FEDERAL UNIVERSITY OF SANTA CATARINA

DEPARTMENT OF CHEMICAL ENGINEERING AND FOOD ENGINEERING

POSTGRADUATE PROGRAM IN CHEMICAL ENGINEERING

TALITA CORRÊA NAZARETH

BIOSURFACTANT PRODUCTION BY *Bacillus subtilis* ATCC 6051 USING BREWERY WASTE AS A CARBON SOURCE

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Thesis submitted to the Postgraduate Program in Chemical Engineering at the Federal University of Santa Catarina to obtain a Doctor degree in Chemical Engineering.

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O presente trabalho em nível de doutorado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

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Certificamos que esta é a **versão original e final** do trabalho de conclusão que foi julgado adequado para obtenção do título de Doutora em Engenharia Química.

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Florianópolis, 2019.

To my husband Conrado and my
parents, José and Fátima, for all their love,
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RESUMO

No cenário atual, a busca por tecnologias limpas foi intensificada devido ao aumento da conscientização ambiental e à criação de legislações ambientais mais rigorosas, buscando viabilizar a substituição dos processos industriais tradicionais por processos biológicos. A síntese de biomoléculas tem sido foco de muitos estudos na literatura, nos quais os biossurfactantes têm se destacado devido às suas vantagens frente aos surfactantes obtidos por processos químicos. Essas vantagens são: alta biodegradabilidade, baixa toxicidade, redução da tensão superficial e a possibilidade de serem produzidos por fontes renováveis. Atualmente, a produção de biossurfactantes apresenta projeções de valorização de mercado nos próximos anos. No entanto, os altos custos de produção e purificação dificultam a produção em escala industrial, sendo o substrato um dos fatores que mais encarece o processo. Nesse contexto, este trabalho teve como objetivo a produção de biossurfactante por *Bacillus subtilis* ATCC 6051 em meio submerso, utilizando resíduo de cervejaria (Trub) como fonte alternativa de carbono. O trabalho consistiu em estudar os efeitos dos parâmetros de processo na produção de biossurfactante. Para tanto, foi utilizado um Planejamento Fatorial Completo (FFD), no qual os fatores Trub (% v/v), extrato de levedura (g L^{-1}) e agitação (rpm) apresentaram efeito estatisticamente significativo na tensão superficial (ST) para o primeiro planejamento (2^4). A concentração máxima de surfactina obtida foi de 62,74 mg L^{-1}. Com o segundo planejamento experimental refinado (2^2), foi possível obter um modelo de regressão quadrática adequado para descrever a variável resposta. Também foi avaliado o efeito da adição de metais (ferro, potássio, magnésio e manganês) no meio de cultura definido na primeira etapa, no qual o estudo indicou que todas as soluções metálicas adicionadas ao mesmo tempo exerceram influência sobre a ST. Além disso, o efeito sinérgico das soluções combinadas no meio de cultura promoveu a produção de surfactina, uma vez que a concentração máxima de surfactina obtida foi de 210,11 mg L^{-1}, após 28 h de cultivo, enquanto no ensaio sem metais foi de 121,20 mg L^{-1} após 40 h. A produção de surfactina também foi avaliada em biorreator de tanque agitado acoplado a um coletor de espuma, no qual a agitação (vvm) e a aeração (rpm) foram estudadas por um FFD (2^2). Somente a agitação mostrou efeito significativo na concentração de surfactina e o modelo mostrou um valor do coeficiente de regressão (R^2) de 0,99915. A concentração máxima de surfactina alcançada foi de 239,734 mg L^{-1}, comprovando que o biossurfactante foi produzido com sucesso em maior escala. A atividade antimicrobiana da surfactina contra *P. aeruginosa* DSM 3227, *E. coli* ATCC 25922, *S. aureus* DSM 20231 e *S. epidermidis* DSM 28319 foi avaliada pela técnica de microdiluição em placa de 96 poços. A atividade antimicrobiana foi observada contra todas as cepas, atingindo a maior inibição (100%) para *P. aeruginosa* (500 μg mL^{-1} de tratamento). Uma redução logarítmica de 3,91 foi alcançada para *P. aeruginosa* e, para *S. aureus* e *S. epidermidis*, foram exibidas de 1 a 2 reduções logarítmicas após o tratamento. Nos ensaios antibiofilme contra *P. aeruginosa*, a maior inibição para co-incubação, anti-adesivo e ruptura foi de 79,80 (400 μg mL^{-1}), 58,81 (350 μg mL^{-1}) e 44,94 (700 μg mL^{-1}), respectivamente. Este estudo fornece evidências de que a surfactina produzida a partir de um substrato de baixo custo pode ser um biocida promissor devido às suas habilidades antimicrobiana, antiadesiva e antibiofilme contra patógenos.

Palavras-chave: Surfactina. Biossurfactante. Resíduos de cervejaria. Planejamento experimental. Atividade antimicrobiana. Atividade antibiofilme.

RESUMO EXPANDIDO

Introdução

Os surfactantes são moléculas anfipáticas que apresentam atividade de superfície e possuem aplicações em diversos segmentos industriais, sendo utilizados na produção de detergentes, óleos lubrificantes, formulações farmacêuticas, agroquímicos, dentre outros. Contudo, os surfactantes obtidos por via química, a partir de derivados do petróleo, provocam maior impacto ambiental quando comparados com os biossurfactantes, que são sintetizados por microrganismos. Além disso, os biossurfactantes possuem alta biodegradabilidade e baixa toxicidade frente aos surfactantes sintéticos. A produção de biossurfactante encontra-se limitada em virtude dos altos custos de produção, principalmente em relação à fonte de substrato e processos de purificação. Dessa forma, estudos têm sido realizados com o intuito de reduzir custos de produção através da utilização de substratos de baixo custo, obtidos a partir de resíduos agroindustriais. Assim, diante do cenário crescente de cervejarias artesanais no Brasil, os resíduos obtidos durante o processo produtivo possuem carbono e nitrogênio em sua composição, sendo portanto, favoráveis a processos biológicos e promissores para a produção de biossurfactantes. Além disso, a produção de biossurfactantes é influenciada pelas condições de processos, onde fatores como composição do meio de cultivo, pH, temperatura, agitação e aeração, influenciam no rendimento da produção. Diante da baixa toxicidade dos biossurfactantes, suas aplicações na indústria alimentícia e em áreas biomédicas têm sido relevantes, visto que estas biomoléculas têm apresentado atividades antimicrobiana e antibiofilme. A atividade antimicrobiana está relacionada com a capacidade das moléculas de biossurfactantes interagirem com a membrana celular bacteriana, causando a sua solubilização, levando a sua ruptura e morte celular. Assim, os biossurfactantes são promissores para atuarem contra microrganismos patogênicos, que são responsáveis por diversas infecções. A atividade antibiofilme ocorre através da inibição da formação de biofilme quando os microrganismos são expostos às moléculas de biossurfactantes, sendo afetados pela ação antimicrobiana e pela remoção do material polimérico extracelular (EPS), que são produzidos pelos microrganismos para proteção da comunidade celular em formação, impedido que o biofilme se consolide. Além disso, biossurfactantes possuem atividade antiadesiva, impedindo que o biofilme se fixe a superfícies pré-condicionadas com a biomolécula, possuindo também a capacidade de romper biofilmes maduros.

Objetivos

Este trabalho teve como objetivo avaliar a produção de biossurfactante em cultivo submerso por *Bacillus subtilis* ATCC 6051 utilizando o resíduo de cervejaria (Trub) como fonte alternativa de carbono. Para isto, buscou-se determinar as condições nutricionais e de cultivo que interferem significativamente no processo de produção de biossurfactante através de técnicas de planejamento experimental e avaliar a influência da suplementação de meio de cultura com soluções metálicas. Após a otimização dos parâmetros de processo e de cultivo, buscou-se avaliar a ampliação da produção em biorreator de tanque agitado e aerado em escala de bancada, estudando o impacto da agitação e aeração no processo biológico e no rendimento de biossurfactante. Após a extração do biossurfactante produzido, buscou-se caracterizá-lo através de espectrometria de massa e avaliar a sua estabilidade, em relação à tensão superficial e índice de emulsificação, frente a variações de pH e temperatura. Por fim, buscou-se avaliar aplicações para o biossurfactante através do estudo das atividades antimicrobiana e antibiofilme contra cepas patogênicas.

Metodologia

Reativação e cultura estoque do microrganismo: a cepa liofilizada de *B. subtilis* ATCC 6051 foi reidratada e a suspensão de células foi transferida para um tubo de ensaio contendo 5 mL de caldo nutriente (Nutrient Broth Medium - NB), e incubada em agitador orbital (160 rpm, 24 h). Após a incubação, a suspensão bacteriana foi redistribuída de forma asséptica em tubos criogênicos contendo glicerol (20% v/v) e meio NB, sendo posteriormente estocada a -80 °C (1° geração). A cultura estoque foi obtida por meio da transferência do conteúdo de um tubo criogênico de primeira geração para um erlenmeyer contendo 50 mL de meio de manutenção, sendo este incubado a 30 °C e 160 rpm durante 24 h, sendo criopreservada a -20 °C. Sempre quando necessário, um tubo da cultura estoque era descongelado e utilizado para a preparação do inóculo.
Resíduo de cervejaria: a fonte de carbono utilizada no processo foi resíduo de cervejaria (Trub), sendo este gentilmente cedido pela Fábrica de Cerveja Artesanal Kairós, localizada em Florianópolis - SC. O pH do Trub foi de 5,73 e sua composição foi realizada por análise elementar (C) e método enzimático-colorimétrico (N). Presença de metais (Fe, K, Mg e Mn) foi quantificada por Espectrometria de Absorção Atômica.
Otimização do meio de cultura e de parâmetros de processo na produção de biossurfactante em frascos agitados: Neste trabalho, diferentes fatores foram avaliados para identificar quais deles tiveram efeitos

significativos na produção de biossurfactante. Os ensaios foram realizados em cultivo submerso em frascos agitados contendo 100 mL de meio de produção (PM), composto por (g L^{-1}): CaCl$_2$ (0.1), KH$_2$PO$_4$ (1.0), MgSO$_4$.7H$_2$O (0.5) e NaCl (0.1). Além desses compostos, Trub, peptona e extrato de levedura foram adicionados em diferentes concentrações, de acordo com o Planejamento Fatorial Completo - FFD (2^4), representado pelos seguintes níveis: Trub - TB (%v/v): 2 (-1), 6 (0), 10 (+1); Extrato de Levedura – YE (g L^{-1}): 4 (-1), 5 (0), 6 (+1); Peptona – PB (g L^{-1}): 0.6 (-1), 0.7 (0), 0.8 (+1) e Agitação – AG (rpm): 100 (-1), 150 (0), 200 (+1). Os frascos foram inoculados (5% v/v) com cultura ajustada em OD$_{600}$ 0.85, e pH inicial de 7.0, sendo incubados durante 72 h. Um novo FFD (2^2) foi realizado, tendo como variáveis independentes o extrato de levedura (YE) e a peptona (PB), e a tensão superficial (ST) como variável resposta. A seleção das variáveis independentes e da amplitude de seus níveis foi baseada nos resultados obtidos no FFD (2^4) apresentados anteriormente. Os níveis dos fatores estudados foram: Extrato de Levedura – YE (g L^{-1}): 2 (-1), 7 (0), 12 (+1); Peptona – PB (g L^{-1}): 0.4 (-1), 0.9 (0), 1.4 (+1). Os frascos contendo meio PM e Trub (2% v/v), e também contendo YE e PB de acordo com FDD (2^2), foram incubados a 30 °C e 200 rpm. Semelhante ao FFD (2^4) apresentado anteriormente, o processo durou 72 horas. Efeito da suplementação de meio de cultura com soluções metálicas na produção de biossurfactante em frascos agitados: a influência dos metais (micronutrientes) na produção de biossurfactante foi avaliada através da adição de soluções de ferro, potássio, magnésio e manganês ao meio de cultura otimizado. Três concentrações (mM) diferentes foram avaliadas para cada composto metálico, apresentados a seguir: FeSO$_4$ (0.008, 1.2 e 4.0); KH$_2$PO4 (5.0, 10.0 e 30.0); MnSO$_4$ (0.01, 0.1 e 0.3) e MgSO$_4$ (0.04, 0.6 e 2.4). Produção de biossurfactante em biorreator de tanque agitado (STR): estudo dos efeitos de aeração e agitação: A produção de biossurfactante foi realizada em biorreator de 5 L, com 2 L de volume de trabalho, equipado com eletrodos de pH e pO$_2$. Soluções de HCl e NaOH, ambas a 1 M, foram automaticamente adicionadas ao meio para garantir que o pH fosse mantido em 7,0. Cada ensaio durou 24 h e um coletor de espuma estéril foi instalado no reator, no qual a espuma foi canalizada através de uma saída superior do reator. O inóculo de *B. subtilis*, previamente ajustado (OD$_{600}$ 0,85), foi adicionado ao meio de cultura (10% v/v). O FFD (2^2) foi realizado com aeração (AR) e agitação (AG) como variáveis independentes, e concentração de surfactina (SF) como variável resposta. Os níveis dos fatores avaliados foram: Aeração – AR (vvm): 0.5 (-1), 1.0 (0), 1.5 (+1); Agitação – AG (rpm): 150 (-1), 225 (0), 300 (+1). O coeficiente volumétrico de transferência de oxigênio (K$_L$a) foi medido de acordo com o Método Dinâmico. Extração do biossurfactante: O biossurfactante foi extraído por centrifugação (9000 rpm, 20 min), seguida de precipitação ácida (pH 2.0). O biossurfactante precipitado foi centrifugado, lavado com água acidificada (pH 2,0) e ressuspenso em água Milli-Q. O pH da solução foi ajustado para 7,0, liofilizado, pesado e armazenado a -18 °C. Caracterização estrutural de biossurfactante: o biossurfactante foi caracterizado por espectrometria de massa por ionização e dessorção a laser assistida por matriz (Maldi-Tof). Tensão superficial e concentração de micelas crítica (CMC): a tensão superficial do sobrenadante isento de células foi medida usando um tensiômetro digital pelo método da placa de platina (Wilhelmy). A CMC do biossurfactante parcialmente purificado foi determinada por medidas de tensão superficial de diluições sucessivas de solução aquosa de biossurfactante. Posteriormente, foi construído um gráfico de tensão superficial *versus* concentração de biossurfactante, no qual a CMC corresponde ao ponto central de inflexão da curva. Estabilidade do biossurfactante: a estabilidade foi avaliada pela influência de diferentes condições de temperatura (25 °C, 45 °C, 65 °C, 85 °C e 100 °C) e pH (entre 2,0 e 10,0) na capacidade do biossurfactante em reduzir a tensão superficial e na sua atividade emulsificante. Para avaliar a estabilidade térmica, soluções de biossurfactantes (30 mg L^{-1}, pH 7.0) foram incubadas por 24 h. O efeito do pH foi estudado através da preparação de soluções de biossurfactantes (30 mg L^{-1}), com diferentes valores de pH, incubadas a 25 °C por 24 h. Em ensaio paralelo, foram adicionados 2 mL de todas as amostras preparadas nos ensaios de estabilidade aos tubos de ensaio contendo 2 mL de n-dodecano. Os tubos foram agitados em vórtex e incubados de acordo com a respectiva condição predeterminada e, após isto, foi calculado o índice de emulsificação (E$_{24}$). Determinação da concentração de biossurfactante: a concentração de surfactina foi determinada por uma curva de calibração construída usando medidas de tensão superficial (método da placa de platina Wilhelmy) de amostras contendo diferentes concentrações de surfactina padrão comercial (Sigma-Aldrich, 98%). Determinação da concentração de biomassa: a concentração de biomassa foi expressa em massa seca (g L^{-1}) após determinação do valor de absorbância (600 nm) e sua correlação com a massa seca por uma curva de calibração (R^2 = 0,9981) construída. Determinação da concentração de substrato (glicose): foi adotado o método calorimétrico Fenol-Sulfúrico, utilizando glicose para a construção da curva de calibração (R^2 = 0,9975). Aplicação antimicrobiana: a concentração inibitória mínima (MIC) foi realizada em placa de 96 poços. O biossurfactante bruto foi dissolvido em caldo Mueller Hinton (MHB) para se obter diferentes concentrações de tratamento para cada cepa, conforme a seguir: *P. aeruginosa* DSM 3227 (500–100 µg mL^{-1}), *E. coli* ATCC 25922 (800–50 µg mL^{-1}), *S. aureus* DSM 20231 (800-50 µg mL^{-1}) e *S.*

epidermidis DSM 28319 (800-100 μg mL^{-1}). O inóculo foi padronizado ajustando OD$_{600}$ para um valor correspondente a 10^8 CFU mL^{-1}. As placas foram incubadas a 37 °C por 24 h e foram realizadas diluições em série para cada concentração. O meio diluído foi replicado em Mueller Hinton Agar (MHA) para se obter a redução logarítmica e a inibição percentual. <u>Aplicação antibiofilme</u>: os testes foram realizados em placa de poliestireno com 24 poços e poços não tratados foram usados como controle. Em todos os experimentos, a absorbância do inóculo de *P. aeruginosa* DSM 3227 a 600 nm correspondeu a um valor equivalente a 10^8 CFU mL^{-1}. O potencial do biossurfactante em impedir a formação de biofilme foi avaliada por meio duas técnicas diferentes: co-incubação e antiadesivo. Nos ensaios de co-incubação, 1 mL de uma variedade de concentrações de biossurfactante (250–500 μg mL^{-1}) dissolvido em NB foi inoculado (5%, v/v), seguido de incubação por 24 h. A atividade antiadesiva foi testada pré-revestindo os poços com soluções de biossurfactante preparadas em tampão fosfato-salino PBS (250–500 μg mL^{-1}), na qual 1 mL de cada concentração foi adicionado no poço correspondente e incubado por 24 h a 40 °C. Após decorrido o tempo de adsorção, a placa foi esterilizada por 3 h sob luz UV. Posteriormente foi adicionado 1 mL de cultura padronizada, seguido de incubação por 24 h a 37 °C. O ensaio de ruptura por biossurfactante de um biofilme pré-formado, foi realizado através da adição de 1 mL de cultura padronizada em cada poço. A placa foi incubada por 24 h a 37 °C para o desenvolvimento do biofilme. Após o período de incubação, as células planctônicas foram descartadas e o biofilme foi lavado com PBS. 1 mL de meio fresco com diferentes concentrações (200–700 μg mL^{-1}) de biossurfactante foi adicionado e incubado por 24 h a 37 °C. No final de todos os experimentos de atividade antibiofilme, o conteúdo das placas foi corado com cristal violeta (0,1%) por 15 minutos à temperatura ambiente. Posteriormente, 1 mL de ácido acético (30%, v/v) foi adicionado em cada poço e a absorbância do conteúdo do poço foi medida em 575 nm, para a realização do cálculo de porcentagem de inibição. <u>Análise estatística</u>: a análise estatística dos dados foi realizada usando o Statistica 7.0 (StatSoft Inc, EUA). Análise de variância (ANOVA) e teste de falta-de-ajuste foram utilizados para verificar a adequação do modelo de regressão empírica. Além disso, os resultados foram comparados pelo teste de Tukey, ao nível de significância de 5%. Nos ensaios de aplicação do biossurfactante, os dados foram analisados por ANOVA, e as médias foram comparadas com o teste de Duncan (5% de probabilidade).

Resultados e Discussão
<u>Efeito de parâmetros nutricionais e de processo na produção de biossurfactante por *B. subtilis* ATCC 6051</u>: Nos resultados obtidos para o FFD (2^4), a concentração mais alta de biossurfactante foi de 100,76 mg L^{-1}. Para os ensaios com maiores valores de concentração de biossurfactante, notou-se que os fatores TB e AG permaneceram constantes no nível mais baixo, de 2% (v/v) e 100 rpm, respectivamente. O efeito dos fatores na ST foi analisado pelo diagrama de Pareto, que mostrou que Trub (TB), extrato de levedura (YE), agitação (AG) e a interação entre eles têm efeito estatisticamente significativo na tensão superficial. A peptona (PB) não teve efeito significativo, assim como a interação entre ela e os demais fatores estudados. Com o intuito de visualizar o comportamento do processo, foi realizada uma cinética adotando a agitação e o extrato de levedura no nível positivo, e a concentração de peptona e Trub no nível negativo, conforme as conclusões obtidas a partir dos resultados da ANOVA e do diagrama de Pareto. A maior concentração de biossurfactante obtida foi de 62,74 mg L^{-1} após 76 h de processo e a CMC foi possivelmente alcançada entre 15 e 28 h. A redução repentina na tensão superficial ocorreu durante a fase exponencial do crescimento celular, sugerindo que a produção de biossurfactante pode estar associada ao crescimento microbiano. De acordo com a análise do FFD (2^4), verificou-se que o Trub teve um efeito predominante e seu uso mais apropriado na produção de biossurfactante estaria no nível (-1). Além disso, a agitação mostrou o segundo maior efeito predominante. Um novo planejamento foi elaborado com as variáveis independentes YE e PB, uma vez que PB apresentou *valor-p* próximo a 0,05. Para isso, a faixa dos níveis dos fatores foi ampliada e os valores de Trub e agitação foram mantidos constantes a 2% (v/v) e 200 rpm, respectivamente. A máxima concentração de biossurfactante obtida foi de 96,56 mg L^{-1}, para a concentração de extrato de levedura no nível (+1). No ponto central, a maior concentração de biomassa foi alcançada, sugerindo que esta é a melhor condição para o crescimento microbiano. O efeito dos fatores sobre a variável resposta foi analisado através do diagrama de Pareto, que mostrou que apenas a concentração de extrato de levedura teve um efeito significativo na tensão superficial. A interação entre as duas variáveis independentes também foi estatisticamente significativa, sugerindo que o efeito combinado entre os fatores exerce influência na produção de biossurfactante. De acordo com as análises do FFD (2^2), foi realizado um estudo cinético da produção de biossurfactante. As condições adotadas foram as do ponto central (CP), pois seriam utilizadas quantidades consideravelmente mais baixas de reagentes. Um aumento significativo na concentração de biossurfactante (121,20 mg L^{-1}) ocorreu após 40 h de cultivo. Além disso, foi observado um perfil de produção associado ao crescimento celular e a concentração micelar crítica foi atingida entre 28 e 40 h de cultivo. <u>Efeito da</u>

suplementação do meio de cultura com soluções de metais na produção de biossurfactante em frascos agitados: a adição individual de soluções metálicas ao meio de cultura levou à produção de biossurfactante, uma vez que foram obtidos baixos valores de tensão superficial da amostra diluída (1:10, ST^{-1}). Realizou-se ANOVA para verificar se os valores de ST^{-1} para os ensaios com soluções metálicas diferiam estatisticamente dos valores de ST^{-1} da amostra controle (sem metais). Como resultado, os ensaios com metais podem ser considerados diferentes da amostra controle, uma vez que as concentrações estudadas para cada metal apresentaram efeito significativo. Além disso, foi realizado o teste de Tukey, através do qual se constatou que as concentrações das soluções de $MnSO_4$ não diferiam entre si. Fato semelhante pode ser observado para as concentrações de soluções de $MgSO_4$ e KH_2PO_4, o que sugere o uso de valores mais baixos de concentração para esses compostos visando reduzir o uso de reagentes. As concentrações de 0,008 e 1,2 mM de soluções de $FeSO_4$ diferiram entre si. O comportamento do processo foi avaliado por meio da adição de soluções de todos os metais ao meio de cultura, buscando verificar a presença de efeitos sinérgicos para melhorar a produção de biossurfactante. Elevadas concentrações de biossurfactante foram obtidas após 30 h de cultivo, atingindo um valor (210.11 mg L^{-1}) quase duas vezes maior que a concentração máxima de biossurfactante obtida na cinética sem a presença de metais no meio. Além disso, a CMC foi alcançada apenas entre 9 e 15 h de processo, enquanto na cinética anterior foram necessárias entre 28 e 40 h. Este resultado é bastante interessante, pois houve um aumento significativo na concentração de biossurfactante em pouco tempo. Assim, a combinação de metais no meio de cultura foi satisfatória para a produção de surfactina.
Produção de biossurfactante em biorreator de tanque agitado: estudo dos efeitos de aeração e agitação: a influência da agitação e aeração na produção de biossurfactante foi avaliada por meio de um FFD (2^2) com aeração (AR) e agitação (AG) como variáveis independentes. A concentração máxima de biossurfactante alcançada foi de 239,74 mg L^{-1}, o que corresponde à condição de aeração e agitação no nível (-1) dos fatores estudados. Além disso, observou-se que este ensaio apresentou a maior concentração celular. A ANOVA indicou que apenas a agitação teve um efeito significativo na SF, com *valor-p* menor que 0,05. O diagrama de Pareto indicou que a agitação teve um efeito negativo, sugerindo que altos valores de agitação não são favoráveis ao processo. A aeração e a interação entre os fatores estudados não tiveram efeito significativo na SF. Os dados experimentais foram ajustados ao modelo de regressão quadrático, que não apresentou efeito significativo no teste de falta-de-ajuste, mostrando um valor do coeficiente de regressão (R^2) de 0,99915.
Atividade antimicrobiana: a maior porcentagem de inibição para *P. aeruginosa* foi de 100% usando 500 μg mL^{-1} de biossurfactante bruto, sendo que essa concentração representa a concentração bactericida mínima (MBC). Um efeito menor do biossurfactante foi observado contra *E. coli*, com apenas 6,69% de inibição alcançada com 800 μg mL^{-1} de tratamento. Para as cepas gram-positivas, como *S. aureus* e *S. epidermidis*, as maiores porcentagens de inibição, usando 800μg mL^{-1}, foram 18,56% e 24,44%, respectivamente.
Atividade antibiofilme: o biossurfactante apresentou atividade antibiofilme nos três tratamentos realizados, sendo o ensaio de co-incubação o mais eficiente, pois inibiu a formação de biofilme em 79.80% com 400 μg mL^{-1} de tratamento. Também foi possível prevenir a formação de biofilme maduro (58,81%) em uma superfície pré-revestida com biossurfactante (350 μg mL^{-1}). Neste estudo, o biossurfactante também foi empregado para romper um biofilme pré-existente de *P. aeruginosa*, atingindo 44,94% de inibição com tratamento de 700μg mL^{-1} de biossurfactante.

Conclusões
Trub foi utilizado com sucesso na produção de surfactina por *B. subtilis* ATCC 6051. O extrato de levedura (YE) teve um efeito significativo na tensão superficial, sugerindo que concentrações mais elevadas de YE estão associadas a uma maior produção de biossurfactante. Além disso, a adição de metais combinados no meio de cultura foi eficaz no aumento da produção de surfactina, sendo três vezes maior que a produção em meio de cultura sem a presença de soluções metálicas. A produção de biossurfactante em biorreator foi eficiente, visto que uma elevada concentração de biossurfactante foi obtida. Além disso, o coletor de espuma foi eficiente em separar o biossurfactante do meio líquido, promovendo um aumento da sua produção. Efeito bactericida em *P. aeruginosa* foi constatado ao se utilizar biossurfactante e efeito inibitório no crescimento bacteriano foi verificado em todas as cepas avaliadas (*E. coli*, *S. aureus* e *S. epidermidis*). Além disso, o biossurfactante apresentou atividade antibiofilme contra *P. aeruginosa*.

Palavras-chave: Surfactina. Biossurfactante. Resíduos de cervejaria. Planejamento experimental. Atividade antimicrobiana. Atividade antibiofilme.

ABSTRACT

In the current scenario, the search for clean technologies has been intensified due to the increase of environmental awareness and the creation of stricter environmental legislation, seeking to enable the replacement of traditional industrial processes for biological processes. The synthesis of biomolecules has been the focus of many studies in the literature, in which biosurfactants have been outstanding due to their advantages over surfactants obtained by chemical processes. These advantages are high biodegradability, low toxicity, surface tension reduction, and the possibility of being produced by renewable sources. Currently, the production of biosurfactants is active and with projections of market appreciation in the coming years. However high production and purification costs have made production at industrial scale difficult and the substrate is one of the most expensive factor. In this context, this work aimed at the production of biosurfactant by *Bacillus subtilis* ATCC 6051 in submerged medium using brewery residue (Trub) as an alternative carbon source. The work consisted of studying the effects of process parameters on biosurfactant production. For this purpose, a Full Factorial Design (FFD) was used, in which the factors Trub (% v/v), yeast extract (g L^{-1}), and agitation (rpm) presented a statistically significant effect on surface tension (ST) for the first design (2^4). The maximum surfactin concentration obtained was 62.74 mg.L^{-1}. With the second refined experimental design (2^2), it was possible to obtain a suitable quadratic regression model to describe the response variable. The effect of the addition of metals in the culture medium defined in the first stage was evaluated (Iron, Potassium, Magnesium and Manganese). The study indicated that all added metal solutions at the same time, exerted influence on ST. In addition, the synergistic effect of the combined solutions on the culture medium promoted the production of surfactin, since the maximum surfactin concentration obtained was 210.11 mg L^{-1}, after 28 h of cultivation, while for the non-metal assay, it was 121.20 mg L^{-1} after 40 h. The surfactin production was also evaluated in stirred tank bioreactor coupled with a foam collector, in which agitation (vvm) and aeration (rpm) was studied by a FFD (2^2). Only agitation showed a significant effect on surfactin concentration and the model showed a regression coefficient (R^2) value of 0.99915. The maximum surfactin concentration reached was 239.74 mg L^{-1}, proving that biosurfactant was successfully produced on a larger scale. The antimicrobial activity of surfactin against *P. aeruginosa* DSM 3227, *E. coli* ATCC 25922, *S. aureus* DSM 20231 and *S. epidermidis* DSM 28319 was evaluated by microdilution technique in 96-well plate. The antimicrobial activity was observed against all the strains, achieving the highest inhibition (100%) for *P. aeruginosa*, at 500 μg mL^{-1}. A log reduction of 3.91 was achieved for *P. aeruginosa* and, to *S. aureus* and *S. epidermidis* were exhibited from 1 to 2 log reductions after treatment. In the antibiofilm assays against *P. aeruginosa*, the highest inhibition for co-incubation, anti-adhesive and disruption was 79.80 (400 μg mL^{-1}), 58.81 (350 μg mL^{-1}) and 44.94 (700 μg mL^{-1}), respectively. This study provides evidence that surfactin produced from a low-cost substrate can be a promising biocide due to its antimicrobial, anti-adhesive and antibiofilm abilities against pathogens.

