UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
CENTRO DE BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

Otimização e caracterização de biossurfactantes produzidos por *Bacillus amyloliquefaciens* MO13 para aplicação em estratégias avançadas de biorremediação.

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Dissertação submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do Grau de Mestre em Biologia Celular e Molecular

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Porto Alegre, Outubro de 2019

O presente trabalho foi desenvolvido no Laboratório de Fungos de Importância Médica e Biotecnológica situado no Centro de Biotecnologia da Universidade Federal do Rio Grande do Sul. Para seu desenvolvimento, este trabalho contou com o fomento da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e Programa CIMAR (CAPES n°: 23038.001433/2014-64). Parte desse trabalho foi realizado no Laboratório de Ecologia Microbiana e Biotecnologia no Centro de Biociências situado na Universidade Federal do Ceará.

AGRADECIMENTOS

Agradeço primeiramente o autor arquitetural de toda estrutura atômica ou energética universal. Que trouxe forma ao amórfico e preenchimento ao vazio. Que criou todas as coisas de forma perfeita e agradável. Obrigado Deus por ser meu consolador que sempre esteve ao meu lado em todos os instantes, sendo eles bons ou ruins.

Agradeço aos meu pais Carlos e Valdete e aos meus irmãos Lucas e Felipe e todos os meus parentes e família por terem me dado suporte quando necessitei. Mesmo a saudade sendo imensa pude sentir de longe e saudá-los com mensagens e ligações. Sem vocês eu não traçaria meu caminho até aqui, desde meu nascimento até hoje.

Agradeço a Loeci, Jandir, Rodrigo, William, Adriano, Juciane, Samuel, Andrielle, Ruan, Hallan, Vanessa, Felipe, Teresinha,Teres, José, Carol, Inajara, Adroaldo, Douglas, Raquel, Isaac e Gabriela por fazerem parte da minha história de vários anos no Rio Grande do Sul.

Agradeço à minha orientadora Dr Marilene Henning Vainstein e co-orientadora Dr Vânia Maria Maciel Melo por toda ajuda, suporte e ideias ao longo do desenvolvimento desse trabalho. Aos professores Augusto, Charley e Lívia por estarem sempre dispostos a me ajudarem em minhas dúvidas. Aos meus colegas Douglas, Amanda e Evandro que me auxiliaram na elaboração de experimentos e desenvolvimento dos mesmos. Aos colegas de outros grupos de pesquisa Lais, Cassiane, Vanessa, Rafael, William, Mateus, Augusto, Nicolau, Ane, Camila, Uriel, Júlia, Eamin e Heryk que convivem diariamente comigo no centro de biotecnologia. Obrigado pela amizade e ajuda.

Agradeço a João, Rubens, Daniele, Louise, Jeise, Manoela, Andressa, Giulia, Raquel, Diego, Guilherme, Marco, Thomas, Léo, Vic, Cleiton, Paola, Marcel, Richard, Sarah, Lívia, Aline, Fernanda e Vinícius pela irmandade e por todas as orações e ajuda que me deram em várias situações.

Agradeço às instituições UFRGS e UFC pelo suporte estudantil para realização desse trabalho, assim como os órgão de fomento à pesquisa CAPES e CNPQ pela bolsa concedida nesse tempo.

Agradeço a todos que de alguma forma fizeram ou fazem parte da minha vida e me auxiliaram em qualquer aspecto inerente à minha vida.

“Ubi Bene Ibi Patria”

J. R. R. Tolkien

SUMÁRIO

LISTA DE ABREVIATURAS

BSF	**Biossurfactante**
CG-FID	**Cromatografia Gasosa com Detector de Ionização de Chamas**
CMC	**Concentração Micelar Crítica**
SDS	**Dodecil-sulfato de sódio**
g	**Gramas**
g/L	**Gramas por Litro**
°C	**Graus Celsius**
h	**Horas**
E_{24}	**Índice de Emulsificação**
L	**Litro**
Lpm	**Litros de ar por minuto**
LB	***Luria-Bertani***
m/v	**Massa por volume**
µL	**Microlitro**
mg	**Miligramas**
mL	**Mililitros**
mM	**Milimolar**
mN/m	**MiliNewton por metro**
min	**Minutos**
pH	**Potencial de hidrogênio iônico**
ppm	**Parte por milhão**
rpm	**Rotação por minuto**
vvm	**Volume de ar por volume de meio**
YPD	***Yeast Peptone Dextrose***

RESUMO

Biosurfactantes (BSFs) são moléculas que apresentam estruturas hidrofóbicas e hidrofílicas produzidas por uma multiplicidade de organismos. Essas moléculas podem ser aplicadas nas áreas farmacêutica, cosmética, alimentícia e com abordagens de biorremediação e recuperação de petróleo. No entanto, os custos de produção desses compostos ainda são um obstáculo para aplicações em grande escala. Assim, são necessários métodos que visam melhorar a produção de BSFs buscando aumentar a eficiência através de relação de custo-benefício mais favorável. Neste trabalho, utilizando a linhagem *Bacillus amyloliquefaciens* MO13, parâmetros essenciais para produção de BSFs foram otimizados para alcançar concentrações máximas de BSFs. O desenvolvimento microbiano, consumo de substrato, concentração de BSFs e tensão superficial foram avaliados e suas curvas analisadas. A concentração da fonte de carbono, temperatura, pH, agitação e aeração no cultivo microbiano foram otimizadas para biorreatores empregando-se um planejamento fatorial esférico de composto central, possibilitando encontrar-se as condições ótimas de cultivo. Estimava-se uma produção de BSFs de 17,92 g/L a partir de testes em biorreatores em condições ótimas de cultivo e utilizando glicose como fonte de carbono. Quando testada atingiu 18,16 g/L de BSFs. Para o caso de glicerina residual como fonte de carbono, foram estimados 14,4 g/L de BSFs e quando testada atingiu 13,56 g/L de BSFs. Ambas equações com seus valores críticos tiveram desvios menores que 15% entre valores observados e estimados, validando o modelo. Testes qualitativos para os BSFs produzidos foram todos positivos e a concentração micelar crítica (CMC) foi de 10 mg/L reduzindo a tensão superficial até 30 mN/m, demonstrando assim o potencial uso dos BSFs para biorremediação. Além disso, a caracterização química do perfil de BSFs foi realizada por espectrometria de massa de alta resolução, revelando cinco isoformas de surfactinas e duas isoformas de iturinas. A otimização na produção de BSFs produzidos por *B. amyloliquefaciens* MO13 em biorreatores representa um grande avanço na produção de BSFs, uma vez que a concentração de BSFs em biorreatores foi 6 vezes maior do que produções em frascos

cônicos. Além disso os BSFs produzidos possuem grande potencial de aplicação na recuperação avançada de petróleo, na indústria farmacêutica, alimentícia e cosmética.

ABSTRACT

Biosurfactants (BSFs) are molecules that have hydrophobic and hydrophilic structures produced by many organisms. These molecules can be applied in pharmaceutical, cosmetic, food and bioremediation and oil recovery approaches. However, the production costs of these compounds are still an obstacle for large scale applications. Thus, methods to improve BSF production are needed in order to increase efficiency through a more favorable cost-benefit ratio. In this work, essential parameters for BSF produced by *Bacillus amyloliquefaciens* MO13 were optimized to reach maximum BSF concentration. Microbial development, substrate consumption, BSF concentration and surface tension were evaluated and their curves analyzed. The carbon source concentration, temperature, pH, agitation and aeration in the microbial culture were optimized for bioreactors using a spherical factorial design of central compost, allowing to find the optimum culture conditions. It was estimated a production of 17.92 g/L BSFs from bioreactor tests under optimum culture conditions and using glucose as carbon source. When tested reached 18.16 g /L BSFs. For residual glycerin as carbon source, 14.4 g/L BSFs were estimated and when tested reached 13.56 g/L BSFs. Both equations with their critical values had a deviation lower than 15% between observed and estimated values, which validate the model. Qualitative tests for the BSFs produced were all positive and the critical micellar concentration (CMC) was 10 mg/L reducing surface tension up to 30 mN/m, thus demonstrating the potential use of BSFs for bioremediation. In addition, the chemical characterization of the BSFs profile was performed by high resolution mass spectrometry, revealing five surfactin isoforms and two iturine isoforms. The optimization in BSF production produced by *B. amyloliquefaciens* MO13 in bioreactors represents a major advance in the production of BSFs, since the concentration of BSFs in bioreactors was 6 times higher than those in conical flasks. In addition, the BSFs produced have great potential application in advanced oil recovery, in the pharmaceutical, food and cosmetic industries.

1. INTRODUÇÃO

1.1 Recursos energéticos

Nas últimas décadas, observando a Terra do espaço, percebemos plenamente que vivemos em uma nave espacial que não pode pousar e não pode atracar em qualquer lugar para ser reabastecido ou reparado. Viajamos sozinhos no Universo e só podemos confiar nos recursos disponíveis na superfície ou no porão do nosso planeta e na energia proveniente do Sol. Também percebemos que a Terra é um sistema de partes intrincadamente conectadas e que as atividades humanas podem afetar os ciclos biogeoquímicos. De fato, nosso planeta entrou em uma nova época; o Antropoceno, caracterizado por um aumento dramático do tamanho da pegada ecológica humana (Armaroli and Balzani, 2010).

A energia está incorporada em qualquer tipo de mercadoria e é necessária para produzir qualquer tipo de serviço. O que torna a vida moderna aparentemente tão fácil em comparação com a de nossos antepassados, ou mesmo com a de bilhões de pessoas que ainda vivem na pobreza, é um fluxo constante de recursos baratos que não durará para sempre e também aprendemos que seu uso causou e ainda está causando sérios danos à atmosfera da Terra. Os combustíveis fósseis contribuíram indiretamente para estabelecer disparidades e questões na sociedade humana: quase metade do suprimento total de energia primária é consumida por cerca de 10% da população que vive nos países ricos, enquanto os 25% mais pobres consomem menos de 3% de energia global (Armaroli and Balzani, 2010).

Atualmente todo mundo quer ter mais e mais energia, uma atitude com uma variedade de problemas emaranhados. Quando ocorre um blecaute em um país por qualquer motivo, a solução proposta pelos políticos que buscam ser (re) eleitos é a de fabricar novas usinas de energia. Muitos economistas parecem acreditar que o bem-estar se correlaciona com o consumo de energia, que os preços da energia refletem todos os custos significativos e que quaisquer problemas sociais podem ser resolvidos pelo aumento do crescimento econômico, mas vários cientistas estão convencidos de

que a tecnologia resolverá problemas de energia. Um desses problemas é o uso do petróleo como principal recurso energético do mundo (Armaroli and Balzani, 2010).

As pressões ambientais decorrentes da exploração, processamento, distribuição e combustão de combustíveis fósseis são múltiplas (Epstein et al., 2002; O'Rourke and Connolly, 2003) e, com elas, consequências novas e inesperadas. Por exemplo, a gigantesca erupção do vulcão de lama em no oeste de Java, que começou em 2006, pode ter sido desencadeada por atividades de perfuração para exploração de gás (American Association of Geologists, 2003; Marshall, 2008). Provavelmente menos imprevisível foi a observação de que a extração de petróleo e gás é amplamente responsável pelo abaixamento dos deltas dos rios em todo o mundo. Essas áreas ecologicamente frágeis sofreram submersão temporária de pelo menos 260.000 km^2 durante a última década e podem ser mais afetadas pelo aumento do nível do mar induzido pelo aquecimento global (Syvitski et al., 2009; Bohannon, 2010).

1.2 Poluição ambiental

Segundo Deblonde et al. (2011) atualmente milhares de indústrias, agricultores e a população em geral utilizam a água, tendo um papel importante na questão dos poluentes em ambientes aquáticos e solo. As práticas indústrias, agrícolas e residenciais geram muitos poluentes, alterando o ciclo da água, poluindo o meio ambiente e causando uma preocupação global ligada ao seu eventual impacto na vida selvagem e na saúde humana. Por 20 anos, muitos artigos relataram a presença de novos compostos, chamados "poluentes emergentes", em águas residuais e ambientes aquáticos (Pham and Proulx, 1997; Vogelsang et al., 2006; Rosal et al., 2010). Poluentes emergentes são novos produtos ou produtos químicos sem regulação e cujos efeitos no ambiente e na saúde humana são ainda desconhecidos. A diretiva sobre tratamento da água da UE 2000/06/CE anunciou no Anexo X uma lista de 33 substâncias que incluem metais, pesticidas, ftalatos, hidrocarbonetos aromáticos policíclicos e desreguladores endócrinos que devem ser removidas com o objetivo de manter a qualidade e preservar o bom estado da água (Deblonde et al., 2011). As autoridades devem prestar especial atenção à sua descarga industrial na água, mas também devem garantir a segurança da população. O regulamento REACH, estabelecido em 2007 na Europa, visa identificar

produtos químicos perigosos em águas e substituí-los por outros menos perigosos. Desde sua fundação, foi proibido o lançamento de três ftalatos (DEHP, DBP e BBP) classificados como cancerígenos, tóxicos e persistentes para o ambiente (Deblonde et al., 2011). Ente os hidrocarbonetos poluentes estão os hidrocarbonetos de petróleo, que atualmente são considerados uma fonte única de energia porque milhares de produtos consumidos diariamente vêm do petróleo. Alega-se que um aumento na produção de petróleo gera dois fenômenos: primeiro, o nível de poluição ambiental que pode afetar a saúde pública e, segundo, o declínio na produção de petróleo nas próximas décadas, com o esgotamento do petróleo ao redor do mundo pelo seu excessivo consumo, que pode afetar o nível econômico do petróleo nos países produtores (Frumkin et al., 2009). Assim, são necessários mais estudos para atender os requisitos do mercado nas próximas décadas. Os impactos na saúde humana causados por derramamentos de óleo se espalharam para além das águas, plantas, animais e habitats, afetando também a saúde das comunidades costeiras em todo o local do acidente. Os impactos variaram de acordo com o tipo de atividade econômica regional anterior, diminuindo a renda local, mas também novas oportunidades de negócios (Griffiths, 2012; Fan et al., 2015; Gould et al., 2015; Hansel et al., 2015). O derramamento de óleo impacta diretamente as áreas econômicas relacionadas com as indústrias de pesca e frutos do mar, turismo e petróleo e gás. Estudos demonstram que a população local que depende dessas indústrias como fonte de renda, e que tiveram perda de receita com o derramamento de óleo eram mais propensas a se sentir ansiosos ou deprimidos, a ingerir maiores quantidades de bebidas alcóolicas ou a ter mais pensamentos suicidas do que outros residentes. Pessoas com vínculos com a pesca tiveram maior probabilidade de apresentar níveis mais altos de estresse do que indivíduos que dependiam de outras áreas afetadas pelo derramamento (Morris et al., 2013; Fan et al., 2015; Mayer et al., 2015; Osofsky et al., 2015).

Um exemplo muito conhecido de acidente com derramamento de petróleo é o ocorrido com o *Deep Water Horizon* no Golfo do México, produzindo um profundo impacto na economia e na segurança ambiental, que ainda é o foco da atenção (Xu et al., 2018). Embora se esteja cada vez mais preocupados com os efeitos tóxicos da poluição por petróleo em seres humanos e animais nas áreas afetadas (Díez et al., 2007; Mason et al., 2012), os fortes impactos tóxicos dos hidrocarbonetos de petróleo nas

comunidades microbianas afetadas são frequentemente negligenciados (Rivers et al., 2013; Overholt et al., 2016). Labud et al. (2007) relataram que os hidrocarbonetos de petróleo inibiram o desenvolvimento de muitos microrganismos. Em experimentos de exposição ao diesel, os efeitos primários da toxicidade reduziram a riqueza de espécies, uniformidade e diversidade filogenética, com a comunidade resultante sendo fortemente dominada por algumas espécies, principalmente *Pseudomonas*. Essas mudanças estavam relacionadas à interrupção do ciclo do nitrogênio, com espécies e genes funcionais envolvidos na nitrificação sendo significativamente reduzidos (van Dorst et al., 2014). Cerniglia et al. (1983) investigaram a toxicidade do naftaleno, 1-metilnaftaleno e 2-metilnaftaleno, bem como seus derivados oxigenados para células bacterianas de *Agmenellum quadruplicatum*, e verificaram que esses compostos não produziam efeitos inibitórios significativos no crescimento bacteriano. No entanto, os derivados fenólicos e quinônicos de naftaleno inibiram o crescimento bacteriano. Outro estudo relatou que certos intermediários metabólicos com solubilidade relativamente alta, produzida a partir da degradação de hidrocarbonetos de petróleo por bactérias, podem ter citotoxicidade maior que as moléculas-mãe e, portanto, danificar as bactérias (Hou et al., 2018). Por conseguinte, enquanto algumas bactérias sensíveis aos hidrocarbonetos de petróleo são bastante inibidas após a exposição a hidrocarbonetos de petróleo, outras são estimuladas pois podem degradar eficientemente estes hidrocarbonetos, bem como bactérias que podem tirar vantagem dos metabólitos intermediários citotóxicos. No entanto, a limpeza dos poluentes do óleo de petróleo, contando apenas com o metabolismo energético desses microrganismos endógenos, leva décadas para ocorrer; portanto, é necessário desenvolver medidas de intervenção para acelerar o processo de degradação.

1.3 Remediação de locais degradados

Até o momento, é conhecida uma variedade de métodos para a remediação de ambientes impactados por compostos recalcitrantes, como água, água subterrânea, águas residuais industriais, solos, sedimentos e lodos. É mais conveniente dividi-los em três categorias principais: primeiro, métodos físicos, como lavagem do solo,

encapsulamento ou eletrocinese, segundo métodos químicos, como solidificação, precipitação ou troca iônica, e terceiro, métodos biológicos (Bradl and Xenidis, 2005).

1.3.1 Remediação física

As técnicas de remediação física incluem lavagem do solo, vitrificação, encapsulamento de áreas contaminadas por camadas verticais e horizontais impermeáveis, eletrocinese e sistemas de barreira permeáveis.

A lavagem do solo é uma técnica amplamente usada para remover metais pesados e poluentes orgânicos dos solos. A maioria das etapas do processo de lavagem de solo não foi desenvolvida para a remediação de solos contaminados, mas tem sido usada há muito tempo na indústria de processamento mineral. O principal princípio da lavagem do solo é uma classificação seletiva de compostos altamente contaminados, seguida pela separação de fase sólida/líquida da suspensão restante. Para a limpeza destes compostos, podem ser utilizados processos alternativos como flotação, lixiviação ou separação magnética de alto gradiente. A lavagem do solo não ataca diretamente os poluentes, mas separa diferentes frações do solo com alto teor de contaminantes das frações do solo com baixo teor de contaminantes. Em geral, os contaminantes concentram-se na fração fina da panícula. A fração grosseira pouco contaminada pode ser reutilizada, enquanto a fração altamente contaminada deve ser submetida a tratamento adicional. O processo de lavagem de solos consiste em duas etapas principais, a liberação úmida e a unidade de classificação (Wilichowski, 2001).

A vitrificação é um processo pelo qual os materiais são reunidos em vidro ou substâncias semelhantes a vidro (Reddi et al., 2000). O vidro é caracterizado por sua não cristalinidade e rigidez, além de sua porosidade muito limitada. Para a remediação de solos e resíduos, a vitrificação pode ser usada tanto como técnica *in situ* quanto como técnica *ex situ*. O processamento e aquecimento de solo ou lixo escavado é mais fácil de controlar do que o processo *in situ*, mas é desvantajoso devido à maior exposição se forem tratados contaminantes radioativos ou dispersivos. A vitrificação utiliza calor produzido por diferentes fontes, que destrói contaminantes orgânicos por pirólise ou combustão e funde metais inorgânicos (incluindo elementos radioativos) na estrutura do

vidro. A formação de vidro requer a disponibilidade de elementos componentes, o que pode não ser sempre o caso em meios contaminados. Nesses casos, aditivos para melhoria da formação de vidro podem ser adicionados ao meio deficiente.

O cercamento de áreas contaminadas é comumente usado em casos de contenção ou prevenção de poluição (Philip, 2001). A maioria dessas técnicas foi adaptada ao uso em engenharia ambiental a partir do estanque à água em poços de construção (Bradl and Xenidis, 2005). O princípio básico é a construção subterrânea de uma barreira vertical impermeável para permitir a contenção de gases e líquidos. Foi desenvolvida uma variedade de métodos de construção, como paredes de lama de corte usando principalmente lamas de cimento-água de bentonita, paredes finas, paredes de estacas pranchas, paredes de estacas chatas, cortinas de rejunte, paredes de injeção e barreiras congeladas (Meggyes and Pye, 1995).

A descontaminação eletrocinética ou a eletrorremediação de locais poluídos é uma tecnologia promissora de tratamento in situ, especialmente para solos de grão fino (Chandra et al., 2013). Os fenômenos eletrocinéticos têm sido aplicados a propósitos ambientais desde a década de 1990 (Bruell et al., 1992; Acar and Alshawabkeh, 1993; Probstein and Hicks, 1993).

Os eletrodos são colocados no solo e uma corrente elétrica direta é aplicada, o que induz movimentos de íons para seus respectivos eletrodos. Os três principais fenômenos eletrocinéticos que ocorrem são eletro-osmose, eletromigração e eletroforese (Probstein, 1994), enquanto os principais mecanismos físicos, que transportam contaminantes no campo elétrico, são eletro-migração e eletro-osmose em solos de grão fino, e eletromigração e eletroforese em solos de grão grosso.

Geralmente, as águas subterrâneas contaminadas são remediadas retirando as águas subterrâneas do aquífero, tratando a água poluída em uma estação de tratamento de águas subterrâneas e, em seguida, alimentando a água tratada no aquífero novamente ou descarregando-a (tecnologia de bombear e tratar). No entanto, a experiência prática mostra que essa tecnologia é muito ineficiente, especialmente no tratamento de águas subterrâneas contaminadas por contaminantes orgânicos, como hidrocarbonetos clorados, óleos minerais, hidrocarbonetos aromáticos policíclicos ou benzenos, toluenos, etilbenzenos e xilenos (Mackay and Cherry, 1989). Essa ineficiência

é causada pela falta de homogeneidade natural subterrânea, pelo fluxo não homogêneo e pela distribuição irregular de contaminantes, bem como pela baixa solubilidade do contaminante e pela lenta difusão do contaminante que contaminou a matriz rochosa por muito tempo (Teutsch et al., 1996). Como consequência, a remediação hidráulica por sistemas ativos, isto é, sistemas que requerem uma entrada permanente de energia, só é eficaz em regiões de alta permeabilidade. As regiões de baixa permeabilidade de um aquífero não são influenciadas por este procedimento.

