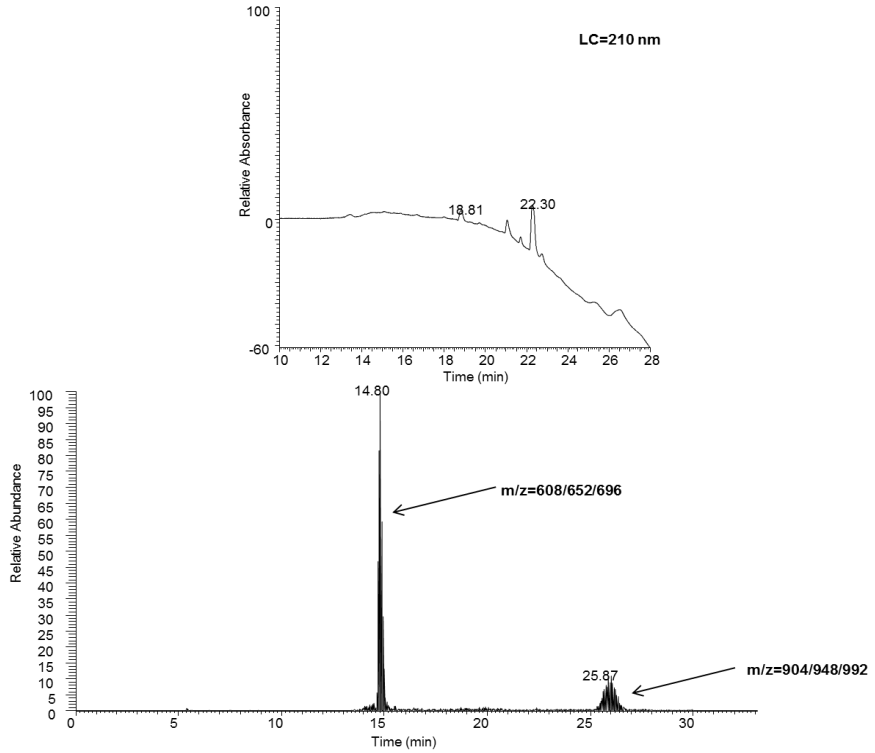

Figure 6. Ion extracted chromatogram obtained from the LC-MS analysis of the biosurfactant produced by *Wickerhamomyces anomalus* CCMA 0358.

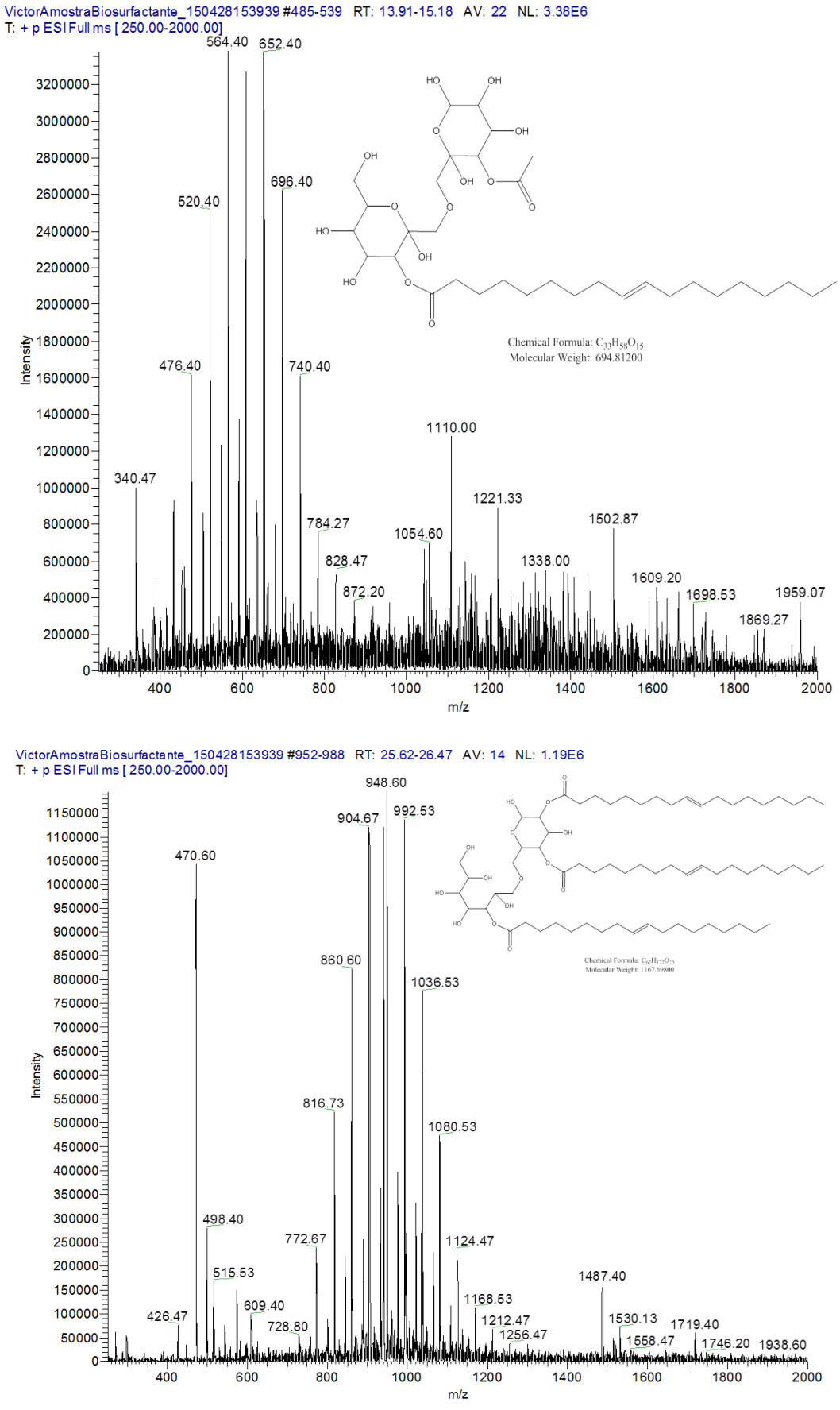

Figure 7. Mass spectra of the glycolipids identified in the peaks at 15 min **(A)** and 26 min **(B)**.