Keywords: Surfactin. Biosurfactant. Brewery waste. Experimental design. Antimicrobial activity. Antibiofilm activity.

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SUMMARY

1 INTRODUCTION

In recent years, biotechnology has been gaining attention in the industrial scenario due to the necessity for processes that seek not only better production yields with low costs, but which aim at reducing environmental impacts through the reduction or reuse of generated waste. Moreover, the development of new biotechnologies to obtain products that already exist in the market has stood out against the advantages offered in the classic industrial processes, which often cause huge environmental impacts.

Surfactants are a class of chemicals used in various sectors in the current industry and, in the 1990s, demand for surfactants increased by about 300% in the US chemical industry, with world production above 3 million tons per year. Most of the commercial surfactants available are chemical surfactants, obtained from petroleum derivatives. However, rapid advances in biotechnology and increased environmental awareness among consumers combined with stricter legislation have boosted the production of biological surfactants as potential alternatives to existing products (BANAT *et al*., 2000).

The global market for biosurfactants was estimated at US$ 14.5 million in 2016 and the estimate for the year 2020 is US$ 17.1 million (TRANSPARENCY MARKET RESEARCH, 2016). Currently, the industrial scale production of biosurfactants is directed to sophorolipids, rhamnolipids, mannosylerythritol lipids (MEL), among others including fatty acids, glycolipids and polymeric surfactants (TRANSPARENCY MARKET RESEARCH, 2016). Due to lenient regulations on the use of bio-based products, it is expected that Europe becomes a leader in the consumption of microbial biosurfactant products, becoming the fastest growing region by 2020 (TRANSPARENCY MARKET RESEARCH, 2016).

Although already inserted in an industrial scale, the biosurfactants production is limited due to the high cost of production, mainly in relation to the use of substrates and purification processes (SINGH *et al*., 2018). According to Makkar *et al.* (2011), the production of biosurfactants depends on the abundance and the low cost of substrate, since this represents 30% of the final product value.

Millions of tons of waste are generated in breweries, leading to the accumulation of large amounts of biomass that are harmful to the environment if appropriate measures are not taken. This wasted material, consisting mainly of spent grains, Trub and spent yeast, has significant amounts of carbon and nitrogen that can be exploited as additives in biological processes (dos SANTOS MATHIAS *et al*., 2015; FĂRCAŞ *et al*., 2017)

In addition, the optimization of the culture medium and growth conditions are essential to increase the yield of the biosurfactant production process. Thus, many researches have sought to identify factors that influence biosurfactant production by optimizing parameters that are generally present in the culture medium or operating conditions (HUANG *et al.*, 2015; MOUAFI *et al.*, 2016; HA *et al.*, 2018).

Biosurfactants have shown antimicrobial and antibiofilm abilities, being an attractive alternative to application in medicine and cosmetics field, since their formulations require substances that prevent and inactivate the growth of microorganisms that may alter the stability of the product or cause infections in the user. Besides, this type of application requires non-toxic substances that do not cause hypersensitivity reactions to the user (WILKINSON; MOORE, 2011). Another important aspect is, in recent years, the indiscriminate use of antibiotics has led to the selection of multi-resistant microbial strains to traditional antibiotics, making it necessary to search for new substances capable of eliminating these microorganisms (PINTO *et al.*, 2010).

In this context, this work proposes the use of agroindustrial residues as an alternative carbon source in the production of biosurfactants, being possible to combine the treatment and adequacy of the residues generated with the production of high added-value compounds with antimicrobial and antibiofilm properties.

1.2 OBJECTIVES

1.1.1 General objectives

This work aims to evaluate the production of biosurfactant in submerged cultivation by *Bacillus subtilis* ATCC 6051 using brewery residue (Trub) as an alternative carbon source.

1.1.2 Specific objectives

• Determination of the nutritional and cultivation conditions that interfere in the biosurfactant production process through experimental design techniques;

• Evaluation of the influence of culture medium supplementation with metal solutions on biological surfactant production;

• Evaluation of the kinetic behavior of the best-established process conditions;

• Evaluation of biosurfactant production in a bench-top bioreactor, as well as, study the influence of agitation and aeration on production by experimental design technique;

• Evaluation of the antimicrobial and antibiofilm applications of biosurfactant produced against pathogenic strains;

• Characterization of the biosurfactant produced and evaluation of its stability against pH and temperature.

2 LITERATURE REVIEW

This chapter presents fundamental concepts and the classification of biosurfactants as well as the factors that influence their production. In addition, topics are addressed focusing on the use of brewery waste as a carbon source in bioprocesses and the applications of biosurfactants.

2.1 SURFACTANTS AND SURFACE TENSION

Surfactants are molecules consisting of a hydrophobic and a hydrophilic moiety in which the apolar moiety is generally a hydrocarbon chain and the polar moiety may be ionic, nonionic or amphoteric. Due to the presence of these two groups in the same molecule, surfactants tend to preferentially diffuse at the interfaces between fluid phases with different degrees of polarity, such as oil-water and air-water (NITSCHKE; PASTORE, 2002).

Surfactant molecules have different structures due to their tendency to associate when in the presence of water or non-polar solvents. According to Randhawa (2014), these molecules are capable of forming micelles, reverse micelles, bilayers and vesicles (Figure 1). The micelles are formed after saturation of the liquid surface by surfactant monomers, causing the reduction of surface tension.

Figure 1 - Schematic representation of some organized structures of some surfactants.
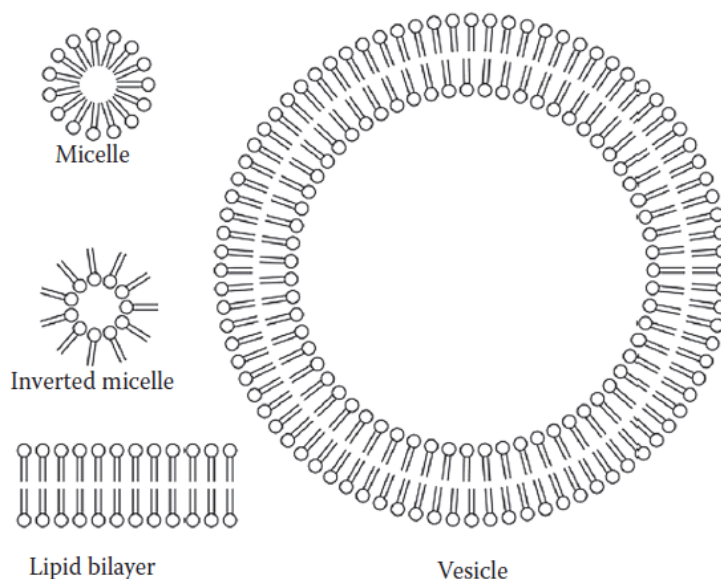
Source: Randhawa (2014).

Surface tension is defined as the measure of surface free energy per unit area needed to bring a molecule from the bulk phase to the surface. The effectiveness of a surfactant is usually determined by its ability to reduce the surface and interfacial tension of systems (MULLIGAN, 2005).

According to Galabova *et al.* (2014), an efficient surfactant is able to reduce the surface tension of water from 72 to 35 mN m^{-1} and the interfacial tension between water and n-hexadecane from 40 to 1 mN m^{-1}. The interfacial or surface tension decreases as surfactant monomers are added to the solution until they reach critical micellar concentration (CMC). Above CMC, no further reduction in surface or interfacial tension is observed. In CMC, surfactant monomers begin to associate spontaneously forming an aggregate structure (Figure 2). At this point, when the CMC was reached, the addition of new monomers results in the formation of new micelles. The micelles are in dynamic equilibrium with the dissolved surfactant monomers, which remain at constant concentration after CMC is reached (PATIST *et al.,* 2002).

Figure 2 - Relationship between surface tension and surfactant concentration.
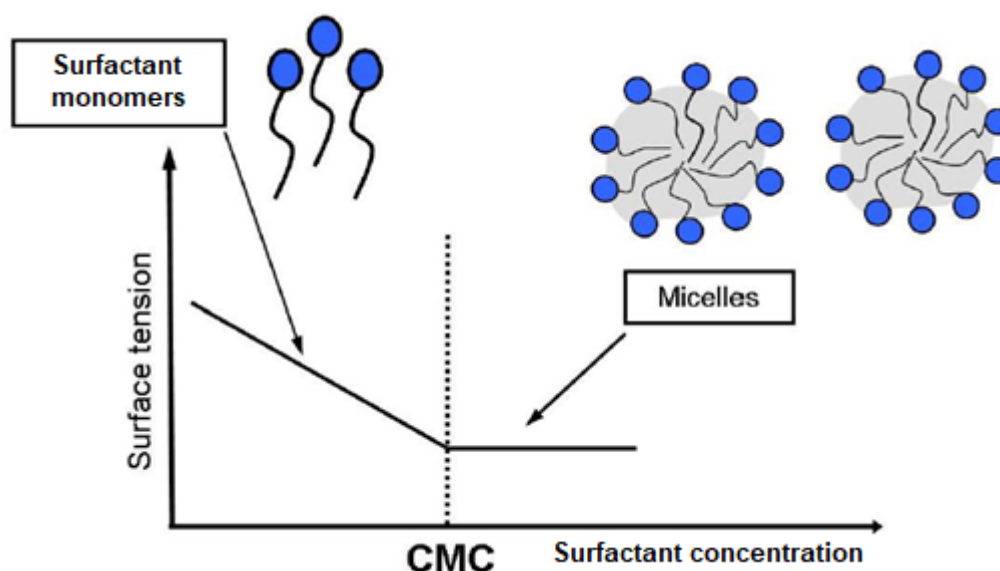
Source: adapted from Pacwa-Płociniczak *et al.* (2011).

2.2 BIOSURFACTANTS

Biosurfactants consist of metabolic by-products of bacteria, fungi and yeast and can be secreted into the culture medium or integrated into the cell wall. Among the producing microorganisms, bacteria are the major responsible for such production (de ARAÚJO *et al.*, 2013) and much of the research found in the literature is linked to microorganisms of the genus *Pseudomonas, Bacillus* and *Acinetobacter* (FRANZETTI *et al.*, 2010).

Many anionic and nonionic biosurfactants are known. In contrast, cationic biosurfactants have been described very rarely, probably because they have toxic effects, as cationic surfactants in general (HAUSMANN; SYLDATK, 2015).

Surfactants can play key roles in the survival of the microorganisms that generated them since, in order to multiply, colonize or disconnect from an ecological niche, microorganisms use cell wall-bound surfactants to regulate cell surface properties. Thus, when they wish to disconnect from a location according to their needs in order to find new habitats with higher nutrient availability or cling to surfaces, surfactants are released by microorganisms to perform these activities. Other activities demonstrated by various biosurfactants are facilitating nutrient transport, antibiotic activity and uptake of hydrophobic compounds. In addition, biosurfactant production can improve emulsification and solubilization of hydrocarbon substrates, thereby facilitating the growth of the microorganism in the medium in question (NITSCHKE; PASTORE, 2002).

Some microorganisms are capable of producing biosurfactants in the presence of various substrate types. Differences between carbon sources lead to changes in the structure and properties of the biosurfactant produced, which favors its different applications (CAMPOS *et al.*, 2014).

According to Lourenço *et al.* (2017), the hydrophobic and hydrophilic moiety of biosurfactant molecules are synthesized by two independent metabolic pathways, which are influenced by the nature of the carbon source. Biosynthesis of both hydrophilic and hydrophobic moiety occurs via the *de novo* pathway (Figure 3), where complex molecules are synthesized from simple structures (amino acids) or through induction of the substrate. These two parts are then grouped, being linked by a glycosidic bond, ester bond or amide bond, forming the amphipathic structure.

Figure 3 - Potential biosynthetic pathways for biosurfactant production.
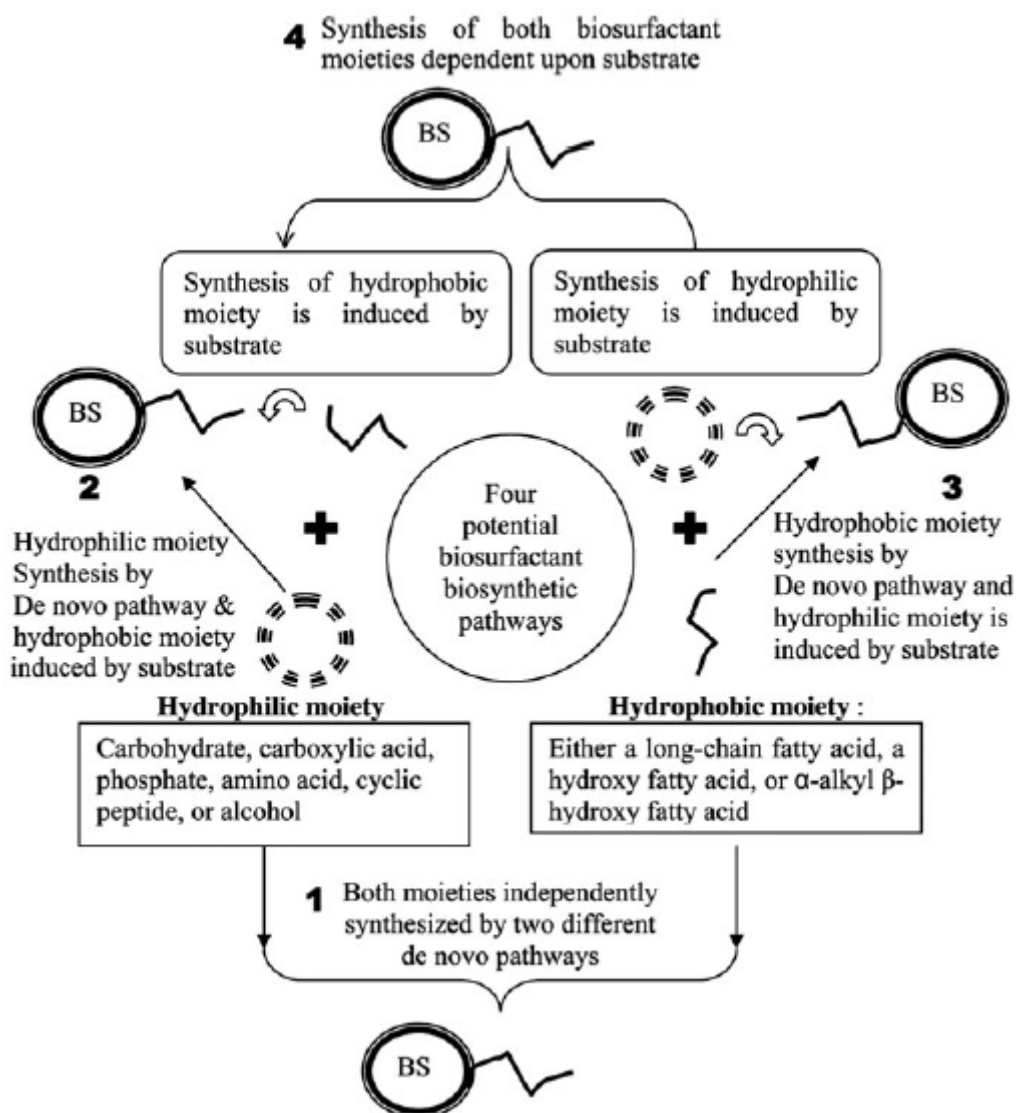
The glycolytic pathway, which is responsible for hydrophilic portion formation, and the lipogenic pathway, which is responsible for lipid formation, are essentially supplied by microbial metabolism when carbohydrates are used as the sole energy source (Figure 4).

Glucose is degraded to form intermediates of the glycolytic pathway, including glucose 6-phosphate, which is a major precursor for the synthesis of carbohydrates in the hydrophilic portion of the surfactant. In the synthesis of the hydrophobic portion, glucose is oxidized to pyruvate and later converted to acetyl-coenzyme A. This last one reacts with an intermediate of the Krebs cycle (oxaloacetate), producing malonyl-CoA and later in fatty acid, which will be used in the synthesis of lipids (LOURENÇO 2017; SANTOS *et al.*, 2016).

Figure 4 - Metabolic routes to synthesis of biosurfactant precursors with use of carbohydrates as substrate. Key enzymes for control of carbon flow: (A) phosphofructokinase; (B) pyruvate kinase; (C) isocitrate dehydrogenase.
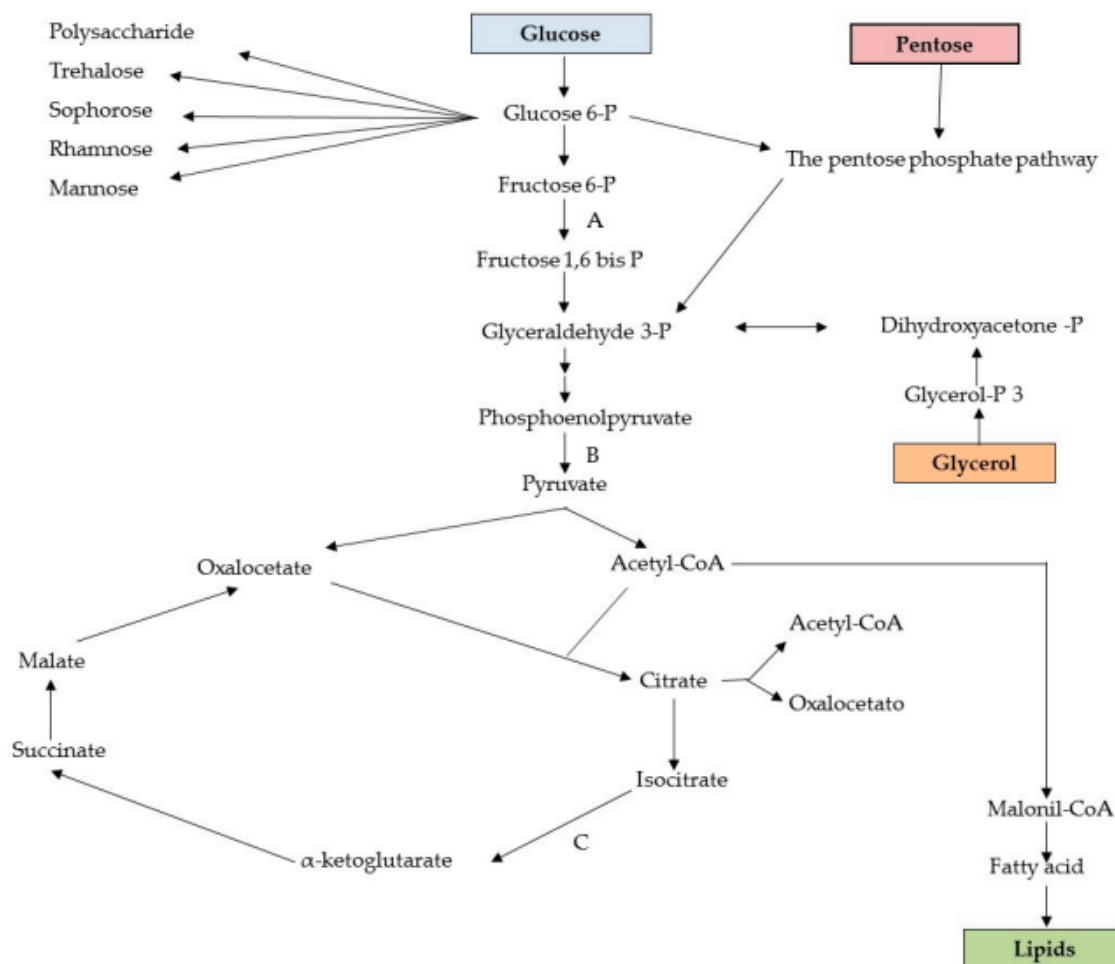
Source: Santos *et al*. (2016).

2.2.1 Classification of Biosurfactants

Biosurfactants are divided according to their chemical nature, in which they may be low or high molecular weight molecules, or according to their microbial origin. Five biosurfactant classes are found in the literature, which are: 1) Glycolipids, 2) lipopeptides and lipoproteins, 3) fatty acids, neutral lipids, and phospholipids, 4) polymeric and 5) particulates. Major classes of low molecular weight surfactants include glycolipids, lipopeptides, and phospholipids, while those of high molecular weight include polymeric and particulate surfactants (DESAI; BANAT, 1997).

Glycolipids are carbohydrates combined with a long chain of aliphatic acids or hydroxyaliphatic acids. It is noteworthy that those produced by *Pseudomonas* strains have received great attention due to their remarkable emulsifying and surfactant properties (DEEPIKA *et al*., 2016). Among glycolipids, the best known are rhamnolipids, sophorolipids, and trehalose lipids (DESAI; BANAT, 1997).

Lipopeptide and lipoprotein surfactants are well known for their antibiotic activities, in which the surfactants produced by *Bacillus sp*. are the best characterized. Such surfactants have fatty acid-associated peptides. The peptide portion of the molecule may be neutral or anionic and the amino acids are generally arranged in a cyclic structure. According to Fonseca *et al.* (2007), the lipopeptides produced by *Bacillus subtilis* are particularly interesting because they have an intense surface activity. Likewise, this biomolecule is effective in reducing surface tension to 27 mN m^{-1} even at low biosurfactant concentrations (0.05 g L^{-1}).

Some examples of surfactants produced by *Bacillus sp*. are surfactin, iturine, fengicin, lichenisine, mycosubtilisine and bacillomycin (KIM *et al*., 2010). Natural surfactins are a mixture of isoforms A, B, C and D (Figure 3), which have various physiological properties, being classified according to the differences in their amino acid sequences. They also possess at least eight depsipeptides with the number of carbon atoms between 13 and 16 as part of the ring system (SHALIGRAM *et al*., 2010). Surfactin is a high value-added compound costing R$ 7137.00 per 50 mg (Sigma-Aldrich).

Figure 3 - Structure of surfactin A, B, C, D (a), and lipohexapeptide (b).

a)

```
        L-Asp ——— D-Leu ——— L-Leu ——— O
       /                                |
  L-Val                               HC—R
       \                                |
                                       CH₂
        D-Leu ——— L-Leu ——— L-Glu ——— C=O
```

$$\text{surfactin A} \quad R = -(CH_2)_7 - \underset{\underset{CH_3}{|}}{\overset{\overset{CH_3}{|}}{CH}}$$

$$\text{surfactin B} \quad R = -(CH_2)_8 - \underset{\underset{CH_3}{|}}{\overset{\overset{CH_3}{|}}{CH}}$$

$$\text{surfactin C} \quad R = -(CH_2)_9 - \underset{\underset{CH_3}{|}}{\overset{\overset{CH_3}{|}}{CH}}$$

$$\text{surfactin D} \quad R = -(CH_2)_{10} - \underset{\underset{CH_3}{|}}{\overset{\overset{CH_3}{|}}{CH}}$$

b)

```
        L-Asp ——— D-Leu ——— L-Leu ——— O          CH₃
       /                                |          |
  L-Val                              HC(CH₂)₉ — CH
       \                                |          |
                                       CH₂        CH₃
        D-Leu ——————————— L-Glu ——— C=O
```
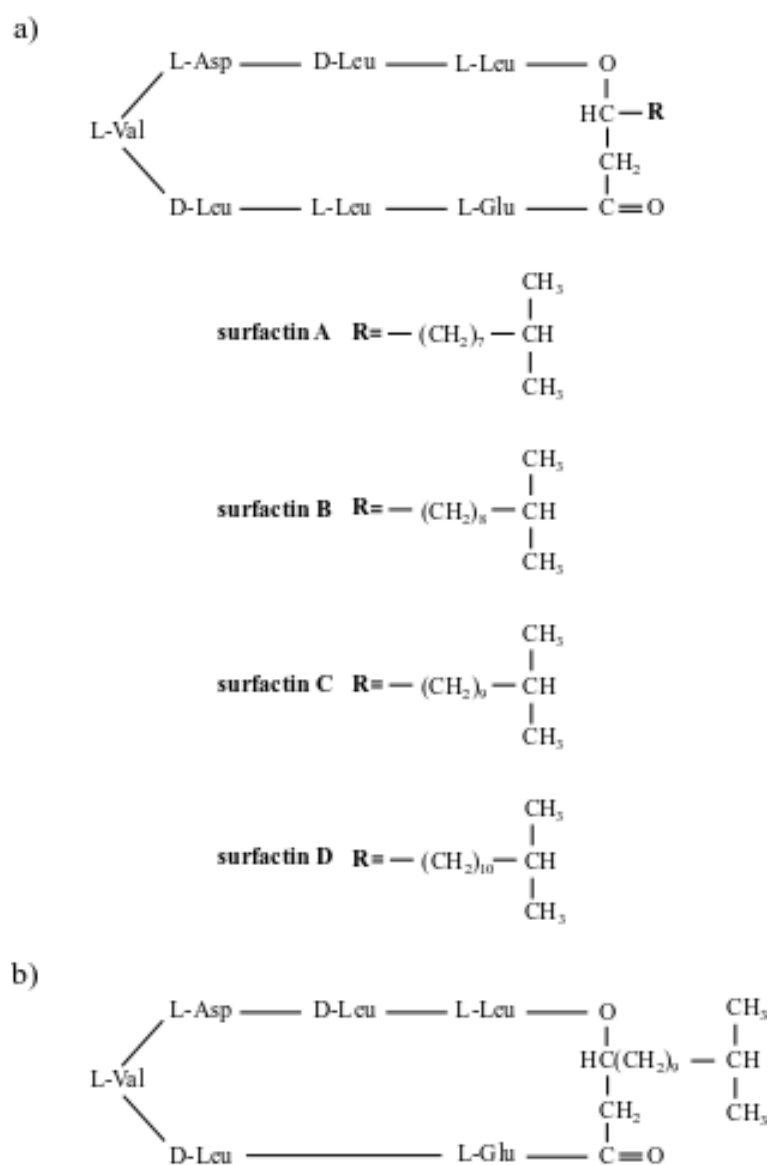
Source: Shaligram *et al*. (2010).

Many bacteria and yeast produce large amounts of phospholipid-like fatty acids and surfactants during growth in the presence of n-alkanes (DESAI; BANAT, 1997). As an example, *Acinetobacter sp.*, which produces double chain vesicles with small polar head areas called phosphatidylethanolamine, can be cited. This substance is capable of promoting the formation of alkane microemulsions in water (KAPPELI; FINNERTY, 1979).

Neutral lipids comprise fatty acids, triacylglycerols and mycolic acids and most of these lipids have some degree of surfactant activity. Polymeric biosurfactants are fatty acids covalently linked to polysaccharides. Among the most studied polymeric biosurfactants are

emulsan, liposan and manoprotein. In emulsan, in which *Acinetobacter* is the genus best known as a producer, fatty acids are linked to a heteropolysaccharide backbone (NITSCHKE; PASTORE, 2002).

Particulate biosurfactants are microbial cells and vesicles that exhibit high surface hydrophobicity. Certain microorganisms are considered properly biosurfactants, in which we can mention species of cyanobacteria and some pathogens such as *S. aureus* and *Serratia sp*. Bacteria of the genus *Acinetobacter sp.* produce extracellular vesicles that exhibit alkane uptake activities into the cell (NITSCHKE; PASTORE, 2002). Table 1 shows examples of biosurfactant types and their respective producing microorganism.

Table 1 - Type of biosurfactant and producer microorganism

Biosurfactant	Microorganism	Reference
Glycolipids		
Rhamnolipids	*Pseudomonas putida*	Johann *et al*. (2016)
	Pseudomonas aeruginosa	Moya Ramírez *et al*. (2016)
Sophorolipid	*Starmerella bombicola*	Maddikeri *et al*. (2015)
	Candida bombicola	Davery *et al*. (2010)
Trealolipids	*Rhodococcus actinobacteria*	Kuyukina *et al*. (2015)
	Rhodococcus erythropolis	Zaragoza *et al*. (2013)
Lipopeptides and lipoproteins		
Lipid-peptide	*Staphylococcus xylosus*	Keskin *et al*. (2015)
Surfactin	*Bacillus subtilis*	Maass *et al*. (2015)
Viscosine	*Pseudomonas fluorescens*	Portet-koltalo *et al*. (2013)
Neutral lipids and phospholipids		
Neutral lipids	*Bacillus subtilis*	Cooper and Goldenberg (1987)
Phospholipids	*Staphylococcus hominis*	Rajeswari *et al*. (2016)
Polymeric surfactants		
Emulsan	*Acinetobacter calcoaceticus*	Kim *et al*. (2000)
Liposan	*Yarrowia lipolytica*	Amaral *et al*. (2006)

Source: author.

2.2.2 Factors affecting biosurfactant production

Biosurfactants are produced as a mixture of homologues, in which the composition depends on the strain and culture age. Factors such as pH, temperature and process conduction are important in the quality and quantity of biosurfactant produced. In addition, other factors, such as the nature of the carbon and nitrogen sources used as well as the presence of phosphorus, iron, manganese, and magnesium in the reaction medium, also influence the process (BANAT, 1995).