1.3.2 Remediação química

As técnicas de remediação química para a remoção de metais pesados em águas subterrâneas contaminadas e águas residuais incluem a precipitação de metais dissolvidos, troca de íons, floculação e processos de filtro de membrana, como micro e ultrafiltração e osmose reversa. Ao lidar com solos contaminados, podem ser utilizados processos de estabilização e solidificação (Koshlaf and S Ball, 2017). Em meios fluidos, metais pesados ocorrem como íons dissolvidos ou ligados a partículas coloidais. Uma distinção básica deve ser feita entre os processos de precipitação e floculação. A precipitação é um processo no qual há uma transferência de fase de componentes iônicos solúveis para uma fase iônica não solúvel. A floculação é um processo no qual pequenos sólidos não dissolvidos de tamanho coloidal são agregados em bandos sólidos maiores. Esses bandos são então separados mecanicamente do fluido por sedimentação, centrifugação ou flotação (Förstner, 1998). A solidificação visa a redução da mobilidade dos metais pesados, prendendo-os ou imobilizando-os no solo, injetando ou misturando agentes imobilizadores no solo contaminado. Além de reduzir a solubilidade química, os processos de estabilização e solidificação podem ser utilizados para aumentar a resistência mecânica e reduzir a permeabilidade do solo (Koshlaf and S Ball, 2017).

Na precipitação, os íons metálicos dissolvidos reagem com os precipitantes adicionados, formando compostos insolúveis. Esses sólidos sedimentam e podem ser removidos do líquido sobrenadante por diferentes técnicas de separação sólido / líquido. Os principais parâmetros químicos, que são importantes no processo de precipitação,

são pH e concentração. Em geral, os metais pesados tendem a ser apresentados na forma iônica a baixos níveis de pH, enquanto tendem a precipitar quando o pH é elevado. Os metais pesados podem ser precipitados como hidróxidos, sulfetos, carbonatos e outros insolúveis (Koshlaf and S Ball, 2017).

Trocadores de íons são comumente usados para a remoção de metais pesados, especialmente quando se trata de águas residuais da indústria de processamento de metais. O tratamento de superfície, como a galvanoplastia, produz águas contendo cianeto, cobre, níquel e cádmio. O princípio básico de um trocador de íons é uma matriz ou resina carregada de contra-íons dissociáveis. Os trocadores de íons mais comuns são feitos de poliestireno entrelaçado e resinas de poliacrilato ou condensação feitas de fenol e formaldeído (Förstner, 1998). Para minimizar as emissões de metais e as águas residuais, os sistemas de ciclo fechado são ideais (Cushnie and Jr., 1985; Patterson, 1985).

Como foi mencionado acima, uma distinção clara deve ser feita entre precipitação e floculação. A floculação é usada para transformar as partículas coloidais suspensas em uma forma para que possam ser separadas mecanicamente da solução sobrenadante com a ajuda de floculantes. As partículas coloidais primárias têm um diâmetro típico de partícula na faixa de 10 a 7 cm (Lagaly, 2007). Para entender os mecanismos de floculação, as diferentes forças que atuam em um sistema coloidal devem ser levadas em consideração.

A retenção de panículas por filtração é uma técnica amplamente utilizada no tratamento de águas residuais e baseia-se em interações físicas entre as partículas e o meio granular. As técnicas de filtragem estão disponíveis para tamanhos de partículas entre 1 mm e 0,1 m. Existem diferentes processos de filtração, que podem ser utilizados, como filtragem por pressão, filtragem por gravidade, filtros de camada única, camada dupla ou tripla, filtragem a jusante ou a montante e filtros de superfície (Kunz, 1995). Todos os filtros devem ser controlados regularmente com lavagem frequente. Os filtros de membrana são usados sempre que um gradiente de pressão ou um campo elétrico força a solução através de uma membrana (Cartwright, 1988).

Solidificação e estabilização (S/S) são processos de tratamento que reduzem a mobilidade de contaminantes, aprisionando-os ou imobilizando-os no solo por meios

químicos e / ou físicos. Eles foram inicialmente aplicados para o tratamento de resíduos radioativos e consolidação de lodos industriais. Após mudanças na legislação, elas também foram aplicadas a resíduos perigosos e solos contaminados. No tratamento de Solidificação/Estabilização (S/S), os agentes são misturados ou injetados no solo contaminado para atingir um ou mais dos seguintes objetivos (US Environmental Protection Agency, 1989, 1997; Wiles, 1989).

Primeiro, o manuseio e as características físicas de um material contaminado precisam ser aprimorados pelo tratamento. Segundo, a área de superfície exposta do material contaminado, através da qual a transferência de massa ou a perda de contaminantes pode ocorrer, deve ser reduzida pela formação de uma estrutura cristalina, vítrea ou polimérica, que circunda as partículas do material. Terceiro, o contato entre fluidos de transporte e contaminantes deve ser limitado, reduzindo a permeabilidade do material. Finalmente, a solubilidade de quaisquer constituintes perigosos contidos no material tratado deve ser reduzida pela formação de espécies absorvidas ou precipitados de baixa solubilidade (por exemplo, hidróxidos, carbonatos, silicatos, fosfatos ou sulfuretos).

1.3.2.1 Surfactantes

Para melhorar a biodisponibilidade de hidrocarbonetos de petróleo, uma abordagem promissora é a aplicação de surfactantes (Kleinert et al., 2001), que podem aumentar as taxas de dissolução ou dessorção, levando à solubilização ou emulsificação de poluentes de hidrocarbonetos de petróleo (Varjani and Upasani, 2017). Chen and Hussey (2007) constataram que com a adesão de *Bacillus* sp. o DQ02 em hidrocarboneto aumentou 44% na presença de ramnolipídios e que a degradação do n-hexadecano aumentou 11,6% em comparação com o tratamento na ausência de ramnolipídios. No entanto, alguns surfactantes, como o Corexit 9500, apresentaram impactos adversos em bactérias degradadoras de óleo (Kleindienst et al., 2015b) devido à toxicidade do surfactante em relação a bactérias ou como resultado da competição do surfactante com substratos de hidrocarbonetos (Laha and Luthy, 1991; S. Liu et al., 2016). Em vista disso, a seleção de surfactantes apropriados é de grande importância para a remediação da poluição e a prevenção da mesma.

1.3.3 Biorremediação

De acordo com (Xu et al., 2018), existe uma ameaça constante de contaminação onde quer que o petróleo seja explorado quando associado a uma capacidade insuficiente de lidar com ambientes contaminados por óleo, especialmente em ambientes extremos ou únicos, como regiões polares, áreas do fundo do mar, desertos e áreas úmidas. Embora a poluição por petróleo seja difícil de tratar, as bactérias degradadoras de hidrocarbonetos de petróleo evoluíram como resultado de adaptação ao meio contaminado. Alguns estudos revelam que existe um grande número de bactérias degradadoras de hidrocarbonetos em ambientes ricos em petróleo, como áreas de derramamento de óleo e reservatórios de petróleo (Hazen et al., 2010; Yang et al., 2015), e que sua abundância e quantidade estão intimamente relacionadas aos tipos de hidrocarbonetos de petróleo e aos fatores ambientais circundantes (Fuentes et al., 2016; Varjani and Gnansounou, 2017). Estas bactérias endógenas metabolizam hidrocarbonetos devido às suas necessidades energéticas e de carbono para crescimento e reprodução, bem como à exigência de aliviar o estresse fisiológico causado pela presença de hidrocarbonetos de petróleo no ambiente microbiano a granel, sendo então candidatos ao tratamento de poluentes de petróleo (Margesin et al., 2003; Hazen et al., 2010; Ron and Rosenberg, 2014; Kleindienst et al., 2015a; Lea-Smith et al., 2015; Dombrowski et al., 2016; Dvořák et al., 2017). Estas bactérias podem ser utilizadas para degradar os resíduos produzidos pelas indústrias alimentícia, agrícola, química e farmacêutica e nos últimos anos, o uso de bactérias para lidar com poluentes ambientais tornou-se uma tecnologia promissora devido ao seu baixo custo e natureza ecológica (Guerra et al., 2018). O desenvolvimento e aprimoramento contínuos da tecnologia de remediação microbiana, como sequenciamento de alto rendimento e técnicas microfluídicas, fornecem um novo método para remediar a poluição por hidrocarbonetos de petróleo pois permitem a triagem e identificação de microrganismos funcionais de ambientes contaminados com hidrocarbonetos de petróleo (Jiang et al., 2016; Guerra et al., 2018).

Das bactérias isoladas e identificadas atualmente, as vias de degradação foram estudadas e demonstraram empregar reações oxidantes. O primeiro passo no processo de degradação do óleo de petróleo frequentemente requer a participação de oxigenases ligadas à membrana bacteriana, que requerem contato direto e eficaz entre células bacterianas e substratos de hidrocarbonetos de petróleo. No entanto, essas vias diferem bastante devido às oxigenases específicas encontradas em diferentes espécies bacterianas. Por exemplo, algumas bactérias podem metabolizar alcanos específicos, enquanto outras quebram frações aromáticas ou de resina de hidrocarbonetos. Esse fenômeno está relacionado à estrutura química dos componentes de hidrocarbonetos de petróleo. Estudos recentes identificaram bactérias de mais de 79 gêneros capazes de degradar hidrocarbonetos de petróleo (Tremblay et al., 2017). Muitas dessas bactérias, como *Achromobacter*, *Acinetobacter*, *Alkanindiges*, *Alteromonas*, *Arthrobacter*, *Burkholderia*, *Dietzia*, *Enterobacter*, *Kocuria*, *Marinobacter*, *Mycobacterium*, *Pandoraea*, *Pseudomonas*, *Staphylococcus*, *Streptobacillus*, *Streptococcus* já são estudadas para a degradação de petróleo (Margesin et al., 2003; Chaerun et al., 2004; Jin et al., 2012; Nie et al., 2014; Varjani and Upasani, 2016; Sarkar et al., 2017; Varjani, 2017; Xu et al., 2017). Comunidades com altas porcentagens de *Alkanindiges* sp. no solo foram relatadas e relacionadas com restrições ambientais, como a poluição por diesel (Fuentes et al., 2016). Da mesma forma, algumas bactérias hidrocarbonoclásticas obrigatórias (OHCB), incluindo *Alcanivorax*, *Marinobacter*, *Thallassolituus*, *Cycloclasticus*, *Oleispira* e algumas outras, mostraram uma baixa abundância ou status indetectáveis antes da poluição, mas foram consideradas dominantes após a contaminação do óleo de petróleo (Yakimov et al., 2007). Esses fenômenos sugerem que esses microrganismos são cruciais para a degradação dos hidrocarbonetos de petróleo e influenciam significativamente a transformação e o destino dos hidrocarbonetos de petróleo no meio ambiente. Embora algumas bactérias tenham sido relatadas como tendo um amplo espectro de capacidade de degradação de hidrocarbonetos de petróleo, *Dietzia* sp. utiliza n-alcanos (C6-C40) e outros compostos como únicas fontes de carbono (Wang et al., 2011), porém *Achromobacter xylosoxidans* DN002 funciona bem em uma variedade de hidrocarbonetos monoaromáticos e poliaromáticos (Ma et al., 2015). Até o momento não existem relatos de isolados bacterianos que podem degradar de maneira autónoma o

petróleo. De fato, a maioria das bactérias só podem degradar ou utilizar efetivamente determinados componentes de hidrocarbonetos de petróleo, enquanto outros estão completamente indisponíveis (Chaerun et al., 2004; Varjani, 2017). Isso pode ser atribuído ao fato de que diferentes bactérias endógenas têm diferentes enzimas catalíticas; assim, seus papéis em locais contaminados por óleo também variam amplamente. Isso também implica que a remediação da contaminação por hidrocarbonetos de petróleo requer a ação conjunta de múltiplas bactérias funcionais para obter o melhor efeito de purificação ambiental (Dombrowski et al., 2016). Com base nessa visão, Varjani and Upasani (2016) construíram um Consórcio Bacteriano de Hidrocarbonetos Halotolerante (HUBC) que consiste em isolados de *Ochrobactrum*, *Stenotrophomonas maltophilia* e *Pseudomonas aeruginosa*, que se mostraram bons degradadores de petróleo bruto (3% v/v), com uma alta porcentagem de degradação (83,49%). Tao et al. (2017) utilizaram uma co-cultura definida de um consórcio bacteriano endógeno e de *Bacillus subtilis* exógeno para acelerar efetivamente a degradação do petróleo bruto. Wang et al. (2018) descobriram que um consórcio bacteriano endógeno no acidente com derramamento de petróleo em Penglai (China) teve maior eficiência de degradação do óleo em comparação com bactérias individuais e demonstrou que o consórcio tinha o potencial de biorremediar o petróleo bruto disperso no ecossistema marinho.

Mesmo com vários estudos e com a taxa de degradação de hidrocarbonetos laboratoriais altas, a hidrofobicidade e a baixa solubilidade em água da maioria dos hidrocarbonetos de petróleo, faz com que a taxa de biodegradação seja limitada no ambiente. Isso porque a biodegradação depende da biodisponibilidade limitada de hidrocarbonetos de petróleo para bactérias e o fato de que o contato celular bacteriano com substratos de hidrocarbonetos é um requisito antes da introdução de oxigênio molecular nas moléculas pelas oxigenases funcionais (Vasileva-Tonkova et al., 2008; Hua and Wang, 2014). Por essa razão, as bactérias desenvolveram medidas contra contaminantes do petróleo, como melhorar a capacidade de adesão das células, alterando seus componentes de superfície e secretando um bioemulsificador para melhorar seu acesso aos substratos de hidrocarbonetos alvo (Kaczorek et al., 2012; Krasowska and Sigler, 2014).

As propriedades da superfície bacteriana são essenciais para a biodegradação efetiva de substratos de hidrocarbonetos hidrofóbicos e seus mecanismos de adesão são de grande importância (Zhang et al., 2015). Rosenberg and Ron (1999) verificaram que a adesão de poluentes hidrofóbicos às células bacterianas está relacionada principalmente as fímbrias hidrofóbicas, fibrilas, proteínas da membrana externa e lipídios, bem como a determinadas moléculas pequenas presentes na superfície celular, como a gramicidina S e a prodigiosina. As fímbrias presentes nas superfícies bacterianas foram confirmadas como necessárias para o crescimento de *Acinetobacter sp*. RAG-1, com alcano C16 como fonte de carbono e benéfico para a adesão bacteriana, substratos hidrofóbicos de assimilação e sua atividade metabólica (Rosenberg and Rosenberg, 1985). No entanto, cápsulas bacterianas e vários exopolissacarídeos aniônicos produzem efeitos inibitórios na adesão do substrato hidrocarboneto. Por exemplo, *Bacillus licheniformis* diminui a hidrofobicidade da superfície celular em resposta à exposição a solventes orgânicos e tem pouca afinidade por compostos orgânicos tóxicos (Torres et al., 2011). Embora a adesão bacteriana possa melhorar a biodegradação de hidrocarbonetos hidrofóbicos, não é necessário anexar células bacterianas a substratos direcionados (Abbasnezhad et al., 2011). Isso ocorre porque, em alguns casos, bactérias com alta hidrofobicidade superficial são facilmente agregadas e formam biofilmes, produzindo riscos potenciais como doenças (Doyle, 2000). De fato, não apenas as bactérias hidrofóbicas podem biodegradar poluentes hidrofóbicos. Várias bactérias hidrofílicas resistentes a solventes também são capazes de metabolizar esses poluentes (Heipieper et al., 2007), o que pode ser devido à modificação de lipopolissacarídeos ou porinas da membrana externa da superfície bacteriana (Krasowska and Sigler, 2014). Megharaj et al. (2011) também relataram que as bactérias resistentes a solventes foram as primeiras a colonizar e dominar a remoção de poluentes. Portanto, o uso de microrganismos hidrofílicos no tratamento de poluentes de hidrocarbonetos parece ser mais vantajoso que os microrganismos hidrofóbicos (Obuekwe et al., 2009).

Com tantos microrganismos diferentes, foram desenvolvidos diferentes métodos e estratégias de biorremediação. As mais utilizadas são atenuação natural, bioaumentação, bioestimulação.

1.3.3.1 Atenuação natural

Por definição, a atenuação natural é o processo mais simples de biorremediação pelo qual a população microbiana endógena (bactérias e fungos) elimina ou desintoxica poluentes perigosos para a saúde humana e meio ambiente em formas menos tóxicas para atenuar o local poluído. Durante esse processo, os microrganismos endógenos utilizam os poluentes como fonte de carbono com base em suas vias metabólicas naturais. Essa tecnologia requer apenas o monitoramento do processo. Quando a poluição ocorre, os microrganismos endógenos degradadores de hidrocarbonetos aumentam rapidamente e se adaptam aos poluentes adicionados recentemente, resultando em degradação de contaminantes; no entanto, a diversidade microbiana pode ser reduzida (McKew et al., 2007). Esse processo natural de remediação ocorre na maioria dos locais contaminados e pode ser aplicado em áreas onde outros mecanismos de restauração não podem ser aplicados ou em locais poluídos relativamente (Pilon-smits, 2005). Pesquisas de biodegradação mostraram que a atenuação natural é eficaz em locais contaminados com petróleo, estimando que quase 25% dos solos poluídos com hidrocarbonetos petrogênicos foram tratados através da atenuação natural (Holden et al., 2002). Um estudo comparativo mostrou que a atenuação natural pode ser tão ou mais eficaz que os métodos de bioestimulação e bioaumentação e que os degradadores de hidrocarbonetos de ocorrência natural associados ao próprio óleo são capazes de utilizar hidrocarbonetos sem nenhum aprimoramento (Makadia et al., 2011; Sheppard et al., 2012). A atenuação natural, no entanto, é frequentemente limitada pela disponibilidade de nutrientes. Além disso, comunidades microbianas com alta atividade degradante podem não estar disponíveis no local ou não possuir os genes catabólicos necessários para a degradação completa, portanto, práticas de remediação desenvolvidas são essenciais nesses casos (Koshlaf and S Ball, 2017).

1.3.3.2 Bioaumentação

Um estudo de campo mostrou que a bioaumentação com um consórcio artificial contendo *Aeromonas hydrophila*, *Alcaligenes xylosoxidans*, *Gordonia* sp., *Pseudomonas fluorescens*, *Pseudomonas putida*, *Rhodococcus equi*, *S. maltophilia* e *Xanthomonas* sp.

contribuíram para a alta eficiência de biodegradação (89%) em um tratamento de 365 dias em solos contaminados com óleo diesel (Szulc et al., 2014).

A capacidade da comunidade microbiana no solo de metabolizar poluentes do petróleo é determinada por sua estrutura e diversidade (Rodríguez-Blanco et al., 2010).

Em solos com número insuficiente ou não detectável de microrganismos endógenos degradantes de poluentes, a atenuação natural talvez seja inadequada como estratégia de remediação, portanto outras tecnologias de biorremediação devem ser aplicadas. Um dos métodos alternativos de biorremediação *in situ* é a bioaumentação. Esta aplicação envolve a adição de linhagens únicas ou consórcios de micróbios degradadores de hidrocarbonetos (bactérias ou, em menor grau, fungos) com capacidade catalítica para remediar locais contaminados, a fim de acelerar a biodegradação de compostos orgânicos indesejados. As linhagens que são bioaumentadas são normalmente isolados de ambientes poluídos por hidrocarbonetos de petróleo (Sarkar et al., 2005). A lógica por trás da bioaumentação é que a introdução de microrganismos degradantes de hidrocarbonetos no solo poluído melhora a capacidade biodegradativa da população endógena. Pesquisas relataram que a aplicação de bioaumentação em ambientes marinhos e terrestres contaminados exibia eficiência de tratamento superior (Tang et al., 2010; Kadali et al., 2012; Li et al., 2012; Agnello et al., 2016).

O efeito da introdução de microrganismos exógenos na diversidade e atividade do ecossistema natural ainda precisa ser investigado. Por exemplo, um estudo recente mostrou que a adição de microrganismos exógenos levou a mudanças significativas na composição da comunidade microbiana do solo (Festa et al., 2016). Em alguns casos, os microrganismos degradadores de hidrocarbonetos inoculados não demonstraram qualquer atividade de degradação (Yu et al., 2005; Margesin et al., 2007).

1.3.3.3 Bioestimulação

Uma estratégia de biorremediação amplamente praticada que explora a capacidade dos microrganismos de degradar e/ou desintoxicar poluentes de petróleo no solo é denominada bioestimulação. Este procedimento resulta na estimulação do crescimento e da atividade dos microrganismos endógenos presentes no local

contaminado através da adição de nutrientes, a fim de acelerar a taxa de biodegradação natural (Nikolopoulou and Kalogerakis, 2010). Existem alguns relatos na literatura que altas concentrações de hidrocarboneto de petróleo, contendo cerca de 80% de carbono, podem levar a uma rápida redução na concentração de nutrientes inorgânicos presentes no solo como por exemplo, nitrogênio e fósforo (Alexander, 1999). O nitrogênio é um exemplo de nutriente encontrado em ambientes terrestres de várias formas. É um nutriente essencial para o crescimento e a atividade microbiana do solo, aumentando a taxa de crescimento de células microbianas degradadoras de hidrocarbonetos e, consequentemente, aumentando a taxa de degradação de hidrocarbonetos (Walworth et al., 2007). A bioestimulação geralmente inclui a adição de nutrientes e receptores de elétrons (como P, C, N e O_2) e representa uma tecnologia eficaz para restaurar locais poluídos por óleo e deficientes em nutrientes (Piehler et al., 1999; Li et al., 2007). No entanto, deve-se tomar cuidado com a quantidade de nutrientes adicionados; por exemplo, a adição de quantidades excessivas de nitrogênio pode resultar na inibição da comunidade microbiana do solo (Chaillan et al., 2006). A principal vantagem da bioestimulação é que a biodegradação aprimorada ocorre pelas comunidades microbianas nativas que já se acostumaram com seu ambiente. A bioestimulação das comunidades microbianas nativas do solo que foi poluído por petróleo pode ser alcançada de várias maneiras. Verificou-se que uma ampla gama de agentes orgânicos e inorgânicos, incluindo nutrientes, surfactantes, lodo de esgoto fresco e adubado e adubo, são bioestimuladores de sucesso para a degradação do hidrocarboneto de petróleo (Ros et al., 2010; Sayara et al., 2011; Blyth et al., 2015). Várias experiências de laboratório e de campo baseadas na adição de fertilizantes inorgânicos e orgânicos ao ambiente contaminado mostraram impactos positivos na degradação de hidrocarbonetos; no entanto, uma série de resultados foi relatada. Por exemplo, estimular o solo com fertilização inorgânica de nitrogênio-fósforo-potássio (NPK) e produtos comerciais EAP e Terramend demonstrou estimular a atividade biodegradativa dos microrganismos do solo nativo (Mair et al., 2013). Em outro estudo, a alteração do solo com adubo aumentou o percentual de degradação do hidrocarboneto de petróleo em até 56% em comparação com as amostras de solo com atenuação natural [(15,6%) (Liu et al., 2010)]. Em contraste, outros resultados de pesquisa indicaram que a bioestimulação

não contribuiu significativamente para a degradação de hidrocarbonetos no solo. Por exemplo, alterações incluindo NPK, um extrato de composto e uma cultura de enriquecimento microbiano não mostraram impacto significativo na remediação do óleo diesel. No entanto, nenhuma alteração na concentração de TPH foi observada quando o solo foi tratado com grãos de café ou resíduos hortícolas. Nesse caso, os autores concluíram que os microrganismos preferiam consumir a fonte de carbono prontamente disponível em vez dos hidrocarbonetos de petróleo (Palmroth et al., 2002; Schaefer and Juliane, 2007). Assim, pode ser mais valioso caracterizar a localização poluída, as condições ecológicas e a comunidade microbiana natural, a fim de realizar uma técnica eficaz de biorremediação no campo. Pesquisas substanciais em laboratório e em mesocosmos são essenciais para avaliar o potencial da biorremediação, embora seja necessário reconhecer que fatores ambientais desempenham um papel importante na determinação da taxa de degradação real no campo. Enquanto em ensaios controlados em laboratório, as medições geralmente podem ser interpretadas facilmente; os relacionamentos de causa e efeito costumam ser difíceis de estabelecer nos locais de campo. Na maioria dos casos de biorremediação, os microrganismos podem degradar prontamente o contaminante quando cultivados em ambientes laboratoriais bem controlados; no entanto, evidências de biodegradação em campo são necessárias. Quando microrganismos degradadores são introduzidos em condições ambientais menos hospitaleiras no campo, eles podem não executar as mesmas tarefas e podem ser inibidos devido à predação ou competição por microrganismos autóctones (Suja et al., 2014). Além disso, devido à heterogeneidade do óleo, a avaliação da degradação do hidrocarboneto de petróleo em escala de campo é mais difícil. O processo de biorremediação pode ser influenciado por fatores bióticos e abióticos, bem como pela capacidade dos microrganismos de sobreviver e migrar. Portanto, é necessário realizar análises laboratoriais experimentos anteriores ao processo de limpeza real para avaliar a melhoria do hidrocarboneto na degradação sob condições controladas; isso estabelecerá a credibilidade científica de um determinado procedimento de biorremediação.