ARTIGO 2

Optimization of glycolipid biosurfactant production by *Wickerhamomyces anomalus* CCMA 0358 and its potential application in oil removal from contaminated sand

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Abstract

The composition of the culture medium for biosurfactant production by *Wickerhamomyces anomalus* CCMA 0358 was optimized through response surface methodology. The optimized culture medium contained yeast extract (4.64 g/L), ammonium sulfate (4.22 g/L),glucose (1.39 g/L) and olive oil (10 g/L). Under optimized conditions, the surface tension was reduced up to 31.4 ± 0.4 mN/m after 24 h of growth in assays performed in flasks. Using a 5-L bioreactor, the surface tension of the culture medium was reduced to 29.3 ± 0.4 mN/m after 24 h of growth. In this case, the optimum agitation speed was found to be 500 rpm. It is important to highlight that *W. anomalus* CCMA 0358 is a fast biosurfactant producer (24 h) as compared to other yeast strains previously reported (144-240 h). Besides, the biosurfactant produced by *W. anomalus* CCMA 0358remains stable at high temperatures, over a wide range of salinities (0-300 g NaCl/L), and at pH values between 6 and 12. Furthermore, this biosurfactant allowed the recovery of 20% of crude oil from artificially contaminated sand. Based on these properties, this biosurfactant is considered a promising candidate for several industrial and environmental applications.

Keywords**:** *Wickerhamomyces anomalus*; biosurfactant; response surface methodology; bioremediation.

1 Introduction

Surface-active compounds (surfactants) exhibit a wide variety of applications and are present in nearly every product and aspect of our daily life. They are includedas active ingredients in the formulation of detergents, cleaning and personal care products and cosmetics, and are also used in bioremediation, as well as in agriculture, food, paper, textile and petroleum industries, among others [1-3]. Surfactants are amphipathic molecules, containing at least one hydrophilic and one hydrophobic moiety. Due to their structure, these compounds accumulate at the interface between different phases, including gas, liquid, solid, and fluid phases of different polarities (such as oil/water).As a result, surfactants reduce the surface and interfacial tensions, which confers them properties such as detergency,emulsifying activity, foaming, and ability to solubilize and disperse hydrophobic compounds in aqueous phases [1, 4-6].

The current worldwide production of surfactants is estimated to be approximately 15 million tons per year [3]. Due to their applications, a considerable amount of these compounds is discharged directly into the environment after their use without any treatment.This results in anadverseenvironmental impact, since most of the traditional chemical surfactants commonly used, which are obtained from petrochemical resources, exhibit high toxicity and low biodegradability [7]. Consequently, given the increasing concern regarding the environmental hazard of synthetic surfactants, and in order to address the growing demand for eco-friendly alternatives, research focusing on the production of bio-based surfactants (derived from renewable resources, but not necessarily obtained through fermentation) has increased in the last years. The market for these “green” alternatives to the traditional chemical surfactants is expected to increase up to 2800 million USD by 2023, with a worldwide production around 462 kilo tons per year[8].

Among the bio-based surfactants, biosurfactants, a heterogeneous group of surface-active compounds synthesized by different microorganisms, have emerged as promising alternatives to the synthetic surfactants.In the last years, biosurfactants have received pronounced attention owing to their excellent interfacial activities, low toxicity, high biodegradability, and stability under extreme conditions of temperature, pH and salinity [4, 9]. As a result of these properties, biosurfactants could replace chemical surfactants in many industrial applications. Furthermore, apart from their detergent-like activity, several biosurfactants stand out owing to

their remarkable biological activities (including anti-microbial, anti-adhesive and anti-tumor activities)which have attracted the interest of the pharmaceutical industry due to their potential use as therapeutic agents [6, 10-13].

Glycolipid biosurfactants produced by non-pathogenic yeasts (e.g. sophorolipids and mannosylerythritol lipids (MEL)) have gained considerable attention as they are produced in relatively high amounts (usually 30-130 g/L) when compared with those produced by bacteria (up to 5 g/L) [9, 14-20]. Despite possessing many attractive properties and clear advantages over their synthetic counterparts, the large-scale industrial production and commercialization of biosurfactants are still limited by the relatively low efficiency of their production and recovery processes, as well as the high prices of the culture media used for their synthesis, which results in high production costs. Extensive efforts have been conducted to make them competitive with the existing chemical surfactants, through the optimization of the culture media composition and the culture conditions, the use of agro-industrial wastes and by-products as substrates, and the development of effective and economically viable downstream processes [3, 17-25].

Regarding the optimization of the culture medium composition, the use of statistical experimental designs such as response surface methodology (RSM) offers an advantage when compared with the classical methods of media optimization, which involve changing one variable at a time, keeping the others at fixed levels; these methods are laborious, time-consuming, and do not guarantee the determination of the optimal conditions. RSM is a collection of mathematical and statistical techniques that are useful for the modeling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response. RSM is one of the most efficient optimization strategies which solve multivalent equations simultaneously by using quantitative data obtained from a limited number of properly designed experiments. This methodology has been successfully used by other authors to optimize the composition of the culture medium and the culture conditions to maximize biosurfactant production by different microorganisms [21, 23-25].