Carbon and nitrogen play an important role in lipopeptide production since the C/N ratio has been reported in the literature as a factor affecting surfactin yield (DAVIS *et al*., 1999). According to Fonseca *et al*. (2007), the literature usually adopts the C/N ratio and agitation around 3 and 150 rpm, respectively, for processes that seek surfactin production. For Davis *et al*. (1999), a high amount of surfactin (439 mg L^{-1}) was obtained by *Bacillus subtilis* ATCC 21332 and culture medium with C/N ratio of 11.50. The experiments were conducted in medium containing glucose (40 g L^{-1}) and ammonium nitrate (4 g L^{-1}).

The initial pH value of the culture medium may have a relevant effect on biosurfactant production, and pH control during the process is an essential criterion to maintain production under optimal conditions. Generally, neutral pH favors lipopeptide production and, for *Bacillus subtilis*, the ideal range is between 6.5 and 7.0 (INÉS; DHOUHA, 2015).

Yeh *et al*. (2005) found that high agitation positively affected surfactin production using *B. subtilis* ATCC 21332 and glucose (40 g L^{-1}) as carbon source. However, agitation above 250 rpm caused foam formation and decreased surfactin yield. Fonseca et al. (2007) evaluated the influence of nitrogen sources on *B. subtilis* surfactin production using sucrose (10 g L^{-1}) as a carbon source at different agitations (50, 150 and 250 rpm). The authors found that the 250 rpm agitation was the most favorable for the surfactant obtaining in shake flasks experiments.

Temperature is a relevant parameter in the production of biosurfactants, in which the ideal temperature range most used for lipopeptide production is between 30 and 45°C (AMANI *et al*. 2010; JOSHI *et al*. 2008; MAASS *et al*. 2015). Ohno *et al*. (1995) found that the maximum production of surfactin occurred in *B. subtilis* RB14 at 37 °C. Joshi *et al*. (2008) produced surfactin at 45 °C by *B. subtilis* R1 and molasses or cheese whey as a source of nutrition. At the end of cultivation, the authors found surface tension values close to 29.3 mN m^{-1}.

According to Huang *et al*. (2015), the presence of metal ions (Mn^{2+}, Cu^{2+}, Co^{2+}, Mg^{2+}, Ni^{2+}, Fe^{2+}, Ca^{2+}, Al^{3+}, and Zn^{2+}) in the culture medium for surfactin production has been

investigated, however, Mn^{2+} is the ion that has the greatest effect on increasing surfactin productivity, since it affects nitrogen utilization and K^+ uptake as well as other biochemical functions. On the other hand, potassium ion stimulates surfactin secretion and therefore may increase total surfactin production as well as *B. subtilis* mobility (WEI *et al*., 2007). Mg^{2+} plays an important role on surfactin synthesis, since the active site of *B. subtilis* Sfp protein that activates the peptidyl carrier protein domains of surfactin synthetase accommodates a magnesium ion as a cofactor (WEI *et al*., 2007).

For Cooper *et al*. (1981), who evaluated surfactin production by *B. subtilis* ATCC 21332 (in 4% glucose mineral medium), only $MnSO_4$, $FeSO_4$ and $Fe_2(SO_4)_3$ caused a significant increase in surfactin production. The other compounds such as $MgSO_4$, $CaCl_2$, Na_2HPO_4, KH_2PO_4, $NaNO_3$ and $ZrOCl_2$ had no effect on production. In addition, the authors found that $ZnSO_4$ suppressed microorganism growth. The addition of iron caused an increase in biomass and the addition of manganese sulfate caused a much larger increase in surfactin production when compared to iron salts, without an increase in biomass. In addition, only a small amount of $MnSO_4$ was required to achieve the maximum effect. The authors reported that a concentration of 10^{-6} M manganese is required to obtain a high yield of *B. subtilis* surfactin.

2.3 THE POTENTIAL USE OF A BREWERY WASTE AS SUBSTRATE IN BIOPROCESSES

Beer production has a great worldwide representation both in industrial scale and its production in handcrafted form. According to Dias and Falconi (2018), Brazil is the third largest manufacturer country in the world, with 14 million kl $year^{-1}$ produced, being only behind of China (39.788 kl $year^{-1}$) and the United States (21.775 kl $year^{-1}$). Thus, the waste generated during the beer production deserves attention, seeking ways to reuse it in the manufacture of new products and technologies, and therefore, valorizing the brewing by-products.

During the beer production, the malt starch is ground (mashing stage) and converted into fermentable (maltose and maltotriose) and non-fermentable (dextrins) sugars. In this same step, proteins are partially degraded into polypeptides and amino acids. As a result of the mashing stage, the wort is generated together with spent grains. In the next step, the wort is filtered and boiled, followed by the addition of hops, in which the bitter and aromatic components of the hops give taste and foam stability (FĂRCAŞ *et al*., 2017).

After the boiling stage, the liquid is separated from spent hops and Trub, for further processing. Trub is a product of the wort boiling process (FĂRCAŞ *et al*., 2017). This waste is mainly composed of coagulated proteins (between 50 and 70% of the dry mass) and the presence of reducing sugars (20%), which are related to the high carbon concentration contained in this residue. In general, between 0.2 to 0.4 kg of wet Trub (80 to 90% humidity) is formed for each hectoliter of beer produced (dos SANTOS MATHIAS *et al*., 2015).

In the fermentation stage, fermentable sugars are metabolized by yeast cells, generating ethanol and carbon dioxide. At the end of this stage, most of the cells are collected as spent yeast (FĂRCAŞ *et al*., 2017). Figure 5 illustrates the main waste generated (spent grain, spent hops and Trub and spent yeast) during the brewing process.

Since Trub has nitrogen source and also a relevant percentage of reducing sugars due to the wort loss during its removal, this residue shows potential for use in industrial bioprocesses. In addition, the low C/N ration (6.3) is similar to C/N ratio of microbial cell composition, which may increase the cell multiplication (dos SANTOS MATHIAS *et al*., 2015).

Figure 5 - Schematic representation of the brewing process and the main by-products.
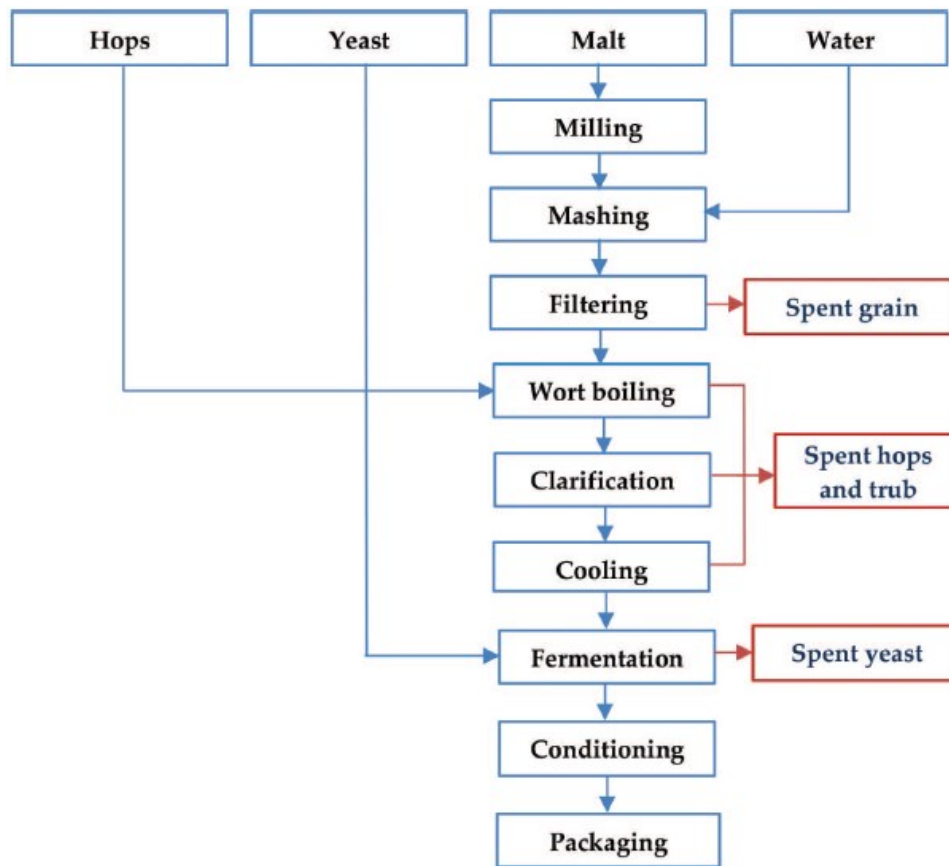
Source: Fărcaş et al. (2017).

2.4 ANTIMICROBIAL AND ANTIBIOFILM ACTIVITIES OF BIOSURFACTANTS

Control of pathogenic microorganisms is essential human health maintenance, since they are responsible for several infectious diseases caused by exposure to contaminated environments, surfaces and food. This has been exacerbated during the past few decades by the indiscriminate use of antibiotics, which has led to the selection of multi-resistant microbial strains to traditional antibiotics, reducing the ability to treat diseases and enhancing the search for new compounds to improve the management of bacterial infections (NIKAIDO *et al*., 2010; PRESTINACI *et al*., 2015).

Bacteria colonization is often influenced by *Quorum Sensing* (QS), a mechanism, responsible for their ability to communicate with each other and to behave as a population. This phenomenon is fundamental for biofilm formation, in which the bacteria remains protected from environmental risks in a self-produced extracellular matrix, with high tolerance to

chemical and physical treatments than planktonic cells forms (DONLAN 2001; JANEK *et al*., 2012; SOLANO *et al*., 2014; DIAZ DE RIENZO *et al*., 2016).

The presence of biofilms may cause serious problems in the field of medicine and food industry, since the bacteria are able to exist on surfaces of hospital equipment and food processing systems. In hospitals, biofilms may result infections in patients with internal medical devices, such as urinary, endotracheal, intravenous, and other types of catheters and implants inserted into over 25% of patients during hospitalization (HAZAN *et al*., 2006; FRACCHIA *et al*., 2010). In food industry, biofilms present on equipment or any related devices in direct contact with food will become a source of contamination, representing a risk to consumers through the transmission of diseases, as well as causing economic losses (ARAÚJO *et al*., 2016).

In this regard, the search for novel natural compounds with biocidal activity against pathogenic microorganisms is an urgent requirement. Due to their low toxicity, biosurfactants have potential applications in food, detergent industry and biomedicine owing to their antibacterial properties (CAMPOS *et al*., 2014). They can also interfere in biofilm development and communication between the cells and may cause rupture of membranes, causing cell lysis, and disruption of the surface properties affecting the adherence of the microorganisms (BANAT *et al*., 2014).

The antimicrobial action of biosurfactants occurs due to the interaction of these compounds with the short chain phospholipids found in the cell plasma membrane (Figure 6), causing an increase in permeability through solubilization, rupture and denaturation (BANAT *et al*., 2014).

Figure 6 - Antimicrobial activity of biosurfactant acting on microbial cell membrane.
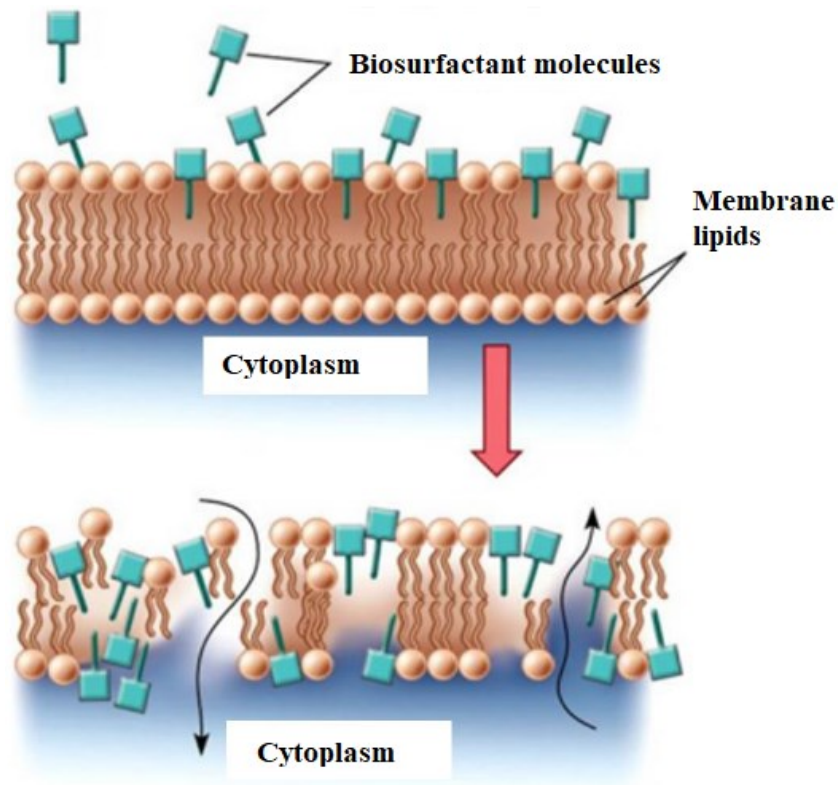
Source: Marcelino (2016).

Fernandes *et al*. (2007) evaluated the antimicrobial activity of biosurfactants synthesized by *B. subtilis* R14 in defined medium containing glucose (40 g L^{-1}). The experiments against multidrug-resistant bacteria were carried out using the agar diffusion method. The authors observed the antimicrobial activity of surfactin against *Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa*, in which the mean halo diameter was 14.2, 13.8 and 9.8, respectively.

In a similar investigation, Isa *et al*. (2017) reported antimicrobial activity of surfactin produced by *B. subtilis* MSH1 in mineral medium containing 4% (w/v) glucose. For *Shigella dysenteriae,* which is a highly contagious pathogenic bacterium responsible for epidemic bacterial dysentery, the inhibition zone diameter was 2.0 ± 0.2 mm at 50 mg L^{-1} of surfactin and increased up to 13.0 ± 0.2 mm with 250 mg L^{-1} of surfactin. When *S. aureus* was treated with biosurfactant, inhibition zones were 2.0 ± 0.17 mm at 50 mg L^{-1} and increased up to 10.5 ± 0.1 mm when treated with 250 mg L^{-1}.

Previous studies have shown the ability of different types of biosurfactants to disrupt and prevent biofilm formation. Elshikh *et al*. (2017) reported treatment of oral pathogens with rhamnolipid showed a reduction of 3–4 log of bacterial viability. *Streptococcus sanguinis* showed 90% of biofilm inhibition when co-incubated with biosurfactant and 65% of biofilm disruption after treatment. In a similar investigation, Dıaz de Rienzo *et al*. (2015) reported the antimicrobial and biofilm disruption of *Cupriavidus necator* ATCC 17699 and *Bacillus subtilis* BBK006 using sophorolipids (5% v/v).

Lipopeptides are the mostly widely known biosurfactants with antimicrobial activity, where surfactin produced by *B. circulans* is the most prominent antimicrobial lipopeptide (DAS *et al*., 2008). The mixture of lipopeptides (surfactin, iturin and fengycin) from *B. subtilis* showed significant anti-adhesive and antibiofilm activities on uropathogenic bacteria (MORYL *et al*., 2015). The effect of surfactin on adhesion and biofilm formation was evaluated by Araújo *et al*. (2016), in which the biosurfactant significantly reduced adhesion of *Pseudomonas fluorescens* ATCC 13525 on polystyrene surfaces (54% of inhibition) and a biofilm formation (73%) on stainless steel surfaces.

2.5 STATE OF ART

Biosurfactants are compounds of wide industrial application and their production is limited due to the high process cost. Thus, the search for renewable production alternatives has been the focus of many studies in the literature. Since the craft beer market is on the rise in Brazil, this work aimed at the use of brewery waste (Trub) as a carbon source since it presents a favorable composition for bioprocesses. Moreover, no work using Trub in surfactin production by *Bacillus subtilis* has been reported in the literature. In this study, we intend to contribute to research aimed at the production of biosurfactant to maximize production through the optimization of nutritional process parameters.

3 MATERIALS AND METHODS

This chapter presents the materials and methods used in the development of this research. The study was divided into two parts: 1 - optimized biosurfactant production and *scale-up* at bench-scale fermenter, and 2 - application for the biosurfactant produced. The experiments were performed at the Laboratory of Mass Transfer (LABMASSA) of the Federal University of Santa Catarina (UFSC) and the Microbiology Laboratory of Ulster University.

3.1 MICROORGANISM: REACTIVATION AND CULTURE STOCK

Bacillus subtilis ATCC 6051 was purchased from the Andre Tosello Foundation culture collection. The lyophilized strain was rehydrated with 0.2 mL of sterile distilled water and then kept at rest for 15 minutes. The cell suspension was transferred to a test tube containing 5 mL of Nutrient Broth (NB) and incubated for 24 h on an orbital shaker at 30 °C and 160 rpm. After incubation, the bacterial suspension was aseptically redistributed into cryogenic tubes containing glycerol (20% v/v) and maintenance medium and then stored at -80 °C. This represents the first generation of microorganisms.

Stock culture was obtained by transferring the contents of a first generation cryogenic tube to a 125 mL shake flask containing 50 mL of NB. The tube was incubated at 30 °C and 160 rpm for 24 h. Similar to the procedure described above, the culture was cryopreserved in different tubes and stored at -20 °C. Whenever required, a stock culture tube was thawed and used for inoculum preparation. Thus, after using the entire stock culture, a new cryogenic tube of the first generation of microorganisms was reactivated, according to the above procedures, thus reducing the risks of strain mutation and contamination during the process.

3.2 BREWERY WASTE

The carbon source used in the process was brewery waste (Trub) obtained after the must cooking step, which was kindly provided by the Kairós Craft Beer Factory, located in Florianópolis - SC. The Trub (pH 5.73) was characterized by elemental analysis (C), Enzymatic-Colorimetric Method (N), and Atomic Absorption Spectrometry (Fe, K, Mn and Mg). The results are shown in Table 2.

Table 2 - Composition of Trub.

Component	Concentration (mg L^{-1})	Method
Fe	< 0.14	Atomic Absorption Spectrophotometer (AA 6300 – SHIMADZU)
Mn	0.3772	
K	426.72	
Mg	74.53	
TOC*	31.50	Total Organic Carbon Analyzer (TOC – SHIMADZU)
NT*	3.45	Enzymatic-Colorimetric (Kit Gold Analisa Diagnostics)

* In g L^{-1}. NT: total nitrogen; TOC: total organic carbon.

3.3 BIOSURFACTANT PRODUCTION BY *Bacillus subtilis* ATCC 6051 IN SUBMERGED CULTIVATION

3.3.1 Optimization of culture medium and process parameters in biosurfactant production in shake flasks

In this work, different factors were evaluated to identify which of them had significant effects on the biosurfactant production. The batches were performed in submerged culture in 250 mL shake flasks containing 100 mL of production medium (PM), based on Maass *et al*. (2015), composed by (g L^{-1}): CaCl$_2$ (0.1), KH$_2$PO$_4$ (1.0), MgSO$_4$.7H$_2$O (0.5) and NaCl (0.1). In addition to these compounds, Trub, peptone and yeast extract were added at different concentrations, according to the Full Factorial Design - FFD (2^4) presented in Table 3.

Table 3 - Values of independent variables for a Full Factorial Design (2^4) in a biosurfactant production by *B. subtilis* ATCC 6051.

Independent variables	Levels		
	(-1)	0	(+1)
Trub (TB) (% v/v)	2	6	10
Yeast Extract (YE) (g L^{-1})	4.0	5.0	6.0
Peptona (PB) (g L^{-1})	0.6	0.7	0.8
Agitation (AG) (rpm)	100	150	200

The inoculum was prepared by adding 2 mL of frozen stocks into 50 mL of NB, and subsequently incubated at 30 °C and 160 rpm. The inoculum was standardized by adjusting its absorbance at 600 nm to 0.85, and it was inoculated in the production medium at a concentration of 5% (v/v). Temperature and initial pH were maintained at 30 °C and 7.0, respectively, according to the conditions favorable to the growth of the microorganism indicated by the supplier. All cultures were performed under aseptic conditions, and the materials and culture medium were autoclaved at 121 °C for 20 min.

The design experiments were performed in triplicate with six central points, totaling 38 runs. Assays between 20 and 35 represent replicates. Cultivation lasted 72 h, and at the end of the batches, cell concentration and pH were measured. The experimental matrix of the FFD performed is presented in Table 4.

Table 4 - Experimental matrix of Full Factorial Design (2^4) for biosurfactant production by *B. subtilis* ATCC 6051. Values are represented in coded and real.

Run	Trub (TB)		Yeast Extract (YE)		Peptone (PB)		Agitation (AG)	
	(%v/v)	Level	(g L^{-1})	Level	(g L^{-1})	Level	(rpm)	Level
1	2.00	(-1)	4.00	(-1)	0.60	(-1)	100	(-1)
2	10.00	(+1)	4.00	(-1)	0.60	(-1)	100	(-1)
3	2.00	(-1)	6.00	(+1)	0.60	(-1)	100	(-1)
4	10.00	(+1)	6.00	(+1)	0.60	(-1)	100	(-1)
5	2.00	(-1)	4.00	(-1)	0.80	(+1)	100	(-1)
6	10.00	(+1)	4.00	(-1)	0.80	(+1)	100	(-1)

Table 4 (Continued) - Experimental Matrix of Full Factorial Design (2^4) for biosurfactant production by *B. subtilis* ATCC 6051. Values are represented in coded and real.

Run	Trub (TB) (%v/v)	Level	Yeast Extract (YE) (g L^{-1})	Level	Peptone (PB) (g L^{-1})	Level	Agitation (AG) (rpm)	Level
7	2.00	(-1)	6.00	(+1)	0.80	(+1)	100	(-1)
8	10.00	(+1)	6.00	(+1)	0.80	(+1)	100	(-1)
9	2.00	(-1)	4.00	(-1)	0.60	(-1)	200	(+1)
10	1000	(+1)	4.00	(-1)	0.60	(-1)	200	(+1)
11	2.00	(-1)	6.00	(+1)	0.60	(-1)	200	(+1)
12	10.00	(+1)	6.00	(+1)	0.60	(-1)	200	(+1)
13	2.00	(-1)	4.00	(-1)	0.80	(+1)	200	(+1)
14	10.00	(+1)	4.00	(-1)	0.80	(+1)	200	(+1)
15	2.00	(-1)	6.00	(+1)	0.80	(+1)	200	(+1)
16	10.00	(+1)	6.00	(+1)	0.80	(+1)	200	(+1)
17	6.00	(0)	5.00	(0)	0.70	(0)	150	(0)
18	6.00	(0)	5.00	(0)	0.70	(0)	150	(0)
19	6.00	(0)	5.00	(0)	0.70	(0)	150	(0)
20	2.00	(-1)	4.00	(-1)	0.60	(-1)	100	(0)
21	10.00	(+1)	4.00	(-1)	0.60	(-1)	100	(0)
22	2.00	(-1)	6.00	(+1)	0.60	(-1)	100	(0)
23	10.00	(+1)	6.00	(+1)	0.60	(-1)	100	(-1)
24	2.00	(-1)	4.00	(-1)	0.80	(+1)	100	(-1)
25	10.00	(+1)	4.00	(-1)	0.80	(+1)	100	(-1)
26	2.00	(-1)	6.00	(+1)	0.80	(+1)	100	(-1)
27	10.00	(+1)	6.00	(+1)	0.80	(+1)	100	(-1)
28	2.00	(-1)	4.00	(-1)	0.60	(-1)	200	(+1)
29	10.00	(+1)	4.00	(-1)	0.60	(-1)	200	(+1)
30	2.00	(-1)	6.00	(+1)	0.60	(-1)	200	(+1)
31	10.00	(+1)	6.00	(+1)	0.60	(-1)	200	(+1)
32	2.00	(-1)	4.00	(-1)	0.80	(+1)	200	(+1)
33	10.00	(+1)	4.00	(-1)	0.80	(+1)	200	(+1)
34	2.00	(-1)	6.00	(+1)	0.80	(+1)	200	(+1)
35	10.00	(+1)	6.00	(+1)	0.80	(+1)	200	(+1)
36	6.00	(0)	5.00	(0)	0.70	(0)	150	(0)
37	6.00	(0)	5.00	(0)	0.70	(0)	150	(0)
38	6.00	(0)	5.00	(0)	0.70	(0)	150	(0)

A new FFD (2^2) was performed, having yeast extract (YE) and bacteriological peptone (PB) as independent variables, and surface tension (ST) as the response variable.

The selection of independent variables as well as the range of their levels were based on the results obtained in FDD (2^4) previously presented. Table 5 shows the factors and their respective levels evaluated.

Table 5 - Values of independent variables for a Full Factorial Design (2^2) in a biosurfactant production by *B. subtilis* ATCC 6051.

Independent Variables	Levels		
	(-1)	(0)	(+1)
Yeast extract (YE) (g L^{-1})	2.0	7.0	12.0
Peptone (PB) (g L^{-1})	0.4	0.9	1.4

The assays were performed in duplicates with two central points. Shake flasks containing PM medium plus Trub (2% v/v), and also containing YE and PB according to FDD (2^2), were incubated at 30 °C and 200 rpm. Similar to FFD (2^4) presented earlier the process lasted 72 hours. Surface tension and pH measurements were taken. The experimental design matrix (2^2) is presented in Table 6.

Table 6 - Experimental matrix of Full Factorial Design (2^2) for biosurfactant production by *B. subtilis* ATCC 6051. Values are represented in coded and real.

Run	Yeast Extract (YE)		Peptone (PB)	
	(g L^{-1})	Level	(g L^{-1})	level
1	2.00	(-1)	0.40	(-1)
2	12.00	(+1)	0.40	(-1)
3	2.00	(-1)	1.40	(+1)
4	12.00	(+1)	1.40	(+1)
5	7.00	(0)	0.90	(0)
6	2.0	(-1)	0.40	(-1)
7	12.00	(+1)	0.40	(-1)
8	2.00	(-1)	1.40	(+1)
9	12.00	(+1)	1.40	(+1)
10	7.00	(0)	0.90	(0)

After the finalization and analysis of the experimental design matrix assays, a time-course of process behavior was evaluated in triplicate under the best conditions determined through the results obtained by the FFD.

3.3.2 Effect of supplementation of culture medium with metals solutions in biosurfactant production in shake flasks

The influence of metals (micronutrients) on the biosurfactant production was evaluated through the addition of iron, potassium, magnesium and manganese solutions to the culture medium. Three different concentrations were evaluated for each metal compound, as presented in Table 7. The concentrations were defined based on the researches done by Gudiña *et al*. (2015), Wei *et al*. (2007), Huang *et al*. (2015), Cooper *et al*. (1981), Wei *et al*. (2002) and Willenbacher *et al*. (2015).

Table 7- Different concentrations of metal solutions used in the production of biosurfactant by *B. subtilis* ATCC 6051.

Solution	Concentration (mM)		
$FeSO_4$	0.008	1.2	4.0
KH_2PO_4	5.0	10.0	30.0
$MnSO_4$	0.01	0.1	0.3
$MgSO_4$	0.04	0.6	2.4

3.3.3 Production of biosurfactant in stirred tank bioreactor: study of aeration and agitation effects.

Biosurfactant production was performed in a 5 L stirred tank bioreactor (BIO-TEC-FLEX, Tecnal) equipped with pH and pO_2 electrodes (Mettler-Toledo International Inc.) and 2 L of working volume. HCl and NaOH solutions, both at 1 M, were automatically added to the medium to ensure that the pH was maintained at 7.0. The inoculum of *B. subtilis*, which was previously adjusted (OD_{600} 0.85) was added to the culture medium (10% v/v). A FFD (2^2) was performed with aeration (AR) and Agitation (AG) as independent variables, and surfactin concentration (SF) as the response variable. The levels were selected based on studies by Sen (1997) and Amani (2010). The design is shown in table 8.

Table 8 - Values of independent variables for a Full Factorial Design (2^2) in a biosurfactant production by *B. subtilis* ATCC 6051 in stirred tank bioreactor.

Independent variables	Levels		
	(-1)	0	(+1)
Aeration (AR) (vvm)	0.5	1.0	1.5
Agitation (AG) (rpm)	150	225	300

Each batch lasted 24 h and a sterile foam collector was installed in the reactor, in which the foam was channelled through an upper reactor outlet. Samples were collected regularly to determine cell concentration, carbon source concentration and surface tension. The experimental design matrix (2^2) is presented in Table 9.

Table 9 - Experimental matrix of Full Factorial Design (2^2) for biosurfactant production by *B. subtilis* ATCC 6051 in stirred tank bioreactor. Values are represented in coded and real.

Run	Aeration (AR)		Agitation (AG)	
	(vvm)	Level	(rpm)	Level
1	0.5	(-1)	150	(-1)
2	1.5	(+1)	150	(-1)
3	0.5	(-1)	300	(+1)
4	1.5	(+1)	300	(+1)
5	1.0	(0)	225	(0)
6	1.0	(0)	225	(0)

The volumetric coefficient of oxygen transfer (K_La) was determined using a dynamic method that consists of the system supply being paralyzed and the agitation almost completely reduced over a short period of time (LOURENÇO, 2017). Thus, a mass balance for oxygen was performed in the ascending period of the dynamic method curve, which is represented by Equation 1:

$$\ln\left(\frac{C_i - C}{C_i - C_0}\right) = -K_La(t - t_0) \qquad (1)$$

Where Ci, Co e C are the dissolved oxygen concentration prior to stopping aeration and agitation at time t_0, and t is the time when aeration returns.