Tomados em conjunto, esses estudos indicam que melhorar o potencial de biodegradação pelo uso de consórcios bacterianos e suas interações com o meio,

através da produção de biomoléculas é uma estratégia razoável e viável para acelerar a eficiência de remoção de hidrocarbonetos de petróleo de ambientes contaminados.

Muitas das biomoléculas produzidas pelas comunidades microbianas para facilitar-se a degradação de hidrocarbonetos são bioemulsificantes e/ou biosurfactantes.

1.3.4 Moléculas de origem microbiana

Os surfactantes microbianos são um grupo estruturalmente diverso de moléculas ativas de superfície e são classificados em dois grupos: o surfactante de baixo peso molecular é chamado de BSF e o de alto peso molecular é chamado de bioemulsificadores (Uzoigwe et al., 2015).

As bactérias produtoras de bioemulsificantes, apresentam dois aspectos fisiológicos a seguir: primeiro, a capacidade de melhorar a complexação e a solubilização de substrato não polares, promovendo a biodisponibilidade dos substratos e segundo, a capacidade de melhorar a afinidade entre superfícies celulares e interfaces óleo-água através do metabolismo, promovendo a deformação do filme de interface óleo-água (Hou et al., 2018). Ben Ayed et al. (2015) relataram que BSFs produzido por *Bacillus amyloliquefaciens* An6 era uma alternativa aos surfactantes sintetizados quimicamente, uma vez que apresentava alta eficiência de solubilização em relação ao óleo diesel (71,54% a 1 g/L), sendo melhor que o SDS e o Tween 80. Portanto a eficiência de degradação do diesel poderia melhorar através da linhagem *B. amyloliquefaciens* An6. No entanto, nem todos os BSFs produzidos por bactérias produtoras de bioemulsificantes podem efetivamente aumentar a taxa de degradação de poluentes (Inakollu et al., 2004). De fato, vários BSFs que estimulam ou inibem a biorremediação de poluentes dependem das propriedades físico-químicas dos surfactantes, tipos de poluentes e características fisiológicas dos microrganismos funcionais (Hua and Wang, 2014).

Sabe-se que os BSFs são capazes de reduzir a tensão superficial e interfacial entre diferentes fases (líquido-líquido, líquido-ar e líquido-sólido), concentração micelar crítica (CMC) e formação estável de emulsões, aumentando a biodisponibilidade de

contaminantes no ambiente para biodegradação em processos de biorremediação (Satpute et al., 2010; Shao et al., 2017). *Pseudomonas* é um gênero conhecido na produção de BSF, seguido por outras espécies. Coelho et al. (2003) relataram produção de BSF por *Pseudomonas* sp. GU 104. Dependendo dos diferentes tipos de fontes de carbono e das condições de desenvolvimento dos microrganismos, diferentes tipos de biosurfactantes são produzidos. Esse fato foi bem comprovado por Desai et al. (1988), onde para a produção de emulsificantes lipídicos de trealose-o-dialquil-monoglicerídeo-proteína por *Pseudomonas fluorescens* ocorreu pelo consumo de hidrocarbonetos, e por de Freitas Ferreira et al. (2019) onde a produção de biosurfactantes era dependente do pH.

1.3.4.1 Fonte de carbono

A fonte de carbono é essencial para o crescimento e desenvolvimento do microrganismo e, consequentemente, para a produção de BSFs. Sua produção varia entre as espécies, incluindo a de bacilos. Os principais grupos de BSFs produzidos pelos bacilos estudados até agora são os ramnolipídeos, lipopeptídeos e soporolipídeos. Sabe-se que o uso de glicose como fonte de carbono para a produção de BSFs em espécies de bacilos pode chegar a 5 g/L (Chen et al., 2015). Estudos sugerem que a mistura de fontes de carbono pode causar um aumento no rendimento de BSF (Santos et al., 2016). Além disso, diferentes tipos de resíduos industriais têm sido utilizados como meio de crescimento para a produção de BSFs para minimizar o custo da incubação. Martins and Martins (2018) relataram que *Corynebacterium aquaticum* apresentou produção eficiente de BSF usando resíduos de peixe e bagaço de vegetais como fonte de carbono. Em outro estudo, resíduos industriais foram utilizados para a produção de um BSF por *C. lipolytica*, o rendimento do complexo proteína-lipídio-carboidrato foi de 4,5 g/L, com uma redução na tensão superficial da água destilada de 71 para 32 mN/m (Rufino et al., 2007). Resíduos agrícolas, como extratos aquosos de resíduos agrícolas, cascas de banana, cascas de laranja, cascas de batata e bagaço, soro de leite, óleos vegetais e o óleo de mostarda são matérias-primas eficazes e de baixo custo que podem ser usados como fontes de carbono para o desenvolvimento microbiano e produção de biosurfactantes (Nitschke et al., 2010; Vandana and Peter, 2014; Kulkarni et al., 2015).

1.3.4.2 Condições de crescimento

As condições físico-químicas de desenvolvimento, como temperatura, pH, velocidade de agitação e aeração, também influenciam a produção de BSFs (Desai and Banat, 1997; Sen and Swaminathan, 1997; Davis et al., 1999; Yeh et al., 2005; Abdel-Mawgoud et al., 2008; Ghribi and Ellouze-Chaabouni, 2011; Chen et al., 2015). As espécies de *Candida* produzem rendimentos máximos de BSFs em uma ampla faixa de pH, como pH 5,7 para *C. glabrata* UCP 1002, pH 7,8 para *Candida* sp. SY16, pH 5,0 para *C. lipolytica* e pH 6,0 para *C. batistae* (Cirigliano and Carman, 1984; Kim et al., 1999; Sarubbo and Campos Takaki, 2006; Konishi et al., 2008). A produção ideal de BSF por espécies de bacilos varia da faixa de pH 6-9 (Sen and Swaminathan, 1997; Wei and Chu, 1998; Abdel-Mawgoud et al., 2008). A temperatura mais favorável para a produção de BSF por diferentes fungos é de 30°C, conforme observado para diferentes espécies de *Candida*. Para bacilos, a temperatura pode variar de 30 a 40°C (Banat, 1993; Sen and Swaminathan, 2005; Yeh et al., 2005; Haddad et al., 2009). A agitação tem um efeito positivo na produção de BSF, mas alguns estudos sugerem que a agitação vigorosa tem um efeito negativo (Cunha et al., 2004; Oliveira et al., 2009; Silva et al., 2010). A aeração desempenha um papel essencial no fornecimento de oxigênio ao microrganismo, porque as funções metabólicas podem ser comprometidas sem ele. A aeração ideal para a produção de BSF de bacilo varia de 0,5 a 1,5 vvm (Sen and Swaminathan, 1997; Yeh et al., 2005). O tempo de incubação também é um fator importante na produção de BSFs podendo variar de dias a semanas (Chen et al., 2015; Santos et al., 2016).

1.4 Mercado global de BSFs

Nas últimas décadas, houve um interesse no uso de surfactantes químicos, principalmente devido às suas diversas aplicações em vários processos industriais na indústria de alimentos, detergentes, recuperação avançada de óleo e biorremediação. A produção global de surfactantes sintéticos atingiu cerca de 13 milhões de toneladas em 2008, gerando uma receita de US$: 700.000. Em 2013, eram 344.068,40 milhões de toneladas e deve chegar a 461.991,67 milhões de toneladas até 2020, crescendo a uma CAGR de 4,3% de 2014 a 2020, sendo expectativa anterior de apenas 3,5% (Markets and Research, 2017; Global Market Insights, 2018). O mercado global de surfactantes

foi estimado em US$ 30,6 bilhões em 2016 e deve alcaçar em 2021 US$ 39,86 bilhões (Markets and Markets, 2016)(Markets and Markets, 2016). Outra pesquisa de mercado projetou o mercado global de BSF em mais de 52,2 bilhões até 2022, com um CAGR de 5,6% entre 2017 e 2022 (Markets and Markets, 2017). Com regulamentos mais rígidos sobre processos industriais mais ecológicos e atendendo a uma demanda enorme, os BSFs formam uma parte importante do mercado de surfactantes. A geração de receita no mercado de BSF ultrapassou US$ 1,8 bilhão em 2016 e deve chegar a US$ 2,6 bilhões até 2023, com o mercado de ramnolipídeos representando 8% do total (Global Market Insights, 2018). Entre os BSFs, os soporolipídios (SLs) tiveram a maior participação no mercado global com a indústria de detergentes, líder no setor de aplicação de produtos. A BASF Cognis (Alemanha) e a Ecover (Bélgica) emergiram como os dois principais fabricantes neste mercado de BSFs. Outros produtores no mercado são MG Intobio, Urumqui Unite, Saraya, Sun Products Corporation, Akzo Nobel, Croda International PLC, Evonik Industries (Alemanha), Mitsubishi Chemical Corporation e Jeneil Biosurfactant (Markets and Research, 2017; Global Market Insights, 2018). No entanto, apesar da alta demanda do mercado, a produção de um BSF não é tão competitiva quanto suas contrapartes sintéticas. Os surfactantes sintéticos custam menos de US$ 2/kg no mercado (Santos et al., 2016; Akbari et al., 2018a; Suh et al., 2019). De acordo com a literatura, o custo de um BSF comercial varia de US$ 2,5 a 6,3/kg para soporolipídeos oferecidos como Sophoron de Saraya (Japão) e Soliance (França) a US$ 13,94/mg para surfactina (98% de pureza) disponível na Sigma Chemical Company, US$ 20–22,7/mg para ramnolipídeos fabricados pelas tecnologias Sigma-Aldrich e AGAE (US) e US$ 50/kg para emulsano RAG-1 comercializado pela Petroferm Research Inc. (Dhanarajan and Sen, 2014; Randhawa and Rahman, 2014; Freitas et al., 2016). Os ramnolipídeos também estão disponíveis na Ecover (Boulogne-sur-Mer, França), na Jeneil Biosurfactant Inc. (Saukville, Wisconsin, EUA) e na Rhamnolipid Holdings Inc. (Nova York, EUA), com um custo de produção de US$ 20/kg em um volume de 20 m^3 e US$ 5/kg quando produzido em uma escala de 100 m^3, aproximando-o do etoxilato ou alquil poliglicósido (US$ 1–3/kg). Portanto, é necessário analisar os custos de produção de BSFs para o desenvolvimento de um processo de fermentação economicamente viável. A Europa aumentou seu mercado em cerca de 53%,

principalmente devido a diretrizes regulatórias mais rigorosas. O aumento da conscientização e da infraestrutura nos países asiáticos os torna um consumidor crescente de BSFs. De acordo com a legislação brasileira, os efluentes industriais de qualquer fonte poluidora só podem ser liberados diretamente no corpo de água receptor, desde que não excedam 20 ppm (0,2%) de conteúdo oleoso (CONAMA 430/2011) e se seguir as diretrizes de outros países a legislação restringirá ainda mais a concentração permitida para os próximos anos.

2. OBJETIVOS

2.1 Objetivo geral

Otimizar a produção e avaliar os BSFs produzidos por *Bacillus amyloliquefaciens* MO13 como estratégia avançada de biorremediação em áreas contaminadas com petróleo.

2.2 Objetivos específicos

- Otimizar a produção de BSFs em biorreatores usando glicose e glicerina residual como fontes de carbono;

- Avaliar a atividade dos BSFs produzidos por meio de testes quantitativos e qualitativos;

- Produzir superfícies de respostas para projetar a condição mais apropriada para a produção de BSFs;

- Identificar os BSFs produzidos.

3. MANUSCRITO

Biosurfactant-ehanced production by *Bacillus amyloliquefaciens* from residual glycerin

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Este manuscrito foi organizado em forma de artigo e formatado segundo as normas da revista *Biotechnology and Bioengineering*, ISSN: 1097-0290, a qual será submetido.

Abstract

Biosurfactants (BSFs) are molecules with hydrophobic and hydrophilic motifs produced by a multiplicity of microorganisms from different carbon sources. These molecules can be applied in several processes, including bioremediation and petroleum recuperation. However, the production costs of these compounds are still a hindrance to large-scale applications. In this work, the central composite design (CCD) model was used to optimize BSFs production by *Bacillus amyloliquefaciens* MO13 strain using residual glycerin as a substrate. The concentration of carbon source (30 g/L), temperature (34 °C), pH (7.2), stirring (239 rpm), and aeration (0.31 Lpm) were standardized for cellular cultivation on bioreactors system (5 L), with the maximum BSFs production reaching 14.4 g/L for residual glycerin. The microbial development, substrate consumption, concentration of BSFs, and surface tension were also evaluated to analyze the bioprocess dynamic. Additionally, chemical identification of BSFs profile was performed by mass spectrometry Q-TOF-MS, revealing five isoforms of surfactins and two isoforms of iturins produced by *B. amyloliquefaciens* MO13. The results obtained represent a significant advance in biosurfactant production using industrial waste as a microbial substrate, since the BSF concentration achieved was significantly higher than previously reported on literature for residual glycerin.

Keywords: Bioprocess, Factorial design, Biosurfactants, *Bacillus amyloliquefaciens*, Bioremediation.

1. Introduction

Several studies currently show that most of the energy market relies on petrochemicals (Farn, 2006; Hayes et al., 2010; Rufino et al., 2014; De Almeida et al., 2016; Akbari et al., 2018b). As a result, the increase of energy demand and consequent fuel transportation may elevate the risk of oil leaks. The oil spill has impacted the fishing, seafood, tourism and oil/gas industries worldwide. Some studies suggest that the economic impact of oil spills could reach billions of dollars. (Deblonde et al., 2011; Randhawa and Rahman, 2014).

Surfactants are chemicals used in various industries like detergents, paints, paper products, pharmaceuticals, cosmetics, petroleum, food and water treatment (Güçlü-Ustündağ and Mazza, 2007; Gupta et al., 2013; Elazzazy et al., 2015; Lu et al., 2017; Mahamallik and Pal, 2017). Commercial surfactants are currently synthetic synthesized from several sources, including petrochemicals, animal fats and vegetable oils. Noteworthy, several microorganisms naturally produce a plethora of biosurfactants (BSFs) that can be employed as a substitutive for synthetized surfactants, in view of reduced toxicity, better biodegradability, stability, specificity and bioavailability (Desai and Banat, 1997; Rosenberg and Ron, 1999; Kosaric, 2001; Piispanen, 2002; Mulligan, 2005; Mohan et al., 2006; Muthusamy et al., 2008; Goel, 2010; Torres et al., 2011; Md, 2012; Boruah and Gogoi, 2013; De et al., 2015). These compounds have an enormous importance in industries but many times they are less safer than BSFs in pharmaceutical, cosmetic and edible oils ones (Nitschke and Costa, 2007; Rahman and Gakpe, 2008; Bhadoriya and Madoriya, 2013; Fracchia et al., 2015).

Several microorganisms produce BSF compounds such as glycolipids (rhamnolipids, soporolipids), lipopeptides (surfactins, iturines, phengicins), phospholipids, polymeric compounds and neutral lipids (Uzoigwe et al., 2015; Geetha et al., 2018). Amongst the prolific producers of BSFs, *Bacillus* spp. have been widely explored, as a notably source of lipopeptides (Rufino et al., 2014; Chaprão et al., 2015). However, the cost for BSFs production on industrial scale still high. In this way, few authors have evaluated as variations in pH, temperature, aeration, stress conditions and carbon source can affect the production of BSFs by *Bacillus* spp. (Desai and Banat, 1997; Bhardwaj et al., 2013). The use of alternative carbon sources as glycerin, vegetal bagasse and others, it is a way to decrease costs in industry (Nitschke et al., 2010; Vandana and Peter, 2014; Kulkarni et al., 2015). Furthermore, cost effective ways to maximize BSFs production still have to be evaluated (Freitas et al., 2016; Santos et al., 2016; Akbari et al., 2018b).

Residual glycerin is a promising residue that can be used as carbon source in many bioprocess and could be used for BSFs production (Oliveira et al., 2009; de Sousa et al., 2014; Binhayeeding et al., 2017). Therefore, it could decrease the costs for BSF production and add value to glycerin as a residue.

During a previous screening for BSF-producing microorganisms a promising isolate of *Bacillus amyloliquefaciens* was found. This strain displayed excellent BSF production and efficiency under different growth conditions (Moro et al., 2018). Therefore, the objective of this work was optimizing the large-scale BSF production process of this strain. Optimum pH, temperature, stirrer and aeration parameters were evaluated, using residual glycerin as a cost-effective carbon source.

2. Material and Methods

2.1 Microorganism and pre-inoculum evaluation

Bacillus amyloliquefaciens MO13 was previously isolated from oil-contaminated soil (Moro et al., 2018) and it is part of the Laboratory of Fungi of Medical and Biotechnological Importance collection (UFRGS, Brazil). In order to evaluate the most appropriate pre-inoculum for pilot scale BSF production, firstly *B. amyloliquefaciens* MO13 was grown in 250 mL erlenmeyers with three different mediums: 50 mL of YPD medium (Yeast extract 10 g/L, Peptone 20 g/L, Glucose 20 g/L), 50 mL of LB (Luria-Bertani) medium (Thermo Fisher Scientific, USA, 20 g/L) and 50 mL LB medium supplemented with glucose (20 g/L) at 200 rpm, 30°C and for 24 h (adapted from Napp et al., 2018). Analysis with residual glycerin were not evaluated carbon source for pre-inoculum.

2.2 Pilot scale culture parameters

For inoculum in 500 mL bioreactors, 2% v/v of pre-inoculums were used and the total volume in each bioreactor was 400 mL. The inoculum was grown for 40 h (Chen et al., 2015). Thus, the minimum saline medium (MSM) for bioreactors were composed of 6 g/L NaH_2PO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L Urea (NH_2CONH_2), 0.24 g/L $MgSO_4$, 0.01 g/L $CaCl_2$, 0.16 g/L $ZnSO_4$, 0.017 g/L $MnSO_4$, 0.27 g/L $FeCl_3$ and 5 g/L $NaNO_3$ (adapted from Sen and Swaminathan, 1997; Davis et al., 1999; Yeh et al., 2005; Abdel-Mawgoud et al., 2008; Ghribi and Ellouze-Chaabouni, 2011; Chen et al., 2015; Farias et al., 2018). MSM was supplemented with 1% v/v, 3% v/v or 5% v/v of saturated glucose

(470 g/L) on pure water previously sterilized by filtration on 22 µm filter (Millipore) in order to evaluate the best glucose concentration for inoculum. Once the best glucose concentration was chosen, experiments using sterile residual glycerin (120°C, 20 min) as carbon source on the same concentration of glucose were performed. *B. amyloliquefaciens* MO13 was grown in 500 mL bioreactors at 20, 25, 30, 35 and 40ºC, pH 6, 6.5, 7, 7.5 and 8, 150, 200, 250, 300 and 350 rpm, 0, 0.1, 0.3, 0.5 and 0.7 liters per minute of air (Lpm) for 40 h. Most of BSF concentration was in the foam during production, so a foam collector was built and allow collect the foam without contaminations (Supplementary Figure 1). The crude BSF were obtained from the supernatant free of cells (9000 rpm, 20 min, 20ºC) and subjected to acid precipitation using HCl 5 M until reach pH 2 and left for 24 h at 4°C. After 24 h, the samples were centrifuged (9000 rpm, 20 min, 9°C), the pellets were dried in an oven at 80°C for 5 hours and their mass measured. All tests were made on duplicates.

2.3 Factorial design

Once the best conditions for pre-inoculum and carbon source concentration were established for BSF production, surface response analyses were generated using 2^4 central composite design (CCD) model. Experiments with 58 different conditions were performed on the 500 mL bioreactors (INFORS, Switzerland) analyzing four varied parameters: temperature (25°C, 30°C and 35°C), agitation (200 rpm, 250 rpm, 300 rpm), aeration (0.1 Lpm, 0.3 Lpm, 0.5 Lpm) and pH (6.5, 7.0, 7.5) for 40 h (Teófilo and Ferreira, 2006). Pareto analysis from central composite design (response surface) was realized using the sotware STATISTICA.

2.4 Large scale BSF production with the parameters obtained through surface response method

Bioreactors of 5 L (INFORS, Switzerland) with a foam collector were used on six assays to validate the equation generated through the surface responses estimation. After 72 h of culture, the BSFs were precipitated (as described above). The resulting material was centrifuged (9000 rpm, 20 min), dried and weighted. The resulting dry pellet was washed twice with acid water (pH 2), centrifuged (9000 rpm, 20 min, 9ºC) and resuspended on water. The pH was adjusted to 7 with NaOH 5 M and the pellet was lyophilized (Pereira et al., 2013).