In this work, in order to increase the biosurfactant production by *Wickerhamomyces anomalus* CCMA 0358, the composition of the culture medium was optimized through RSM. The results obtained were validated in shaken flasks and bioreactor. The stability of the

biosurfactant produced by this yeast at different environmental conditions was studied, as well as its potential applicability in bioremediation.

2 Material and Methods

2.1 Strain and culture conditions

The yeast strain*Wickerhamomyces anomalus* CCMA 0358 (isolated from coffee processing by-products) wasobtained from the Culture Collection of Agricultural Microbiology, CCMA (Department of Biology, Federal University of Lavras, Brazil). The yeast was grown in YEPG medium (10 g/L yeast extract; 20 g/L peptone; 20 g/L glucose; pH 6.5) at 28ºC and 200 rpm.

2.2 Biosurfactant production

For the production of biosurfactant by *W. anomalus* CCMA 0358, a culture medium that was previously established (*data not shown*) was used. This medium consisted of: 4 g/L yeast extract; 2 g/L glucose; 4 g/L ammonium sulfate; and 20 g/L olive oil. The experiments were performed in flasks (500 mL capacity) containing 200 mL of culture medium.Each flask was inoculated with a pre-culture of *W. anomalus* CCMA 0358 (grown overnight in YEPG medium at 28ºC and 200 rpm) to attain an initial cell concentration of 10^7 cells/mL. Subsequently, the flasks were incubated at 28ºC and 200 rpm for 24 h. Samples (4 mL) were taken along the fermentation to evaluate growth and biosurfactant production. Cell growth was determined according to the number of cells counted using a Neubauer improved cell counter (Marienfeld GmbH, Germany). Afterwards, the samples were centrifuged (2700 × *g*, 15 min) and the cell-free supernatants were used to assess biosurfactant production through surface tension measurement, as described below.

2.3 Surface tension measurement

The surface tension of the cell-free supernatants was measured according to the Ring method as described elsewhere [5]. A KRÜSS K6 Tensiometer (KRÜSS GmbH, Germany) equipped with a 1.9cm De Noüy platinum ring was used. All the measurements were performed in triplicate at room temperature (25ºC).

2.4 Culture medium optimization through response surface methodology (RSM)

The composition of the culture medium described above was optimized in order to maximize biosurfactant production by *W. anomalus* CCMA 0358. The concentration of three medium components and the effect of the interactions between them on biosurfactant production were analyzed and optimized by RSM using a central composite design (CCD). The medium components (independent factors) selected to perform the optimization process were yeast extract (X_1), ammonium sulfate (X_2) and glucose (X_3). Each factor was studied at five coded levels (-1.68, -1, 0, +1, +1.68), as shown in Table 1. Statistica 8.0 software was used to generate a set of 17 experiments, which included 3 replicates at the central point (Table 2).

The experiments were performed in flasks (500 mL capacity) containing 200 mL of the different culture media (prepared according to the CCD (Table 2)); all the media were supplemented with 20 g/L of olive oil. The flasks were inoculated and incubated at the same conditions described above.Cell growth and biosurfactant production were evaluated as previously described. The surface tension values obtained for each experimental run were taken as the response variable, and the biosurfactant production was assessed as the surface tension reduction. All experiments were performed in triplicate.

The experimental results of RSM were fitted via the response surface regression procedure, using the following second-order polynomial equation to define the predicted responses in terms of the independent factors:

$$Y = \beta_0 + \sum_i \beta_i X_i + \sum_{ii} \beta_{ii} X_i^2 + \sum_{ij} \beta_{ij} X_i X_j \ (Eq. 1)$$

where Y is the predicted response value; β_0 is the intercept term; β_i are the linear coefficients; β_{ii} are the quadratic coefficients; β_{ij} are the interaction coefficients; and X_i-X_j are the independent factors.

Table 1. Central composite design factors and corresponding variation ranges according to the experimental levels.

	Levels				
Factor	-1.68	-1	0	+1	+1.68
	Concentration (g/L)				
Yeast Extract (X_1)	0.60	2.0	4.0	6.0	7.4
Ammonium Sulfate (X_2)	0.60	2.0	4.0	6.0	7.4
Glucose (X_3)	0.32	1.0	2.0	3.0	3.68

Statistica 8.0 softwarewas used for the analysis of the experimental designs and subsequent regression analysis of the experimental data obtained. The coefficient of determination (R^2) was used as a parameter of quality of the polynomial model equation. The statistical significance of the model equation and the model terms was evaluated through the *F*-test, whereas the *t*-test was used to evaluate the significance of the regression coefficients. The fitted polynomial equation was then expressed in the form of response surfaces to illustrate the individual and combinatorial effect of the factors evaluated on the responses. The point optimization method was used to optimize the level of each factor towards a maximum response (i.e. to predict the optimum concentration of each medium component for maximum surface tension reduction). Subsequently, the model was validated by performing the experimental runs suggested during the statistical optimization of the model (i.e. the combination of different optimized factors which yielded the maximum response).

Table 2. Matrix of the central composite design showing the concentration of the different factors for the experimental design and the results of the experimental runs (predicted and observed).