3.4 EXTRACTION OF BIOSURFACTANT

The biosurfactant was extracted by centrifuging (9000 rpm, 20 min) the culture broth in order to remove suspended solids (cells and solid particles from Trub). Subsequently, the pH of the supernatant was adjusted to 2.0 by adding HCl (4.0 M) and left overnight under refrigeration (4 °C) for precipitate formation. The precipitated biosurfactant was centrifuged (9000 rpm, 20 min), washed twice with acidified water (pH 2.0) and resuspended in Milli-Q® water (Millipore, USA). The pH of the solution was adjusted to 7.0, lyophilized, weighed and stored at -18 °C (MAASS et al., 2015).

3.5 STRUCTURAL CHARACTERIZATION OF BIOSURFACTANT

The biosurfactant was chemically characterized by Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), using a PerSeptive Biosystems Voyager-DE Biospectrometer (Hertfordshire, UK) equipped with a 1 m time-of-flight tube. The system utilized a pulsed nitrogen laser set at 337 nm towards the densest area of the sample/matrix spot. The accelerating voltage was maintained at 20.000V, the grid voltage and guide wire voltages were set at 93% and 0.05% respectively of the accelerating voltage. A solution of α-cyano-4-hydroxycinnamic acid (CHCA) matrix (Sigma Aldrich, UK) with a concentration of 10 mg mL^{-1} was prepared in 80% acetonitrile, 20% water with 0.1% trifluroacetic acid. 10 µL aliquot of sample was mixed with 10 µL of matrix and, subsequently, the samples were spotted on MALDI plate for analysis (CHEBBI *et al.*, 2017).

3.6 SURFACE TENSION AND CRITICAL MICELLE CONCENTRATION (CMC)

The surface tension of the cell-free supernatant was measured using a digital tensiometer (KSV, Sigma 702, Finland) by the Wilhelmy platinum plate method. All measurements were performed in triplicate at 25 ± 2 °C. During the measurement procedure, the plate was washed with ethanol and distilled water, and then flambe.

The determination of the CMC of the partially purified biosurfactant was determined by surface tension measurements of successive dilutions of aqueous biosurfactant solution,

according to the methodology proposed by Sheppard and Mulligan (1987). Subsequently, a graph of surface tension versus biosurfactant concentration was constructed, in which the CMC corresponds to the central point of inflection of the curve.

3.7 BIOSURFACTANT STABILITY

Biosurfactant stability was evaluated by the influence of different temperature and pH conditions on the ability of surfactin to reduce surface tension and in its emulsifying activity.

To evaluate thermal stability, biosurfactant solutions (30 mg L^{-1}) at pH 7.0 were incubated for 24 h at different temperature conditions (25 °C, 45 °C, 65 °C, 85 °C and 100 °C). The effect of pH was studied by preparing biosurfactant solutions (30 mg L^{-1}) with different pH values (between 2.0 and 10.0). The solutions were incubated at 25 °C for 24 h. In parallel assay, 2 mL of all samples prepared as previously described were added to test tubes containing 2 mL of n-dodecane (Sigma-Aldrich). The tubes were vortexed for 2 min and incubated according to their respective predetermined condition (MAASS *et al*., 2015).

The emulsification index (E$_{24}$) was calculated by the height of the emulsified layer divided by the total height of the liquid column. At the end of experiments, surface tension and emulsification index data were obtained (MAASS *et al*., 2015).

3.8 DETERMINATION OF BIOSURFACTANT CONCENTRATION

The concentration of surfactin was determined by a calibration curve constructed using the surface tension measurement (Wilhelmy platinum plate method) of samples containing different concentrations of standard surfactin (Sigma-Aldrich, 98%) (DEBON, 2015). The surface tension method is based on the definition that the surface activity is dependent on the mass concentration of the biosurfactant, becoming an approximate measure of the biosurfactant concentration (RASHEDI *et al*., 2006). Santos da Silva *et al*. (2015) and Sen (1997) also performed similar method of determination of surfactin concentration.

For surface tension values between 72.73 and 42.73 mN m^{-1} the surfactin concentration was calculated by Equation (2) and from that value up to 31.05 mN m^{-1}, Equation (3).

$$\Upsilon = 100.85\, e^{-0.345\, C} \qquad (2)$$
$$R^2 = 0.989$$

$$Y = -1.455C + 45,2 \qquad (3)$$
$$R^2 = 0.953$$

Where:

Y = surface tension (mN m^{-1})

C = surfactin concentration (mg L^{-1})

3.9 DETERMINATION OF THE BIOMASS CONCENTRATION

Biomass concentration was expressed in dry mass (g L^{-1}) after determination of the absorbance value (600 nm) and its correlation with the dry mass by a calibration curve (R^2 = 0.9981) constructed.

To construct the calibration curve (Figure 7), the contents of a stock culture tube were transferred to a shake flask containing NB. The flask was incubated at 30 °C and 160 rpm for 24 h. Subsequently, an aliquot of the growth broth (10 times diluted) was automatically scanned in a spectrophotometer to determine the ideal wavelength to perform the readings. The wavelength of 600 nm was the highest optical density value obtained.

Different dilutions (1: 2, 1: 4, 1: 6, 1: 8, 1:10, and 1:12) of the growth medium were prepared and readings were taken on the spectrophotometer. Since turbidimetry is an indirect method, a direct gravimetry method was used to relate optical density versus cell concentration values. For this, according to Maass *et al*. (2015), five aliquots of growth medium were vacuum filtered on cellulose acetate membrane (with 0.45 μm pores) previously microwaved at low power for 15 minutes. After filtration, the membranes were again microwaved to constant mass.

At the end of the batch, broth aliquots had their absorbance value measured and, when necessary, the broth was diluted to fit the calibration curve interval. Thus, to obtain the actual concentration value, it was multiplied by the dilution factor.

Figure 7 - Calibration curve of *Bacillus subtilis* ATCC 6051 biomass concentration versus optical density.

$$y = 2{,}0769x + 0{,}0212$$
$$R^2 = 0{,}9981$$
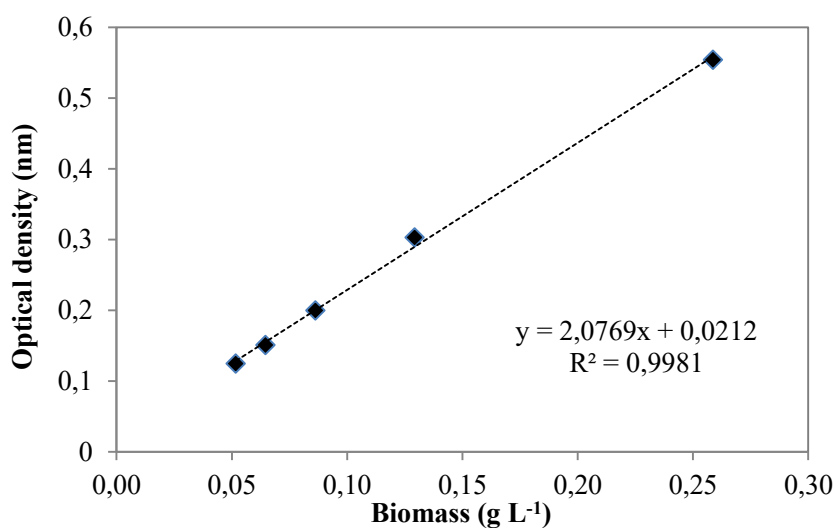
3.10 DETERMINATION OF SUBSTRATE CONCENTRATION (GLUCOSE)

For the determination of the substrate concentration the phenol-sulfuric calorimetric method was used (DUBOIS *et al*., 1956), using glucose for the construction of the standard curve (R^2 0.9975) (Figure 8). The experimental procedure consisted of the transfer of 0.5 mL sample of the culture broth (diluted, when necessary) to test tube. Subsequently 0.5 mL of phenol solution (5% w/v) and 2.5 mL of Sulfuric Acid PA were added to the tube. The tube was carefully shaken and wavelength reading of 490 nm was measured in a spectrophotometer (Femto Cirrus 80).

Figure 8 Calibration curve of glucose concentration
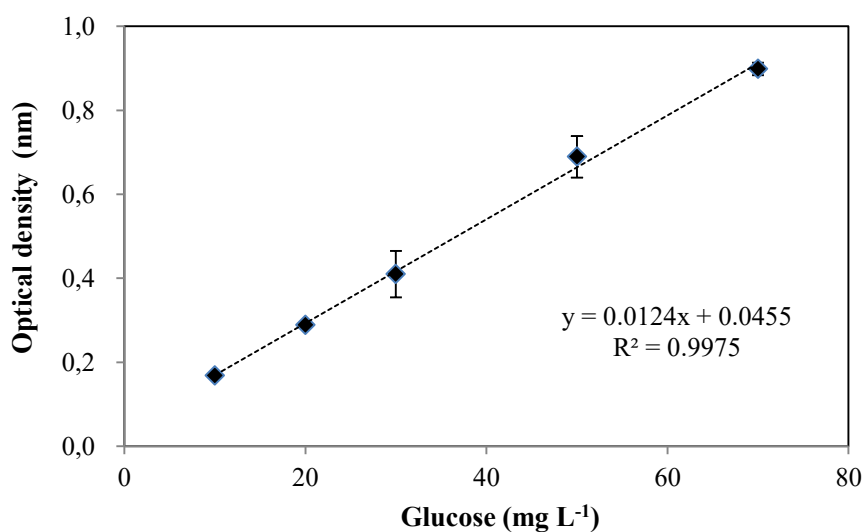
3. 11 MICROORGANISMS

Pseudomonas aeruginosa DSM 3227; *Escherichia coli* ATCC 25922; *Staphylococcus aureus* DSM 20231 and *Staphylococcus epidermidis* DSM 28319 were stored in (1:1) Nutrient Broth medium (NB) with 20% glycerol at -80 °C. Whenever required, the frozen stocks of cells were streaked in Nutrient Agar plates (NA) and incubated for 24 h at 37 °C. After that, the flasks content were stored at 4 °C. To prepare an overnight culture, a loop of a pure culture (streaked on agar plate) was added into 10 mL of NB, and then incubated at 37 °C and 200 rpm.

3.12 INVESTIGATION OF THE ANTIMICROBIAL ACTIVITY OF BIOSURFACTANT

The minimum inhibitory concentration (MIC) was carried out in 96 well-plate. The crude biosurfactant was dissolved in Mueller Hinton broth (MHB) to obtain different concentrations of treatment for each strain, as follows: *P. aeruginosa* DSM 3227 (500–100 µg mL^{-1}), *E. coli* ATCC 25922 (800–50 µg mL^{-1}), *S. aureus* DSM 20231 (800–50 µg mL^{-1}), and *S. epidermidis* DSM 28319 (800–100 µg mL^{-1}).

Control assays were accomplished in a column loaded with broth only and in a second column containing just untreated bacteria. The inoculum was standardized by adjusting OD$_{600}$ to a value corresponding to 10^8 CFU mL^{-1} and added to each well resulting in 5 x10^5 CFU mL^{-1}, approximately per well (CLSI, 2012).

The well-plates were incubated at 37 °C for 24 h and serial dilutions for each concentration were performed. The diluted medium was streaked in Mueller Hinton Agar (MHA) in order to obtain the log reduction and percentage inhibition. All the concentrations were tested in triplicate.

3.13 INVESTIGATION OF THE ANTIBIOFILM ACTIVITY OF THE BIOSURFACTANT

The biofilm formation ability of *P. aeruginosa* DSM 3227 was evaluated according to O'Toole (2011) and, in all the experiments, the absorbance of inoculum at 600 nm corresponded to a value equivalent to 10^8 CFU mL^{-1}. The tests were performed on polystyrene-24-well-plate (Sarstedt) and untreated wells were used as controls.

The potential of the biosurfactant to prevent a biofilm formation was studied using two different techniques: co-incubation and anti-adhesive. In the co-incubation experiments, 1 mL of a range of biosurfactant concentrations (250–500 µg mL^{-1}) dissolved in Nutrient broth (NB) was inoculated (5%, v/v) followed by incubation for 24 h (ELSHIKH *et al*., 2017).

The anti-adhesive activity was tested by pre-coating the wells with solutions of biosurfactant prepared in Phosphate buffered saline - PBS (250–500 µg mL^{-1}) in which 1 mL of each concentration was dispensed in the corresponding well and incubated for 24 h at 40 °C in order to improve the adsorption. After the adsorption time had elapsed, the plate contents were removed and the wells were washed twice with PBS to removed unbound biosurfactant. The plate was sterilized for 3 h under UV light and 1 mL of standardized culture was added followed by incubation for 24 h at 37 °C (ELSHIKH *et al*., 2017).

The antibiofilm activity was also explored in the ability of the biosurfactant to disrupt an existing biofilm (CHEBBI *et al*., 2017). Initially, 1 mL of standardized culture was added in each well and the plate was incubated for 24 h at 37 °C for the biofilm development. After the incubation period, the planktonic cells were discarded and the biofilm was washed twice with PBS. 1 mL of fresh media with different concentrations (200–700 µg mL^{-1}) of biosurfactant was added and incubated for 24 h at 37 °C.

At the end of all experiments, the plates contents were discarded and the wells were washed twice with PBS and stained with crystal violet (0.1%) for 15 minutes at room temperature. Posteriorly, the plates were washed twice with sterilized water and left to dry overnight. 1 mL of acetic acid (30%, v/v) was added in each well and the absorbance of the content of the well was measured at 575 nm (O'TOOLE, 2011).

The percentage of the biofilm inhibition was calculated using the Equation (4):

$$\% \; Biofilm \; inhibition = 100 \; x \left[\frac{(OD_C - OD_t)}{OD_C} \right] \qquad (4)$$

Where ODc and ODt correspond to the optical density of the untreated biofilm and treated biofilm with biosurfactant, respectively.

3.14 IMAGES

Scanning electron microscopy (SEM) was employed to investigate the biofilm of *P. aeruginosa*. The control and biofilm assays (co-incubation, anti-adhesive and disruption) were performed on coverslip as the adhering surface at 450 µg mL^{-1} surfactin concentration. At the end of each experiment, the coverslips were washed with PBS and immersed in 2.5% glutaraldehyde solution for 12 h.

After that, the cells were dehydrated in graded ethanol (50%, 65%, 80%, 95% and 100%) during 10 minutes and in hexamethyldisilazane (HMDS) in ratios of (1:1), (1:2), (1:3) and 100%, during 15 minutes each. The HMDS evaporated overnight and the samples were coated and analysed under SEM (DIAZ DE RIENZO *et al.*, 2015; SARASWATHI *et al.*, 2017).

3.15 STATISTICS

Statistical analysis of the data obtained in the experiments was performed using Statistica 7.0 (StatSoft Inc, USA). Analysis of variance (ANOVA) and *lack of fit* test were used to verify the adequacy of the empirical regression model. In addition, the results were compared using *Tukey* test at a significance level of 5%.

For the antimicrobial and antibiofilm assays, the results were expressed as the mean ± SD (standard deviation) of 3 independent replicates. The data were analyzed using ANOVA (Analysis of variance), and the means were compared with the Duncan's test (5% probability). Significance of variances is indicated as follows: NS (Non-significant), *$p < 0.05$, **$p < 0.05$, ***$p < 0.005$.

4 RESULTS AND DISCUSSION

The results of biosurfactant production by *Bacillus subtilis* ATCC 6051 using brewery waste (Trub) as an alternative carbon source are presented below. In this regard, experimental designs and tests with different concentrations of metal solutions were carried out to evaluate their effect on the productive process. Furthermore, a bench-top bioreactor assays were carried out to study the influence of oxygen on the culture medium for biosurfactant production. Evaluation of antimicrobial and antibiofilm activities of biosurfactant against several pathogens are also presented.

4.1 EFFECT OF NUTRITIONAL AND PROCESS PARAMETERS ON THE BIOSURFACTANT PRODUCTION BY *Bacillus subtilis* ATCC 6051

Biosurfactant production may be affected by cultivation conditions, where carbon, nitrogen and trace element sources are essential to promote production. Thus, researches focusing on the evaluation and optimization of the culture medium for biosurfactant production has been reported in the literature (WEI *et al*., 2007; FONSECA *et al*., 2007; SANTOS *et al*., 2016; MOUAFI *et al*., 2016). Moreover, the influence of process parameters such as pH, temperature and agitation proved to be of great relevance to biological processes (MAKKAR; CAMEOTRA, 2002).

In order to study the biosurfactant production using Trub in the process, a Full Factorial Design - FFD (2^4) was elaborated, having as factors the concentration of Trub (TB), yeast extract (YE), peptone (PB) and agitation (AG). The experiments lasted 72 h and, at the end of the batch, surface tension (ST), cell concentration and pH measurements were taken. The levels of all factors, as well as the results for each assay are presented in Table 10.

Note that the lowest ST value was obtained in assay 3 (27.91 mN m^{-1}) followed by assay 22 (28.06 mN m^{-1}), which corresponds to replicate of assay 3. In addition, successive dilutions (dilution factor equal to 10) were performed to alter the biosurfactant concentration in the samples and, improving data analysis (LOURENÇO, 2017). The highest biosurfactant concentration was 100.76 mg L^{-1} in assay 5. Similar surfactin concentration values were obtained for assays 3, 22, and 24. For the assays mentioned, it is noted that Trub and agitation factors remained constant at the lower level, at 2% (v/v) and 100 rpm, respectively.

Table 10 – Full Factorial Design (2^4) for biosurfactant production with Surface Tension (ST) as response variable. The experimental were performed in duplicates with six central points.

Run	Trub (TB) (%v/v)	Level	Yeast Extract (YE) (g L^{-1})	Level	Peptone (PB) (g L^{-1})	Level	Agitation (AG) (rpm)	Level	ST (mN m^{-1})	ST^{-1}	Surfactin (mg L^{-1})	X (g L^{-1})	pH$_{final}$
1	2.00	(-1)	4.00	(-1)	0.60	(-1)	100	(-1)	33.47	-	8.06	0.321	6.89
2	10.00	(+1)	4.00	(-1)	0.60	(-1)	100	(-1)	48.98	-	2.09	1.386	6.21
3	2.00	(-1)	6.00	(+1)	0.60	(-1)	100	(-1)	27.91	31.09	96.98	1.078	8.06
4	10.00	(+1)	6.00	(+1)	0.60	(-1)	100	(-1)	48.33	-	2.13	1.516	6.53
5	2.00	(-1)	4.00	(-1)	0.80	(+1)	100	(-1)	28.39	30.54	100.76	1.069	8.25
6	10.00	(+1)	4.00	(-1)	0.80	(+1)	100	(-1)	49.29	-	2.08	1.615	6.46
7	2.00	(-1)	6.00	(+1)	0.80	(+1)	100	(-1)	34.01	43.15	24.61	0.537	6.88
8	10.00	(+1)	6.00	(+1)	0.80	(+1)	100	(-1)	47.85	-	2.16	1.819	6.70
9	2.00	(-1)	4.00	(-1)	0.60	(-1)	200	(+1)	29.30	36.49	59.86	2.176	8.61
10	1000	(+1)	4.00	(-1)	0.60	(-1)	200	(+1)	42.02	-	2.54	1.806	6.82
11	2.00	(-1)	6.00	(+1)	0.60	(-1)	200	(+1)	28.69	34.65	72.51	2.499	8.74
12	10.00	(+1)	6.00	(+1)	0.60	(-1)	200	(+1)	36.25	-	6.15	2.170	6.75
13	2.00	(-1)	4.00	(-1)	0.80	(+1)	200	(+1)	28.89	35.41	67.29	1.683	8.66
14	10.00	(+1)	4.00	(-1)	0.80	(+1)	200	(+1)	47.28	-	2.20	1.651	6.24
15	2.00	(-1)	6.00	(+1)	0.80	(+1)	200	(+1)	29.10	33.72	78.90	2.506	8.71
16	10.00	(+1)	6.00	(+1)	0.80	(+1)	200	(+1)	29.25	36.47	60.00	1.468	7.64
17	6.00	(0)	5.00	(0)	0.70	(0)	150	(0)	29.61	35.70	65.29	0.655	7.42
18	6.00	(0)	5.00	(0)	0.70	(0)	150	(0)	29.11	35.65	65.64	0.785	7.64
19	6.00	(0)	5.00	(0)	0.70	(0)	150	(0)	29.14	36.64	58.83	0.566	7.35

ST: Surface Tension; ST^{-1}: Surface tension (diluted 10 times), X: biomass concentration.

Table 10 (Continued) – Full Factorial Design (2^4) for biosurfactant production with Surface Tension (ST) as response variable. The experimental were performed in duplicates with six central points.

Run	Trub (TB) (% v/v)	Level	Yeast Extract (YE) (g L^{-1})	Level	Peptone (PB) (g L^{-1})	Level	Agitation (AG) (rpm)	Level	TS (mN m^{-1})	TS^{-1}	Surfactin (mg L^{-1})	X (g L^{-1})	pH$_{final}$
20	2.00	(-1)	4.00	(-1)	0.60	(-1)	100	(0)	34.50	-	7.35	0.391	6.80
21	10.00	(+1)	4.00	(-1)	0.60	(-1)	100	(0)	49.13	-	2.08	1.607	6.62
22	2.00	(-1)	6.00	(+1)	0.60	(-1)	100	(0)	28.06	31.96	91.00	1.182	8.22
23	10.00	(+1)	6.00	(+1)	0.60	(-1)	100	(-1)	49.88	-	2.04	1.984	6.79
24	2.00	(-1)	4.00	(-1)	0.80	(+1)	100	(-1)	28.28	31.95	91.07	1.035	8.03
25	10.00	(+1)	4.00	(-1)	0.80	(+1)	100	(-1)	50.55	-	2.00	1.576	6.67
26	2.00	(-1)	6.00	(+1)	0.80	(+1)	100	(-1)	29.68	42.09	25.33	0.320	7.18
27	10.00	(+1)	6.00	(+1)	0.80	(+1)	100	(-1)	47.22	-	2.20	1.035	6.44
28	2.00	(-1)	4.00	(-1)	0.60	(-1)	200	(+1)	28.38	37.95	49.83	2.356	8.62
29	10.00	(+1)	4.00	(-1)	0.60	(-1)	200	(+1)	46.62	-	2.24	2.065	6.60
30	2.00	(-1)	6.00	(+1)	0.60	(-1)	200	(+1)	28.86	34.61	72.78	2.197	8.75
31	10.00	(+1)	6.00	(+1)	0.60	(-1)	200	(+1)	34.78	-	7.16	2.951	6.75
32	2.00	(-1)	4.00	(-1)	0.80	(+1)	200	(+1)	28.70	34.85	71.13	1.815	8.42
33	10.00	(+1)	4.00	(-1)	0.80	(+1)	200	(+1)	48.65	-	2.11	1.461	6.26
34	2.00	(-1)	6.00	(+1)	0.80	(+1)	200	(+1)	29.28	33.70	79.04	2.297	8.77
35	10.00	(+1)	6.00	(+1)	0.80	(+1)	200	(+1)	31.32	37.21	54.91	1.555	7.27
36	6.00	(0)	5.00	(0)	0.70	(0)	150	(0)	29.04	35.35	67.70	0.777	7.46
37	6.00	(0)	5.00	(0)	0.70	(0)	150	(0)	29.08	35.40	67.35	0.834	7.62
38	6.00	(0)	5.00	(0)	0.70	(0)	150	(0)	29.15	36.73	58.21	0.846	7.60

ST: Surface Tension; ST^{-1}: Surface tension (diluted 10 times), X: biomass concentration.

The effect of factors on ST can be analyzed by the Pareto chart (Figure 9), which shows that Trub (TB), yeast extract (YE), agitation (AG) and the interaction between them, have statistically significant effect (with 95% reliability), on surface tension. Peptone (PB) had no significant effect, as well as the interaction between it and the others factors studied.

However, it is noted that Trub has the highest positive effect on ST. This means that higher ST values were obtained while the amount of Trub was increased in the culture medium, resulting in a lower biosurfactant concentration. This can also be seen in Table 1, where the highest ST values were obtained in assays with Trub at their highest level (+1). This can be explained by the fact that Trub is a waste and it may contain unidentified substances that are harmful to the process. Nevertheless, low ST values were obtained for assays using Trub at lowest level (-1).

Figure 9 - Pareto chart for ST according to the statistical analysis of the Full Factorial Design (2^4) carried out to evaluated the effect of independent variables (TB, YE, PB and AG) in the biosurfactant production
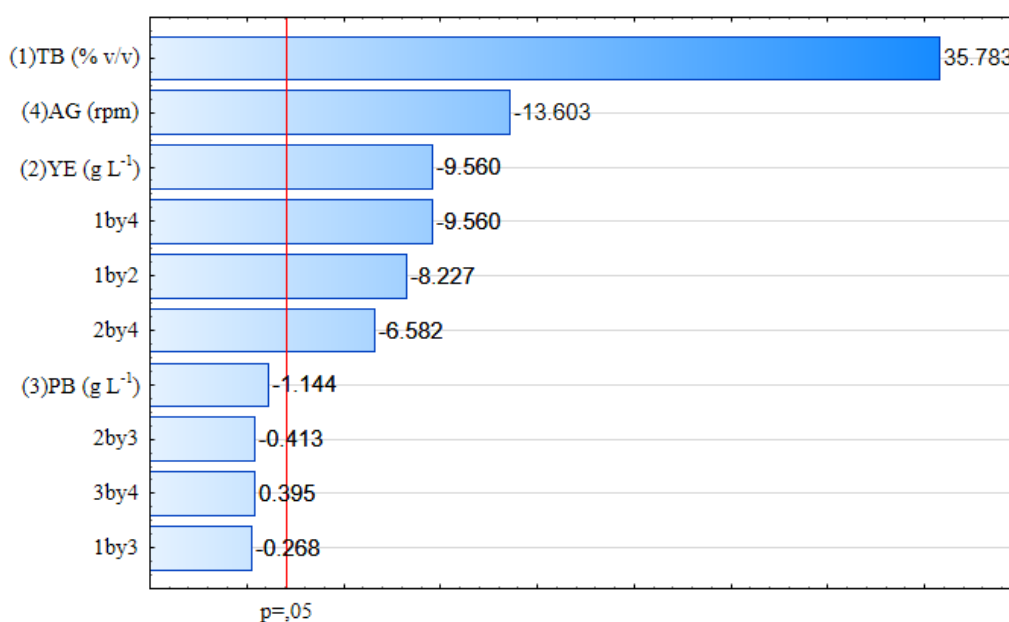
TB (Trub), YE (Yeast extract), PB (Peptone), AG (Agitation). Significant factors at 95% of confidence level.

The experimental data were adjusted to the linear regression model, based on the statistical analysis of the FFD, presenting a coefficient of determination (R^2) of 0.81845. The *p-value* of *lack of fit* was less than 0.05 (Table 11), meaning the model did not fit the data. Thus,

it was not possible to obtain predictions for the behavior of the response variable as a function of independent variables.

This may be justified by the fact that biological processes are difficult to predict, mainly due to the heterogeneity of samples and the existence of uncontrollable factors (LOURENÇO, 2017). Although it was not possible to propose a statistical model, the analysis of the effects of the factors was able to provide important information about the process.

Table 11 – ANOVA for Surface Tension (ST) as a function of studied variables (TB, YE, PB and AG) in biosurfactant production.

Source of variation	SS	df	MS	F	p-value
TB (% v/v)	1680.55	1	1680.55	1280.45	0.000
YE (g L^{-1})	119.97	1	119.97	91.40	0.000
PB (g L^{-1})	1.72	1	1.72	1.31	0.265
AG (rpm)	242.88	1	242.88	185.05	0.000
TB x YE	88.84	1	88.84	67.69	0.000
TB x PB	0.09	1	0.09	0.072	0.790
TB x AG	119.97	1	119.97	91.40	0.000
YE x PB	0.22	1	0.22	0.17	0.683
YE x AG	56.87	1	56.87	43.33	0.000
PB x AG	0.20	1	0.20	0.15	0.696
Lack of fit	485.14	6	80.85	61.60	0.000
Pure error	27.56	21	1.31		
Total SS	2814.03	37			

TB (Trub), YE (Yeast extract), PB (Peptone), AG (Agitation). Significant factors at 95% of confidence level.

As seen in the Pareto chart (Figure 9), the effect of agitation and yeast extract concentration had a negative effect on surface tension, suggesting the biosurfactant production was improved when the conditions of these factors were used at their highest levels. Besides, according to Brumano *et al*. (2017), in general, higher biosurfactant concentration values are associated with higher cell concentration and, for this, the aeration should be increased in the process. This suggests that further agitation will lead to increased cell growth due to culture medium aeration.

Thus, in order to visualize the behavior of the process, a time-course (Figure 10) was performed adopting the agitation and yeast extract at the positive level and the concentration of peptone and Trub at the negative level.

Figure 10 - A time-course profile of biosurfactant production according to the FFD (2^4) at 30 °C and initial pH 7.0. (a) Values as a function of surfactin concentration and (b) as a function of ST.