2.5 Cellular growth

In order to evaluate the viable cells, Colony forming units (CFU/mL) were calculated using a drop plate technique (adapted from Naghili et al., 2013). Briefly, serial dilutions from 10^{-3} until 10^{-14} were performed using sterile NaCl (0.85%) and 10 µL of the dilutions were inoculated on agar plates containing YPD medium. After incubation for 24 h at 30°C, bacterial colonies were counted. CFU/mL counting was performed in biological triplicates.

2.6 Carbon source determination

Glucose levels were determined by colorimetric enzymatic assay using Bioliquid (Laborclin, Brazil). The samples were measured spectrophotometrically at 500 nm and

the results were calculated from the ratio between the test absorbance and the absorbance of the standard, according to manufacturer instructions.

Free glycerol levels present in the inoculum during BSF production were measured (Bondioli and Della Bella, 2005; Keppy et al., 2009; Sartori and Borin, 2011). For calibration curve, tubes with 0.00, 3.75, 7.5, 11.25, 15, 18.75, 22.5, 26.25 and 30 µg/mL of glycerol solution were diluted with working solvent until reached 2 mL. It was added 1.2 mL of a 10 mM sodium periodate solution and homogenized for 30 s. After that, it was added 1.2 mL of a 0.2 M acetylacetone solution, followed by incubation at 70°C and stirring for 1 min. After the reaction time, the samples were immediately cooled by immersing the tubes in water. Finally, the absorbance was recorded in a spectrophotometer set at 410nm.

The samples containing crude BSF followed the same procedure as for the calibration curve. Two blanks were used to subtract the BSF absorption at 410 nm. One blank contained working solvent (10 mM sodium periodate solution and 0.2 M acetylacetone solution) and the second blank contained the working solvent and 6 g/L BSF solution.

Physical characteristics of residual glycerin was carried out by viscosity measurement at 24°C using a Brookfield LVDV-I Prime viscometer equipped with a sample adapter (spindle S-21). Density was determined by measuring the mass of residual glycerin in 5 mL volumetric flasks calibrated at 22°C. The pH was determined in a digital pH meter (Jenway 3505) after dissolving 1.00 ± 0.1 g in 50 mL of deionized water at 23°C. The results of these parameters were necessary to validate the use of residual glycerin according to literature (ASTM D6584 – 10, 2010; EN 14105:2011, 2011).

Evaluation of chemical characteristics of free glycerol in residual glycerin was quantified by Gas Chromatography flame ionization detector (GC-FID) on a PerkinElmer Clarus 600 chromatograph equipped with a built-in auto-sampler coupled automatic injector. Samples were injected (1 µL) in split mode (30:1) with carrier gas (N_2) at constant flow of 1.3 mL/min. Injector and detector temperatures were 300°C. A Petrocol DH 50.2 capillary column (5% phenyl, 95% dimethylpolysiloxane, 50 m, 0.20 mm d.i. x 0.50 mm) was used. The initial oven temperature was 180°C for 1.5 min. The heating ramp ranged from 25ºC/min to 235ºC and remained for 1.5 min, totaling 5.2 min of analysis (ASTM D6584 – 10, 2010; EN 14105:2011, 2011; Hu et al., 2012; Prados et al., 2012; Valerio et al., 2015; Chaurasia and Pal, 2016; Binhayeeding et al., 2017). To determine the equation ($y = ax + b$) and obtain the angular (a) and linear (b) coefficients of the calibration function, internal standard stock solutions (1,2,4-butanotriol) and reference substance (glycerol) were prepared directly in 5 mL volumetric flasks. The volume was filled using pyridine until reached meniscus (Sigma-Aldrich, 99%). GC-FID analysis of the free glycerol content in the treated glycerin was conducted (10 to 50 mg) directly in vials and added to 1 mL with pyridine after undergoing a stoichiometric reaction at a stoichiometric ratio of 3: 1, N-methyl-N-trimethylsilyl trifluoracetamide (MSTFA, 98%; Sigma-Aldrich) glycerol and/or 1,2,4-butanotriol plus 45% MSTFA to ensure product formation. The silylation reaction remained at 60°C for 30 min. The results of these parameters were necessary to evaluate the glycerin purity.

2.7 Qualitative BSFs assays

2.7.1 Drop collapse assay

Drop collapse assay was conducted according to Bodour and Miller-Maier (1998). Crude BSF (20 µL) was added on a flat and clear surface with 1.8 µL of oil. The results were considered negative when the droplet remained after 1 min and considered positive when droplet collapsed.

2.7.2 Oil spread assay

Oil spread assay were performed according to Pornsunthorntawee et al. (2008). Briefly, 50 mL of distilled water was added to a Petri dish, supplied to 200 µL of oil on the water surface and 20 µL of crude BSF was placed in the center of the oil layer. The results were considered positive when displacement of the oil was observed and the negative when it did not happen.

2.7.3 Wettability reversal of rocks assay

In a proportion of 1:20:0.22 calcite:decane:5-cyclohexylpentanoic acid, glass tubes containing 5 g of calcite treated with a mix of a solution of 98.73 mL of decane 1M and 1.14 mL 5-cyclohexylpentanoic acid 0.066 M were utilized for mimic the wettability reversal. Ten mL of a synthetic seawater solution containing 25 ppm of crude BSF were added to each glass tube. The results were considered positive if resembled a positive control (SDS 10 %) and were considered negative if resembled a negative control (synthetic sea water) (Goddard III et al., 2005).

2.8 Quantitative BSF assays

2.8.1 Emusification activity assay

For the measurement of emulsifying activity (E_{24}), the tests were performed according to Cooper and Goldenberg (1987). Two mL of kerosene was mixed with an equal volume of crude BSF, stirring for 2 minutes. After 24 h, the emulsion formed was calculated as a ratio between the height of the emulsion layer (cm) and the total height of the liquid without tube (cm).

2.8.2 Surface tension measurement

The surface tension was measured using a digital tensiometer (Gibertini, Milan, Italy). Twenty mL of the supernatant (crude BSF) were used. The measurements were made by immersion of a cover slip under the surface of the supernatant (ca 1 mm), which was then slowly pulled out, and the maximum force was measured. Distillated water and ethanol 96% were used as standards (Moro et al., 2018).

2.8.3 Critical micellar concentration (CMC)

Critical micellar concentration (CMC) was calculated using different concentrations (0 – 800 mg/L) of the crude BSF. A curve (crude BSF concentration x surface tension) was generated and the inflection point was determined. Surface tension measurements were taken in triplicates at 25°C (Nitschke and Pastore, 2006).

2.9 Mass spectrometry analysis

The crude BSFs obtained from factorial design analysis (item 2.3) in 500 ml bioreactors using the best BSFs production conditions were extracted three times with chloroform/methanol (2:1 v/v) in a separation funnel for extraction of organic compounds

(lower phase). The solvent was evaporated under reduced pressure (45°C, 400 mmHg for 10 min), and the solute was solubilized with a mixture of acetonitrile and deionized water (1:1 v/v). This solution was acidified (0.1% formic acid), filtered through a 0.45 µm PVDF membrane and introduced into the quadrupole time-of-flight mass spectrometer (Q-TOF-MS; Bruker® Daltonics, Impact II) by direct infusion into one flow, at a rate of 180 µL/h. Q-TOF-MS was operated in positive ionization mode under the following conditions: 4500 V capillary, 0.3 bar (N^2) nebulizer, 4 L/min (N^2) drying gas and gas temperature at 200°C. All information was recorded in the mass to load ratio (m/z) range from 50 to 1300 and the mass spectrometer calibration was performed with sodium formate solution. The raw data acquired were processed with DataAnalysis 4.2 software. An error mass deviation of ± 5 ppm was attributed to the elemental composition of the ions (Brenton and Godfrey, 2010; Moro et al., 2018). The results founded were compared with literature (Chen et al., 2008; Ali et al., 2014; Gong et al., 2015).

3. Results

3.1 Pre-inoculum effects on BSF production

In order to understand the influence of different culture media, as a pre-inoculum for BSF production, experiments were conducted employing YPD, LB and LB supplemented with glucose in triplicates. Notably, the pre-inoculum employing YPD displayed better results, reaching three times greater BSF yields when compared to LB supplemented with glucose, after 40 h of incubation. In Figure 1A, it was detected that when YPD was used for pre-inoculum the concentration of BSF reached 15.2 g/L. LB

supplemented with or without glucose, BSF production was 5 g/L and 1 g/L respectively. For residual glycerin, the YPD pre-inoculum had a BSF production of 12.5 g/L. LB supplemented with or without glucose, BSF production was 4 g/L and 1 g/L respectively (Figure 1B). Thus, in light of the results, YPD was chosen to be used as pre-inoculum medium for subsequent experiments.

3.2 Substrate concentration effects on BSF production

In order to understand how different glucose concentrations can affect the production and which concentration would maximize BSF concentration, experiments were conducted employing 10, 30 and 50 g/L of glucose (Figure 2). BSF production, after 40 h of incubation, using 30 g/L and 50 g/L of glucose were equivalent, reaching 2.2 g/L of crude BSF and 43 mN/m of surface tension in 20 h (Figure 2A and Figure 2B). The culture medium containing 10 g/L of glucose did not achieve similar results on BSF production (1.5 g/L) and surface response (66mN/m) (Figure 2C). Therefore, 30 g/L of glucose was chosen to be used for subsequent experiments. The same concentration, 30 g/L, was chosen for residual glycerin for subsequent experiments in order to compare results between glucose and residual glycerin. Results of physical-chemical characterization of residual glycerin are expressed on table 4 on supplementary material.

3.3 Statistical and factorial design results

After evaluating the best conditions for pre-inoculum and different glucose concentrations, factorial experiments were conducted. To evaluate if CCD was the best surface response method, the central points should have the best results. Crude BSF

production was higher for the central conditions (pH 7.0, 30°C, 250 rpm and 0.3 Lpm) using glucose or residual glycerin as carbon source, reaching a BSF production of 14.4 g/L [Table 2 (25 - 29 assays)] and 13.4 g/L [Table 3 (25 - 29 assays)] respectively. With the statistical analysis using the CCD factorial design method, a 3D spatial and contour graph was obtained for the four parameters analyzed: temperature, pH, stirrer and aeration (Supplementary Figures 2 - 15). With the coefficients obtained from statistical analysis, a optimization equation for BSF production was elaborate to reach the maximum production of BSF.

3.4 BSF production optimization

3.4.1 Equation

From the CCD quadratic model statistical results, two equations, one for glucose [1] and other for residual glycerin [2] as sole carbon source, were established using the significant coefficients found on the described assays. The equations were evaluated in order to identify the parameters for the highest BSF production, minimizing the number of experiments to be conducted. In the equation generated for glucose, as sole carbon source, the putative parameters for highest BSF production were: 31°C, 252 rpm, 0.43 Lpm and pH 7.0, expecting a yield of 17.92 g/L of crude BSF. Noteworthy, for glucose, employing these predicted parameters led to an observed yield of 18.16 ± 0.55 g/L (Figure 3). For the equation generated for residual glycerin, as sole carbon source, the putative parameters for highest BSF production were: 34°C, 239 rpm, 0.31 Lpm and pH 7.2,

expecting a yield of 14.4 g/L of crude BSF. For residual glycerin, a yield of 13.56 ± 0.26 g/L was observed when the predicted parameters were applied (Figure 4).

$$BSF = -738{,}84 + 118{,}90A + 0{,}803S + 3{,}44T + 163{,}54p - 75{,}98A^2 - 10{,}7 \cdot 10^{-4}S^2 - 4{,}72 \cdot 10^{-2}T^2 - 10{,}66p^2 + 9{,}24 \cdot 10^{-2}AS - 0{,}35AT - 11{,}95Ap - 2{,}24 \cdot 10^{-3}ST - 3{,}2 \cdot 10^{-2}Sp + 2 \cdot 10^{-3}Tp \quad [1]$$

$$BSF = -532.37 - 52.30A + 0{,}58S + 1{,}57T + 127{,}96p - 77{,}31A^2 - 7{,}3 \cdot 10^{-4}S^2 - 4{,}58 \cdot 10^{-2}T^2 - 10{,}16p^2 - 7{,}32 \cdot 10^{-2}AS - 0{,}16AT - 17{,}92Ap - 5{,}55 \cdot 10^{-3}ST - 6{,}24 \cdot 10^{-3}Sp + 0{,}4Tp \quad [2]$$

BSF = Biosurfactant concentration (g/L)

T: Temperature (°C).

p: pH.

A: Aeration (Lpm).

S: Stirrer (rpm).

The correlation and importance of each variable in BSF production in the equations created can be evaluated by pareto analysis. Pareto graphs are observed in Figure 5 (glucose as carbon source) and Figure 6 (residual glycerin as carbon source). The results for each graph were statistically different. Figure 5 reveals that almost all variables interactions are important while Figure 6 reveals the opposite. The values on the right of

red line are significative and the signal in the front of each value represents its positive or negative influence.

3.4.2 Scale-up

After checking the maximum crude BSF yield in 500 mL reactors, the same established parameters were applied in 5 L bioreactors. Cell development curve and substrate consumption were evaluated for both glucose and residual glycerin substrates for 72 h. The maximum crude BSF production reached 6.3 g/L and total glucose consumption occurred in approximately 38 h (Figure 7). Additionally, for residual glycerin, crude BSF production reached 6.5 g/L and total free glycerol consumption occurred in approximately 60 h (Figure 8).

3.5 Qualitative and quantitative BSF assays

All qualitative and quantitative assays were performed after incubation time using glucose and residual glycerin as carbon source from cell free BSF produced. Noteworthy, BSFs from both cultures displayed similar excellent results in the oil spread assay (Figure 9; Table 1). Furthermore, drop collapse assays showed positive results for both culture media (Figure 10; Table 1). In order to further confirm if the crude BSF extracts could emulsifier, emulsification activity assays were performed using BSF extraction from both culture media. Excellent results were observed for glucose and residual glycerin reaching 85% of emulsification for both (Figure 11; Table 1). Additionally, wettability reversal of rocks assay was also successful for both culture media, being portrayed by calcite decanting after crude BSF extract addiction (Figure 12; Table 1).

3.6 Critical Micellar Concentration (CMC)

BSFs obtained from both culture medium supplemented with glucose or residual glycerin in triplicates exhibited similar results in CMCs evaluation. The curves for the critical micellar concentration displayed the inflection point at 10 mg/L of crude BSF which means that a minimum concentration of 10 mg/L of BSFs are necessary to reduce the surface tension until its minimum (Figure 13). The minimum surface tension was 30 mN/m in both cases.

3.7 Mass spectrometry analysis

High-resolution mass spectrometry was used to identify lipopeptides families of the BSFs produced. Q-TOF-MS direct infusion system was used in positive ionization mode. Two families of long acyclic side-chain cyclic lipopeptides (surfactin and iturin) produced by *B. amyloliquefaciens* MO13 were identified and subjected to the optimized conditions described previously (Table 4). Lipopeptide families were identified by the charge to mass ratio (m/z) of the ions with the sodium adduct [M + Na]+. In the samples containing glucose as carbon source, surfactins were identified by the m/z ions 1016.6215, 1030.6369, 1044.6525, 1058.6680 and 1072.6830, referred to C-13, C-13, C- 14, C-15 and C-16 respectively. The two iturins were identified as C-14 and C-15, with the ions of m/z 1066.6340 and 1080.6488, respectively (Figure 14; Table 4). For samples containing residual glycerin as carbon source, surfactins were identified by the m/z ions 1016.6222, 1030.6377, 1044.6534, 1058.6689 and 1072.6836, which are C-13, C-13, C -14, C-15

and C-16 respectively. The two iturins were identified as C-14 and C-15, with ions m/z 1066.6350 and 1080.6505, respectively (Figure 15; Table 4).

4. Discussion

BSFs have many advantages over synthetic surfactants, as superior tolerance to pH, salinity, pression, and temperature, also displaying low toxicity and high biodegradability (Moro et al., 2018). The BSFs' market has grown in the last years not just in view of these advantages but also because of political environmental laws that try to minimize impacts on environment using compounds less aggressive to environment as biosurfactants. In order to improve BSF production, optimization is necessary, once the BSF costs of production are higher than synthetic surfactant. Several approaches have employed salts to assist in increasing productivity of BSF, since these salts are used as enzymatic cofactors (Liu et al., 2012; Zhao et al., 2013; Willenbacher et al., 2015; Najmi et al., 2018; Hu et al., 2019). Because of this, MSM was adapted to provide better yields of BSF production. During BSF production, most of BSF produced by *B. amyloliquefaciens* MO13 is concentrated in the foam. Besides that, it is reported that the production of foam containing BSF stimulates the production of BSF even more by microrganisms (Razafindralambo et al., 1996; Santos et al., 2016). In order to collect the BSF-rich foam, a foam collector was adapted. The use of defoamers is not recommended, as it would result in a decrease in BSF production. The use of bioreactors instead of the usual conical flasks has advantages such as control of pH, temperature, agitation and aeration rates. Controlling these rates speeds up the optimization process and increases productivity (Chen et al., 2015).

Culture media have a major influence on the types of metabolites produced by microorganisms (Liu et al., 2012; Zhao et al., 2013; Willenbacher et al., 2015; Najmi et al., 2018). *B. amyloliquefaciens* MO13 was tested with three different culture media in order to determine which pre-inoculum resulted in the highest yield of crude BSF. Notably, YPD medium (i.e., a culture medium most commonly used for fungi rather than bacteria) yielded the highest amounts of crude BSF. Several projects aiming for bioreactor's BSF production have employing glucose as a carbon source in amounts of 1, 3 and 5% w/v (Chen et al., 2015; Moro et al., 2018). YPD medium has B-complex vitamins which stimulates bacterial growth, but also high glucose concentrations which could even be toxic to cells (Liu et al., 2012; Najmi et al., 2018). Those combination could be stimulating BSF production, since bacterial stress is related with BSF production (Desai and Banat, 1997; Bhardwaj et al., 2013). One explanation for this is when in contact with glucose, enzymes related to the glycolysis process were transcribed, enabling the use of them to be used in the inoculum. The synthesis of BSFs from the glycolysis process has already been described by some authors and several enzymes participating in their construction are present in the glycolytic pathway (Jung et al., 2012; Willenbacher et al., 2016; Zhi et al., 2017). It is then assumed that the microorganisms that came into contact with YPD have a range of enzymes and proteins more adapted to be inoculated and produce BSFs.

Additionally, several studies have attempted to use a minimal amount of carbon source for maximum BSF production. Concentrations of 1, 3 and 5% w/v of carbon sources in inoculum have already been reported as sufficient concentrations for BSF production (Sen and Swaminathan, 1997; Chen et al., 2015; Sakthipriya et al., 2015; Zhou et al., 2015; Moro et al., 2018). Therefore, glucose concentrations of 1% w/v, 3% w/v and

5% w/v were employed, under the same aeration, agitation, pH and temperature conditions to evaluate BSF production. Notably, BSF concentration was relatively higher prior to 20 h for 5% w/v condition when compared to the 3% w/v condition. However, both conditions reached a maximum production of 2.2 g/L crude BSF at the end of 40 h of cultivation. Accordingly, several authors have described that 30% w/L of glucose is sufficient for BSF production in many bacteria species (Sen and Swaminathan, 1997; Davis et al., 1999; Yeh et al., 2005; Abdel-Mawgoud et al., 2008; Chen et al., 2015; Moro et al., 2018). Furthermore, 1% w/v of glucose did not achieve the same BSF concentration.

As expected, surface tension, measured from free cell supernadant (item 3.5), decreases over time on cultures employing 5% w/v and 30 w/v of glucose as we observe on figure 2. This result corroborates to literature reports (Abdel-Mawgoud et al., 2008; Ghribi and Ellouze-Chaabouni, 2011; Chen et al., 2015; Moro et al., 2018). Notably, surface tension did not decrease on culture employing 1% w/v of glucose. These results can be related to the lower BSF concentration observed. For comparative purposes, the same residual glycerin concentration was used for the inoculum.

The surface response simulation had shown a correlation between the maximum BSF production and the evaluated parameters which can be transformed in a BSF production optimized equation. The relationship between the data is given through R^2, which for glucose was 0.97 and for residual glycerin was 0.85. According to the literature, in cases of optimization, an R^2 up to 0.6 is acceptable due to the difficulty in optimizing a process, as it may vary due to several factors as medium, microrganisms, contaminations and others (Liu et al., 2012; Zhao et al., 2013; Willenbacher et al., 2015; Najmi et al.,

2018; Hu et al., 2019). Choosing composite central design (CCD) - quadratic spherical model and the variations on parameters were appropriate because indicated that the optimization of the BSF production equation would have as parameter values close to the center points. This allows building spatial graphs where it is possible to see the maximum BSF production (Teófilo and Ferreira, 2006). Pareto analysis demonstrate that the results using glucose and residual glycerin were different. We observe that when glucose is used as carbon source, only the interactions S^2, A^2 and Txp are insignificant, while when residual glycerin is used as carbon source all variables interactions besides T^2 are insignificant. This means the importance of temperature when a more complex carbon source is used. In order to metabolize residual glycerin, a higher temperature is necessary due to the complexity of this carbon source. However, when the carbon source is relatively less complex, the variables interactions are more important due to the metabolic pathways that this carbon source can be used. In other words, glucose can only be used as carbon source to produce biosurfactant in a high yield, if there is a fine adjustment in pH, temperature, aeration and stirrer. Therefore, probably *B. amyloliquefaciens* MO13 metabolize residual glycerin for BSF production, while glucose can be used for BSF production or other metabolic pathways. The optimized equation in which glucose was used as carbon source estimated the maximum yield of 18 g/L of crude BSF. These results compared to the literature are promising, since the production of BSFs is usually around 3 - 4 g/L (Sen and Swaminathan, 1997; Davis et al., 1999; Yeh et al., 2005; Abdel-Mawgoud et al., 2008; Chen et al., 2015; Meena and Kanwar, 2015; Hu et al., 2019). There was an increase of 600% in BSF production by optimizating pre-inoculum, carbon source concentration and pH, temperature, stirrer and aeration on inoculum. The

optimized equation using residual glycerin as carbon source estimated the maximum yield of 13 g/L and is also considered a promising value. However, higher concentrations may be related to small-scale production, what generally trend to minimize errors due the cellular behavior, functionality parameters and process definitions (Razafindralambo et al., 1996; Liu et al., 2012; Zhao et al., 2013; Willenbacher et al., 2015; Santos et al., 2016; Najmi et al., 2018; Hu et al., 2019). Achieving values that approach the optimized equations, in small-scale studies, are essential for industrial applications. After small-scale optimization, the same parameters values were employed on 5 L bioreactors, with an increasing on time to evaluate if a higher yield of crude BSF would be obtained. Both productions reached a maximum production of more than 6 g/L of crude BSF. Even with decreasing concentration, 6 g/L is above to other BSF productions between 3-4 g/L (Sen and Swaminathan, 1997; Davis et al., 1999; Yeh et al., 2005; Abdel-Mawgoud et al., 2008; Chen et al., 2015; Meena and Kanwar, 2015; Hu et al., 2019). Another important fact is that residual glycerin can be used as a carbon source for BSF production. The slight increase of surface tension on figure 8 between 0-10 h can be related to the use of glycerin as carbon source. Once this carbon source is more complex than glucose, it takes more time to the cells metabolize glycerin. Therefore, in 0 h the residual glycerin decreased de surface tension, but in 10 h residual glycerin probably was been metabolized by the cells and the medium only contained compounds that increases surface tension. After 10 h, the cells start to produce BSFs which decreased surface tension. However, figure 8 also demonstrate that with residual glycerin BSF production reached 6.5 g/L which shows the potential use of this substrate as carbon source, reaching CMC in 12 h. Since residual glycerin is a waste of the biodiesel industry (Kumar

et al., 2014), it could be reused and thus adding value to the industry and bringing benefits to the environment.