Experiment	Concentration (g/L)			ST (mN/m)	
	X_1	X_2	X_3	Predicted	Observed
1	2	2	1	34.4	33.2
2	6	2	1	31.8	30.9
3	2	6	1	34.2	33.5
4	6	6	1	31.9	30.9
5	2	2	3	34.3	34.3
6	6	2	3	32.1	31.9
7	2	6	3	35.3	35.3
8	6	6	3	33.3	33.5
9	0.6	4	2	38.8	39.5
10	7.4	4	2	35.0	35.7
11	4	0.6	2	30.0	30.9
12	4	7.4	2	30.9	31.4
13	4	4	0.32	32.2	34.0
14	4	4	3.68	33.3	32.9
15	4	4	2	31.9	32.0
16	4	4	2	31.9	31.8
17	4	4	2	31.9	31.6

After optimizing the selected culture medium components (yeast extract, ammonium sulfate and glucose), the effect of different olive oil concentrations (0, 10, 20, 30, 40 and 50 g/L) on the biosurfactant production was studied. The culture conditions used were the same as described above and the experiments were conducted in triplicate.

2.5 Biosurfactant production in bioreactor

A 5-L capacity bioreactor (BIOSTAT® A Fermentor, B. Braun Biotech International GmbH, Germany), equipped with agitation, temperature, pH and dissolved oxygen concentration online measurement and control was used. The experiments were performed at 28ºC, using 2 L of the previously optimized culture medium, with air injected in the bottom of the bioreactor at a constant flow rate of 1 L/min and without pH control. The bioreactor was inoculated with a pre-culture of *W. anomalus*CCMA 0358 (grown overnight in YEPG medium at 28ºC and 200 rpm) to attain an initial cell concentration of 10^7 cells/mL. The effect of different agitation rates (250, 300, 350, 400 and 500 rpm) on biosurfactant production was studied. In order to evaluate cell growth and biosurfactant production, samples (10 mL) were taken at different time points during the fermentation. Cell concentration and surface tension were determined as described above. Whenever required, the culturebroth supernatants were

diluted 10 times withdemineralized water, and the surface tension (ST^{-1}) was measured.All the experiments were performed in triplicate.

2.6 Effect of environmental parameters on biosurfactant activity

The effect of pH, temperature and salinity on the activity of the biosurfactant produced by *W. anomalus*CCMA 0358was studied using the cell-free supernatants obtained at the end of the fermentationconducted with the optimized culture medium in bioreactor, as described by Gudiña and co-workers[26]. The effect of pH on surface activity was studied by adjusting the cell-free supernatants to different pH values (2, 4, 6, 8, 10, 12) using 1 N NaOH or 1 N HCl solutions, and measuring the surface tension as described above. To assess the effect of salinity onbiosurfactant activity, the cell-free supernatants were supplemented with different NaCl concentrations (50, 100, 200 and 300 g/L); subsequently, the surface tension was measured as described above and compared with the values obtained without addition of NaCl. The stability of the biosurfactant at high temperatures was also determined; for that purpose, the cell-free supernatants were incubated at 121ºC for 30 min and then allowed to cool to room temperature; the surface tension was measured and compared to the corresponding values before the heat treatment. All experiments were carried out in triplicate.

2.7 Removal of crude oil from contaminated sand

The potential applicability of the biosurfactant produced by *W. anomalus*CCMA 0358in bioremediation was evaluated using artificially contaminated sand, as described by Gudiña and co-workers [18]. Briefly, samples of 50 g of sand were mixed with 5 g of Arabian Light crude oilin 100 mL flasks and allowed to age at room temperature for 24 h. Afterwards, 50 mL of cell-free supernatant (obtained at the end of the fermentationconducted in bioreactor with the optimized culture medium) were added to each flask. The flasks were incubated at 80 rpm and 37ºC for 24 h.The oil removed was recovered from the surface and transferred to a graduated tube. In order to separate the water recovered together with the crude oil, the tubes were centrifuged (2700 × g, 20 min), and subsequently the volume of crude oil was measured. The amount of crude oil recovered was calculated according to its density (0.837 g/mL). Control assays were performed using demineralized water at the same conditions. All the experiments were carried out in triplicate.

2.8 Statistical analysis

The Statistica 8.0 software was used for all the statistical analysis. The mathematical model that relates the factors optimized in the CCD was determined through a second-order adjustment of the experimental data. All the determinations of surface tension and cell concentration were performed at least three times.

3 Results and Discussion

3.1 Optimization of biosurfactant production using response surface methodology (RSM)

Previous studies demonstrated that *W. anomalus*CCMA 0358 produced a new type of glycolipid biosurfactant using a culture medium containing yeast extract, ammonium sulfate, glucose and olive oil (*data not shown*). In this study, a RSM based on a CCD was used to optimize the concentration of the different medium components in order to maximize biosurfactant production by this yeast strain.Yeast extract (X_1), ammonium sulfate (X_2) and glucose (X_3) were the independent factors selected to perform the optimization process. The corresponding levels for each factor are shown in Table 1. The design matrix (17 experiments) and the corresponding results of RSM experiments to determine the effect of the three abovementioned independent factors on the surface tension (response factor) are shown in Table 2 as observed and predicted values. Regression analysis of the optimization study indicated that yeast extract (X_1) was the only significant factor ($p< 0.1$) on surface tension (Table 3), whereas ammonium sulfate (X_2) and glucose (X_3) were found to be non-significant, as indicated by their high *p* values (Table 3). Moreover, the interactions between the different medium components were found to be non-significant.

Table 3. Coefficient regression values for the model that describes the effects of the medium components in the surface tension.