(a)

(b)
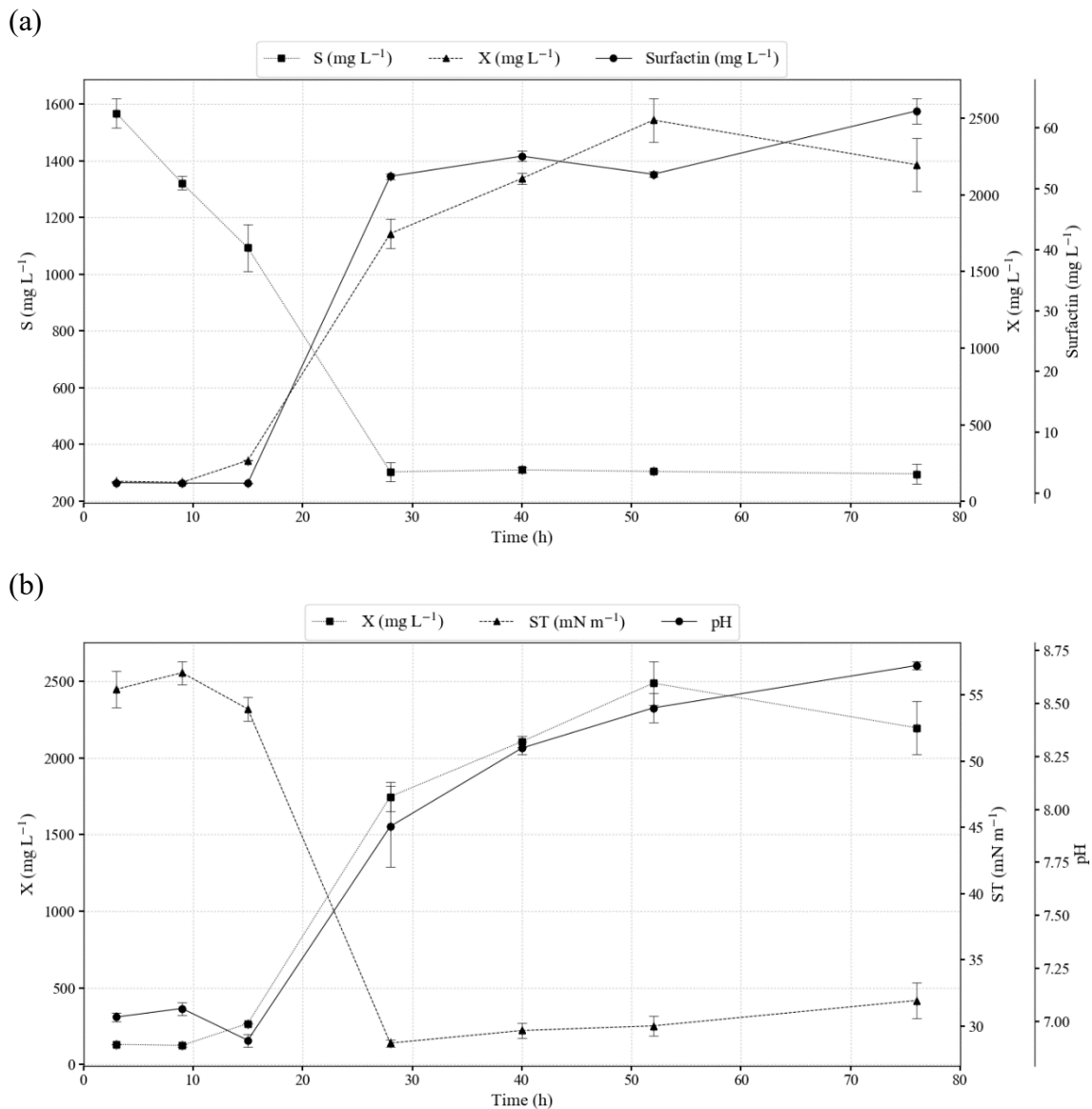
The highest concentration of surfactin obtained was 62.74 ± 2.09 mg L^{-1} after 76h of process and the critical micellar concentration (CMC) was possibly reached between 15 and 28 h (Figure 10).

Figure 10b shows that the sudden reduction in surface tension occurs during the exponential phase of cell growth. This suggests that biosurfactant production may be associated with microbial growth. Similar fact was found by Amani *et al*. (2010), who evaluated biosurfactant production by *Bacillus subtilis, Pseudomonas aeruginosa* and *Bacillus cereus*. The biosurfactant was able to reduce surface tension from 72 to 25 mN m^{-1} after approximately 20 h of cultivation.

According to the analysis of FFD (2^4), it was found that Trub had a predominant effect and its most appropriate use in biosurfactant production would be at the negative level. In addition, agitation showed the second predominant effect, indicating that high aeration values improve the process. A new design was elaborated with independent variables YE and PB, since the PB presented *p-value* close to 0.05 (Figure 9).

For this, the range of factors level was widened and Trub and agitation values were kept constant at 2% (v/v) and 200 rpm, respectively. Table 12 shows the matrix of the new experimental design (FFD, 2^2) and the respective results for each test performed, in which replicates are presented between tests 6 and 9.

Table 12 – Full Factorial Design (2^2) for biosurfactant production with Surface Tension (ST) as response variable. The experimental were performed in duplicates with two central points.

Run	YE		PB		ST (mN m^{-1})	Surfactin (mg L^{-1})	Biomass (mg L^{-1})	pH$_{final}$
	g L^{-1}	Level	g L^{-1}	Level				
1	2	(-1)	0.4	(-1)	29.61	18.43	1587.85	8.11
2	12	(+1)	0.4	(-1)	28.13	94.36	1916.22	8.00
3	2	(-1)	1.4	(+1)	30.01	20.01	1593.14	8.21
4	12	(+1)	1.4	(+1)	27.98	96.56	1895.04	7.91
5	7	(0)	0.9	(0)	28.66	85.98	2726.56	8.41
6	2	(-1)	0.4	(-1)	29.12	20.69	1662.00	8.16
7	12	(+1)	0.4	(-1)	28.32	92.71	1953.30	7.85
8	2	(-1)	1.4	(+1)	30.21	20.22	1614.33	8.09
9	12	(+1)	1.4	(+1)	28.03	92.03	1884.44	7.85
10	7	(0)	0.9	(0)	28.43	90.31	2112.19	8.19

The highest biosurfactant concentration obtained was 96.56 mg L^{-1} in assay 4. Similar values were obtained for runs 2, 7 and 9 (Table 12). For the mentioned tests, it was observed that the concentration of yeast extract remained constant at the highest level tested. At the central point, the highest biomass concentration was achieved, suggesting this is the best condition for the microbial growth (Table 12).

The effect of factors on the response variable was analyzed in the Pareto chart (Figure 11), which shows that only yeast extract concentration had a significant effect on surface tension. This fact was also confirmed by analysis of variance (ANOVA), since the *p*-value is below 0.05 (Table 13). The interaction between both independent variable was also statistically significant, suggesting that the combined effect between factors exert influence on biosurfactant production.

Figure 11 – Pareto chart for ST according to the statistical analysis of the Full Factorial Design (2^2) carried out to evaluated the effect of YE and PB concentration in culture medium in the biosurfactant production
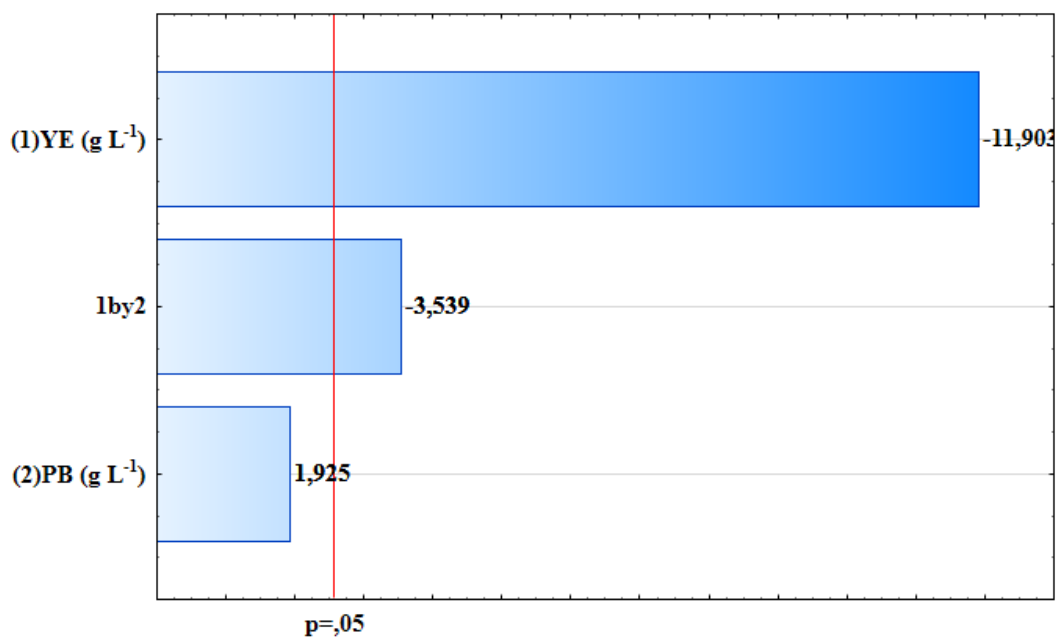
Similar effect was reported by Ponte Rocha *et al*. (2009), who evaluated biosurfactant production by *Bacillus subtilis* LAMI008 and clarified cashew juice as a carbon source. The authors studied the influence of yeast extract supplementation on cell growth and surfactin production and found that the reduction of surface tension was more effective when using yeast

extract in the medium, since supplementation reduced surface tension from 58.95 to 38.10 mN m^{-1}.

The experimental data were adjusted to the quadratic regression model and validated by analysis of variance (ANOVA), as shown in Table 13. The model presented coefficient of determination (R^2) of 0.9334 and adjusted coefficient of determination (R^2Adj) of 0.90018, i.e. 93% of the variations are explained by the model. Moreover, the *lack of fit* test showed no significant result, which indicates agreement between the experimental values and those predicted by the model. Thus, the model is adequate to describe the response variable as a function of the independent variables studied and can be represented by the equation:

$$ST = 29.14155 - 0.07540\,(YE) + 0.93800(PB) - 0.09650(YE \times PB)$$

Table 13 – ANOVA for fitted model for the Surface Tension (ST) as a function of studied variables (Yeast Extract and Peptone) in biosurfactant production.

Source of variation	SS	*df*	MS	F	*p-value*
Yeast Extract (YE)	5.265013	1	5.265013	141.6849	0.000074
Peptone (PB)	0.137813	1	0.137813	3.7086	0.112095
YE x PB	0.4656	1	0.4656	12.5299	0.016567
Lack of fit	0.232562	1	0.232562	6.2584	0.054378
Pure error	0.185800	5	0.037160		
Total SS	6.286800	9			

Significant factors at 95% of confidence level.

From the quadratic regression model, it was possible to construct the response surface and the contour lines as a function of the factors YE and PB. In the surface graph (Figure 12a), it is observed that smaller values of ST are obtained for yeast extract concentrations above 9 g L^{-1}. Figure 12b shows the tendency of response of the dependent variable. When these lines do not present curvature, there is no interaction effect between the independent variables (CALADO; MONTGOMERY, 2003). Thus, there is interaction between the factors due to the curvature in contours (Figure 12b), in which the region that has the lowest surface tension values is found in the concentrations of yeast extract between 8 and 14 g L^{-1}, for any corresponding peptone concentration.

Figure 12 - Response surface (a) and the corresponding contour lines (b) from a Full Factorial Design (2^2), considering Surface Tension (ST) as response variable and Yeast Extract (YE) and Peptone (PB) as factors.

(a)

(b)
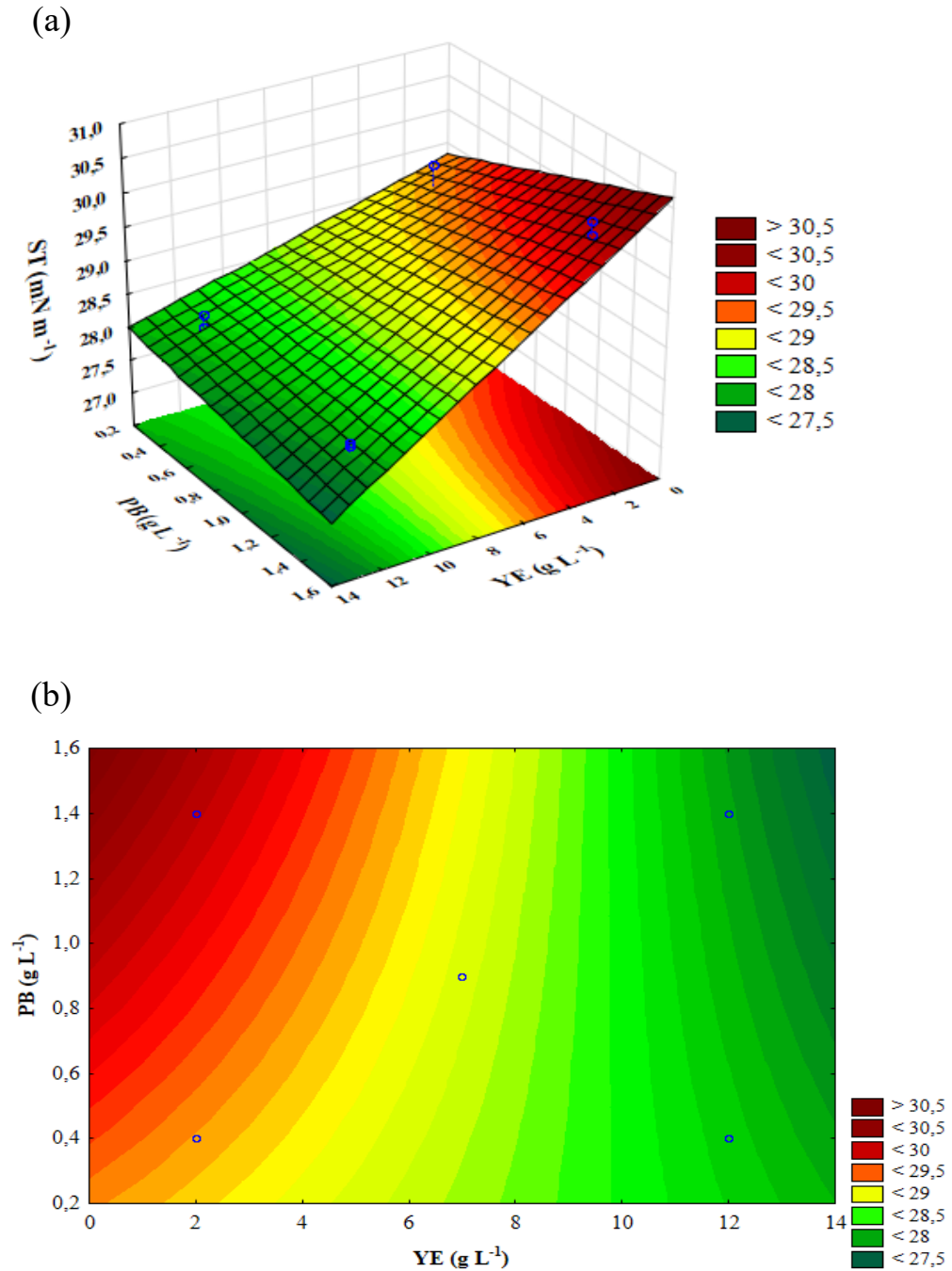
According to the analyses presented for the Full Factorial Design (2^2), a kinetic study (Figure 13) of biosurfactant production was proposed. The conditions adopted were those of the central point (CP), since considerably lower reagent concentrations would be used and the

surfactin concentration obtained would be theoretically similar to that achieved in test 4, which corresponds to the maximum value.

Figure 13 – Time-course profile of biosurfactant production at 30 °C and initial pH 7.0. (a) Values as a function of surfactin concentration and (b) as a function of ST.

a)

b)

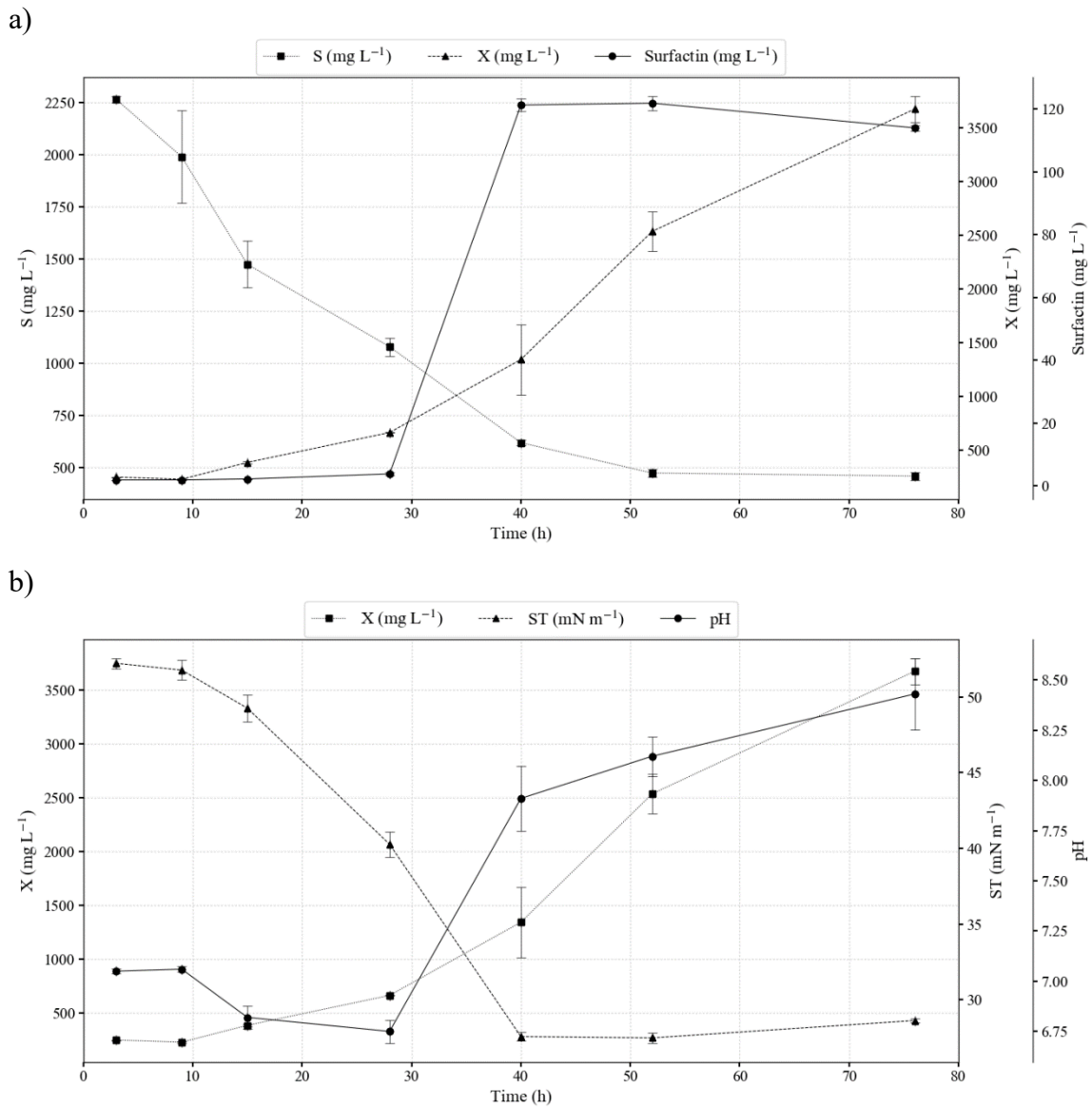Figure 13a shows a significant increase in surfactin concentration (121.20 ± 1.98) after 40 h of cultivation, reaching a maximum concentration of 121.80 ± 2.26 mg L^{-1}. In addition, a production profile associated with cell growth was observed, since biosurfactant production was directly proportional to microbial growth. In *Bacillus sp.*, the expression of the surfactin

genes occurs mostly in the transition from exponential to stationary growth phase and it is associated with increased cell densities (RAAIJMAKERS *et al*., 2010). In Figure 13b, it is noted that the critical micellar concentration was reached between 28 and 40 h of cultivation, and the pH remained close to 8.

A similar result was also reported by Vedaraman and Venkatesh (2011), in which critical micelle concentration was achieved after 48 h for biosurfactant production by *Bacillus subtilis*. The medium was composed by mineral salts and glucose (50 g L^{-1}) and the highest surfactin concentration in the process was 983 mg L^{-1} of surfactin, which is quite higher than in this work, probably due to the high glucose concentration.

4.2 EFFECT OF SUPPLEMENTATION OF CULTURE MEDIUM WITH METALS SOLUTIONS IN BIOSURFACTANT PRODUCTION IN SHAKE FLASKS

The influence of culture medium supplementation with metal solutions on biosurfactant production was evaluated. The metals selected for study were Mg^{2+}, K^+, Mn^{2+} and Fe^{2+}, since they were promising for biosurfactant production in the literature (GUDIÑA *et al*., 2015; WEI *et al*., 2007).

According to Wei *et al*. (2004), an improvement in surfactin production by *Bacillus subtilis* ATCC 21332 was achieved when was used an iron-enriched medium. The authors reported that the addition of Fe^{2+} (4.0 mM) led to an 8-fold increase in cell concentration and 10-fold in surfactin yield when compared to the assay without Fe^{2+} supplementation.

Sen (1997) evaluated the optimization of culture medium to produce biosurfactant by *Bacillus subtilis* DSM 3256 through a composite central design (2^4) with glucose, NH_4NO_3, $FeSO_4$ and $MnSO_4$ as independent variables. The study indicated that NH_4NO_3 and $MnSO_4$ had statistically significant effects on biosurfactant production.

In the present study, solutions of $FeSO_4$, KH_2PO_4, $MnSO_4$ and $MgSO_4$ were added individually to the culture medium and, for each metal, three different concentrations were analysed. Lower surface tension values were obtained, even considering the dilution (1:10). The addition of $FeSO_4$ solution (1.2 mM) resulted in the lowest surface tension value for the metal solution tests (Figure 14).

Figure 14 - Surface Tension (ST^{-1}) values for individual addition of metals in culture medium for biosurfactant production by *Bacillus subtilis* ATCC 6051.
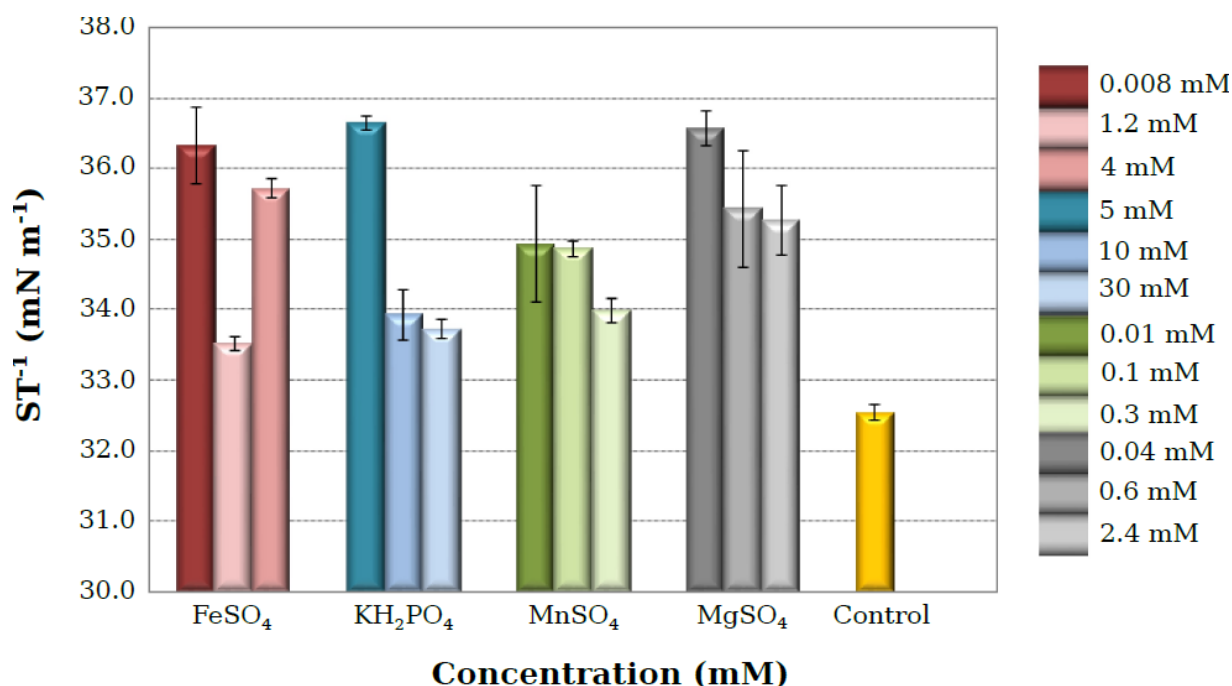
According to the results presented (Figure 14), it is noted that the addition of metal solutions individually to the culture medium leads to biosurfactant production since low values of surface tension of the diluted sample (1:10, ST^{-1}) were obtained.

For a more accurate study, analysis of variance (ANOVA) was performed to verify that the ST^{-1} values for the metal solution assays differed statistically from the ST^{-1} values of the control sample (without metals), as shown in Table 14. Note that all evaluated metals can be considered different to the control sample since the concentrations studied for each metal had significant effect.

On the other hand, a Tukey test was performed (data not shown), with a 95% confidence level, in which it was found that the concentrations of $MnSO_4$ solutions did not differ from each other. A similar fact could be observed for the concentrations of $MgSO_4$ and KH_2PO_4 solutions, which suggests the use of lower concentration values for these compounds to reduce the use of reagents. The 0.008 and 1.2 mM concentrations of $FeSO_4$ solutions differ from each other.

Table 14 - ANOVA for ST^{-1}. Assays with different metal concentrations in the culture medium for biosurfactant production.

Source of variation	SS	df	MS	F	p-value
Between treatments	11271.36	1	11271.36	21524.61	0.0000
[FeSO$_4$]	7.15	2	3.57	6.83	0.0011
Error	3.14	6	0,52		
Between treatments	10577.09	1	10577.09	12413.01	0.0000
[KH$_2$PO$_4$]	10.06	2	5.03	5.90	0.0045
Error	5.11	6	0.85		
Between treatments	10871.19	1	10871.19	45426.49	0.0000
[MnSO$_4$]	2.52	2	1.26	5.26	0.0013
Error	1.44	6	0.24		
Between treatments	11639.89	1	11639.89	35283.39	0.0000
[MgSO$_4$]	2.97	2	1.48	4.49	0.0043
Error	1.98	6	0.33		

According to Wei *et al*. (2007), the addition of metals individually does not always lead to maximum biosurfactant production and the combined effect of factors may be greater than the individual effect on production. The authors got this conclusion after evaluated surfactin production in MSM medium with glucose, exploring the presence of trace elements (K^+, Mg^{2+}, Ca^{2+}, Fe^{2+}, and Mn^{2+}), in which the components of the medium containing one of the five trace elements were removed from MMS one at a time. The authors found that without Mg^{2+} or K^+, the surfactin yield presented 25% of the value obtain from control (complete MSM) and 33% in absence of Mn^{2+} and Fe^{2+}. Without only Fe^{2+} or Mn^{2+}, the surfactin productivity was close to control, achieving 80% of control value, suggesting that correlation between metals is important for promoting surfactin yield.

In this sense, the process behavior was evaluated (Figure 15) by adding solutions of all metals in the culture medium in order to verify the presence of synergistic effects to improve biosurfactant production. Thus, based on the ANOVA (Table 14) and ST values (Figure 14), a 76 h kinetics (Figure 15) was performed using metal solutions with the following

concentrations: $FeSO_4$ (1.2 mM), KH_2PO_4 (5 mM), $MnSO_4$ (0.01 mM) and $MgSO_4$ (0.04 mM). The culture medium was composed by (g L^{-1}): Trub (2%, v/v), $CaCl_2$ (0.1), KH_2PO_4 (1.0), $MgSO_4 \cdot 7H_2O$ (0.5) and NaCl (0.1).

Figure 15 - Time-course profile of biosurfactant production in shake flasks at 30 ° C and initial pH 7.0. (a) Values as a function of biosurfactant concentration and (b) as a function of ST.
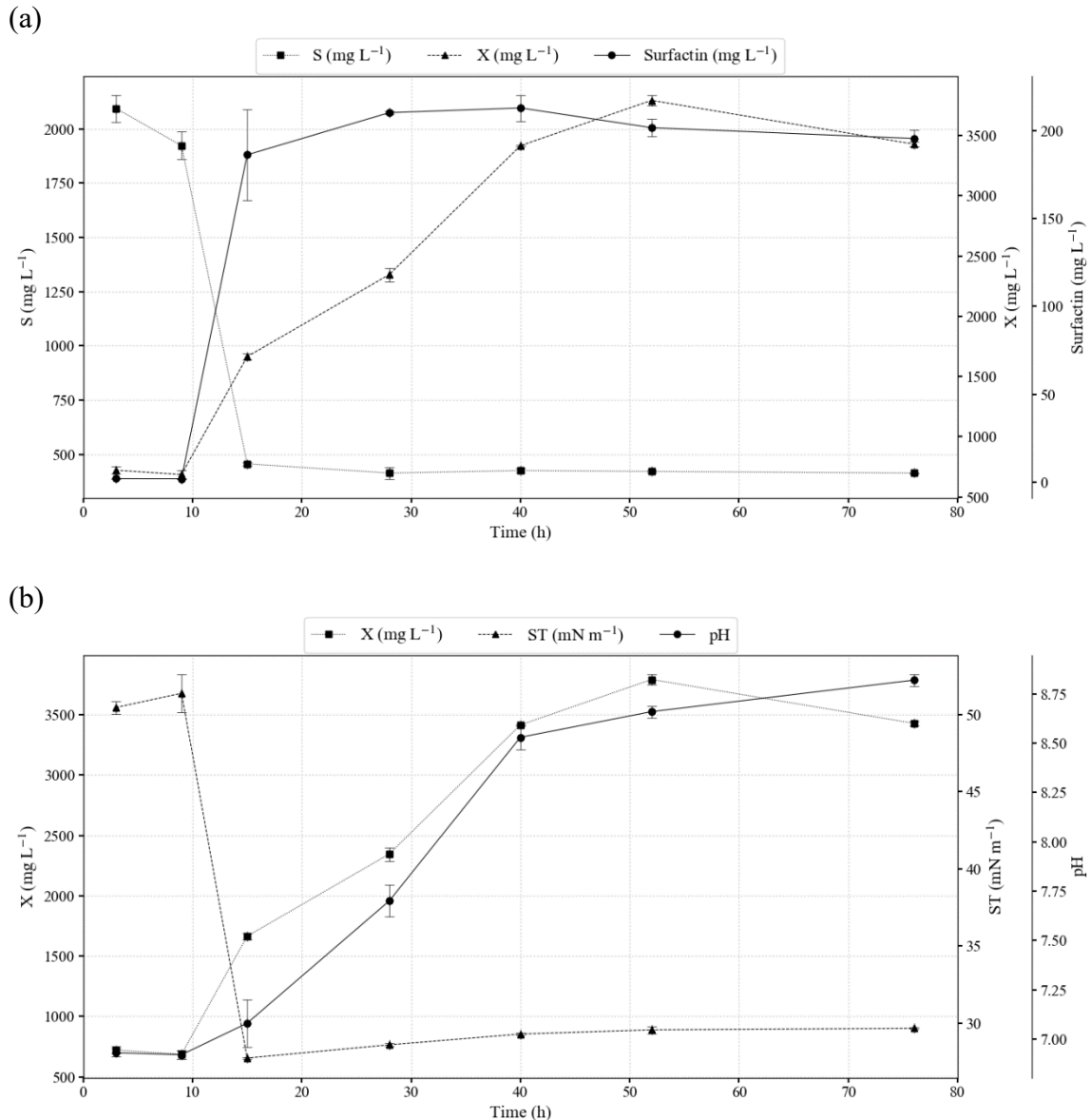
(a)

(b)

Figure 15a shows that high biosurfactant concentrations was obtained after 30 h of cultivation and this value is almost two times higher than the maximum surfactin concentration obtained in the previous kinetics (Figure 13a), without the presence of metals in the medium.