Qualitative tests (such as oil spreading, drop collapse and wettability reversal of rocks) emulsification tests demonstrate the potential of *B. amyloliquefaciens* MO13 BSF for hydrocarbons bioremediations and advanced oil recovery, since the results corroborate with literature (Cooper and Goldenberg, 1987; Morikawa et al., 1993; Sen and Swaminathan, 1997; Bodour and Miller-Maier, 1998; Davis et al., 1999; Youssef et al., 2004; Yeh et al., 2005; Abdel-Mawgoud et al., 2008; Pornsunthorntawee et al., 2008; Sriram et al., 2011; Chen et al., 2015; Meena and Kanwar, 2015; Moro et al., 2018). The oil spreading assay (i.e., measurement of the halo formed by oil scattering) can be used to determine the potential BSF application for bioremediation strategies on areas degraded with hydrocarbons (Pornsunthorntawee et al., 2008; Moro et al., 2018). Noteworthy, the halo formed employing *B. amyloliquefaciens* MO13 BSFs was identical to the positive control. Furthermore, the results of drop collapse assay were also promising. Additionally, the emulsification rates, observed in the emulsification assay, reached 85 %. Emulsifying surfactants are considered efficient when they have an emulsification above 60% (Cooper and Goldenberg, 1987; Sriram et al., 2011). It is observed a decrease of surface tension during BSF production and probably is due the formation of an emulsion that demonstrates the action of *B. amyloliquefaciens* MO13 BSF in contact with hydrocarbons. Furthermore, the wettability reversal of rocks assay demonstrates optimal results in comparison with the literature, where almost all calcite is in the bottom of the tube (Salehi et al., 2006; Wang et al., 2011; de Castilho et al., 2018). Many oil exploration facilities have a significant amount of oil in the rock pores and *B.*

amyloliquefaciens MO13 BSFs could be employed to maximize petroleum extraction, on an advanced microbial oil recovery strategy (Goddard III et al., 2005).

To define the minimum concentration of crude BSF need to reduce the surface tension in distilled water, the quantitative assay of the critical micellar concentration (CMC) was employed. According to the results, BSFs of both conditions had similar behaviors. CMCs was identified at 10 mg/L reaching 30 mN/m and the surface tension was stable on higher concentrations (Figure 13). The surface tension decrease curve observed in the results is typical of surfactants founded in the literature (Zhang and Miller, 1992; Nitschke and Pastore, 2006; Ferhat et al., 2011). The lower the ratio between concentration and surface tension the better for industrial applications, since the lower the costs for concentrating the BSFs. Most BSFs currently used have a critical micellar concentration of between 1 - 40 mg/L of BSF for 1 - 35 mN/m surface tension (Moro et al., 2018). Therefore, *B. amyloliquefaciens* MO13 BSFs have the potential to be used for bioremediation on soils and water in degraded areas with hydrocarbons since their CMC is appropriate for this application (Zhang and Miller, 1992; Nitschke and Pastore, 2006; Ferhat et al., 2011; Moro et al., 2018).

In order to characterize the composition of BSFs, obtained in the cultures of *B. amyloliquefaciens* MO13, a high resolution mass spectrometry approach was employed. Families of surfactins and iturines were detected, corroborating to previous studies showing the same pattern of molecules in *Bacillus* spp. cultures (Chen et al., 2008; Ali et al., 2014; Gong et al., 2015; Pereira et al., 2018). The same class of surfactins identified here, has already been identified previously by our group (Moro et al., 2018) and it has been already known as BSFs that play an important role in bioremediation processes

(Chen et al., 2008; Ali et al., 2014; Gong et al., 2015; Pereira et al., 2018). Both identified surfactins and iturines form micelles with oil allow a faster hydrocarbons degradation by bioavailability of the oil since the microbiota can access this oil readily and use these compounds as carbon source. Besides that, the BSFs identified could also be employed on food, pharmaceutical and cosmetics industries (Zhang, 2012; Deleu et al., 2013; Mandal et al., 2013; Meena and Kanwar, 2015; Aslam et al., 2019; de Freitas Ferreira et al., 2019; Ding et al., 2019).

5. Conclusions

- On a smaller scale, the best conditions for optimizing BSF production were identified.
- On a large scale the already stablished conditions were further standardized and the BSF evaluation assays were performed.
- Characterization and identification of the compounds present in BSF.
- Qualitative and quantitative results for the production of *Bacillus amyloliquefaciens* MO13 show that the production was adequate and the BSFs produced are promising for use in oil contaminated areas and for application in the pharmaceutical, cosmetic and food industry areas.

Conflict of Interest

The authors declare that there are no conflicts of interests

Acknowledgments

This work was supported by grants from the Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal do Ensino Superior (CAPES). AS and MHV have been supported by grant number [302637/2017-6] and [307191/2016-8], respectively. This study is part of the Programa CIMAR from CAPES n°: 23038.001433/2014-64. The authors would like to thank to Nicolau Sbaraini Oliveira and Lívia Kmetzch for supporting manuscript preparation.

Author's contributions

HAB, APN, JESP, VMMM and MHV conceived and designed the experiments. HAB, APN, JESP, AD performed the experiments. HAB wrote the manuscript. HAB, APN, JESP, VMMM and MHV provided editorial comments and helpful discussion. All authors read and approved the final version of the manuscript.

Table 1: Qualitative and emulsification tests for BSFs produced from 500 ml bioreactors after 40 hours at 31°C, 252 rpm, 0.43 Lpm and pH 7.0 and 34°C, 239 rpm, 0.31 Lpm and pH 7.2 using glucose and residual glycerin as carbon source respectively.

Carbon Source	Oil spread assay[3]	Drop collapse assay[3]	Emulsification (E_{24}) assay	Reversal Wettability of rocks[3]
Negative Control[1]	-	-	0%	-
Positive Control[2]	+++	+++	NA[4]	+++
Glucose	+++	+++	85%	+++
Residual Glycerin	+++	+++	85%	+++

[1] Negative control: MSM
[2] Positive control: SDS 10%

[3] Symbols means: (-) no results, (+) average results, (++) good results, (+++) excellent results.
[4] NA: Non-available.

Table 2: Crude BSF concentration, CFU/ml and surface tension results using 30g/L glucose as carbon source.

Experimental tests					Observed (Estimated[1])		
Assay	pH	T[2] (°C)	Stir[3] (rpm)	Aer[4] (Lpm)	Crude BSF Conc (g/L)[5]	CFU/mL[6]	Surface Tension (mN/m)
1	6.5	25	200	0.1	0.30 (-0.74)	1.6E+11	28.2
2	7.5	25	200	0.1	4.93 (5.93)	2.3E+11	37.7
3	6.5	35	200	0.1	3.07 (1.90)	2.7E+11	28.3
4	7.5	35	200	0.1	12.03 (8.60)	3E+11	28.6
5	6.5	25	300	0.1	1 (0.50)	2.2E+11	28.6
6	7.5	25	300	0.1	2.81 (3.94)	1.4E+11	37.35
7	6.5	35	300	0.1	0.53 (0.91)	4E+10	27.5
8	7.5	35	300	0.1	1.61 (4.37)	1.7E+11	29.3
9	6.5	25	200	0.5	0.99 (0.68)	1.7E+10	41
10	7.5	25	200	0.5	0.93 (2.58)	1.1E+11	37.25
11	6.5	35	200	0.5	1.02 (1.92)	9E+10	28
12	7.5	35	200	0.5	0.9 (3.84)	2.1E+11	26.8
13	6.5	25	300	0.5	0.16 (5.63)	4E+10	70.2
14	7.5	25	300	0.5	0.67 (4.29)	9E+10	29.3

15	6.5	35	300	0.5	3.18 (4.63)	7E+10	63.25
16	7.5	35	300	0.5	0.22 (3.31)	6E+10	28.2
17	6.0	30	250	0.3	0.24 (2.91)	9E+10	28.2
18	8.0	30	250	0.3	10.45 (3.28)	1.5E+11	43.2
19	7.0	20	250	0.3	0.66 (2.64)	3.7E+11	29
20	7.0	40	250	0.3	9.82 (3.36)	9E+10	29.1
21	7.0	30	150	0.3	6.41 (3.14)	1E+11	67.3
22	7.0	30	350	0.3	6.03 (4.81)	1.2E+11	29.05
23	7.0	30	250	0.0	0.66 (0.32)	8E+10	29.3
24	7.0	30	250	0.7	3.82 (5.68)	2.3E+11	31.2
25	7.0	30	250	0.3	14.45 (14.41)	2.6E+11	28.6
26	7.0	30	250	0.3	14 (14.41)	1E+11	28.6
27	7.0	30	250	0.3	14.2 (14.41)	2E+10	27.7
28	7.0	30	250	0.3	14.4 (14.41)	4.1E+11	27.6
29	7.0	30	250	0.3	15 (14.41)	2.6E+11	27.6

[1]Values estimated from factorial design equation (see section 2.3)

[2]Temperature

[3]Stirrer

[4]Aeration

[5]Crude BSF Concentration (g/L)

[6]Colony Formation Unity per mL

Table 3: Crude BSF concentration, CFU/ml and surface tension results using 30g/L residual glycerin as carbon source.

Experimental tests					Observed (Estimated[1])		
Assay	pH	T[2] (°C)	Stir[3] (rpm)	Aer[4] (Lpm)	Crude Bio Conc (g/L)[5]	CFU/mL[6]	Surface Tension (mN/m)
1	6.5	25	200	0.1	2.4 (2.53)	1.6E+11	28.2
2	7.5	25	200	0.1	0.77 (-1.09)	2.3E+11	37.7
3	6.5	35	200	0.1	11.13 (7.35)	2.7E+11	28.3
4	7.5	35	200	0.1	6.46 (7.80)	3E+11	28.6
5	6.5	25	300	0.1	5.17 (6.15)	2.2E+11	28.6
6	7.5	25	300	0.1	2.12 (1.90)	1.4E+11	37.35
7	6.5	35	300	0.1	4.66 (5.42)	4E+10	27.5
8	7.5	35	300	0.1	7.79 (5.25)	1.7E+11	29.3
9	6.5	25	200	0.5	0.59 (-0.35)	1.7E+10	41
10	7.5	25	200	0.5	2.36 (2.90)	1.1E+11	37.25
11	6.5	35	200	0.5	2.30 (3.82)	9E+10	28
12	7.5	35	200	0.5	15.63 (11.17)	2.1E+11	26.8
13	6.5	25	300	0.5	0.38 (0.34)	4E+10	70.2
14	7.5	25	300	0.5	2.67 (2.97)	9E+10	29.3
15	6.5	35	300	0.5	0.57 (-1.03)	7E+10	63.25
16	7.5	35	300	0.5	4.51 (5.69)	6E+10	28.2
17	6.0	30	250	0.3	2.31 (3.81)	9E+10	28.2

18	8.0	30	250	0.3	0.68 (1.35)	1.5E+11	43.2
19	7.0	20	250	0.3	4.05 (6.73)	3.7E+11	29
20	7.0	40	250	0.3	5.38 (4.87)	9E+10	29.1
21	7.0	30	150	0.3	0.62 (0.09)	1E+11	67.3
22	7.0	30	350	0.3	4.92 (7.62)	1.2E+11	29.05
23	7.0	30	250	0.0	1.02 (1.42)	8E+10	29.3
24	7.0	30	250	0.7	2.75 (4.52)	2.3E+11	31.2
25	7.0	30	250	0.3	15.25 (13.47)	2.6E+11	28.6
26	7.0	30	250	0.3	9.95 (13.47)	1E+11	28.6
27	7.0	30	250	0.3	16.02 (13.47)	2E+10	27.7
28	7.0	30	250	0.3	15.38 (13.47)	4.1E+11	27.6
29	7.0	30	250	0.3	10.74 (13.47)	2.6E+11	27.6

[1]Values estimated from fractional design equation (see section 2.3); [2]Temperature; [3]Stirrer; [4]Aeration; [5]Crude BSF Concentration (g/L); [6]Colony Formation Unity per mL

Table 4: BSFs characterization by high resolution mass spectrometry.

Structure	Molecular formula	[M+Na]+	Glucose Observed [M+Na]+	Error (ppm)	R. Glycerin Observed [M+Na]+	Error (ppm)
Surfactins						
C13 (Val - 7)	$C_{50}H_{88}N_7O_{13}$	1016.6440	1016.6215	2.2	1016.6222	2.2
C13 (Leu - 7)	$C_{51}H_{90}N_7O_{13}$	1030.6596	1030.6369	2.2	1030.6377	2.6

C14 (Leu - 7)	$C_{52}H_{92}N_7O_{13}$	1044.6753	1044.6525	2.3	1044.6534	2.2
C15 (Leu - 7)	$C_{53}H_{94}N_7O_{13}$	1058.6909	1058.6680	2.3	1058.6689	3.8
C16 (Leu - 7)	$C_{54}H_{96}N_7O_{13}$	1072.7066	1072.6830	2.4	1072.6836	2.3
Iturins						
C14 (Asn - 1)	$C_{54}H_{89}N_7O_{13}$	1066.6625	1066.6340	2.8	1066.6350	2.7
C15 (Asn - 1)	$C_{55}H_{91}N_7O_{13}$	1080.6995	1080.6488	5.0	1080.6505	4.9

Figures

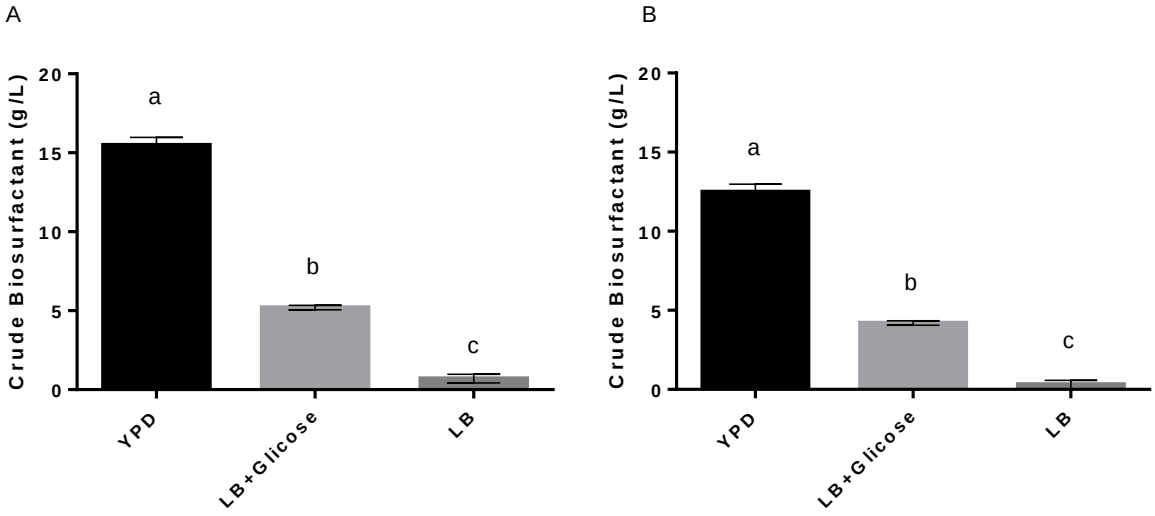

Figure 1. Pre-inoculum effects on BSF production using glucose 3% w/v (A) and residual glycerin 3% w/v (B) as carbon source for inoculum. Three different pre-inoculums were tested to observe which one would be more appropriated to achieve best results on BSF production. Conditions for BSF production were 30°C, pH 7.0, 250 rpm and 0.3 Lpm. All the tests were made in triplicates. The results are present in MEAN + SD. The results demonstrate significative statistical difference among the samples with $p < 0.05$. The different letters represent statistical difference by one-way ANOVA followed by Tukey test.

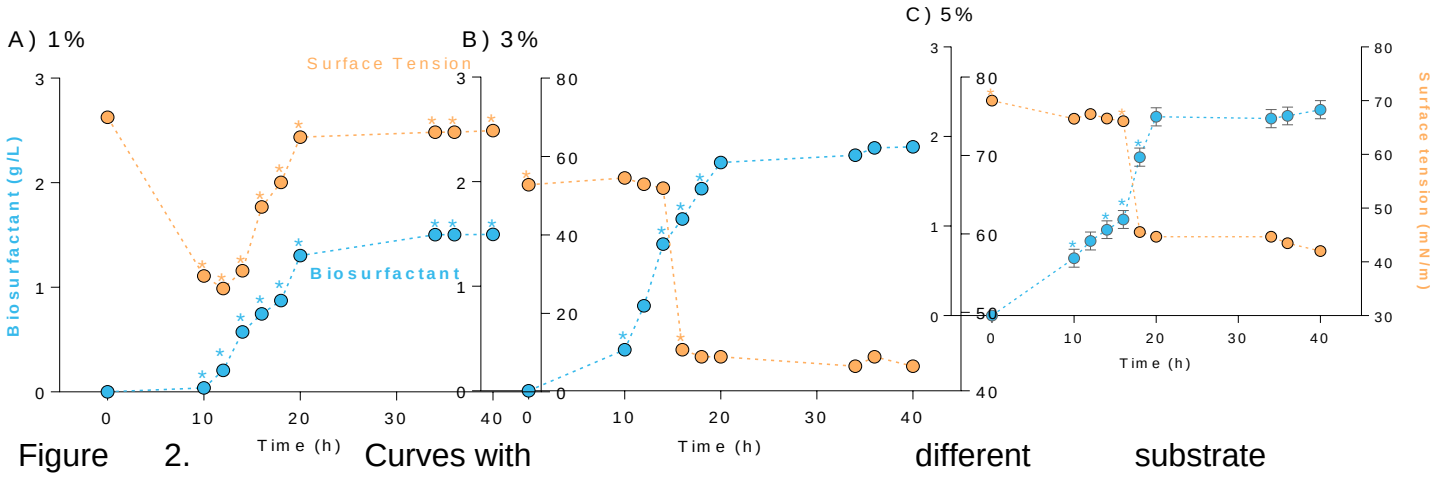

Figure 2. Curves with different substrate concentrations 50 g/L of glucose (A), 30 g/L of glucose (B) and 10 g/L of glucose (C) at 30°C, pH 7.0, 250 rpm and 0.5 Lpm. The results are present in MEAN + SD. The results demonstrate some significative statistical difference among the samples with $p < 0.05$. The symbol (*) represent statistical difference by two-way ANOVA followed by Tukey test.

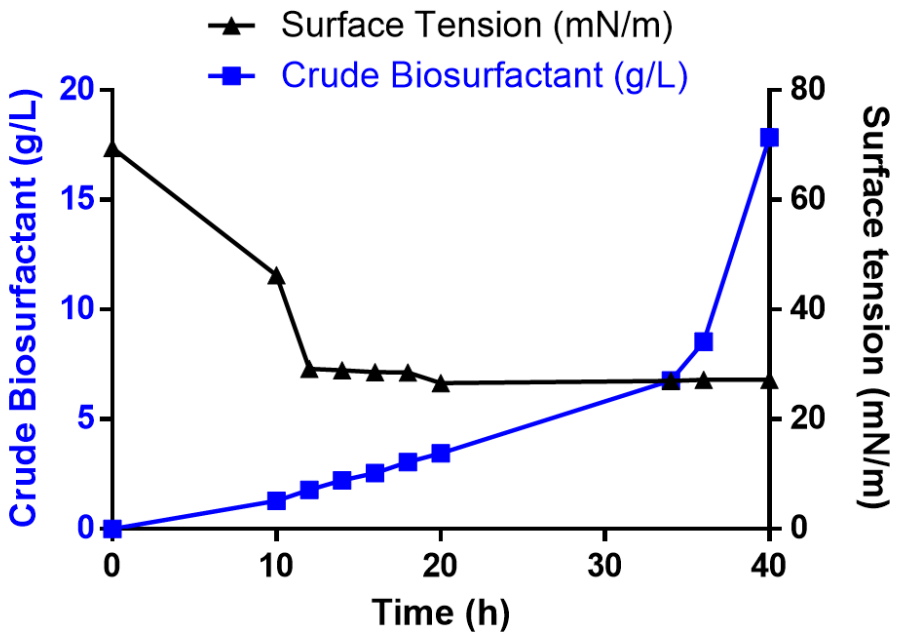

Figure 3. BSF production curve after optimization of the equation using glucose as carbon source, after 40 hours at 31°C, 252 rpm, 0.43 Lpm and pH 7.0. All tests were made in triplicates

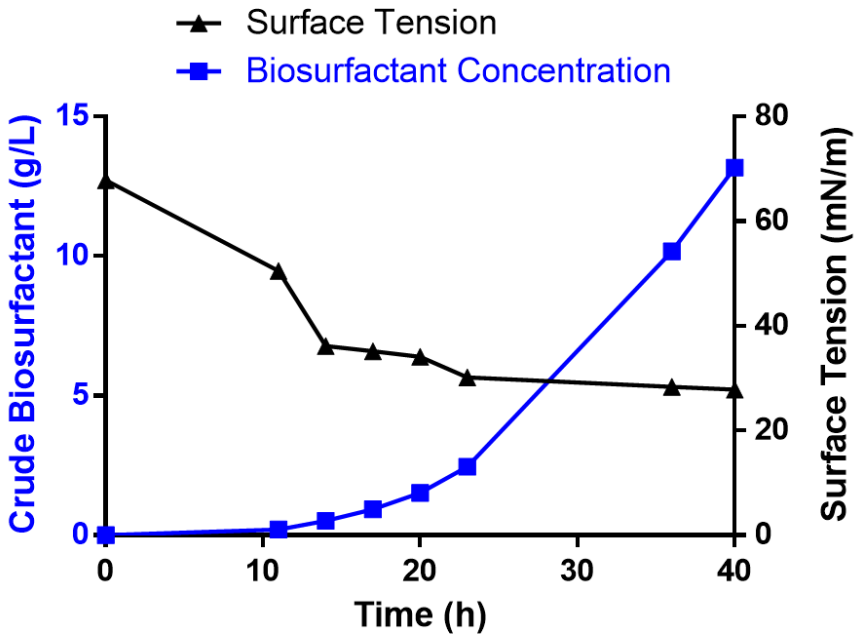

Figure 4. BSF production curve after equation optimization using residual glycerin as carbon source, after 40 hours at 34°C, 239 rpm, 0.31 Lpm and pH 7.2. All tests were made in triplicates.