Factor	Terms	Regression coefficients	Standard error	*p*-value
Yeast Extract (X_1)	Linear	-1.1238	0.31865	0.009641
Ammonium sulfate (X_2)	Linear	0.2777	0.31865	0.412291
Glucose (X_3)	Linear	0.3446	0.31865	0.315358
X_1X_2	Interactive	0.0312	0.41616	0.942244
X_1X_3	Interactive	0.0812	0.41616	0.850755
X_2X_3	Interactive	0.2937	0.41616	0.503088
X_1^2	Squared	1.8169	0.35106	0.001287
X_2^2	Squared	-0.4860	0.35106	0.208688
X_3^2	Squared	0.3288	0.35106	0.380125

The results herein gathered were then fittedto a second-order polynomial equationthat describes the surface tension (response factor) based on the concentration of the significant medium components:

$$Y = 31.8106 - 1.1238\,X_1 + 1.8169\,X_1^2 \ (Eq.2)$$

where Y is the surface tension, and X_1 the concentration of yeast extract (the only component that was found to be significant). As ammonium sulfate and glucose were found to be non-significant, they were not included in the model equation.The regression equation obtained from ANOVA showed that R^2 (coefficient of determination) was 0.8792, indicating that 87.9% of the variability in the responses could be explained by the second-order polynomial equation given above (Eq. 2).

To establish the optimum level of each independent factor for maximum biosurfactant production, three-dimensional response surface plots were constructed by plotting the response (surface tension) as a function of two independent factors at a time, while maintaining the value of the other factor at its central level (Figure 1). Finally, the optimum levels of yeast extract(X_1 = 4.64 g/L), ammonium sulfate (X_2 = 4.22 g/L)and glucose(X_3 = 1.39 g/L) that allow the highest biosurfactant production (i.e. the lowest surface tension value, estimated to be 31.5 mN/m) can be obtained from the minimization of the previous equation.

Figure 1. Response surface representing the effect of medium components (yeast extract, ammonium sulfate and glucose) on the surface tension, which is the indicative of the production of biosurfactant. Only yeast extract was found to significantly affect the surface tension. **(A)** Yeast extract and ammonium sulfate; **(B)** Yeast extract and glucose and **(C)** Ammonium sulfate and glucose.

In order to validate the optimum culture medium predicted by the model, *W.anomalus*CCMA0358 was grown in flasks at the same conditions previously described, using a culture medium with the following composition: 4.64 g/L yeast extract; 4.22 g/L ammonium sulfate; 1.39 g/L glucose; and 20 g/L olive oil. The initialsurface tension of this culture medium(49.0 ± 0.1mN/m)was reduced to31.1 ± 0.1mN/m after 24 h of growth, which is very similar to the surface tension value predicted by the model (31.5mN/m). Hence, it can be concluded that the model adequately adjusts the experimental data and clearly describes the effect of the culture medium composition on the biosurfactant production, as the experimental values obtained are in accordance with the expected values determined by the model.

Several authors reported the effect of yeast extractas nitrogen source (at concentrations between 3 and 10 g/L) for the production of biosurfactants by yeast strains belonging to the genera *Candia* and *Pseudozyma* [16, 17, 27, 28]. For instance, Konishi and co-workers [16] reported that growth and MELproduction by *Pseudozyma hubeiensis* SY62 increased as the concentration of yeast extract increased from 1 to 10 g/L. However, yeast extract does not always have a positive effect on biosurfactant production. Daverey and Pakshirajan [15] reported that yeast extract at concentrations higher than 2 g/L reducedthe sophorolipid production by *Starmerella bombicola* NRRL Y-17069 (in this case, the highest sophorolipid production occurred under nitrogen-limiting conditions). It has also been reported that supplementing the culture medium with yeast extract at a concentration of 10 g/L did not have any effect on the sophorolipid production by *C. bombicola* ATCC 22214 [29].

In our previous assays, the different culture media were supplemented with olive oil at a concentration of 20 g/L. To further optimize the biosurfactant production by *W.anomalus*CCMA0358, additional experiments were performed using the optimized culture medium supplemented with olive oil at different concentrations (0-50 g/L). The assays were performed using the conditions previously described. From Table 4, it can be seen that the

culture media supplemented with olive oil at concentrations between 10 and 40 g/L led to similar surface tension values (around 31 mN/m) after 24 h of growth. For that reason, to reduce the production costs of this biosurfactant, an olive oil concentration of 10 g/L was selected to perform the following experiments.

Table 4. Effect of olive oil concentration on biosurfactant production. Surface tension values (ST, mN/m) obtained in cultures performed with *Wickerhamomyces anomalus* CCMA 0358 grown in the optimized culture medium supplemented with different concentrations of olive oil (0-50 g/L). The assays were performed at 28ºC and 200 rpm for 24 h. Results represent the average of three independent experiments ± standard deviation.

Olive oil concentration (g/L)	ST (mN/m)
0	40.6 ± 0.1
10	31.4 ± 0.4
20	31.2 ± 0.1
30	31.4 ± 0.3
40	31.7 ± 0.6
50	34.1 ± 0.1

Moreover, these results demonstrated that the presence of a hydrophobic carbon source (in this case olive oil) in the culture medium is essential for biosurfactant production by this yeast strain. The assays performed without olive oil led to surface tension values considerably higher when compared with those observed with the other culture media containing olive oil at different concentrations (Table 4). The results herein obtained are in accordance with the general notion in the literature, as it has been widely reported that biosurfactant production by yeasts usually requires the simultaneous presence of a hydrophilic and a hydrophobic carbon source in the culture medium [15-17, 20, 27-29]. As an exception,Monteiro and co-workers [30] reported that the maximum glycolipid biosurfactant production by *Trichosporon montevideense* CLOA72 was achieved using sunflower oil (20 g/L) as the sole carbon source.

Furthermore, it has to be pointed out that the concentration of the hydrophobic carbon source used in this study was considerably lower when compared with other works, where oleic acid, cotton seed oil, olive oil, coconut oil or soybean oil at concentrations between 75 and 150 g/L were added to the culture medium [15-17, 27, 29].