Moreover, CMC (Figure 15b) was achieved between 9 and 15 h of process only, whereas in the previous kinetics between 28 and 40 h were required (Figure 15b). This result is quite interesting since there was a significant increase in biosurfactant concentration in a short time. Thus, the combination of metals in the culture medium was satisfactory for surfactin production.

According to Sen (1997), the concentration of $MnSO_4$ and $FeSO_4$ have a direct relationship with surfactin concentration. In addition, through the Taguchi method, the author found out that the interaction between $MnSO_4$ and $FeSO_4$ is strongly significant ($p > 0.018$). Makkar and Cameotra (2002) state that metal supplementation significantly affects cell growth and biosurfactant production by *B. subtilis* MTCC 2423 and the effect of multiple use of metal cations on the medium is more effective in producing biosurfactant than using them individually.

4.3 PRODUCTION OF BIOSURFACTANT IN STIRRED TANK BIOREACTOR: STUDY OF AERATION AND AGITATION EFFECTS

Oxygen transfer in aerobic process is essential for cell maintenance, where an efficient agitation and aeration system is required for oxygen transfer in the gas phase to the liquid phase, in which the oxygen will reach the cells in suspension, allowing the reaction (SCHMIDELL, 2001). In addition, it was reported in the literature that sufficient supply of dissolved oxygen and mechanical agitation has improved lipopeptide production (JOSHI *et al.*, 2013; HA *et al.*, 2018).

In order to properly size this system, it is important to consider the low oxygen dissolution in the liquid (7 mgO_2 L^{-1}, 1 atm, 35 °C) and the fact that high agitation rates may lead to cell shear and foam formation (SCHMIDELL, 2001), which may result in poor surfactin productivity (HA *et al.*, 2018).

In this sense, the influence of agitation and aeration on biosurfactant production was evaluated through an FFD (2^2) with aeration (RA) and agitation (AG) as independent variables. The design results are presented in Table 15.

Table 15 – Full Factorial Design (2^2) for biosurfactant production in Stirred Tank Bioreactor. Biosurfactant concentration (SF) is the response variable. The experimental were performed with two central points.

Run	Aeration (AR)		Agitation (AG)		Biosurfactant*	X
	(vvm)	Level	(rpm)	Level	(mg L^{-1})	(g L^{-1})
1	0.5	(-1)	150	(-1)	2159.84	2753.00
2	1.5	(+1)	150	(-1)	1689.68	2509.00
3	0.5	(-1)	300	(+1)	562.89	2557.10
4	1.5	(+1)	300	(+1)	454.98	2348.70
5	1.0	(0)	225	(0)	1255.84	2431.07
6	1.0	(0)	225	(0)	1198.13	2285.11

X: biomass concentration; *biosurfactant concentration in foam collected after 24 h batch cultivation.

The maximum biosurfactant concentration reached in foam was 2159.84 mg L^{-1} in test 1, which corresponds to the aeration and agitation condition at the lowest level of the studied factors. For the same assay, biosurfactant concentration considering the working volume of fermentation was 239.74 mg L^{-1}. Furthermore, it is observed that assay 1 has the highest cell concentration, suggesting that surfactin production is associated with cell growth, as seen in previous kinetics presented in this thesis (Figure 15a).

Similar biosurfactant concentration was achieved by Willenbacher *et al*. (2014), who evaluated surfactin production by six different *Bacillus* strains in a 2.5 L bench-top fermenter containing medium composed by glucose (4%) and mineral salts. A foam trap was also employed and the system was operated in cascade mode, in which the agitation was initially adjusted to 300 rpm and PO$_2$ was not allowed falling below 10%. The maximum surfactin concentration achieved was 2.39 ± 0.90 g L^{-1} for *B. subtilis* ATCC 21332, which is probably greater than in this work due to the use of a higher concentration of pure glucose in culture medium.

Even though bioreactor aeration and agitation system favored production, this result suggests that the use of a foam collector may be an interesting alternative for downstream isolation and purification since a concentrated biosurfactant extract was obtained at the end of the batch. Besides, it is also known that surfactin product preferentially distributed toward the foam fraction and then it concentrated on the foam. In addition, another important reason for removing the foam is to overcome product inhibition (YEH *et al*., 2006).

Chen *et al*. (2006) evaluated surfactin production by *B. subtilis* BBK006 in mineral medium with 0.2% of glucose. The bioreactor was equipped with a foam fractionation column and a collection flask. PO2 was maintained above 20% of saturation. The authors reported that surfactin concentration in the foam was around 50 times greater than in the culture medium inside the fermenter (2253 mg L^{-1}) at the end of the batch. Similar observation, even that in greater proportion, was also noticed in this work, in which the concentration in the fermenter vessel was 5.04 mg L^{-1} after 24 h, while the concentration in the foam was 2159.84 in assay 1 (Table 15).

On the other hand, the condition where the maximum agitation and aeration was employed (test 4), the lowest concentration of surfactin in foam obtained (454.98 mg L^{-1}) was observed, which may be related to the excessive foam formed during the process, causing loss of culture medium, limiting production. This is also confirmed by the product yield coefficient (Yp/s), wherein test 1 it is 1.34 while in test 4 it is 0.32. Yp/s values > 1 indicate that the carbon source was preferably directed to product formation.

Analysis of variance (ANOVA) indicated that only agitation has a significant effect on surfactin concentration (SF), with *p*-value less than 0.05 (Table 16). In the Pareto chart, it is also possible to see that agitation had a negative effect, suggesting that high agitation values are not favorable to the process. Aeration and interaction between factors had no significant effect on SF (Figure 16).

Table 16 – ANOVA for fitted model for the biosurfactant concentration (SF) as a function of studied variables (Aeration and Agitation).

Source of variation	SS	*df*	MS	F	*p-value*
Aeration (AR)	83540	1	83540	50.167	0.089291
Agitation (AG)	2004564	1	2004564	1203.782	0.018344
AR x AG	32808	1	32808	19.702	0.141071
Lack of fit	137	1	137	0.082	0.822162
Pure error	1665	1	1665		
Total SS	2122713	5			

Significant factors at 95% of confidence level.

The experimental data were adjusted to the quadratic regression model, which did not present a significant *lack of fit* test (Table 16), showing a regression coefficient (R^2) value of 0.99915. The equation of the model as a function of SF is presented as follows:

$$SF = 4176.384 - 832.418\,(AR) - 11.854\,(AG) + 2.415\,(AR\;x\;AG)$$

Figure 16 - Pareto chart for SF according to the statistical analysis of the Full Factorial Design (2^2) carried out to evaluate the effect of AR and AG in the biosurfactant production in Stirred Tank bioreactor.
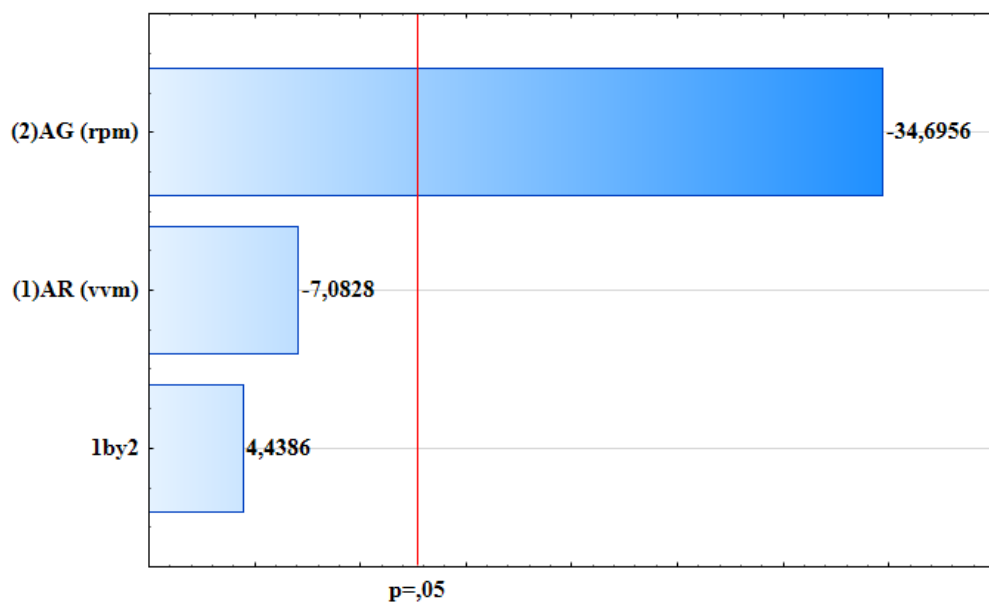
The model explains 99% of the variation in surfactin concentration, thus the response surface and contour lines were constructed (Figure 17a and b) for a better view of the response variable profile as a function of independent variables. It is noted that higher biosurfactant concentration values were obtained for agitation between 150 and 180 rpm. Aeration has less impact on production since higher surfactin concentrations are achieved at aeration rates between 0.4 and 1 vvm. In addition, the contour lines (Figure 17b) show little interaction between the independent variables.

Figure 17 - Response surface (a) and the corresponding contour lines (b) from a Full Factorial Design (2^2), considering biosurfactant concentration (SF) as response variable and Aeration (AR) and Agitation (AG) as factors.
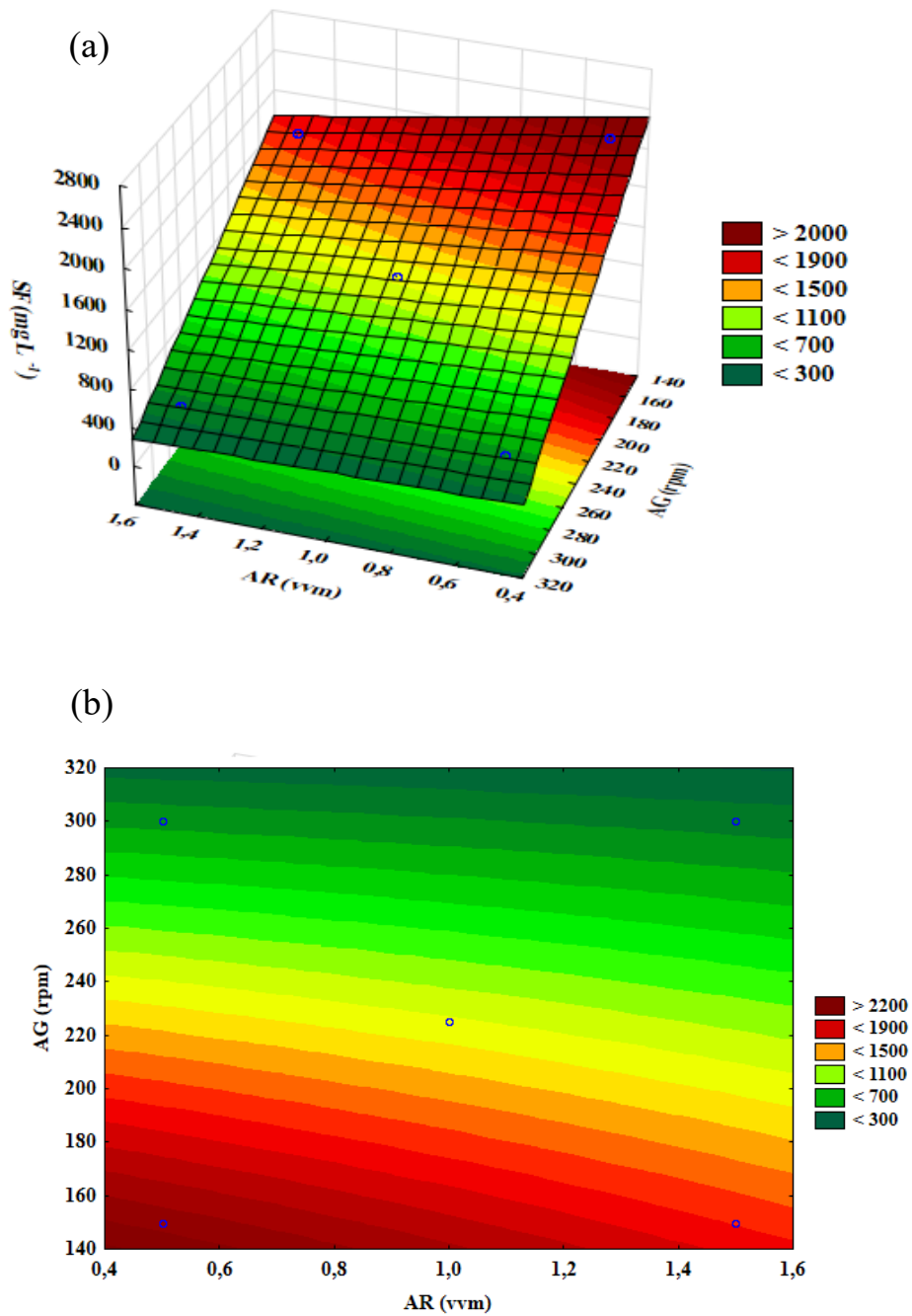
The oxygen transfer coefficient (K_La) is often used as an essential design parameter for bioprocess scale-up, hence its optimal value needs to be determined. The K_La can be manipulated by the aeration and agitation rate (YEH *et al*., 2006).

In order to evaluate oxygen transfer during the tests presented in Table 15, the K_La values were determined at times 3, 9 and 24 h and their possible relationship with biosurfactant volumetric productivity was evaluated (Table 17).

Table 17 - K_La and volumetric biosurfactant productivity (VS) values obtained at different cultivation times for the Full Factorial design (2^2).

Run	Aeration (vvm)	Agitation (rpm)	K_La (h^{-1})			VS (mg L^{-1}h^{-1})
			3 h	9 h	24 h	
1	0.5	150	16.76	*	45.41	89.99
2	1.5	150	26.21	*	*	70.40
3	0.5	300	115.00	108.04	58.24	23.45
4	1.5	300	156.68	99.34	105.34	18.95
5	1.0	225	74.81	145.70	76.23	52.32

* High cell growth phase.

In Table 17, it is observed that for the increase of aeration from 0.5 to 1.5 vvm, keeping the agitation at 150 rpm, the K_La value increases 1.5 times after 3 hours of process, but a decrease in biosurfactant volumetric productivity is observed. When evaluating an increase in agitation from 150 to 300 rpm, maintaining constant aeration at 0.5 vvm, an expressive increase of approximately seven times in the K_La value is observed.

This may have been due to the increase in surface area of mass transfer since an increase in agitation causes larger bubbles to rupture, turning them into smaller bubbles and in greater amounts. Even the K_La presented a high value (test 3), it is noted that the volumetric productivity of biosurfactant decreased. This may be justified by foaming due to high agitation, causing 28.5% of the medium to be dragged out of the reactor until the end of the batch.

The presence of biosurfactant in the liquid medium reduces the surface tension and therefore favors the formation of smaller diameter bubbles. This causes an increase in the surface area, leading to an increase in K_La. However, surfactants can also have a negative effect on K_La as they may stick to the bubble surface making mass transfer difficult (LOURENÇO,

2017). This may have occurred in test 1, in which a large amount of surfactant was generated, and lower K_La values were obtained when compared to the other tests.

4.4 ANTIMICROBIAL AND ANTIBIOFILM ACTIVITIES OF BIOSURFACTANT
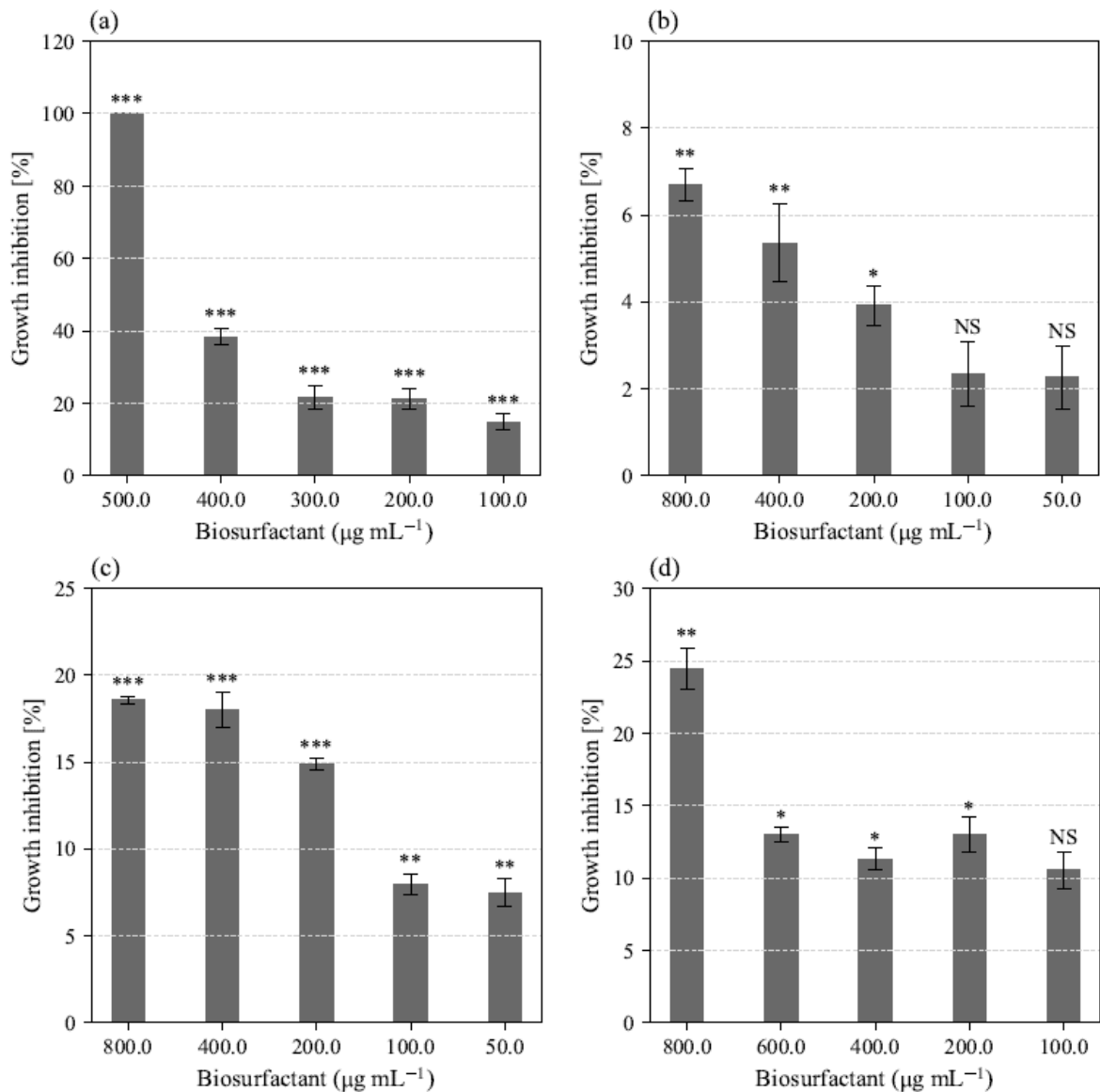
4.4.1 Antimicrobial Activity

The antimicrobial activity of the crude biosurfactant was tested against different microorganisms, which the highest percentage of inhibition for *P. aeruginosa* was 100% using 500 µg mL^{-1} of crude biosurfactant (Figure 18a). This concentration represents the minimum bactericidal concentration (MBC) which is the lowest concentration capable to eliminate a microorganism i.e. not revivable under in a fresh sterile medium (DAS; SEN, 2008).

This phenomenon can also be confirmed by the ratio MBC/MIC, which is 2.5. To ratios ≤ 4.0, the agent can be considered bactericidal (JOSEPH *et al*., 2015). The bacterial growth remained constant for concentrations between 400 and 200µg mL^{-1}, i.e. the lower value considered the MIC. This result also suggests the biosurfactant presents bacteriostatic effect at lower concentrations.

For the gram-positive strains, as *S. aureus* and *S. epidermidis*, the highest percentages of inhibition using 800µg mL^{-1} (Figure 18c and 18d, respectively) were 18.56 ± 0.24% and 24.44 ± 1.46%, respectively. Quite a minor effect of biosurfactant was observed on *E. coli* with only 6.69 ± 0.37% inhibition achieved with 800 µg mL^{-1} of crude biosurfactant (Figure 18b).

Chebbi *et al*. (2017) evaluated the antimicrobial activity of certain types of biosurfactants, including the lipopeptide lichenisin A, produced by *B. licheniformis* and, similarly to this work, showed an inhibitory effect. The study was carried out in 96 well-plate containing biosurfactant (0.0125-25 mg mL^{-1}) diluted in Mueller-Hinton broth. After 24 h of incubation, the MIC was 1.25 mg mL^{-1} for *P. aeruginosa* strain W10, 2.50 mg mL^{-1} for *E. coli* ATCC 25922 and 1.25 mg mL^{-1} for *S. aureus* ATCC 43300.

Figure 18 - Antimicrobial activity of different concentrations of crude biosurfactant (surfactin) against (a) *P. aeruginosa*; (b) *E. coli*; (c) *S. aureus* and (d) *S. epidermidis*. Values are represented as means ± SD (n = 3).

These results are also in agreement with previous findings of Fanaei *et al*. (2018) studies, in which the antimicrobial activity of crude lipopeptide (surfactin) was evaluated against *S. aureus* and *E. coli* using disc diffusion method. The biosurfactant did not inhibit the growth of *E. coli* while showing inhibitory effect against *S. aureus*, with MIC_{50} of 70 µg mL^{-1}. Similarly, Santos da Silva *et al*. (2015) reported the antimicrobial activity of surfactin (2.3g L^{-1}) produced by *Bacillus sp*. culture ITP-001. However, no antimicrobial effects were observed on *S. aureus* ATCC-6533 while a significant inhibiting was detected with *E. coli* CCT-0355.

Sudarmono *et al.* (2019) reported that surfactin produced by *B. amyloliquefaciens* presented antimicrobial activity against *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922, showing inhibition zone diameter of 20.0, 15.7 and 11.4 mm, respectively. The MIC was determined by resazurin assay in a 96 well-plate and, for *P. aeruginosa* to be higher than 1024 μg mL^{-1}.

The biocidal properties of surfactin observed in this work are very promising. The surfactin showed potential antimicrobial activity against both Gram-positive and Gram-negative bacteria. The variation in the structural composition of surfactin can be the main reason for antimicrobial activity, since it can significantly influence the physicochemical properties and physiological activities, including interaction with the microbial membrane. The composition of surfactin is highly dependent on strain, culture condition, and growth medium composition (SUDARMONO *et al.*, 2019).

While, limited information is available about the antimicrobial activity of surfactin against emerging pathogenic microorganisms. Several studies in the literature evaluate the antimicrobial activity through the agar diffusion tests, which does not allow indicating values of concentrations of antimicrobial agents and their respective inhibitory effect on microorganism growth (SANTOS DA SILVA *et al.*, 2015; BERNAT *et al.*, 2016). In our study, we carried out a throughput assay using 96-well plates, allowing us to evaluate the effect of different biosurfactant concentrations on the test microorganisms.

According to Kaczorek *et al.* (2018), the biosurfactant acts in cellular phospholipid membrane, which is responsible for protecting their inner plasma membrane and cell wall from external toxic compounds (SANA *et al.*, 2018), causing permeability due to the penetration of biosurfactant molecules through hydrophobic interactions. This permeability leads to a release of small metabolites, ions, enzymes, and supplementary substances from the cells and allows small molecules to enter cells and inhibiting their metabolism.

Antimicrobial activity of surfactin is based on permeabilization of the cell membrane of the microorganisms due to the accumulation of biosurfactant on the microbial cell, causing its disintegration through the formation of pores in the cell membrane, inducing an increase in Ca^{2+} and H^+ flux in the cells (NDLOVU *et al.*, 2017). Moreover, the activity of surfactin is influenced by the concentration, since the biosurfactant is able to penetrate the membrane even at low concentrations, owing to the fact it is miscible with phospholipids, forming mixed micelles. At moderate concentrations of surfactin, the formation of ion-conducting pores in the

membrane increases and, at high concentrations, the membrane is ruptured due to the detergency effect (GRAU *et al.*, 1999).

A log reduction was observed (Table 18) for all microorganism tested, achieving the highest reduction for *P. aeruginosa* (3.91 ± 0.23). This result shows the biocidal nature of surfactin, since it caused > 3-log reduction as compared to the untreated bacteria after 24h of treatment, as suggested by (PANKEY; SABATH, 2004). *S. aureus* and *S. epidermidis* showed 1 to 2 log reductions after treatment, suggesting that surfactin has limited antimicrobial effects towards them (Table 18).

Table 18 – Antimicrobial activity of crude surfactin based on colony forming unit. The values represent means ± SD.

Bacteria	Biosurfactant ($\mu g\ mL^{-1}$)	Log reduction	Inhibition (%)
P. aeruginosa	400	3.91 ± 0.23	99.99
E. coli	800	0.57 ± 0.07	73.08
S. aureus	400	1.67 ± 0.14	97.86
S. epidermidis	800	2.04 ± 0.38	99.09

4.4.2 Antibiofilm Activity

The antibiofilm activity of crude surfactin was evaluated against *P. aeruginosa* and performed in three different ways: co-incubation, anti-adhesive and disruption. Optical density values were significantly different for the biofilm in the presence of the different treatments with respect to the control ($p < 0.005$).

The co-incubation assay was the most efficient, reducing biofilm formation by 79.80 ± 0.91% when using 400$\mu g\ mL^{-1}$ biosurfactant treatment. It is noted that percent inhibition remained between 72.14 ± 0.78 and 79.29 ± 0.89% for the other surfactin treatments and this inhibitory effect may have been caused by the antimicrobial activity of surfactin (Figure 19a). Such inhibition of biofilm formation in co-incubation treatment has been described by Sriram *et al.* (2011), who evaluated the antibiofilm activity of surfactin produced by *Bacillus cereus* NK1 against *P. aeruginosa*. The assay was performed in polystyrene 96 well-plate with Brain Heart Infusion broth (BHI) using 0.1 – 15 mg mL^{-1} biosurfactant concentrations. The highest

percentage of inhibition reported was $54.21 \pm 0.04\%$ at 15mg mL^{-1}. It is difficult to compare results in the literature, since fermentation for biosurfactants production provides a mixture of homologues, which can present different percentages of inhibition when used to inhibit biofilm of the same lineage of microorganisms (ARAÚJO *et al.*, 2013).

The anti-adhesive experiment revealed the highest inhibition of 58.81 ± 2.85 % by pre-coating the surface with 350µg mL^{-1} crude surfactin (Figure 19b), suggesting that it is capable to modify the physico-chemical properties of the surface reducing adhesion and inhibiting biofilm formation. Moreover, lipopeptides alter the hydrophobicity of the bacterial surface and, consequently, alter the adhesion mechanism of the microorganisms. Its effects depend on the initial bacterial hydrophobicity, as well as the type of lipopeptide and its concentration, which may increase or decrease the hydrophobicity of the bacterial surface due to being more or less hydrophobic (AHIMOU *et al.*, 2000). The anti-adhesive activity of biosurfactants has been described in previous reports, in which Janek *et al.* (2012) present the ability of Pseudofactin II (0.5mg mL^{-1}), a cyclic lipopeptide, to prevent biofilm formation on polystyrene surface of *E. coli*, *Enterococcus faecalis*, *Enterococcus hirae, S. epidermidis*, *Proteus mirabilis* and *Candida albicans*. Araujo *et al.* (2016) observed that surfactin, at 0.50% (w/v), significantly reduced adhesion of *Listeria monocytogenes* on polystyrene surfaces when used at higher concentrations, reaching values of up to 54% inhibition. For, *Pseudomonas fluorescens*, the highest inhibition was only 17.1%.