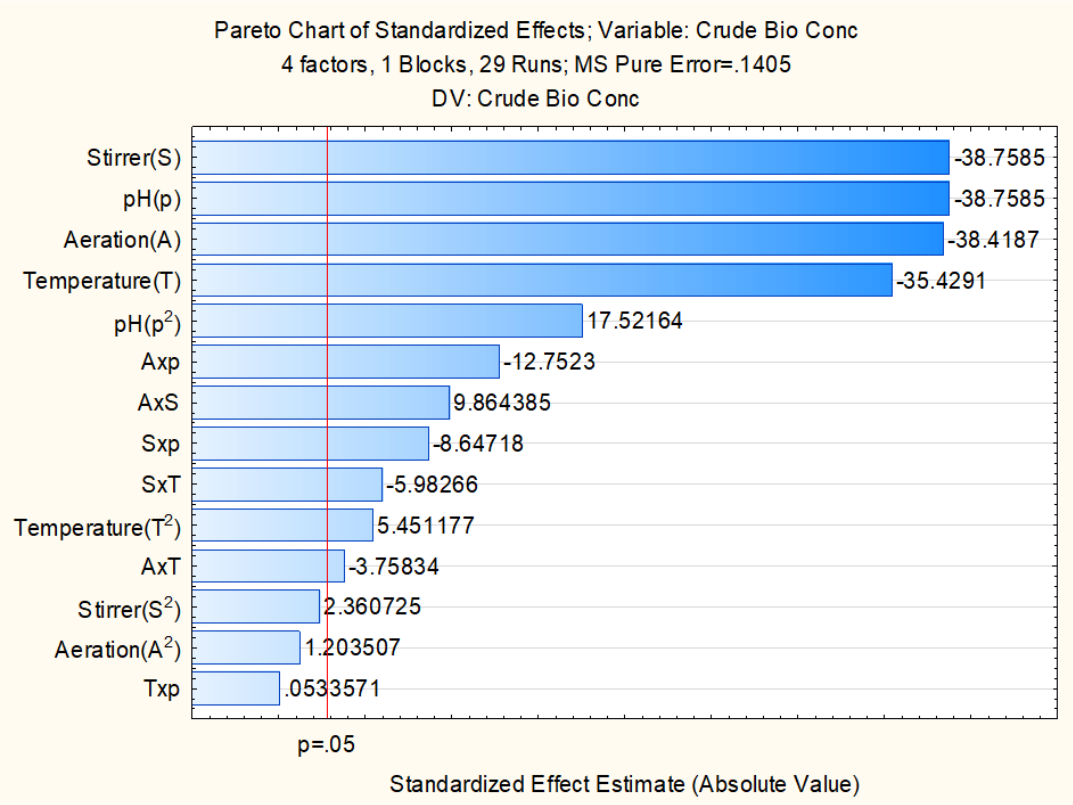

Figure 5. Central composite analysis on experiments using glucose as carbon source. Pareto chart showing the influence of each variable in the whole system. Values on the right of the red line are considerable significant. The increase on the value parameter has a negative influence when there is a minus (-) and vice versa. The analysis contains the interaction of p, T, S, A, p^2, T^2, S^2, A^2, pxT, pxS, pxA, TxS, TxA and SxA. The results demonstrate some significative statistical difference among the variables with $p < 0.05$ by two-way ANOVA.

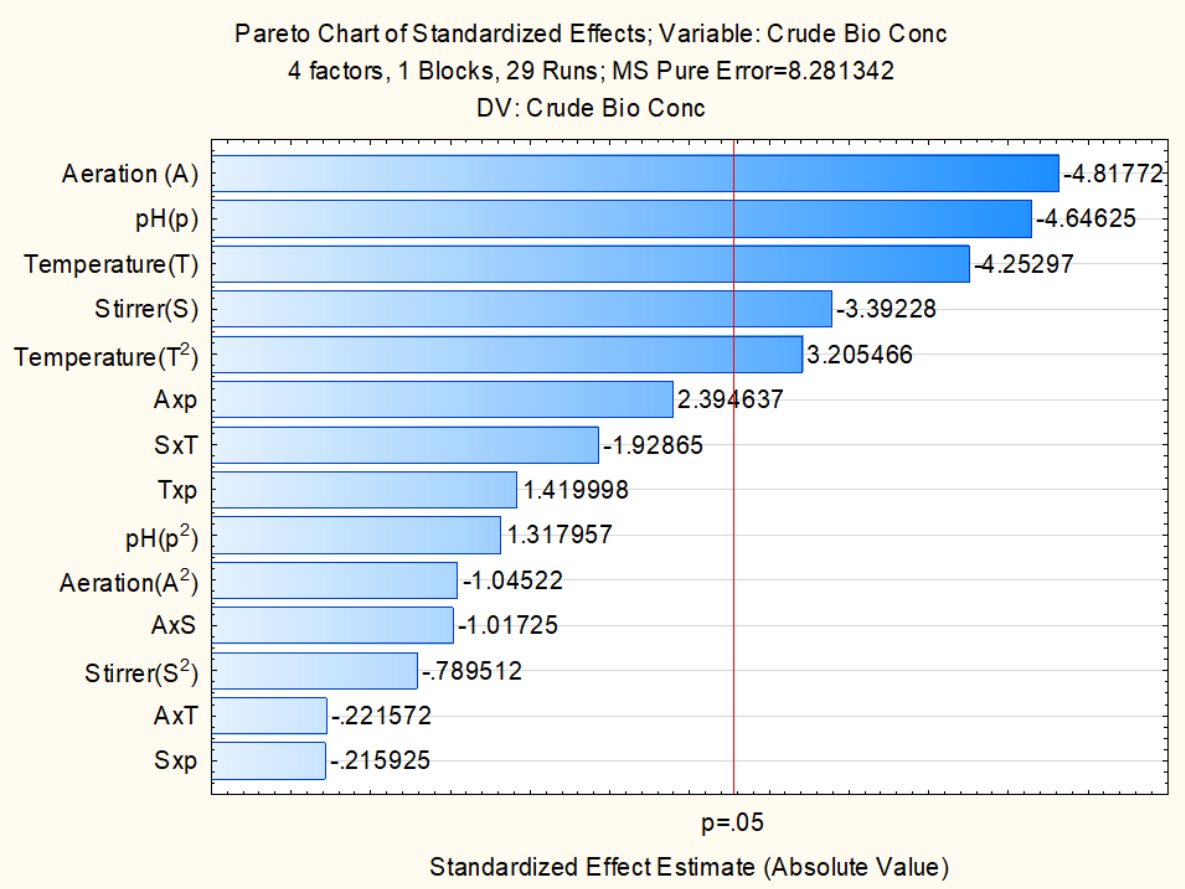

Figure 6. Central composite analysis on experiments using residual glycerin as carbon source. Pareto chart showing the influence of each variable in the whole system. Values on the right of the red line are considerable significant. The increase on the value parameter has a negative influence when there is a minus (-) and vice versa. The analysis contains the interaction of p, T, S, A, p^2, T^2, S^2, A^2, pxT, pxS, pxA, TxS, TxA and SxA. The results demonstrate some significative statistical difference among the variables with $p< 0.05$ by two-way ANOVA.

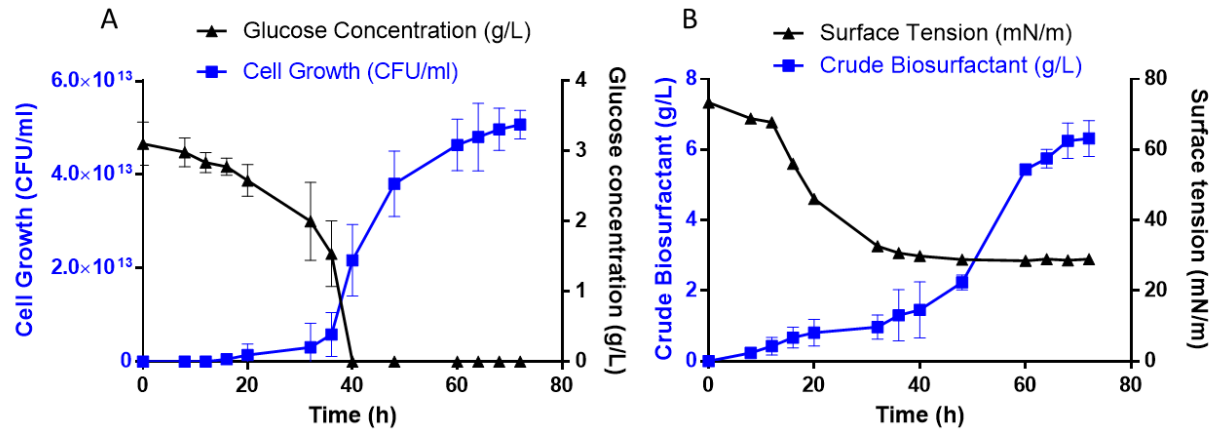

Figure 7. Cell growth and glucose consumption curves (A), crude BSF concentration and surface tension curves (B). Cell culture conditions were 31°C, 252 rpm, 0.43 Lpm and pH 7.0 for 72 h.

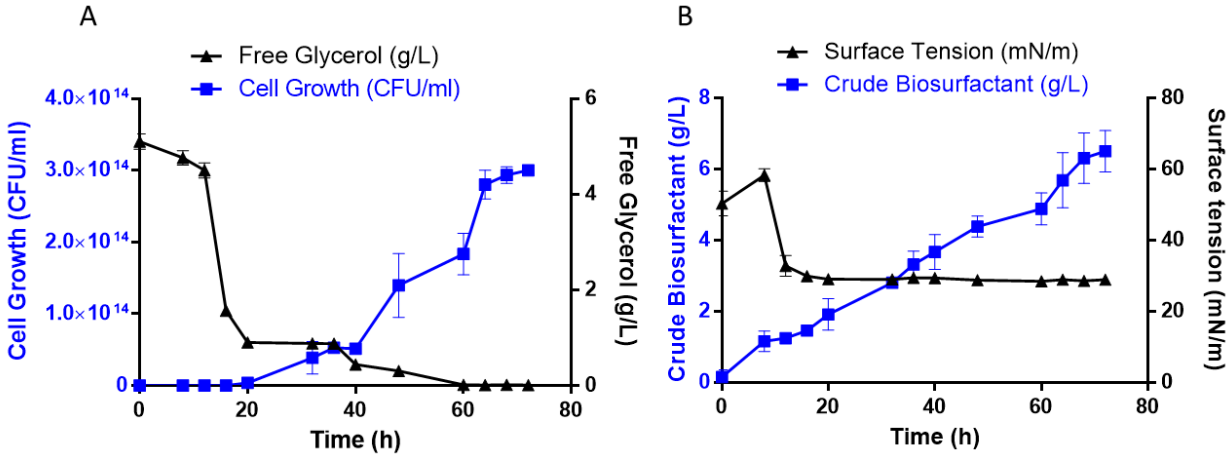

Figure 8. Cell growth and free glycerol consumption (A), crude BSF concentration and surface tension curves (B). Cell culture conditions were 34°C, 239 rpm, 0.31 Lpm and pH 7.2 for 72 h.

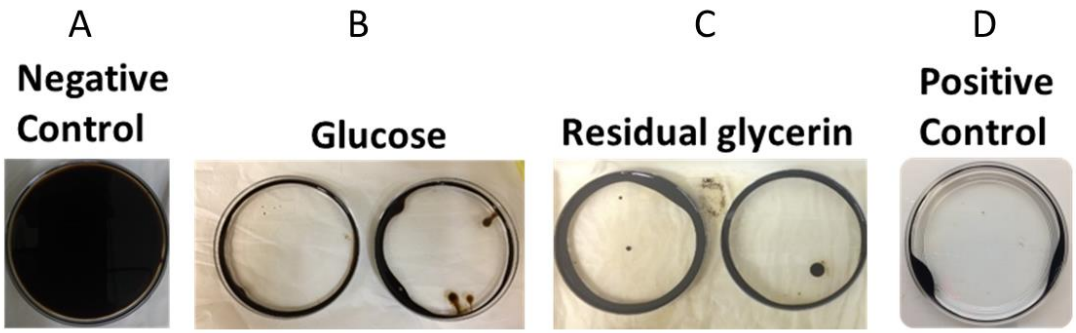

Figure 9. Oil spread assay. Positive results for both experimental conditions using glucose (B) and residual glycerin (C) as carbon source to produce BSFs. Negative control: MSM (A). Positive control: SDS 10% (D).

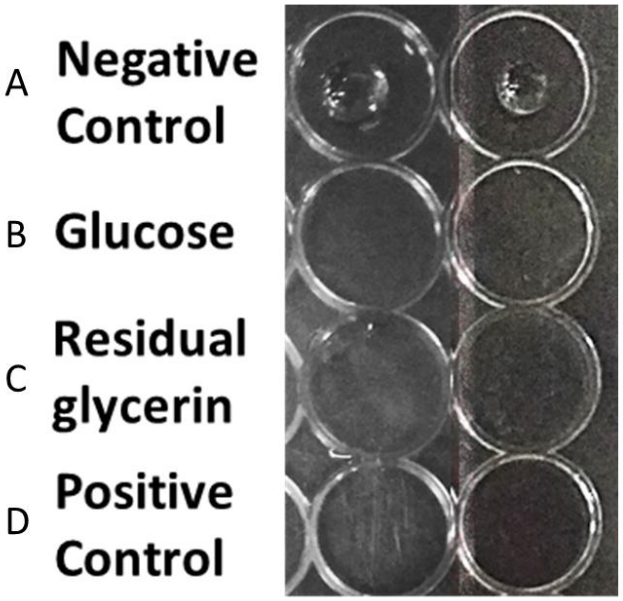

Figure 10. Drop collapse assay. Positive results for both experimental conditions using glucose (B) and residual glycerin (C) as carbon source to produce BSFs. Negative control: MSM (A). Positive control: SDS 10% (D).

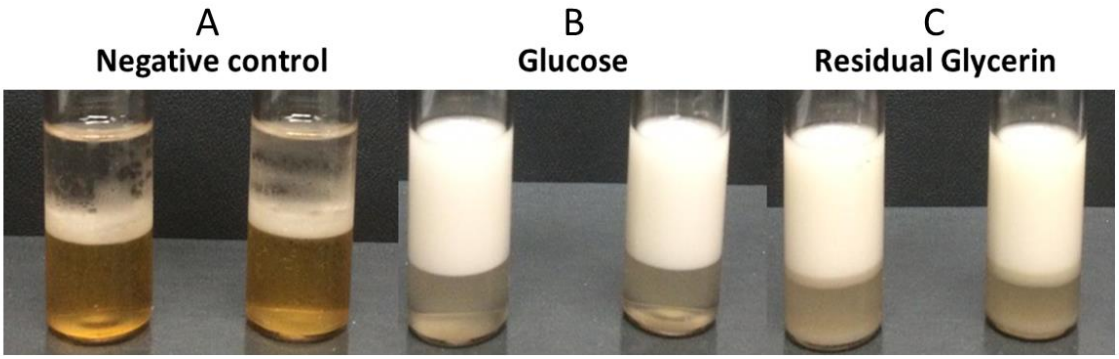

Figure 11. Emulsification activity assay. Positive results for both experimental conditions using glucose (B) and residual glycerin (C) as carbon source to produce BSFs. The emulsification achieves 85% for both conditions. Negative control: MSM (A).

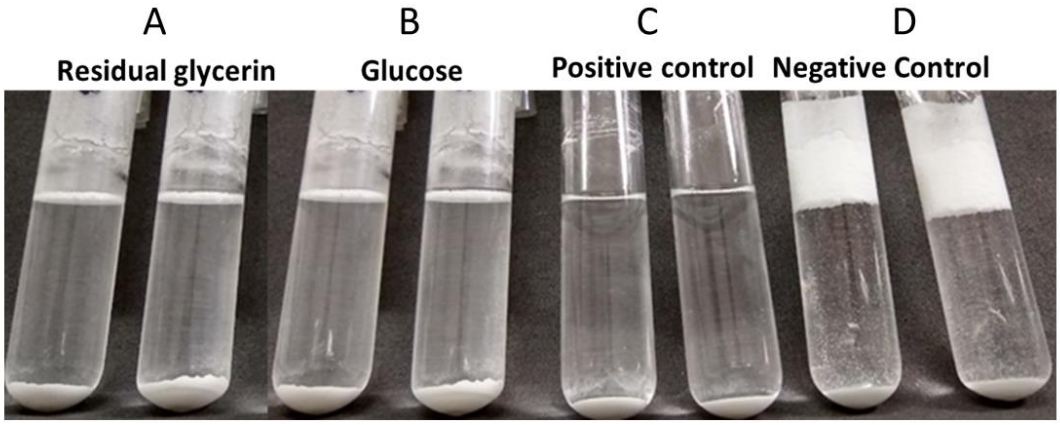

Figure 12. Wettability reversal of rocks assay. Positive results for both experimental conditions using glucose (A) and residual glycerin (B) as carbon source to produce BSFs. Negative control: synthetic sea water (A). Positive control: SDS 10% (D).

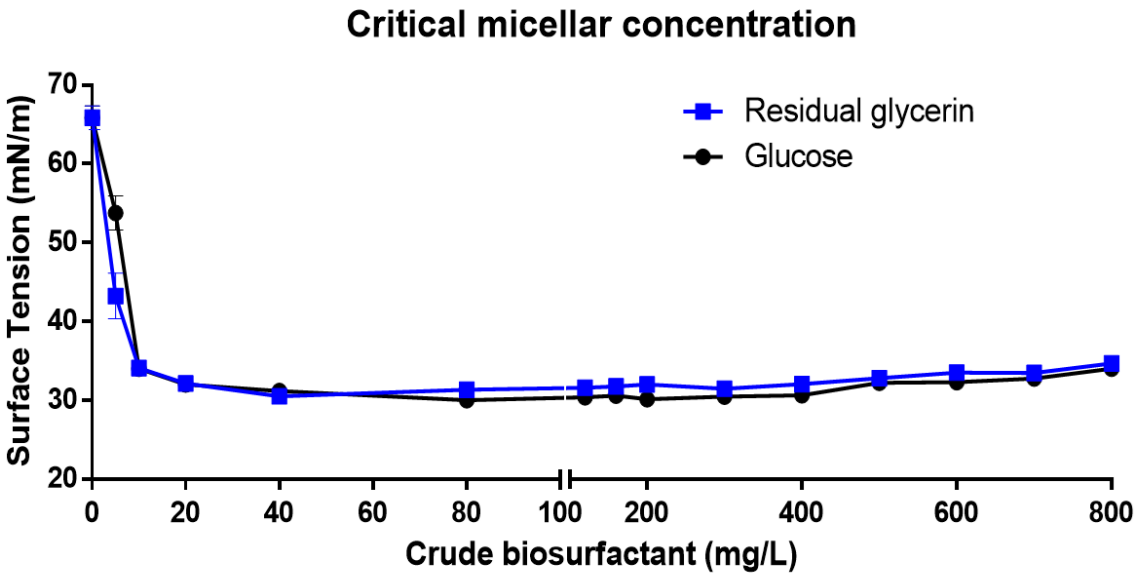

Figure 13. Critical micellar concentration (CMC) curves obtained from different concentrations using glucose and residual glycerin as substrates. It was used distilled water for the tests. All the tests were made in triplicates. As we observed both curves had similar tendencies.

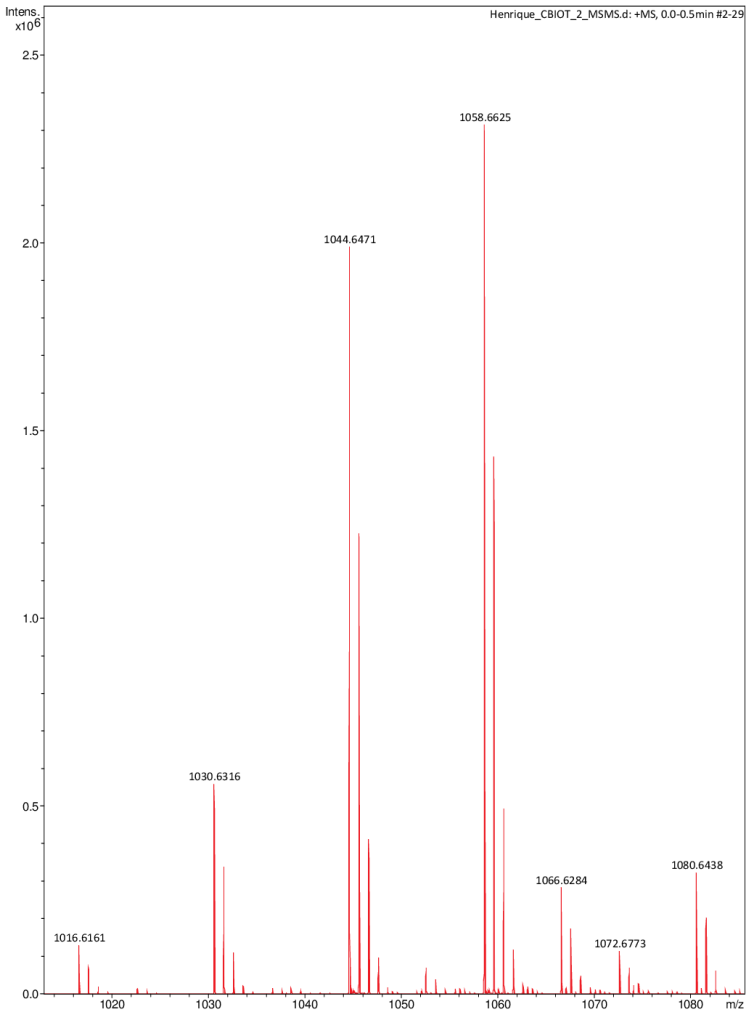

Figure 14. Glucose substrate results for Q-TOF-MS mass spectrum in positive ionization mode of m/z [M + Na] + ions observed up to 5 ppm mass error of surfactins and iturines produced by *Bacillus amyloliquefaciens* MO13 in MSM medium at 31°C, 252 rpm, 0.43 Lpm and pH 7.0 for 72 h.