3.2 Biosurfactant production in bioreactor

Growth and biosurfactant production by *W. anomalus* CCMA 0358 were evaluated in a 5-L bioreactor aiming at an increase of the production scale.The culture medium used in these assays was the optimized in the previous section, and the effect of the agitation speed (250-500 rpm) on biosurfactant production (assessed as the reduction of the surface tension of the culture medium) was evaluated. In all cases, air was injected into the culture medium at a constant flow rate of 1 L/min (0.5 vvm (volume of air per culture volume and minute)).

Table 5. Surface tension values (ST and ST^{-1}, mN/m) and cell concentrations (cells/mL) obtained with *Wickerhamomyces anomalus* CCMA 0358 grown in bioreactor using the optimized culture medium at different agitation speeds (250-500 rpm). ST: surface tension of the cell-free supernatant; ST^{-1}: surface tension of the cell-free supernatant diluted 10 times with demineralized water. The results presented correspond o the values obtain e datth e optimumtim e foreach condition (24 h). Results represent the average of three independent experiments ± standard deviation.

Agitation speed (rpm)	ST (mN/m)	ST^{-1} (mN/m)	Growth (cells × 10^8/mL)
250	34.5 ± 1.0	42.2 ± 0.7	4.7 ± 0.1
300	32.8 ± 0.5	39.9 ± 0.3	4.5 ± 0.2
350	31.2 ± 0.4	38.0 ± 0.9	2.7 ± 0.2
400	31.0 ± 0.2	38.4 ± 0.3	2.0 ± 0.1
500	29.3 ± 0.4	33.9 ± 0.1	2.0± 0.2

The results obtained (Table 5) indicated that a higher agitation speed resulted in a lower surface tension value at the end of the fermentation. The initial surface tension of the culture medium was 49.1 ± 0.1 mN/m, and the lowest surface tension value (29.3 ± 0.4 mN/m) was achieved in fermentations performed at 500 rpm, after 24 h of growth. However, as it can be seen from Table 5, an increase in the agitation speed led to a decrease in the number of cells at the end of the fermentation.

Similarly to the results herein obtained, other authors reported that a high agitation speed is more favorable for biosurfactant production by different yeast strains; agitation rates between 350 and 550 rpm have been commonly used for biosurfactant production by different yeasts in bioreactor, with air-flow rates between 1 and 2 vvm [15, 17, 20]. On the contrary, Santos and co-workers [23] optimized the culture conditions for biosurfactant production by *Candida lipolytica* UCP0988 in bioreactor through RSM, and concluded that the highest biosurfactant

production was achieved with the lowest agitation speed tested (200 rpm) and without aeration.

One parameter that contributes to the production costs of biosurfactants is the fermentation time. In the case of *W. anomalus* CCMA 0358, the lowest surface tension value (i.e. the highest biosurfactant production) was achieved after 24 h of growth. However, in most of the yeast strains reported in the literature, longer incubation times (usually 144-240 h) are required to achieve the maximum biosurfactant production [15, 17, 20, 23]. This represents an important advantage of *W. anomalus* CCMA 0358 over other commonly used yeasts.

Figure 2 shows the evolution of growth and surface tension for the fermentation performed in bioreactor at 500 rpm. As it can be seen, up to 14 h of fermentation there is an inverse relationship between both parameters, indicating a growth-associated biosurfactant production. After that point, the number of cells decreased, whereas the surface tension remained constant up to 24 h. Afterwards, a slight increase in the surface tension was observed, probably due to a partial degradation of the biosurfactant. On the contrary, the production of sophorolipids by several yeast strains occurs mainly during the stationary growth phase [20, 29]. However, Monteiro and co-workers [30] reported a partially growth-associated biosurfactant production by *Trichosporon montevideense* CLOA72 (70% of the biosurfactant was produced during the exponential growth phase).

Figure 2. Evolution of growth (cells/mL) and surface tension (ST, mN/m) in fermentations performed with *Wickerhamomyces anomalus* CCMA 0358 grown in bioreactor using the optimized culture medium at 28ºC and 500 rpm. Results represent the average of three independent experiments ± standard deviation.

The great advantage of the present study when compared with otherspreviously reported is the faster (24 h) biosurfactant production achieved with *W. anomalus* CCMA 0358.

3.3 Effect of temperature, pH and salinity on biosurfactant activity

The applicability of biosurfactants can be conditioned by their stability at different temperatures, salinities and pH values. For instance, the stability of biosurfactants at high temperatures is of great importance for their use in the food and oil industries, as well as for

their incorporation in laundry detergents, as these processes are usually associated to high temperatures. The biosurfactant produced by *W. anomalus* CCMA 0358 was found to be stable at high temperatures. The surface tension of the cell-free supernatant remained constant (around 29 mN/m) after incubation at 121ºC for 20 min, indicating no loss of activity. In a similar way, other biosurfactants produced by yeasts were not affected by incubations at high temperatures (80-120ºC) [15, 22, 27, 28, 30].

Regarding the effect of salinity, NaCl concentrations as high as 300 g/L did not have a negative effect on biosurfactant activity, as it can be seen from the surface tension values obtained (Table 6). The lowest surface tension value (28.2 ± 0.5 mN/m) was obtained for a NaCl concentration of 50 g/L. This property makes this biosurfactant useful for applications involving high salinities, such as bioremediation of marine environments.Other biosurfactants produced by yeasts have been reported to remain stable at NaCl concentrations up to 100 g/L [27], 120 g/L [22], 150 g/L [28], 200 g/L [15] and 300 g/L [30].