The disruption of biofilm of *P. aeruginosa* using surfactin has not been reported yet. In this study, the biosurfactant was also employed to disrupt a pre-existing biofilm, achieving $44.94 \pm 6.19\%$ of inhibition at surfactin concentration of 700µg mL^{-1}. The percentage of inhibition remained similar for treatments between 200 and 500µg mL^{-1} (Figure 19c). The inhibition may have been induced by the removal of extracellular polymeric substances (EPS) and the destruction of microcolonies, caused by the biosurfactant (DIAZ DE RIENZO *et al.*, 2016). The EPS plays an important role in the biofilm resistance, as it hinders contact of the microorganism with the antimicrobial agent (ARAÚJO *et al.*, 2013). Dıaz de Rienzo *et al.* (2016) pointed out that *P. aeruginosa* biofilms were disrupted by rhamnolipids at concentrations between 0.5 and 0.4 g L^{-1}.

Figure 19 - Antibiofilm activity of crude biosurfactant (surfactin) against *P. aeruginosa* DSM 3227 at different concentrations: (a) Co-incubation, (b) Anti-adhesive and (c) Disruption. Values are represented as means $\pm$ SD (n = 3).
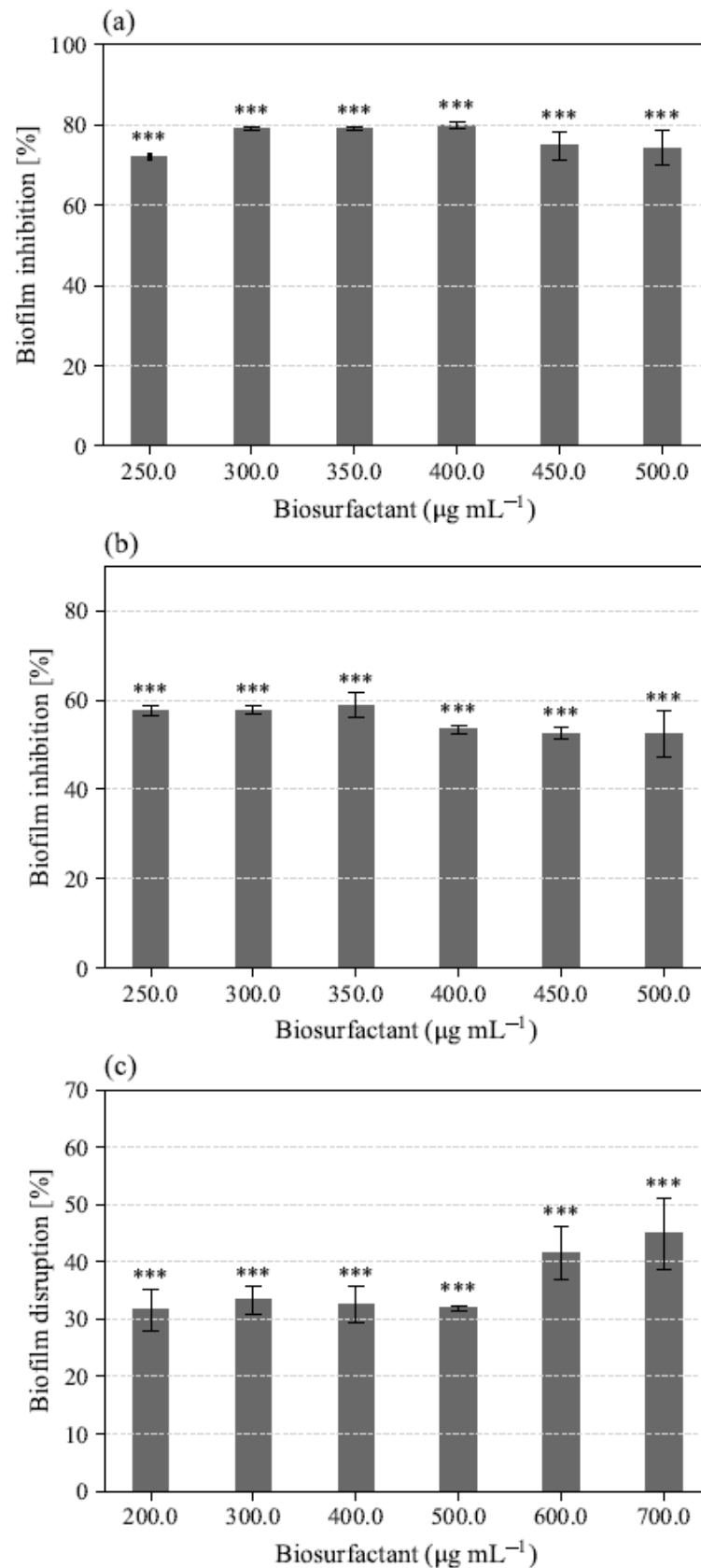
SEM examinations of *P. aeruginosa* before and after treatment were carried to visualize the effect of the surfactin (450 µg mL^{-1}) on biofilm formation and disruption. SEM analysis showed changes in biofilm morphology and topography as a result of treatment with the surfactin.

In the co-incubation assay, where cells were treated with surfactin for 24 h, the inhibition in biofilm formation was visible with changes in cell morphology observed. This effect was probably due to the antimicrobial activity of surfactin, which did not allow the full development of the biofilm. Furthermore, treated cells appear visually shorter than control cells (Figure 20b). Similar observation was also reported by Dengle-Pulate *et al.* (2014), in which *E. coli* cells had reduced size after treated with sophorolipids.

In the anti-adhesive test, the presence of free-living cells, in planktonic form was observed (Figure 20c). However, they do not form a biofilm, suggesting that the coating with biosurfactant was effective in inhibiting biofilm formation. Similar observation was reported by Araujo *et al*. (2016), who evaluated anti-adhesion activity of rhamnolipids (0.50%, w/v) against *P. fluorescens*, with a higher percentage of inhibition of 79%. The authors reported the growth of planktonic cells practically did not differ from the control, suggesting that biosurfactants do not affect planktonic growth and they are adsorbed to polystyrene surfaces when used as surface conditioners.

In the rupture test, the biofilm was treated for 24 h with culture medium containing biosurfactant. After the treatment, regions of rupture within the biofilm were observed as well as the presence of cell in monolayers, while in the control the cells were distributed in multilayers (Figure 20d). Damage in the cell membrane and outpouring of cellular cytoplasm after cell disruption is noticeable (Figure 20e).

Figure 20 - Scanning electron microscopy (SEM) images of *P. aeruginosa* DSM 3227. a) Control shows biofilm formation after 24 h of incubation. Co-incubation (b), Anti-adhesive (c) and Disruption treatment (d and e).
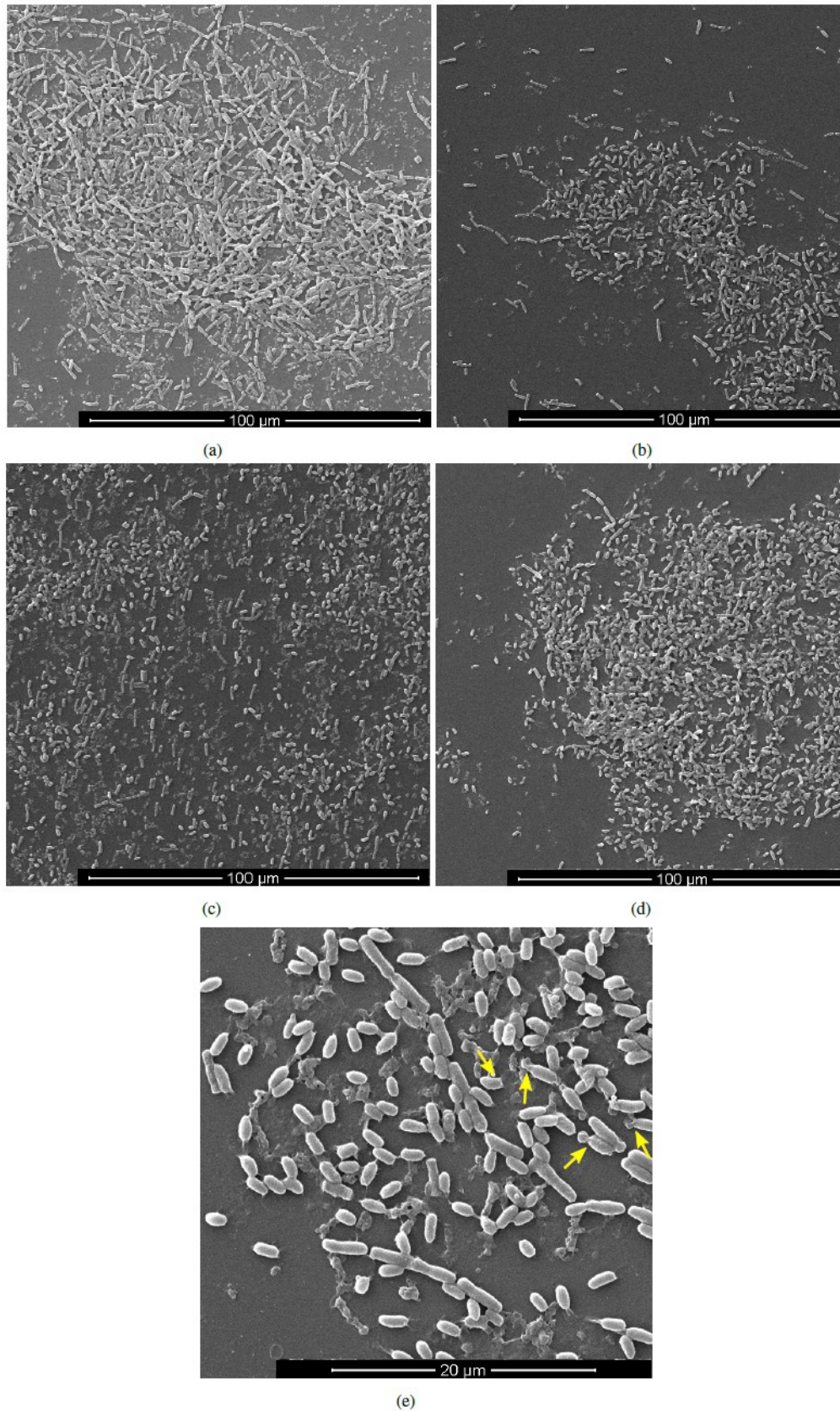
4.5 BIOSURFACTANT CHARACTERIZATION AND STABILITY

Critical micellar concentration (CMC) is an appropriate indicator of the biosurfactant efficiency, since biosurfactants with low CMC values can be considered a good surfactant (Pacwa-Plociniczak *et al.*, 2011).

The CMC of the biosurfactant was determined by a plot of ST *vs.* log of surfactin concentration (Figure 21), with a value of 15 mg L^{-1}. This value is close to those presented in the literature, in which Felix *et al*. (2019) presented a CMC of 12.5 mg L^{-1} for surfactin synthesized by *B. subtilis* using clarified cashew apple juice as carbon and energy source. In addition, the CMC reported in this work is also a satisfactory when compared to commercial standard surfactin (Sigma-Aldrich, 98% purity), whose CMC is between 7.5 and 20 mg.L^{-1}, depending on methods.

Figure 21 - The surface tension values of different concentrations of the surfactin. The intercept point represents the estimated CMC concentration.
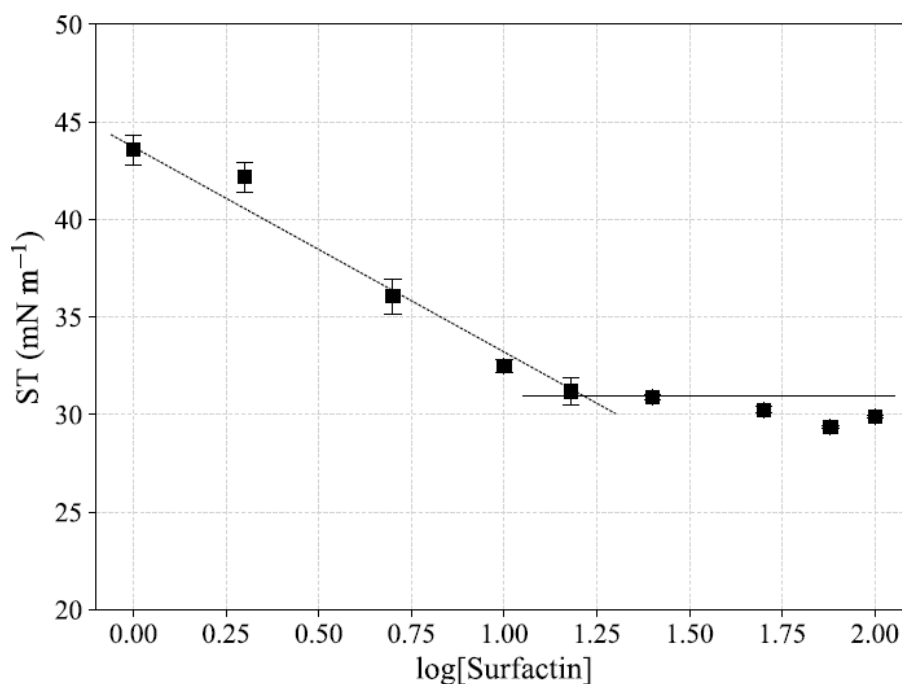
Biosurfactant stability was evaluated under different pH and temperature conditions for 24 h. A solution with a concentration of 2xCMC (30 mg L^{-1}) had its pH adjusted to different values (2, 5, 7, 8 and 10) and, after the incubation period at 25 °C, surface tension (ST) and

emulsification index (E_{24}) measurements were taken. To evaluate the effect of temperature on biosurfactant stability, 2xCMC neutral pH solutions were incubated at different temperatures (25 °C, 45 °C, 65 °C, 85 °C and 100 °C). Similarly to the pH stability test, ST and E_{24} measurements were taken.

Variations in pH values influenced the surface tension, presenting lower ST values (34.74 ± 0.85 mN m-1) for pH 7.0 solution (Figure 22a), which was expected since the biosurfactant is very soluble in this condition. At pH values of 2.0 (48.47 ± 0.52 mN m^{-1}) and 5.0 (46.12 ± 0.37 mN m^{-1}), an increase in surface tension occurred, probably due to precipitation of the biosurfactant in the solution (AL-WAHAIBI *et al*., 2014).

In addition, the biosurfactant showed high ability to stabilize emulsions in n-dodecane (Figure 22a), presenting higher emulsification index (56.45 ± 2.28%), also at pH 7.0. pH values of 2.0 and 5.0 are ineffective in stabilizing emulsions, since emulsification rates were 0.00 and 11.29 ± 2.28, respectively. It is noted that pH values close to neutral intensify the emulsifying activity of the biosurfactant, since an index of 51.61 ± 4.56% was obtained for pH 8.0, while for pH 10.0, this value dropped to 14.52 ± 2.28%.

The biosurfactant was stable under temperature variations (Figure 22b) between 65 and 100 ° C, showing ST values of 34.78 ± 0.53 and 32.61 ± 0.40 mN m^{-1}, respectively. The highest ST value presented was 55.24 ± 0.65 mN m^{-1} at 25 ° C. The biosurfactant was effective on emulsifying activity even incubated at different temperatures, with rates between 54.84 and 67.74%.

The values obtained in this study are in agreement with those found in the literature, where Felix *et al*. (2019) presented emulsification rates between 67.5% and 55.2% for biosurfactant (500 mg L^{-1}) produced *Bacillus subtilis* LAMI005 using cashew apple juice as substrate. Similarly, from a 1 g L^{-1} solution of semipurified surfactin produced by *B. subtilis*, E_{24} values in 69% n-hexadecane were found (NITSCHKE; PASTORE, 2006).

Figure 22 - Effect of pH (a) and temperature (b) on biosurfactant stability produced by *Bacillus subtilis* ATCC 6051.

(a)
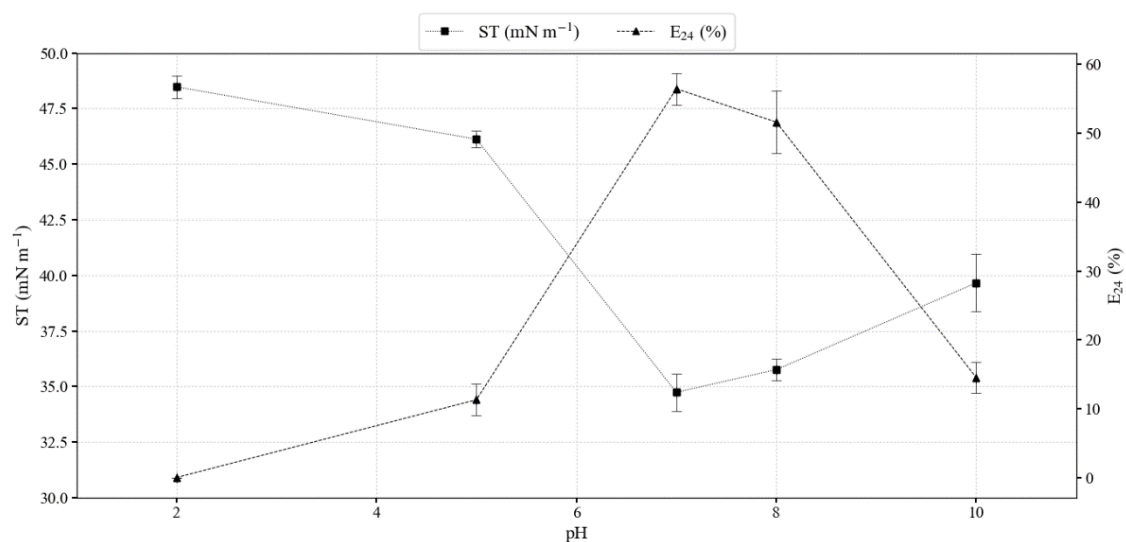
(b)
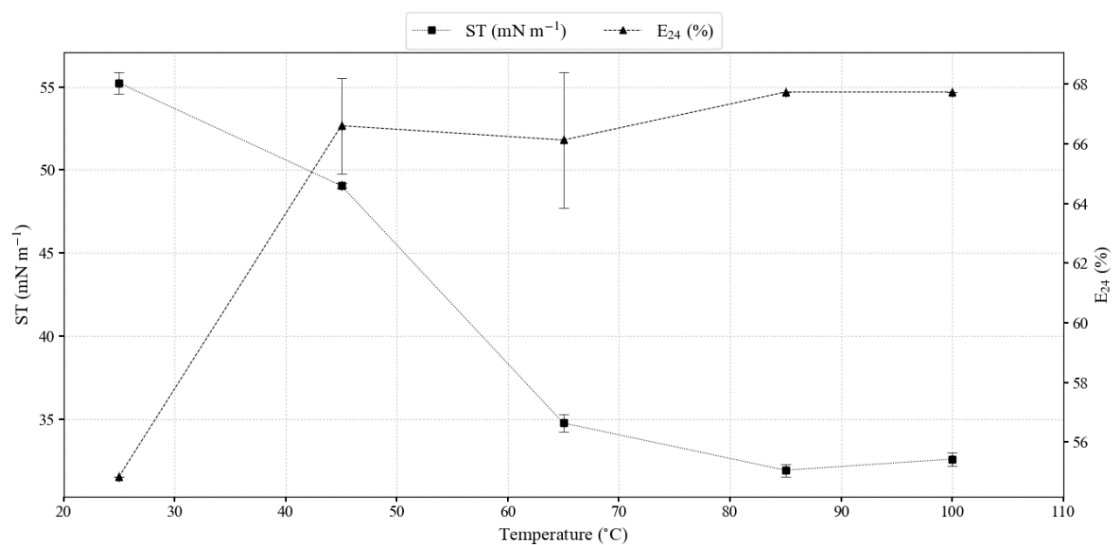
In order to confirm the presence of surfactin in the biosurfactant extract, a MALDI-TOF analysis was performed in positive mode. The spectral (Figure 22) revealed a cluster containing several molecules mainly observed at m/z 1074.6, 1058.6, 1008.6, 1030.6, 1046.6, indicating a lipopeptide with mixture of structural analogs from surfactin class (VATER *et al*., 2002; PEREZ *et al*., 2017; DIMKIC′ *et al*., 2017).

According to Geetha *et al*. 2010, it is well known that surfactin occurs naturally as a mixture of homologous, in which the length and branches of fatty acid side chains as well as the amino acid substitutions in the peptide ring are the differences in these structures.

The different isoforms of surfactin were ranging from C13 to C15, whereas the highest peak (m/z 1074.6) corresponded to potassium adduct of surfactin C15, followed by the second highest peak, showing a $[M+Na]^+$ ion at m/z 1058.6. The remaining mentioned peaks correspond to C13 surfactin (PEREZ *et al*., 2017; DIMKIC´ *et al*., 2017).

Figure 23 - MALDI-TOF mass spectrum in positive mode of crude biosurfactant from *Bacillus subtilis* ATCC 6051. The spectrum shows data for surfactin C15 (1058.6, 1074.6), surfactin C14 (1022.6), surfactin C13 (1008.6, 1030.6, 1046.6) and surfactin C12 (994.6).
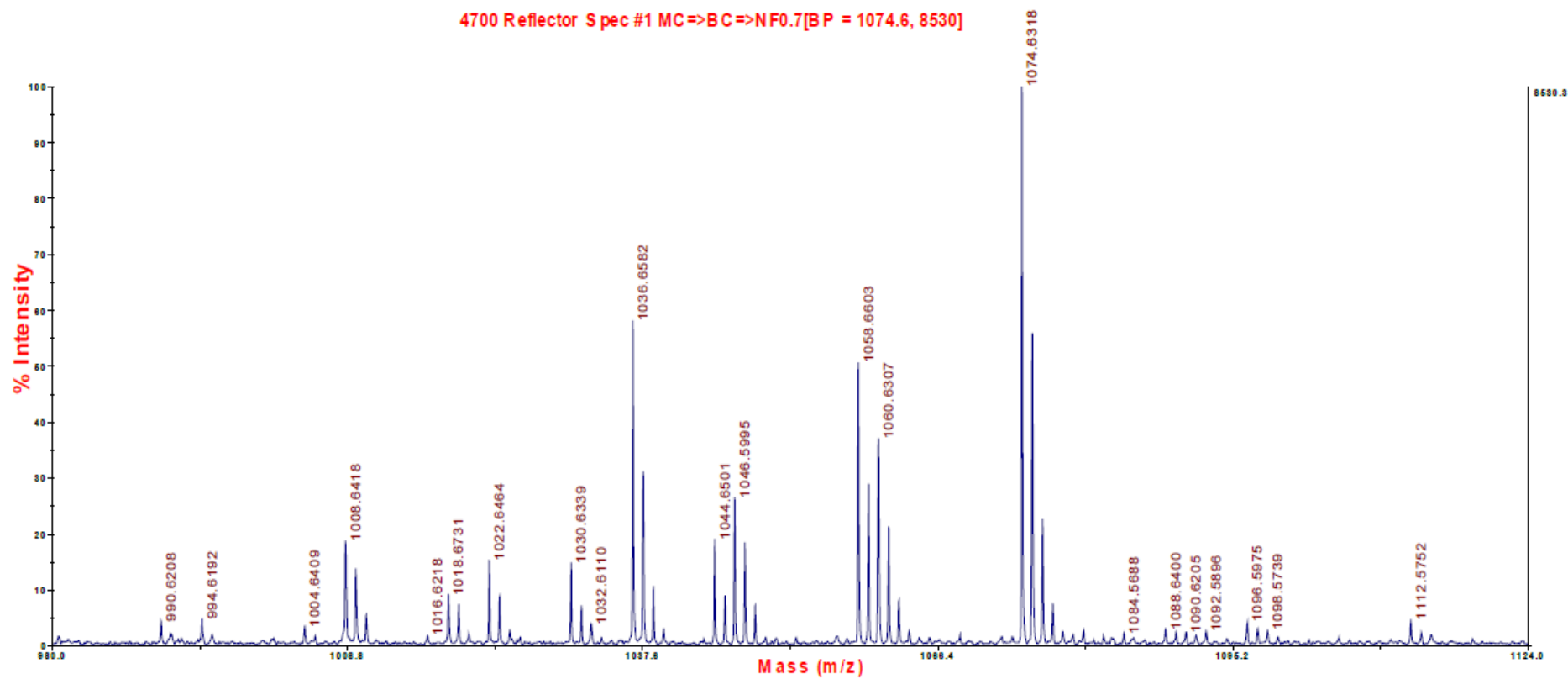
6 CONCLUSIONS

The *Bacillus subtilis* ATCC 6051 microorganism was able to grow in medium containing brewery residue and produce high surface activity biosurfactant.

The effects of nutritional and process parameters on biosurfactant production were evaluated by experimental design. The highest surfactin concentration value obtained in the full factorial design (2^4) was 100.76 mg L^{-1}. In this design, all evaluated factors had a significant effect on surface tension, including their interaction, except peptone concentration. Through the complete factorial design (2^2), it was possible to propose a quadratic regression model with regression coefficient (R^2) of 0.93. Moreover, only the yeast extract concentration had a significant effect on the surface tension values. The highest surfactin concentration value achieved in this experimental design was 96.56 mg L^{-1}.

The effect of supplementation of the culture medium with metal solution caused surfactin production, as low surface tension values were achieved for the addition of individual solutions in the culture medium.

The synergistic effect of the addition of all metal solutions in the culture medium promoted surfactin production since, in the kinetic study, the highest surfactin concentration achieved was 210.11 ± 0.85 mg L^{-1}, obtained after 28 hours of cultivation. This value is almost two times higher than the maximum surfactin concentration (121.20 ± 1.98 mg L^{-1}) obtained in the kinetics without metals in the medium after 40 h of cultivation.

The surfactin production was also evaluated in stirred tank bioreactor coupled with a foam collector. Only agitation showed a significant effect on surfactin concentration and the maximum surfactin concentration reached was 239.74 mg L^{-1}. The model showed a regression coefficient (R^2) value of 0.99915.

Surfactin inhibited the growth of all microorganisms tested. The bactericidal effects were highest against *P. aeruginosa.* In addition, surfactin was also effective against *P. aeruginosa* biofilm, presenting the highest inhibition (79.80%) in the co-incubation assay, using a biosurfactant solution at 400µg mL^{-1}. Therefore, the cost-effective production of surfactin together with antimicrobial and antibiofilm activity makes it relevant for biomedical applications.

These results prove the feasibility of using brewery waste in the production of biosurfactant by *Bacillus subtilis* ATCC 6051 and that the optimization of process parameters is fundamental to achieve high surfactin productivity.

REFERENCES

AHIMOU, F.; JACQUES, P.; DELEU, M. Surfactin and iturin A effects on *Bacillus subtilis* surface hydrophobicity. **Enzyme and Microbial Technology**, v. 27, n. 10, p. 749–754, 2000.

AL-WAHAIBI, Y.; JOSHI, S.; AL-BAHRY, S.; ELSHAFIE, A.; AL-BEMANI, A.; SHIBULAL, B. Biosurfactant production by *Bacillus subtilis* B30 and its application in enhancing oil recovery. **Colloids and Surfaces B: Biointerfaces**, v. 114, p. 324– 333, 2014.

AMANI, H.; MEHRNIA, M. R. Scale up and Application of Biosurfactant from *Bacillus subtilis* in Enhanced Oil Recovery. **Applied Biochemistry and Biotechnology**, v. 162, n. 2, p. 510–523, 2010.

AMARAL, P.F.F.; SILVA, J.M.; LEHOCKY, M.; BARROS-TIMMONS, A.M.V; COELHO, M.A.Z.; MARRUCHO, I.M.; COUTINHO, J.A.P. Production and characterization of a bioemulsifier from *Yarrowia lipolytica*. **Process Biochemistry**, v. 41, n. 8, p. 1894–1898, 2006.

ARAUJO, L.V.; FREIRE, D.M.; NITSCHKE, M. Biosurfactants: anticorrosive, antibiofilm and antimicrobial properties. **Quimica Nova**, v. 36, n. 6, p. 848–858, 2013.

ARAUJO, L.V.; GUIMARÃES, C.R.; MARQUITA, R.L.; DA S.; SANTIAGO, V. M. J.; DE SOUZA, M. P.; NITSCHKE, M.; FREIRE, D. M. G. Rhamnolipid and surfactin: Anti-adhesion/antibiofilm and antimicrobial effects. **Food Control**, v. 63, p. 171–178, 2016.

BANAT, I.M. Biosurfactants Production and Possible uses in Microbial Enhanced Oil Recovery and Oil Pollution Remediation: A Review. **Bioresource Technology**, v. 51, n. 1, p. 1-12, 1995.

BANAT, I.M.; MAKKAR, R.S.; CAMEOTRA, S.S. Potential commercial applications of microbial surfactants. **Applied Microbiology and Biotechnology**, v. 53, n. 5, p. 495–508, 2000.

BANAT, I.M.; DIAZ DE RIENZO, M.A.D.; QUINN, G.A. Microbial biofilms: biosurfactants as antibiofilm agents. **Applied Microbiology and Biotechnology**, v. 98, n. 24, p. 9915–9929, 2014.

BERNAT, P.; PARASZKIEWICZ, K.; SIEWIERA, P.; MORYL, M.; PŁAZA, G.; CHOJNIAK, J. Lipid composition in a strain of *Bacillus subtilis*, a producer of iturin A lipopeptides that are active against uropathogenic bacteria. **World Journal of Microbiology and Biotechnology**, v. 32, n. 157, 2016.