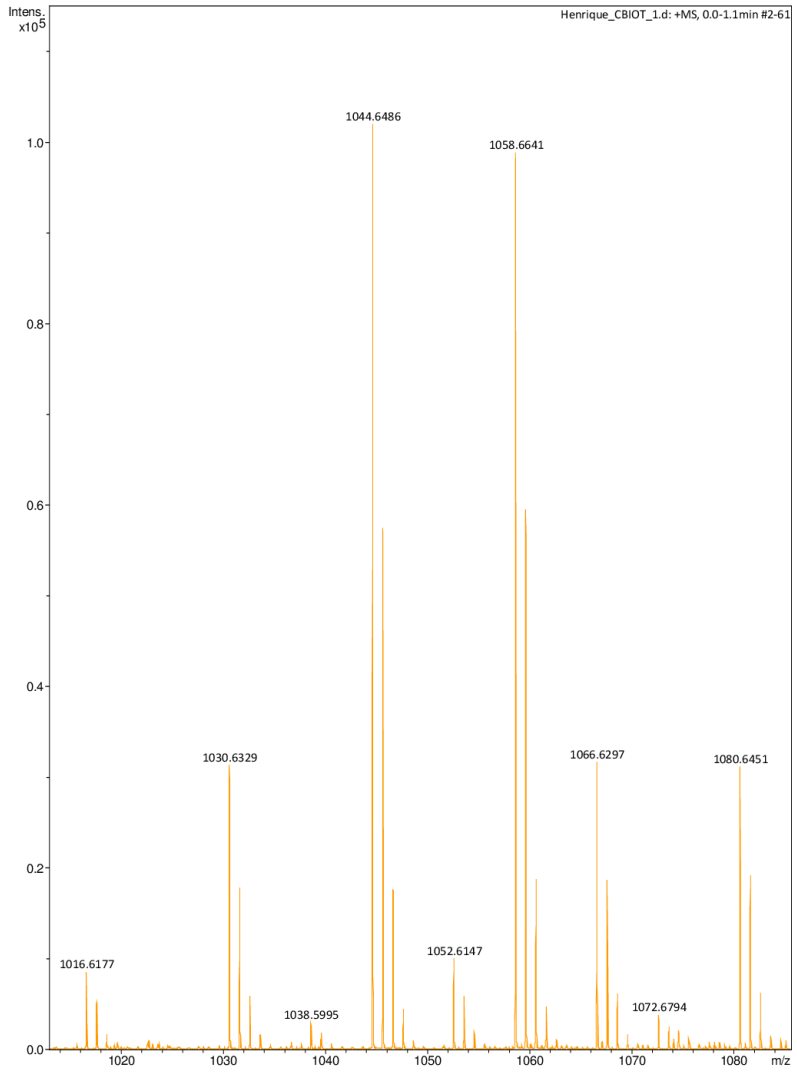

Figure 15. Residual glycerin substrate results for Q-TOF-MS mass spectrum in positive ionization mode of m/z [M + Na] + ions observed up to 5 ppm mass error of surfactins and iturines produced by *Bacillus amyloliquefaciens* MO13 in MSM medium at 34°C, 239 rpm, 0.31 Lpm and pH 7.2 for 72 h.

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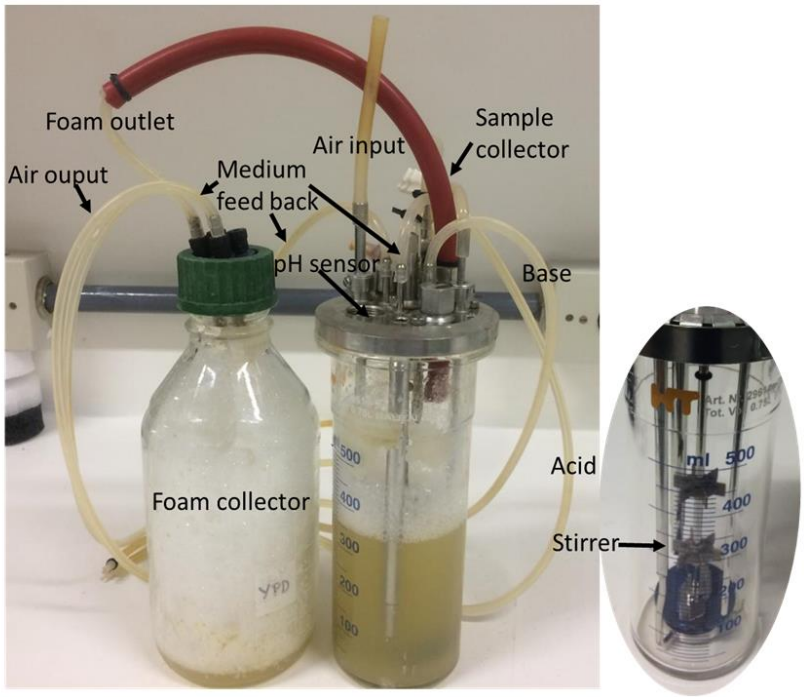
Supplementary material

Table S1. Residual glycerin physical-chemical characterization.

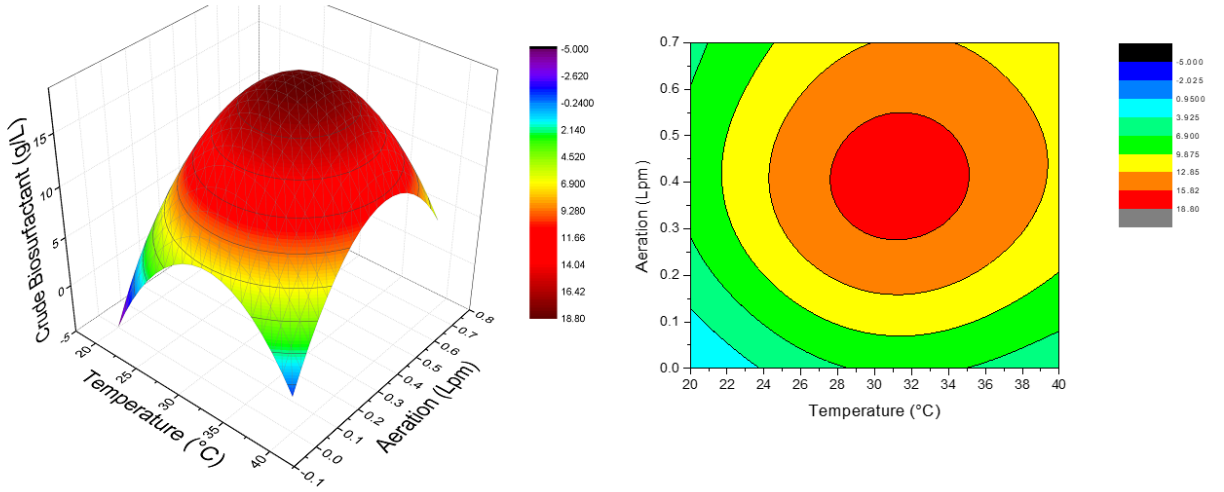
Component	Viscosity (mPa.s)	Density (g/mL)[(1)]	pH[(2)]	Free glycerol (%)[(3)]
Residual glycerin	232	1.263 ± 0.006	5.74 ± 0.02	78.9 ± 0.3
Glycerol[(d)]	887	1.254 ± 0.004	6.42 ± 0.03	-

(1), (2) and (3) are expressed in triplicates ± standard deviation.

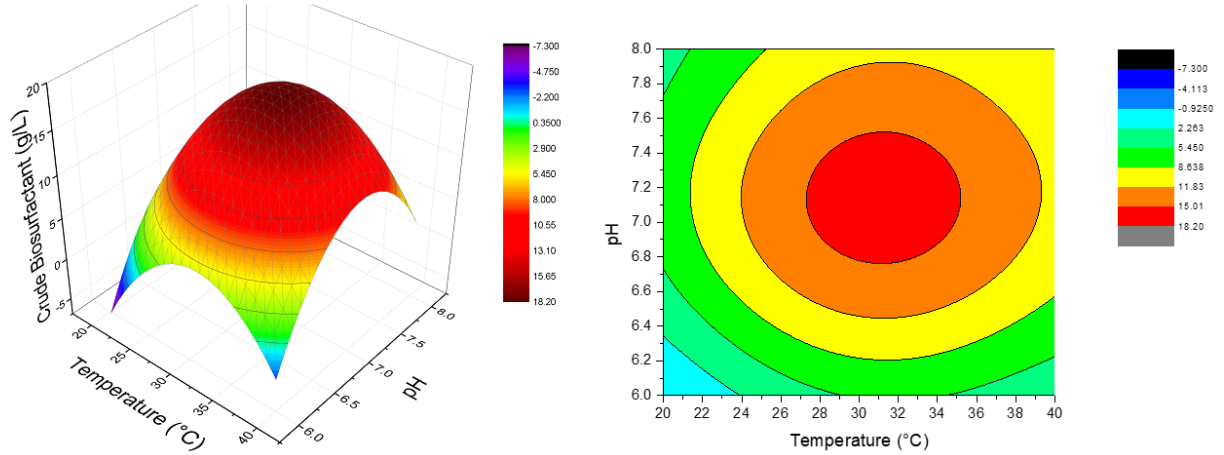
(4) Glycerol (Sigma-Aldrich, 99,5 %).



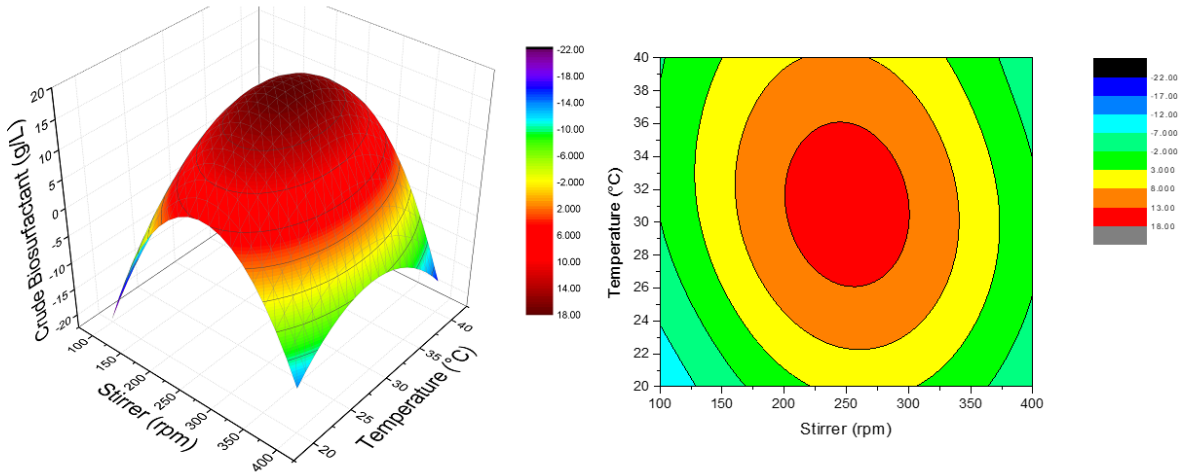
Supplementary Figure 1: Foam collector built by group in order to evaluate the BSF production without any losses of BSFs on the foam.



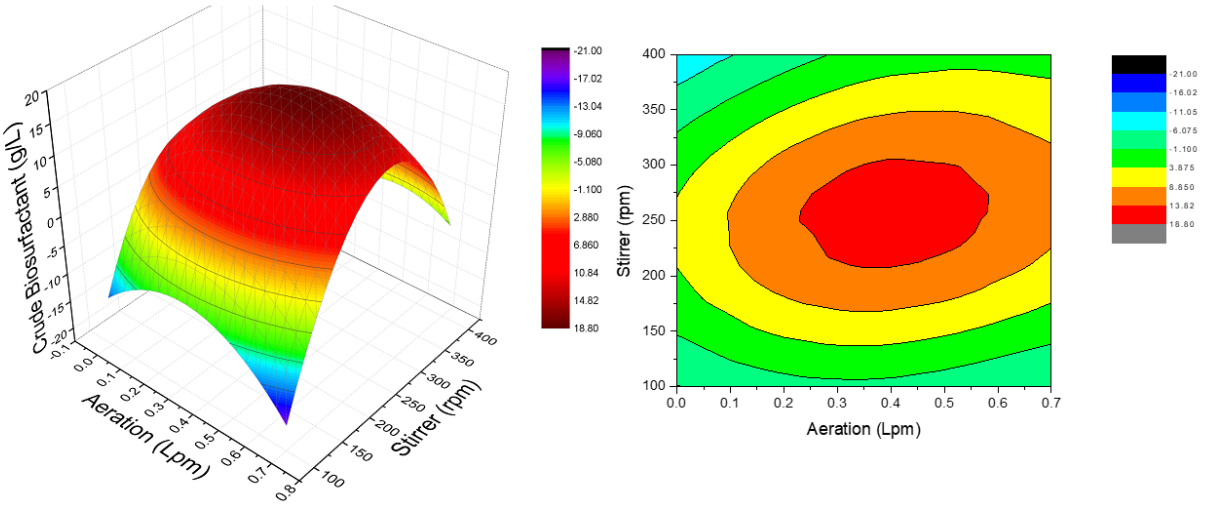
Supplementary Figure 2: Surface response for samples using 30 g/L of glucose as carbon source. 3D spatial graph (Z: Crude biosurfactant (g/L), Y: Aeration (Lpm), X: Temperature (°C)) and Contour graph (Temperature (°C) x Aeration (Lpm)) show the peak center as a hot spot condition to produce the maximum biosurfactant quantity.



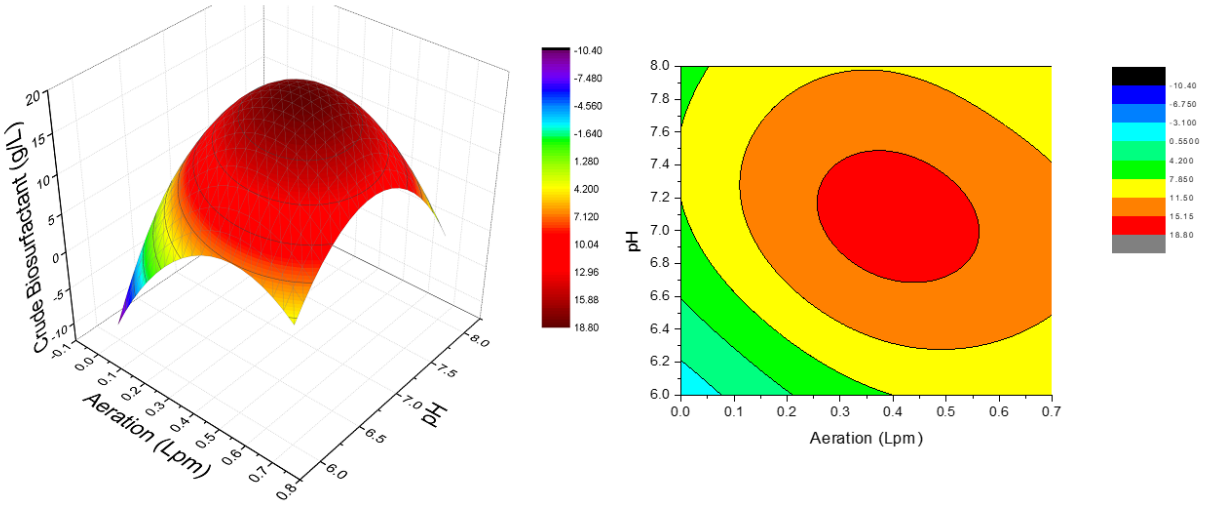
Supplementary Figure 3: Surface response for samples using 30 g/L of glucose as carbon source. 3D spatial graph (Z: Crude biosurfactant (g/L), Y: pH, X: Temperature (°C)) and Contour graph (Temperature (°C) x pH) show the peak center as a hot spot condition to produce the maximum biosurfactant quantity.



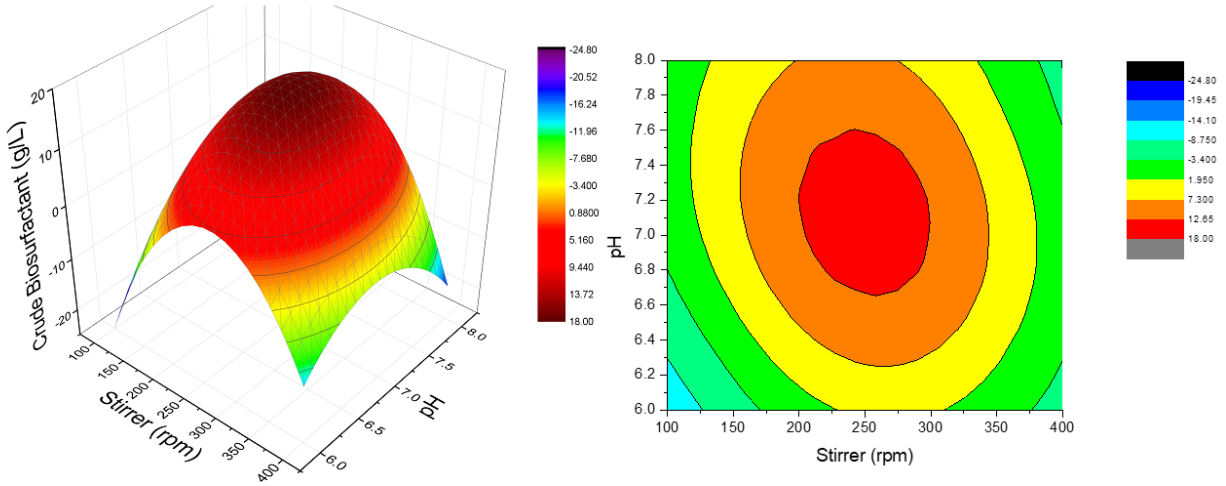
Supplementary Figure 4: Surface response for samples using 30 g/L of glucose as carbon source. 3D spatial graph (Z: Crude biosurfactant (g/L), Y: Temperature (°C), X: Stirrer (rpm)) and Contour graph (Stirrer (rpm) x Temperature (°C)) show the peak center as a hot spot condition to produce the maximum biosurfactant quantity.



Supplementary Figure 5: Surface response for samples using 30 g/L of glucose as carbon source. 3D spatial graph (Z: Crude biosurfactant (g/L), Y: Stirrer (rpm), X: Aeration (Lpm)) and Contour graph (Aeration (Lpm) x Stirrer (rpm)) show the peak center as a hot spot condition to produce the maximum biosurfactant quantity.



Supplementary Figure 6: Surface response for samples using 30 g/L of glucose as carbon source. 3D spatial graph (Z: Crude biosurfactant (g/L), Y: pH, X: Aeration (Lpm)) and Contour graph (Aeration (Lpm) x pH) show the peak center as a hot spot condition to produce the maximum biosurfactant quantity.

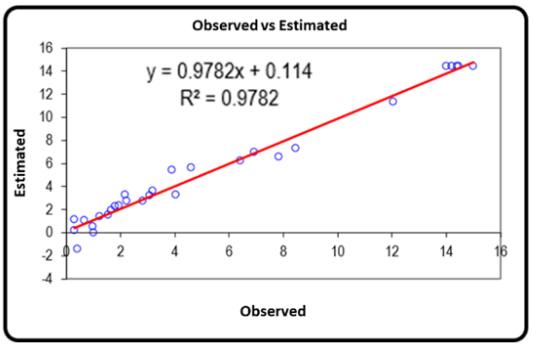


Supplementary Figure 7: Surface response for samples using 30 g/L of glucose as carbon source. 3D spatial graph (Z: Crude biosurfactant (g/L), Y: pH, X: Stirrer (rpm)) and Contour graph (Stirrer (rpm) x pH) show the peak center as a hot spot condition to produce the maximum biosurfactant quantity.