Table 6. Effect of NaCl concentration on surface activity. Surface tension values (ST, mN/m) obtained with the cell-free supernatants from *Wickerhamomyces anomalus* CCMA 0358 (grown in bioreactor using the optimized culture medium) supplemented with different NaCl concentrations. The surface tension values were determined at room temperature (25ºC).Results represent the average of three independent experiments ± standard deviation.

[NaCl] (g/L)	ST (mN/m)
0	29.3 ± 0.4
50	28.2 ± 0.5
100	28.9 ± 0.1
200	29.2 ± 0.3
300	29.1 ± 0.2

Regarding the effect of pH, the biosurfactant produced by *W. anomalus* CCMA 0358was more stable under neutral and alkaline conditions (pH 6-12), where the surface tension remained almost constant (Table 7). On the contrary, at acidic pH values, a slight increase in the surface tension values was observed, meaning that the biosurfactant was less active in those conditions. The stability of this biosurfactant at pH values between 6 and 12 is useful for its application in laundry detergent formulations, which pH is usually in the range of 9-12 [25]. Other biosurfactants produced by yeasts remained stable at pH values between 2 and 12

[27, 28]. In other cases, a partial loss of activity was observed at high pH values (pH 12) [15, 30], whereas other biosurfactants remained stable only at pH values between 6 and 10 [22].

Table 7. Effect of pH on surface activity. Surface tension values (ST, mN/m) obtained with the cell-free supernatants from *Wickerhamomyces anomalus* CCMA 0358 (grown in bioreactor using the optimized culture medium) at different pH values. The surface tension values were determined at room temperature (25ºC).Results represent the average of three independent experiments ± standard deviation.

pH	ST (mN/m)
2	34.7 ± 0.9
4	33.9 ± 0.7
6	31.5 ± 0.8
8	30.7 ± 0.3
10	31.5 ± 0.6
12	30.5 ± 0.6

3.4 Bioremediation assays

The biosurfactant produced by *W. anomalus* CCMA 0358 was evaluated for its ability to remove crude oil from artificially contaminated sand. The cell-free supernatant obtained at the end of the fermentation performed using the optimized culture medium allowed the recovery of about 20 ± 4% of the oil impregnated in the sand when compared with the control assays(performed with demineralized water at the same conditions). One advantage of biosurfactants for application in bioremediation is their possible use without purification (i.e. as cell-free supernatants), which contributesto increase their competitiveness with chemical surfactants, as the purification steps can account for up to 60-70% of their total production costs [3].

The potential use of biosurfactants produced by several yeasts in bioremediation has been reported by other authors. The biosurfactants produced by *C. lipolytica* UCP0988 and *Candida glabrata* UCP 1002 were found to remove between 19% and 43% (depending on the biosurfactant concentration and the sand particle size)of motor oil from contaminated sand when compared with a control performed with demineralized water[22, 27]. Moreover, sophorolipid biosurfactants produced by *C. bombicola* ATCC 22214 recovered up to 27% of crude oil in core-flooding experiments [28]. The results herein obtained were similar to those

reported for the lipopeptide biosurfactant surfactin produced by *Bacillus subtilis* #573, whichled to recoveries between 19 and 26% (depending on the culture medium used to grow the microorganism) of crude oil from contaminated sand in similar assays [9, 19].

These results, together with the stability of this biosurfactant at high temperatures,as well as over a wide range of salinities and pH values, make it a promising candidate for application in bioremediation processes.

4 Conclusion

The yeast strain *W. anomalus* CCMA 0358 was previously found to produce a new type of glycolipid biosurfactant. As a result of the optimization of the culture medium composition through RSM, lower surface tension values were achieved, thus suggesting a higher biosurfactant production. Biosurfactant production using the optimized culture medium was validated both in flasks and bioreactor. In both cases, the highest biosurfactant production was achieved quickly (after 24 h). The excellent surface tension reducing ability exhibited by this biosurfactant, together with its stability under extreme environmental conditions, as well as its ability to recover crude oil from contaminated sand, makes it a potential candidate for application in bioremediation or in the petroleum industry, as an alternative to the traditional chemical surfactants.

Acknowledgements

This study was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UID/BIO/04469/2013 unit and COMPETE 2020 (POCI-01-0145-FEDER-006684); and by BioTecNorte operation (NORTE-01-0145-FEDER-000004) funded by the European Regional Development Fund under the scope of Norte2020 - Programa Operacional Regional do Norte. The authors also thank the FCT for the financial support under the scope of the Project RECI/BBB-EBI/0179/2012 (FCOMP-01-0124-FEDER-027462).

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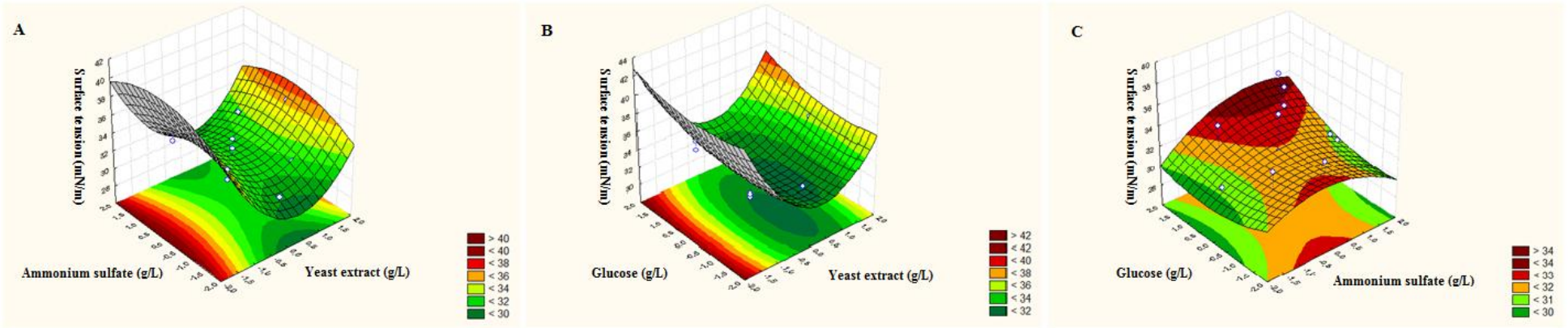