BRUMANO, L.P.; ANTUNES, F.; SOUTO S.G.; DOS SANTOS J.C.; VENUS J, SCHNEIDER, R.; DA SILVA S.S. Biosurfactant production by *Aureobasidium pullulans* in stirred tank bioreactor: New approach to understand the influence of important variables in the process. **Bioresource Technology**, v. 243, p. 264 - 272, 2017.

CALADO, V.; MONTGOMERY, D.C. Design of Experiments using Statistica. 260 f. Rio de Janeiro: E-Papers Editorial Services, 2003.

CAMPOS, J.M.; STAMFORD, T.L.; SARUBBO, L. Production of a Bioemulsifier with Potential Application in the Food Industry. **Applied Biochemistry and Biotechnology**, v. 172, n. 6, p. 3234 - 3252, 2014.

CHEBBI, A.; ELSHIKH, M.; HAQUE, F.; AHMED, S.; DOBBIN, S.; MARCHANT, R.; BANAT, I.M. Rhamnolipids from *Pseudomonas aeruginosa* strain W10; as antibiofilm/antibiofouling products for metal protection. **Journal of Basic Microbiology**, v. 57, n. 5, p. 364–375, 2017.

CHEN, C.; BAKER, S.; DARTON, R. Batch production of biosurfactant with foam fractionation. **Journal of Chemical Technology and Biotechnology**, v. 82, p. 329–333, 2006.

CLINICAL AND LABORATORY STANDARDS INSTITUTE - CLSI. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically ; Approved Standard — Ninth Edition. In Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard - Ninth Edition (Vol. 32), 2012.

COOPER, D.G.; GOLDENBERG, B.G. Surface-active agents from two *Bacillus* species. **Applied and Environmental Microbiology**, v. 53, n. 2, p. 224–229, 1987.

COOPER, D.G.; MACDONALD, C.R.; DUFF, S. J. B.; KOSARIC, N. Enhanced Production of Surfactin from *Bacillus subtilis* by Continuous Product Removal and Metal Cation Additions. **Applied and Environmental Microbiology**, v. 42, n. 3, p. 408–412, 1981.

DAS, P.; MUKHERJEE, S.; SEN, R. Antimicrobial potential of a lipopeptide biosurfactant derived from a marine *Bacillus circulans*. **Journal of Applied Microbiology**, v. 104, n. 6, p. 1675–1684, 2008.

DAVEREY, D.; PAKSHIRAJAN, K. Sophorolipids from Candida bombicola using mixed hydrophilic substrates: Production, purification and characterization. **Colloids and Surfaces B: Biointerfaces**, v. 79, p. 246–253, 2010.

DAVIS, D. A.; LYNCH, H. C.; VARLEY, J. The production of Surfactin in batch culture by *Bacillus subtilis* ATCC 21332 is strongly influenced by the conditions of nitrogen metabolism. **Enzyme and Microbial Technology**, v. 25, n. 3, p. 322–329, 1999.

DEBON, J. Production of biosurfactant by *Bacillus subtilis* ATCC 21332 under anaerobic condition. 165 p. Doctoral Thesis - Chemical Engineering Course, Federal University of Santa Catarina, Florianópolis, 2015.

DEEPIKA, K.V.; KALAM, S.; SRIDHAR, P.R.; RAO, A.; BRAMHACHARI, V. Biocatalysis and Agricultural Biotechnology Optimization of rhamnolipid biosurfactant production by mangrove sediment bacterium *Pseudomonas aeruginosa* KVD-HR42 using response surface methodology. **Biocatalysis and Agricultural Biotechnology**, v. 5, p. 38–47, 2016.

DENGLE-PULATE, V.; CHANDORKAR, P.; BHAGWAT, S.; PRABHUNE, A.A. Antimicrobial and SEM studies of sophorolipids synthesized using lauryl alcohol. **Journal of Surfactants and Detergents**, v. 17, n. 3, p. 543–552, 2014.

DESAI, J. D; BANAT, I. M. Microbial Production of Surfactants and their Commercial Potential. **Microbiology and Molecular Biology Reviews**, v. 61, n. 1, p. 47–64, 1997.

DIAS, M. DE O.; FALCONI, D. The Evolution of Craft Beer Industry in Brazil**. Journal of Economics and Business**, v. 1, n. 4, p. 618-626, 2018.

DIAZ DE RIENZO, M.A.; BANAT, I.M.; DOLMAN, B.; WINTERBURN, J.; MARTIN, P.J. Sophorolipid biosurfactants: Possible uses as antibacterial and antibiofilm agent. **New Biotechnology**, v. 32, n. 6, p. 720- 726, 2015.

DIAZ DE RIENZO, M. A.; STEVENSON, P. S.; MARCHANT, R.; BANAT, I.M. *Pseudomonas aeruginosa* biofilm disruption using microbial surfactants. **Journal of Applied Microbiology**, v. 120, n. 4, p. 868–876, 2016.

DIMKIC, I.; STANKOVIC, S.; NIŠAVIC, M.; PETKOVIC, M.; RISTIVOJEVIC, P.; FIRA, D.; BERIC, T. The profile and antimicrobial activity of *Bacillus* lipopeptide extracts of five potential biocontrol strains. **Frontiers in Microbiology**, v. 8, p. 1–12, 2017.

DONLAN, R.M. Biofilm formation: a clinically relevant microbiological process. **Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America**, v. 33, n. 8, p. 1387–1392, 2001.

DOS SANTOS MATHIAS, T.R.; ALEXANDRE, V.M.F.; CAMMAROTA, M.C.; DE MELLO, P.P.M.; SÉRVULO, E.F.C. Characterization and determination of brewer's solid wastes composition. **Journal Institute of Brewing**, v. 121, p. 400–404, 2015

DUBOIS, M.; GILLES, K. A.; HAMILTON, J. K.; REBERS, P. A.; SMITH, F. Colorimetric Method for Determination of Sugars and Related Substances. **Analytical Chemistry**, v. 28, n. 3, p. 350–356, 1965.

ELSHIKH, M.; FUNSTON, S.; CHEBBI, A.; AHMED, S.; MARCHANT, R.; BANAT, I.M. Rhamnolipids from non-pathogenic *Burkholderia thailandensis* E264: Physicochemical characterization, antimicrobial and antibiofilm efficacy against oral hygiene related pathogens. **New Biotechnology**, v. 36, p. 26 -3 6, 2017.

FANAEI, M.; EMTIAZI, G. Microbial assisted (*Bacillus mojavensis*) production of bio-surfactant lipopeptide with potential pharmaceutical applications and its characterization by MALDI-TOF-MS analysis. **Journal of Molecular Liquids**, 268, 707–714, 2018.

FĂRCAȘ, A.C.; SOCACI, S.A.; MUDURA, E.; DULF, F.V.; VODNAR, D.C.; TOFANĂ, M.; SALANȚĂ, L.C. Exploitation of Brewing Industry Wastes to Produce Functional Ingredients. **Brewing Technology**, 2017.

FELIX, A.K.; MARTINS, J.J.L.; LIMA ALMEIDA, J.G.; GIRO, M.; CAVALCANTE K.F.; MACIEL MELO, V.M.; AGUIAR, R.S. Purification and characterization of a biosurfactant produced by *Bacillus subtilis* in cashew apple juice and its application in the remediation of oil-contaminated soil. **Colloids and Surfaces B: Biointerfaces**, v. 175, p. 256–263, 2019.

FERNANDES, P.A.V.; ARRUDA, I.R.DE, SANTOS, A.F.DOS.; ARAÚJO, A.A.DE.; MAIOR, A.M.S.; XIMENES, E.A. Antimicrobial activity of surfactants produced by *Bacillus subtilis* R14 against multidrug-resistant bacteria. **Brazilian Journal of Microbiology,** v. 38, n. 4, p. 704–709, 2007.

FONSECA, R.R.; SILVA, A.J.R.; DE FRANÇA, F.P.; CARDOSO, V.L.; SÉRVULO, E. F.C. Optimizing Carbon/Nitrogen Ratio for Biosurfactant Production by a *Bacillus subtilis* Strain. **Applied Biochemistry and Biotechnology**, v. 136, n. 1, p. 471–486, 2007.

FRACCHIA, L.; CAVALLO, M.; ALLEGRONE, G.; MARTINOTTI, M.G. A Lactobacillus-derived biosurfactant inhibits biofilm formation of human pathogenic *Candida albicans* biofilm producers. **Technology and Education Topics in Applied Microbiology and Microbial Biotechnology**, v. 3, p. 827–837, 2010.

FRANZETTI, A.; TAMBURINI, E.; BANAT, I. M. Applications of Biological Surface Active Compounds in Remediation Technologies. In: Sen, R (Ed). **Advances in Experimental Medicine and Biology vol. 672**. Austin: Landes Bioscience, ch. 9, p. 121-134, 2010.

GALABOVA, D.; SOTIROVA, A.; KARPENKO, E.; KARPENKO, O. Role of Microbial Surface-Active Compounds in Environmental Protection. **The Role of Colloidal Systems in Environmental Protection**, ch. 3, p. 41-83, 2014.

GEETHA, I.; MANONMANI, A.M.; PAILY, K.P. Identification and characterization of a mosquito pupicidal metabolite of a *Bacillus subtilis* subsp*. subtilis* strain. **Applied Microbiology and Biotechnology**, v. 86, p. 1737–1744, 2010.

GRAU, A.; GÓMEZ FERNÁNDEZ, J.C.; PEYPOUX, F.; ORTIZ, A. A study on the interactions of surfactin with phospholipid vesicles. **Biochimica et Biophysica Acta - Biomembranes**, v. 1418, n. 2, p. 307–319, 1999.

GUDIÑA, E.J.; FERNANDES, E.C.; RODRIGUES, A.I.; TEIXEIRA, J.A.; RODRIGUES, L.R. Biosurfactant production by *Bacillus subtilis* using corn steep liquor as culture medium. **Frontiers in Microbiology**, v. 6, n. 59, p. 1–7, 2015.

HA, S.; KIM, H.M.; CHUN, H.H.; HWANG, I.M.; LEE, J.H.; KIM, J.C.; PARK, H.W. Effect of oxygen supply on surfactin production and sporulation in submerged culture of *Bacillus subtilis* Y9. **Applied Sciences (Switzerland)**, v. 8, n. 1660, p. 1 - 10, 2018.

HAUSMANN, R.; SYLDATK, C. Types and Classification of Microbial Surfactants. In: KOSARIC, N; VARDAR-SUKAN, F (Ed). **Biosurfactants Production and Utilization—Processes, Technologies, and Economics.** Nova York: CRC, ch 1, p. 5-17, 2015.

HAZAN, Z.; ZUMERIS, J.; JACOB, H.; RASKIN, H.; KRATYSH, G.; VISHNIA, M.; LAVIE, G. Effective prevention of microbial biofilm formation on medical devices by low-energy surface acoustic waves. **Antimicrobial Agents and Chemotherapy**, v. 50, n. 12, p. 4144–4152, 2006.

HUANG, X.; LIU, J.; WANG, Y.; LIU, J.; LU, L. The positive effects of Mn^{2+} on nitrogen use and surfactin production by *Bacillus subtilis* ATCC 21332. **Biotechnology and Biotechnological Equipment**, 29(2), 381–389, 2015.

INÈS, M.; DHOUHA, G. Lipopeptide surfactants: Production, recovery and pore forming capacity. **Peptides**, v.71, p. 100–112, 2015.

ISA, M.; SHANNAQ, M.; MOHAMED, N.; HASSAN, A.; AL-SHORGANI, N.; HAMID, A. Antibacterial Activity of Surfactin Produced by *Bacillus subtilis* MSH1. **Transactions on Science and Technology**, v. 4, n. 3, p. 402–407, 2017.

JANEK, T.; ŁUKASZEWICZ, M.; KRASOWSKA, A. Antiadhesive activity of the biosurfactant pseudofactin II secreted by the Arctic bacterium *Pseudomonas fluorescens* BD5. **BMC Microbiology**, v. 12, n. 24, p. 1 - 9, 2012.

JOHANN, S.; SEILER, T.; TISO, T.; BLUHM, K.; BLANK, L. M.; HOLLERT, H. Science of the Total Environment Mechanism-specific and whole-organism ecotoxicity of mono-rhamnolipids. **Science of the Total Environment**, 548–549, 155–163, 2016.

JOSEPH, N.; MATCHAWE, C.; PATRICE, D.N., KASALI, M.; YANN, N.J.; KWETCHE, F.; RENÉ, P. Characterization and evaluation in vitro of the antibacterial activity of tannins from *Garcinia brevipedicellata* (Bark. G) Hutch. & Dalz. **Journal of Pharmacognosy and Phytochemistry**, v. 4, n. 2, p. 81 – 85, 2015.

JOSHI, S.; BHARUCHA, C.; JHA, S.; YADAV, S.; NERURKAR, A.; DESAI, A.J. Biosurfactant production using molasses and whey under thermophilic conditions. **Bioresource Technology**, v. 99, p. 195–199, 2008.

JOSHI, S.J.; GEETHA, S.J.; YADAV, S.; DESAI, A.J. Optimization of Bench-Scale Production of Biosurfactant by *Bacillus Licheniformis* R2. **APCBEE Procedia**, v. 5, p. 232–236, 2013.

KACZOREK, E.; PACHOLAK, A.; ZDARTA, A.; SMUŁEK, W. The impact of biosurfactants on microbial cell properties leading to hydrocarbon bioavailability increase. **Colloids and Interfaces**, v. 2, n. 35, p. 1 - 22, 2018.

KAPPELI, O.; FINNERTY, W. Partition of alkane by an extracellular vesicle derived from hexadecane-grown *Acinetobacter*. **Journal of Bacteriology**, v. 140, n. 2, p. 707–712, 1979.

KESKIN, O.N.; HAN, D.; DEVRIM, A.; CANDAN, O.; UMU, O.; TEKINAY, T. Production and structural characterization of biosurfactant produced by newly isolated *staphylococcus xylosus* STF1 from petroleum contaminated soil. **Journal of Petroleum Science and Engineering,** v. 133, p. 689 – 694, 2015.

KIM, P.; OH, D-K.; LEE, J-K.; KIM, S-Y.; KIM, J-H. Biological modification of the fatty acid group in an emulsan by supplementing fatty acids under conditions inhibiting fatty acid biosynthesis. **Journal of Bioscience and Bioengineering**, v. 90, n. 3, p. 308-312, 2000.

KIM, P.; JAEWON, R.; YOUNG HWAN, K.; YOUN-TAE, C. Production of biosurfactant lipopeptides iturin a, fengycin, and surfactin a from *Bacillus subtilis* CMB32 for Control of Colletotrichum gloeosporioides. **Journal of Microbiology and Biotechnology**, v. 20, n. 1, p. 138 – 145, 2010.

KUYUKINA, M.S.; IVSHINA, I.B.; BAEVA, T.A.; KOCHINA, O. A.; GEIN, S.V; CHERESHNEV, V.A. Trehalolipid biosurfactants from nonpathogenic *Rhodococcus actinobacteria* with diverse immunomodulatory activities. **New Biotechnology**, v. 32, n. 6, p. 559 – 568, 2015.

LOURENÇO, L.A. Production of fungal biosurfactants by solid and submerged cultivation using hydrophobic substrates. 145 p. Doctoral Thesis - Chemical Engineering Course, Federal University of Santa Catarina, Florianópolis, 2017.

MAASS, D.; MOYA RAMÍREZ, I.; GARCÍA ROMÁN, M.; JURADO ALAMEDA, E.; ULSON DE SOUZA, A.A.; BORGES VALLE, J.A.; ALTMAJER VAZ, D. Two-phase olive mill waste (alpeorujo) as carbon source for biosurfactant production. **Journal of Chemical Technology & Biotechnology**, 91(7), 1990–1997, 2015.

MARCELINO, P. Production of second generation biosurfactants by yeast in sugarcane bagasse hemicellulosic hydrolyzate. 157p. Doctoral Thesis - Industrial Biotechnology Course, University of São Paulo, Lorena, 2016

MADDIKERI, G.L.; GOGATE, P.R.; PANDIT, A.B. Improved synthesis of sophorolipids from waste cooking oil using fed batch approach in the presence of ultrasound. **Chemical Engineering Journal**, 263, 479–487, 2105.

MAKKAR, R.S; CAMEOTRA, S.S. Effects of various nutritional supplements on biosurfactant production by a strain of *Bacillus subtilis* at 45°C. **Journal of Surfactants and Detergents**, v. 5, n. 1, p. 11 – 17, 2002.

MAKKAR, R.S.; CAMEOTRA, S.S.; BANAT, I.M. Advances in utilization of renewable substrates for biosurfactant production. **AMB Express**, v. 1, n. 1, p. 1 – 19, 2011.

MORYL, M.; SPĘTANA, M.; DZIUBEK, K.; PARASZKIEWICZ, K.; RÓŻALSKA, S.; PŁAZA, G.A.; RÓŻALSKI, A. Antimicrobial, antiadhesive and antibiofilm potential of lipopeptides synthesised by *Bacillus subtilis*, on uropathogenic bacteria. **Acta Biochimica Polonica**, v. 62, n. 4, p. 725 - 732, 2015.

MOUAFI, F. E.; ABO ELSOUD, M.M.; MOHARAM, M.E. Optimization of biosurfactant production by *Bacillus brevis* using response surface methodology. **Biotechnology Reports**, v. 9, p. 31 – 37, 2016.

MOYA RAMÍREZ, I.M; VAZ, D.A; BANAT, I.M; MARCHANT, R; ALAMEDA, E. J; ROMÁN, M. G. Hydrolysis of Olive Mill Waste to enhance Rhamnolipids and Surfactin Production. **Bioresource Technology**, v. 205, p. 1-6, 2016.

MULLIGAN, C.N. Environmental applications for biosurfactants. **Environmental Pollution**, v. 133, n. 2, p. 183 – 198, 2005.

NDLOVU, T.; RAUTENBACH, M.; VOSLOO, J. A.; KHAN, S.; KHAN, W. Characterisation and antimicrobial activity of biosurfactant extracts produced by *Bacillus amyloliquefaciens* and *Pseudomonas aeruginosa* isolated from a wastewater treatment plant. **AMB Express**, v. 7, n. 108, p. 0363-8, 2017.

NIKAIDO, H. Multidrug Resistance in Bacteria. **Annual Review of Biochemistry**, v. 78, n. 2, p. 119 – 146, 2010.

NITSCHKE, M.; PASTORE, G. Biossurfactantes: propriedades e aplicações. **Química Nova**, v. 25, n. 5, p. 772 – 776, 2002.

NITSCHKE, M.; PASTORE, G. Biosurfactant Production by *Bacillus subtilis* Using Cassava-Processing Effluent. **Applied Biochemistry and Biotechnology**, v. 112, n. 7, p. 163 – 172, 2004.

NITSCHKE, M.; PASTORE, G. M. Production and properties of a surfactant obtained from *Bacillus subtilis* grown on cassava wastewater. **Bioresource Technology,** v. 97, p. 336- 341, 2006.

O'TOOLE, G.A. Microtiter Dish Biofilm Formation Assay. **Journal of Visualized Experiments**, v. 47, p. 10 – 11, 2011.

OHNO, A; ANO, T; SHODA, M. Effect of temperature on production of lipopeptide antibiotics, iturin a and surfactin by a dual producer, *Bacillus subtilis* RB14, in solid-state fermentation. **Journal of Fermentation and Bioengineering**, v. 80, n. 5, p. 517 – 519, 1995.

PACWA-PŁOCINICZAK, M.; PŁAZA, G.A.; PIOTROWSKA-SEGET, Z.; CAMEOTRA, S.S. Environmental applications of biosurfactants: Recent advances. **International Journal of Molecular Sciences**, v. 12, n. 1, p. 633–654, 2011.

PANKEY G.A.; SABATH, L.D. Clinical relevance of bacteriostatic versus bactericidal activity in the treatment of gram-positive bacterial infections. **Clinical Infectious Diseases**, v. 39, n. 5, p. 755 – 756, 2004.

PATIST, A.; KANICKY, J.R.; SHUKLA, P.K.; SHAH, D.O. importance of micellar kinetics in relation to technological processes. **Journal of Colloid and Interface Science**, v. 245, n. 1, p. 1 – 15, 2002.

PEREZ, K.J.; VIANA, J.DOS S.; LOPES, F.C.; PEREIRA, J.Q.; DOS SANTOS, D. M.; OLIVEIRA, J. S.; NARDI, R M.D. *Bacillus spp.* isolated from puba as a source of biosurfactants and antimicrobial lipopeptides. **Frontiers in Microbiology**, v. 8, n. 61, p. 1 - 14, 2017.

PINTO, T.; KANEKO, T.; PINTO, A. Controle Biológico de Qualidade de Produtos Farmacêuticos, Correlatos e Cosméticos. 3. ed. [S.l.: s.n.], 2010. 780 p.

PONTE ROCHA, M.V.; GOMES BARRETO, R.V.; MELO, V.M.M.; BARROS GONÇALVES, L.R. Evaluation of cashew apple juice for surfactin production by *Bacillus subtilis* LAMI008. **Applied Biochemistry and Biotechnology**, v. 155, p. 366–378, 2009.

PORTET-KOLTALO, F.; AMMAMI, M.T.; BENAMARB, A.; WANGB, H.; LE DERFA, F.; DUCLAIROIR-POC, C. Investigation of the release of pahs from artificially contaminated sediments using cyclolipopeptidic biosurfactants. **Journal of Hazardous Materials**, v. 261, p. 593– 601, 2013.

PRESTINACI, F.; PEZZOTTI, P.; PANTOSTI, A. Antimicrobial resistance: a global multifaceted phenomenon. **Pathogens and Global Health**, v. 109, n. 7, p. 309 – 318, 2015.

RAAIJMAKERS, J. M.; DE BRUIJN, I.; NYBROE, O.; ONGENA, M. Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. **FEMS Microbiology Reviews**, v. 34, n. 6, p. 1037 – 1062, 2010.

RAJESWARI, V.; PRIYADARSHINI, S.K.; SARANYA, V.; SUGUNA, P.; SHENBAGARATHAI, R. Immunostimulation by phospholipopeptide biosurfactant from *Staphylococcus hominis* in Oreochromis mossambicus. **Fish and Shellfish Immunology**, v. 48, p. 244 – 253, 2016.

RANDHAWA, K. Biosurfactants Produced by Genetically Manipulated Microorganisms: Challenges and Opportunities. Biosurfactants, In: KOSARIC, N; VARDAR-SUKAN, F (Ed). **Biosurfactants Production and Utilization—Processes, Technologies, and Economics**. Nova York: CRC, 2015. ch 4, p. 49 -72, 2014.

RASHEDI, H.; JAMSHIDI, E.; ASSADI, M. M.; BONAKDARPOUR, B. Biosurfactant production with glucose as a carbon source. **Chemical and Biochemical Engineering Quarterly**, v. 20, n. 1, p. 99 – 106, 2006.

SANA, S.; DATTA, S.; BISWAS, D.; SENGUPTA, D. Assessment of synergistic antibacterial activity of combined biosurfactants revealed by bacterial cell envelop damage. **Biochimica et Biophysica Acta - Biomembranes**, v. 1860, n. 2, p. 579–585, 2018.

SANTOS DA SILVA, M.T., SOARES, C.M.F., LIMA, A.S.; SANTANA, C.C. Integral production and concentration of surfactin from *Bacillus sp*. ITP-001 by semi-batch foam fractionation. **Biochemical Engineering Journal**, v. 104, p. 91 – 97, 2015.

SANTOS, D.K.; RUFINO, R.D.; LUNA, J.M.; SANTOS, V.A.; SARUBBO, L.A. Biosurfactants: multifunctional biomolecules of the 21st century. **International Journal of Molecular Sciences**, v. 18, n. 17, p. 401, 2016.

SARASWATHI, V.; KAMARUDHEEN, N.; BHASKARA RAO, K.V., SANTHAKUMAR, K. Biofilm inhibition formation of clinical strains of *Pseudomonas aeruginosa mutans*, photocatalytic activity of azo dye and GC–MS analysis of leaves of *Lagerstroemia speciosa*. **Journal of Photochemistry and Photobiology B: Biology**, v.169, p. 148–160, 2017.

SATPUTE, S.K.; BHUYAN, S.S.; PARDESI, K.R.; MUJUMDAR, S.S.; DHAKEPHALKAR, P.K.; SHETE, A.M.; CHOPADE, B.A. Molecular Genetics of Biosurfactant Synthesis in Microorganisms. In: Sen, R (Ed). **Advances in Experimental Medicine and Biology** vol. 672. Austin: Landes Bioscience, ch 2, p. 14 – 41, 2010.

SCHMIDELL, W. Agitação e aeração em biorreatores. In: SCHMIDELL, W.; LIMA, U. DE A.; AQUARONE, E.; BORZANI, W. **Biotecnologia Industrial**. São Paulo: Edgard Blucher, v.2, 2001. p. 277–330.

SEN, R. Response surface optimization of the critical media components for the production of surfactin. **Journal of Chemical Technology and Biotechnology**, v. 68, n. 3, p. 263 – 270, 1997.

SHALIGRAM, N.S.; SINGHAL, R.S. Surfactin - a review on biosynthesis, fermentation, purification and applications. **Food Technology and Biotechnology**, v. 48, n. 2, p. 119 – 134, 2010.

SHEPPARD, J.D.; COOPER, D.G. The effects of a biosurfactant on oxygen transfer in a cyclone column reactor. **Journal of Chemical Technology & Biotechnology**, v. 48, p. 325–336, 1990.

SINGH, P.; PATIL, Y.; RALE, V. Biosurfactant production: emerging trends and promising strategies. **Journal of Applied Microbiology**, v. 126, n. 1, p. 2–13, 2018.

SOLANO, C.; ECHEVERZ, M.; LASA, I. Biofilm dispersion and quorum sensing. **Current Opinion in Microbiology**, v. 18, n. 1, p. 96 – 104.

SRIRAM, M.I.; KALISHWARALAL, K.; DEEPAK, V.; GRACEROSEPAT, R.; SRISAKTHI, K.; GURUNATHAN, S. Biofilm inhibition and antimicrobial action of lipopeptide biosurfactant produced by heavy metal tolerant strain *Bacillus cereus* NK1. **Colloids and Surfaces B: Biointerfaces**, v. 85, n. 2, p. 174 – 181, 2011.

SUDARMONO, P.; WIBISANA, A.; LISTRIYANI, L.W.; SUNGKAR, S. Characterization and synergistic antimicrobial evaluation of lipopeptides from *Bacillus amyloliquefaciens* Isolated from Oil-Contaminated Soil. **International Journal of Microbiology**, Article ID 3704198(1-8), 2019.

TRANSPARENCY MARKET RESEARCH. Demand for microbial biosurfactants to rise due to growing use of bio-based cleaning products, REPORTS TMR. [S.l.], 2016. Available in: <https://www.transparencymarketresearch.com/pressrelease/global-microbial-biosurfactants-market.htm>.

VATER, J.; WILDE, C.; FRANKE, P.; MEHTA, N.; CAMEOTRA, S.S. Matrix-assisted laser desorption ionization–time of flight mass spectrometry of lipopeptide biosurfactants in whole cells and culture filtrates. **Applied and Environmental Microbiology**, v. 68, n. 12, p. 6210 – 6219, 2002.

VEDARAMAN, N.; VENKATESH, N. Production of surfactin by *Bacillus subtilis* MTCC 2423 from waste frying oils. **Brazilian Journal of Chemical Engineering**, v. 28, n. 2, p. 175 – 180, 2011.

WEI, Y.; CHU, I. Mn^{2+} improves surfactin production by *Bacillus subtilis*. **Biotechnology Letters**, v. 24, n. 6, p. 479 – 482, 2002.

WEI, Y.; WANG, L.; CHANG, J. Optimizing iron supplement strategies for enhanced surfactin production with *Bacillus subtilis*. **Biotechnology Progress**, v. 20, n. 3, p. 979–983, 2004.

WEI, Y.H.; LAI, C.C.; CHANG, J.S. Using Taguchi experimental design methods to optimize trace element composition for enhanced surfactin production by *Bacillus subtilis* ATCC 21332. **Process Biochemistry**, v. 42, n. 1, p. 40 – 45, 2007.

WILKINSON, J.; MOORE, R. Harry's Cosmeticology. 7. ed. [S.l.]: Chemical Publishing New York, 2011. 934 p.

WILLENBACHER, J.; ZWICK, M.; MOHR, T.; SCHMID, F.; SYLDATK, C.; HAUSMANN, R. Evaluation of different *Bacillus* strains in respect of their ability to produce Surfactin in a model fermentation process with integrated foam fractionation. **Applied Microbiology and Biotechnology**, v. 98, n. 23, p. 9623 – 9632, 2014.

WILLENBACHER, J.; RAU, J.T.; ROGALLA, J.; SYLDATK, C.; HAUSMANN, R. Foam-free production of Surfactin via anaerobic fermentation of *Bacillus subtilis* DSM 10T. **AMB Express**, v. 5, n. 1, 2015.

YEH, M.; WEI, Y.; CHANG, J. Enhanced production of Surfactin from *Bacillus subtilis* by addition of solid carriers. **Biotechnology Progress**, v. 21, n. 4, p. 1329 – 1334, 2005.

YEH, M.; WEI, Y.; CHANG, J. Bioreactor design for enhanced carrier-assisted surfactin production with *Bacillus subtilis*. **Process Biochemistry**, v. 41, n. 8, p. 1799 – 1805, 2006.

ZARAGOZA, A; TERUEL, J.A; ARANDA, F.J; ORTIZ, A. Interaction of a trehalose lipid biosurfactant produced by *Rhodococcus erythropolis* 51T7 with a secretory phospholipase A2. **Journal of Colloid and Interface Science**, v. 408, p. 132–137, 2013.