Figure 1. Response surface representing the effect of medium components (yeast extract, ammonium sulfate and glucose) on the surface tension, which is the indicative of the production of biosurfactant. Only yeast extract was found to significantly affect the surface tension. **(A)** Yeast extract and ammonium sulfate; **(B)** Yeast extract and glucose and **(C)** Ammonium sulfate and glucose

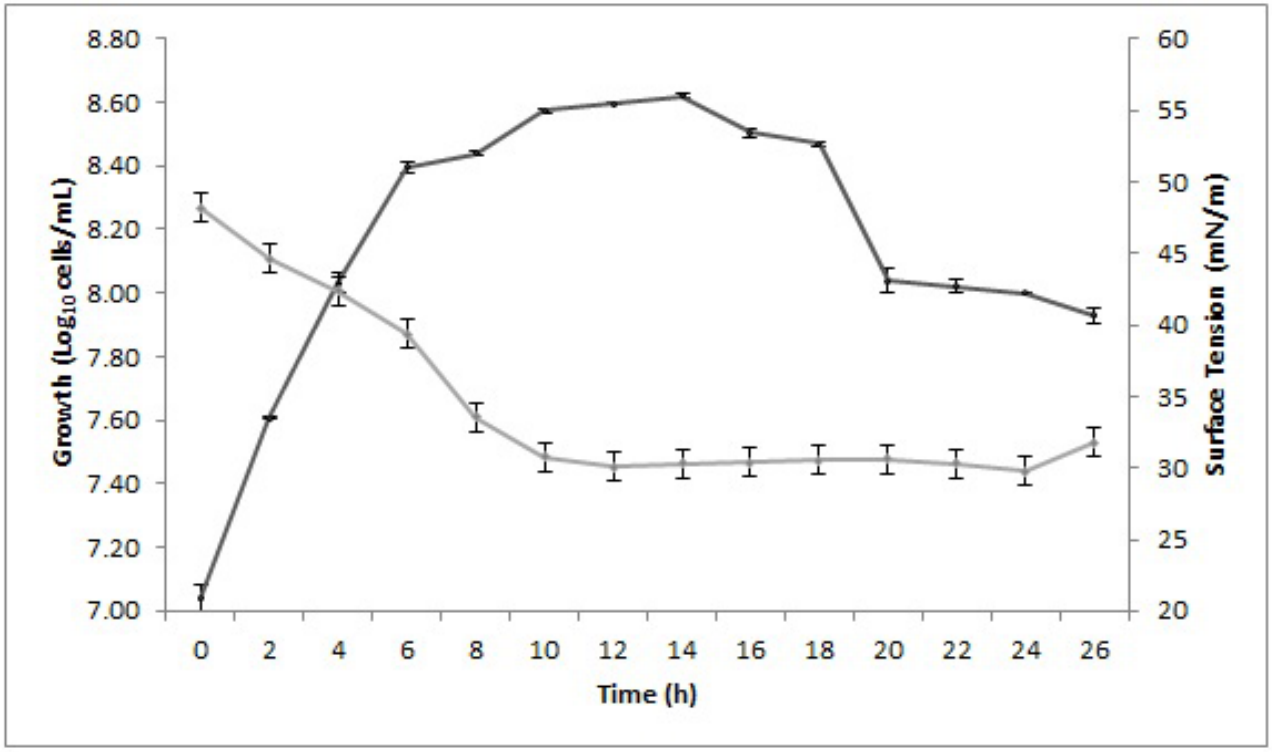

Figure 2. Evolution of growth (cells/mL) and surface tension (ST, mN/m) in fermentations performed with *Wickerhamomyces anomalus* CCMA 0358 grown in bioreactor using the optimized culture medium at 28ºC and 500 rpm. Results represent the average of three independent experiments ± standard deviation.

Considerações finais

A seleção de leveduras produtoras de biossurfactantes associada à otimização do processo fermentativo, são os fatores chaves para aumentar o rendimento da produção de biossurfactantes e diminuir os custos.

De acordo com este estudo, a levedura *Wickerhamomyces anomalus* CCMA 0358, mostrou-se produtora de um glicolipídeo, ainda não descrito anteriormente na literatura, onde apresentou atividade tensoativa e não apresentou atividade emulsionante. O meio de cultivo utilizado para a produção continha azeite de oliva, que mostrou-se excelente precursos para a produção.

Outro dado interesse deste trabalho, é que a maior redução da tensão superficial foi observada após 12 horas de fermentação, diminuindo desta forma o tempo de fermentação e mostrando-se mais rentável para a indústria. Foi observada atividade antimicrobiana do biossurfactante e na recuperação do petróleo de areia contaminada.

Por fim, podemos concluir que o presente estudo apresenta grande contribuição para os estudos com biossurfactantes, uma vez que a levedura *Wickerhamomyces anomalus* CCMA 0358 ainda não havia sido relatada como produtora de biossurfactantes, e o glicolipídeo caracterizado não foi descrito pela literatura.