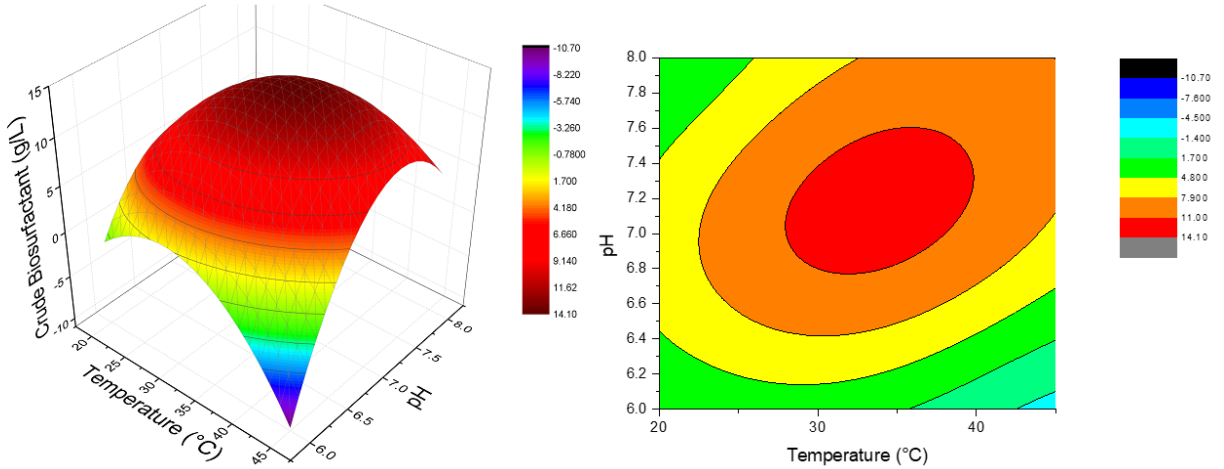
Variance analysis – Quadratic model

FV	SQ[1]	nGL[2]	MQ[3]	Fcalc.[4]	*p*
Regression	617.074	14	44.0767	2.73473	0.034947
Residues	225.643	14	16.1174		
F. adjust	225.643	10	22.5643		
Error	0.562	4	0.1405		
Total	842.717	28			

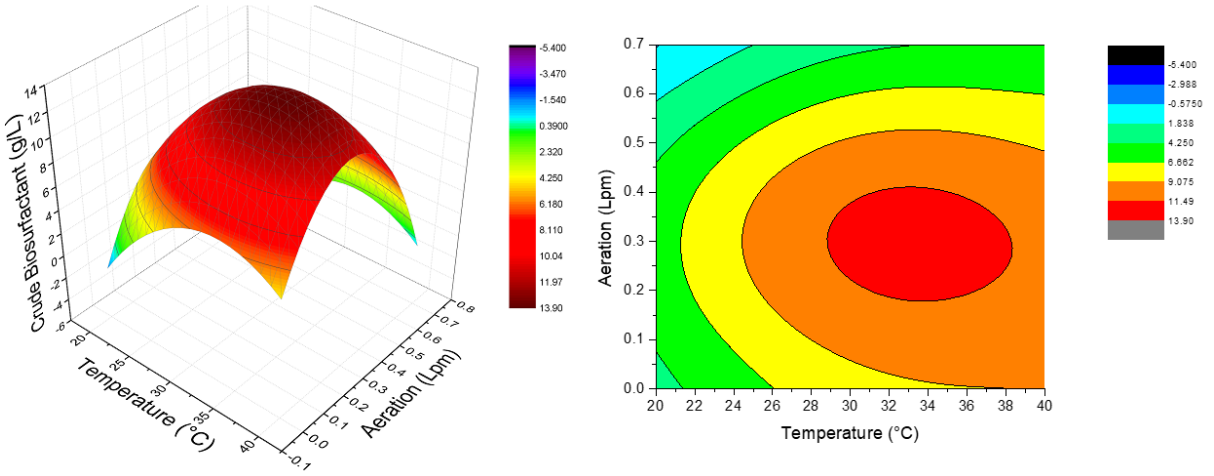
[1]Soma dos quadrados
[2]Número de graus de liberdade
[3]Média da soma dos quadrados
[4]Valor *F* calculado



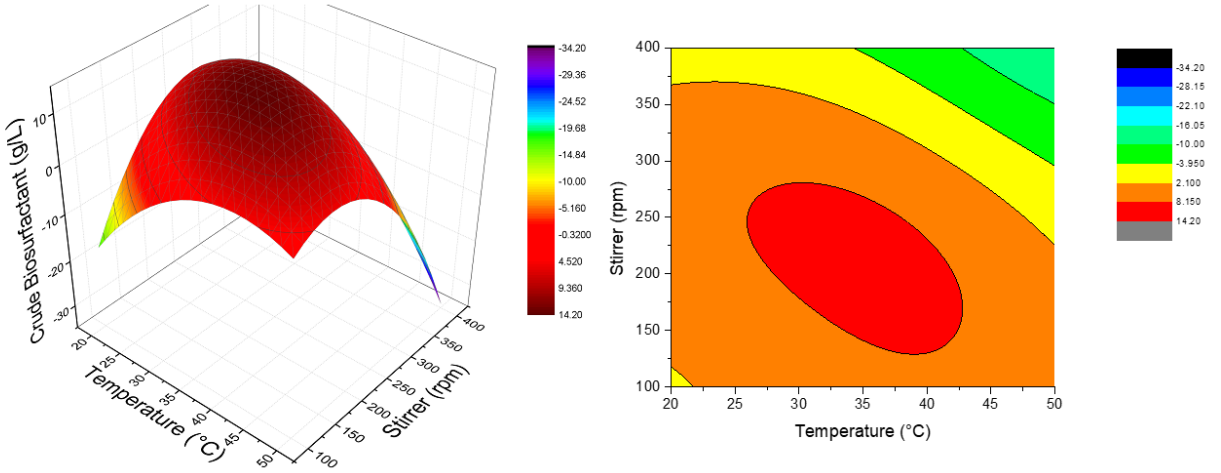
Supplementary Figure 8: F test, p value and R^2 from statistical analysis of CCD quadratic model using 30g/L of glucose as carbon source. The results were obtained through ANOVA and EXCEL.



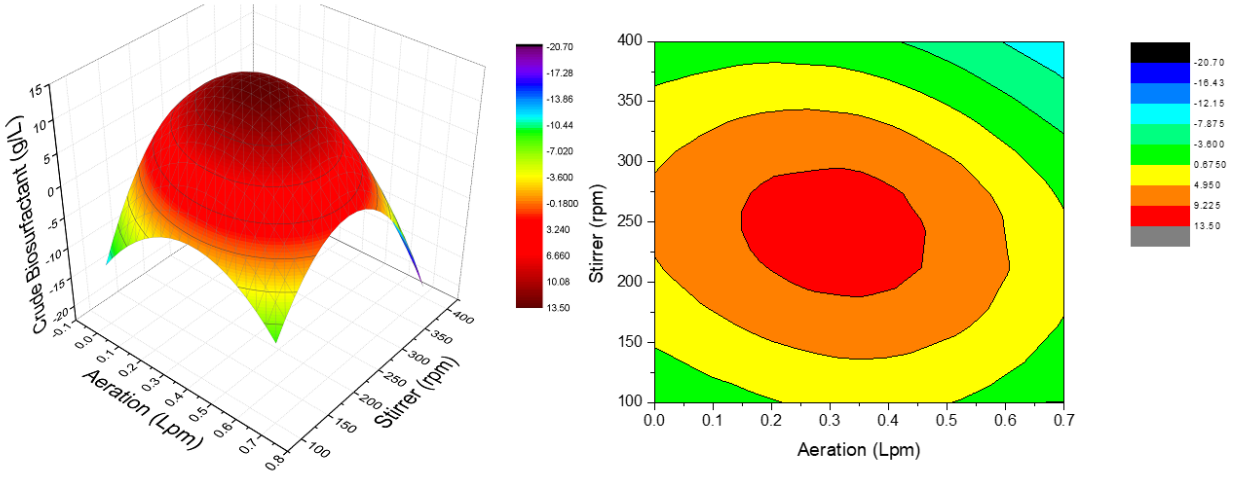
Supplementary Figure 9: Surface response for samples using 30 g/L of residual glycerin as carbon source. 3D spatial graph (Z: Crude biosurfactant (g/L), Y: pH, X: Temperature (°C)) and Contour graph (Temperature (°C) x pH) show the peak center as a hot spot condition to produce the maximum biosurfactant quantity.



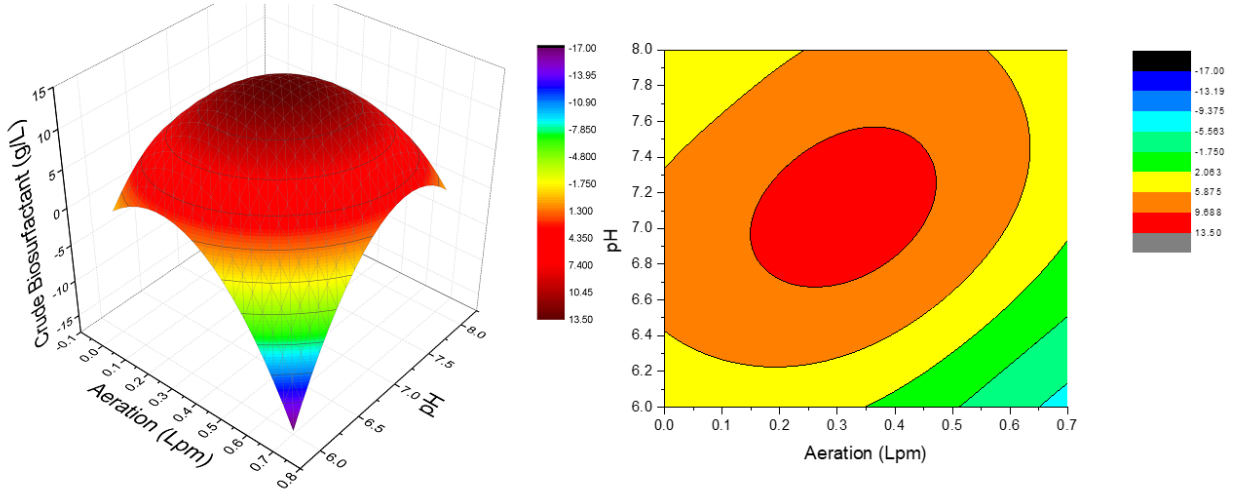
Supplementary Figure 10: Surface response for samples using 30 g/L of residual glycerin as carbon source. 3D spatial graph (Z: Crude biosurfactant (g/L), Y: Aeration (Lpm), X: Temperature (°C)) and Contour graph (Temperature (°C) x Aeration (Lpm)) show the peak center as a hot spot condition to produce the maximum biosurfactant quantity.



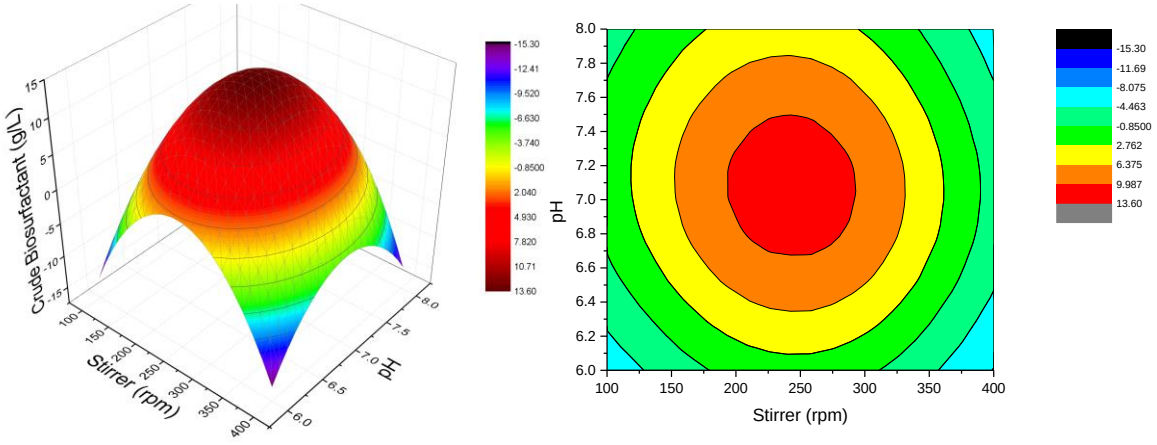
Supplementary Figure 11: Surface response for samples using 30 g/L of residual glycerin as carbon source. 3D spatial graph (Z: Crude biosurfactant (g/L), Y: Stirrer (rpm), X: Temperature (°C)) and Contour graph (Temperature (°C) x Stirrer (rpm)) show the peak center as a hot spot condition to produce the maximum biosurfactant quantity.



Supplementary Figure 12: Surface response for samples using 30 g/L of residual glycerin as carbon source. 3D spatial graph (Z: Crude biosurfactant (g/L), Y: Stirrer (rpm), X: Temperature (°C)) and Contour graph (Temperature (°C) x Stirrer (rpm)) show the peak center as a hot spot condition to produce the maximum biosurfactant quantity.



Supplementary Figure 13: Surface response for samples using 30 g/L of residual glycerin as carbon source. 3D spatial graph (Z: Crude biosurfactant (g/L), Y: pH, X: Aeration (Lpm)) and Contour graph (Aeration (Lpm) x pH) show the peak center as a hot spot condition to produce the maximum biosurfactant quantity.

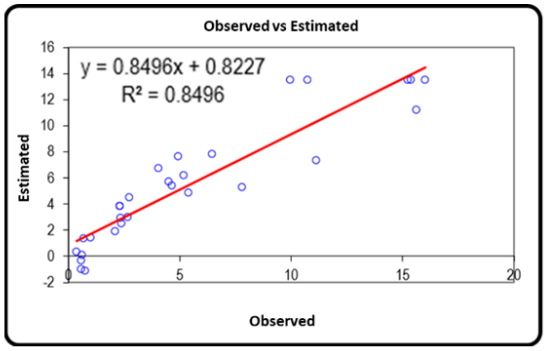


Supplementary Figure 14: Surface response for samples using 30 g/L of residual glycerin as carbon source. 3D spatial graph (Z: Crude biosurfactant (g/L), Y: pH, X: Stirrer (rpm)) and Contour graph (Stirrer (rpm) x pH) show the peak center as a hot spot condition to produce the maximum biosurfactant quantity.

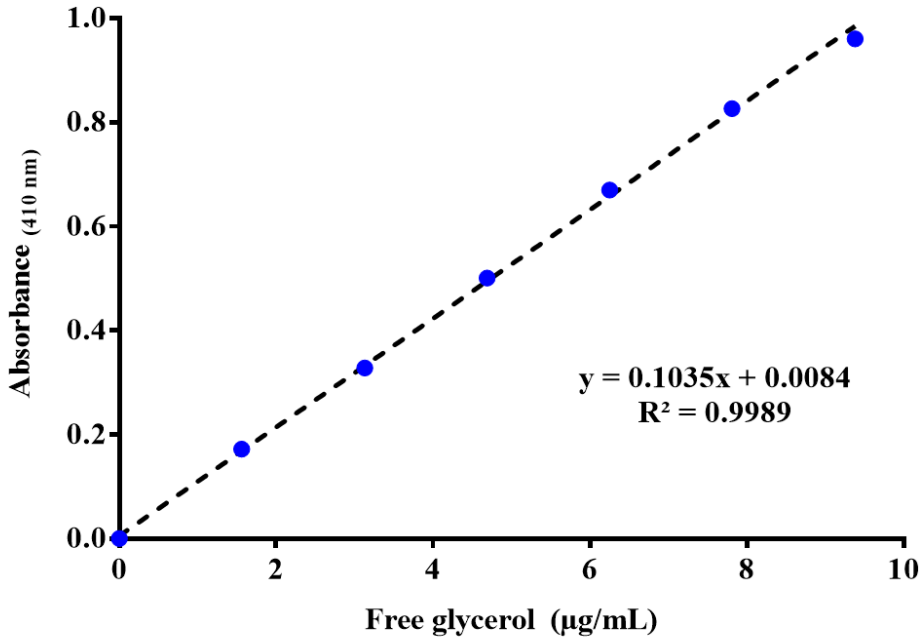
Variance analysis – Quadratic model

FV	SQ[1]	nGL[2]	MQ[3]	Fcalc.[4]	*p*
Regression	617.087	14	44.0776	5.65024	0.001287
Residues	109.214	14	7.80102		
F. adjust	109.214	10	10.9214		
Error	33.1254	4	8.28134		
Total	726.301	28			

[1]Soma dos quadrados
[2]Número de graus de liberdade
[3]Média da soma dos quadrados
[4]Valor *F* calculado



Supplementary Figure 14: F test, p value and R^2 from statistical analysis of CCD quadratic model using 30g/L of residual glycerin as carbon source. The results were obtained through ANOVA and EXCEL.



Supplementary Figure 15: Calibration curve for free glycerol measurement.

4. DISCUSSÃO GERAL

A biorremediação é um processo que tem provado ser muito promissor, já que suas aplicações vão muito além de solucionar problemas ambientais, mas também o impacto no ambiente ao se utilizar dessa técnica é menor do que remediações mecânicas e químicas (Hazen et al., 2010; Yang et al., 2015; Xu et al., 2018). Porém como qualquer outra área, necessita de avanços tecnológicos e de custos. A utilização de biomoléculas produzidas por diversos organismos tem ajudado a acelerar o processo de biodegradação de poluentes persistentes em ambientes aquáticos e no solo, porém o custo de produção dos mesmos ainda se encontra alto. No presente trabalho realizou-se uma série de experimentos para avaliar o potencial uso de BSFs produzidos por *Bacillus amyloliquefaciens* MO13 como estratégia de biorremediação.

Os BSFs têm muitas vantagens em relação aos surfactantes sintéticos como tolerância a pH alto, salinidade, temperatura de pressão, baixa toxicidade e alta biodegradabilidade (Desai and Banat, 1997; Rosenberg and Ron, 1999; Kosaric, 2001; Piispanen, 2002; Mulligan, 2005; Mohan et al., 2006; Muthusamy et al., 2008; Goel, 2010; Torres et al., 2011; Md, 2012; Boruah and Gogoi, 2013; De et al., 2015). Além disso, o mercado global de BSFs cresceu nos últimos anos e políticas ambientais têm abordado o uso de biomoléculas em vez de surfactantes sintéticos (Freitas et al., 2016; Santos et al., 2016; Akbari et al., 2018b).

A otimização do processo de produção de BSFs por *B. amyloliquefaciens* MO13 foi realizada desde o pré-inóculo. Sabe-se que meios de cultivo diferentes induzem a produção de proteínas e enzimas diferentes nos microrganismos (Zhao et al., 2013; Willenbacher et al., 2015; Liu et al., 2015; Najmi et al., 2018). Por essa razão diferentes meios de cultivo foram testados no pré-inóculo. O gênero *Bacillus* é relativamente fácil de cultivar e pode crescer em diversos meios de cultura. Os meios YPD e LB são amplamente utilizados como meios de crescimento de microrganismos e apesar de LB ser mais utilizado para bactérias, o meio YPD do pré-inóculo teve influência diferente nos resultados finais de produção de BSFs, proporcionando uma produção final de BSFs maior que o meio LB no pré-inóculo. Isso provavelmente ocorreu pelo fato de que o meio YPD possui uma quantidade de glicose relativamente alta, o que poderia até mesmo ser

tóxico para célula (Liu et al., 2015; Najmi et al., 2018). Porém as células não morreram, mas ao contrário, ao entrar em contato com a glicose, enzimas referentes ao processo de glicólise foram transcritas possibilitando o uso das mesmas para serem utilizadas no inóculo. A síntese de BSFs a partir do processo de glicólise já foi descrita por alguns autores e várias enzimas participantes na construção dos mesmos estão presentes na via glicolítica (Jung et al., 2012; Willenbacher et al., 2016; Yan et al., 2017). Assume-se então que os microrganismos que entraram em contato com YPD tem uma gama de enzimas e proteínas mais adaptadas para serem inoculadas e produzirem BSFs.

Assim como o pré-inóculo, o meio do inóculo foi adaptado para induzir a produção de BSFs ao seu máximo. Diversos sais já foram reportados como sendo essenciais para o aumento de produção de BSFs (Zhao et al., 2013; Willenbacher et al., 2015; F. Liu et al., 2016; Najmi et al., 2018; Hu et al., 2019). Estes são necessários para o metabolismo de microrganismos sendo utilizados como co-fatores enzimáticos e agentes quelantes auxiliando no processo de síntese. Além disso, o tipo de substrato utilizado no inóculo tem grande influência no desenvolvimento celular. Concentrações excessivas e insuficientes podem causar um desbalanço energético celular e diminuição da produção de biomoléculas. Os testes de concentração de substrato foram essenciais para estabelecer uma relação de custo/benefício chegando á concentração de 3% de glicose que é corroborado por outros autores (Sen and Swaminathan, 1997; Chen et al., 2015; Sakthipriya et al., 2015; Zhou et al., 2015; Moro et al., 2018). Poderia se esperar que uma maior quantidade de glicose geraria uma maior produção de BSFs devido a abundância de substrato, porém o que se tem notado é que a escassez de substratos simples ou uma quantidade grande de substratos complexos aumenta a produção de BSFs. Isso se deve ao fato de as biomoléculas produzidas servirem para diminuir a tensão superficial entre a fonte de carbono e o ambiente aquoso e dessa forma o microrganismo pode utilizar a fonte de carbono mais rapidamente e de forma facilitada (Sen and Swaminathan, 1997; Davis et al., 1999; Yeh et al., 2005; Abdel-Mawgoud et al., 2008; Chen et al., 2015; Moro et al., 2018). A utilização de outras fontes de carbono é incentivada não só pelo fato dito anteriormente, mas também pela diminuição de custos. A utilização da glicerina como fonte de carbono permitiu não somente a produção de BSFs, mas agrega valor à um resíduo da indústria de combustíveis. Em comparação

com glicose, a glicerina usada como fonte carbono no inóculo nos biorreatores proporcionou uma maior produção de BSFs. Esse fato é corroborado em outros trabalhos que já utilizaram diversos resíduos e tiveram uma produção aumentada de BSFs (Rufino et al., 2007; Nitschke et al., 2010; Randhawa and Rahman, 2014; Kulkarni et al., 2015; Santos et al., 2016; Martins and Martins, 2018).

Muitas vezes o processo de síntese dos BSFs é visivelmente observado através da produção de espuma, sendo proporcional a sua produção. Logo a maior parte dos BSFs pode se concentrar na espuma, sendo necessário coletar essa espuma produzida (Razafindralambo et al., 1996; Santos et al., 2016). A utilização de anti-espumantes causa uma diminuição da produção, não sendo sugerida em casos de excesso de produção de espuma. O coletador de espuma construído permitiu a coleta total de espuma sem haver contaminações e sem gerar pressões internas no biorreator que pudessem estressar as células e diminuir a produção de BSFs. Isso também facilitaria produções industriais de BSFs em larga escala já que a maior concentração de BSFs estaria na espuma e seria necessário somente a coleta da mesma que subiria pelo biorreator.

A escolha de se utilizar biorreatores em vez de testes em frascos erlenmeyers se deve ao fato de se controlar mais variáveis durante a produção. Foi possível controlar pH, temperatura, agitação e aeração o que permitiu uma produção maior de BSFs. Esses parâmetros são essenciais para a otimização da produção de BSFs de diversos microrganismos e também o tiveram nesse trabalho (Chen et al., 2015). Ao avaliar-se tais parâmetros, escolheu-se uma estratégia estatística de resposta de superfície, que já vem sido usada em outros trabalhos (Teófilo and Ferreira, 2006). O método de composto central (CCD) utilizado encaixou-se substancialmente nos resultados encontrados. Isso foi observado nos resultados estatísticos, gráficos espaciais e a relação R^2 entre os resultados reais e os encontrados na equação gerada a partir da resposta de superfície. Observamos que os pontos centrais da técnica tiveram os maiores resultados de produção de BSFs, o que permitiu a construção de uma equação com abrangência significativa dos parâmetros avaliados. Os valores dos parâmetros encontrados para otimizar a produção de BSFs são próximos ao da literatura (Chen et al., 2015), porém as diferenças permitiram uma produção maior do que a encontrada na maioria dos

trabalhos. Já foi reportado produções de 0-5 g/L de BSFs aproximadamente para diversas escalas de produção (Sen and Swaminathan, 1997; Davis et al., 1999; Yeh et al., 2005; Abdel-Mawgoud et al., 2008; Ghribi and Ellouze-Chaabouni, 2011; Chen et al., 2015; Meena and Kanwar, 2015). Alcançar uma concentração de 6,5 g/L se utilizando um resíduo industrial como a glicerina em 72 horas de produção é promissor se pensarmos em escalonamento industrial. Além disso manter os parâmetros de pH, temperatura, agitação e aeração não seriam relativamente mais custosos que outros processos de produção de surfactantes sintéticos que já existem na indústria (Razafindralambo et al., 1996; Zhao et al., 2013; Willenbacher et al., 2015; F. Liu et al., 2016; Santos et al., 2016; Najmi et al., 2018; Hu et al., 2019).

Os BSFs produzidos foram, além de serem otimizados, testados em sua eficácia como auxiliadores de biorremediação. Os testes qualitativos de espalhamento do óleo, colapso da gota, emulsificação (E_{24}) e inversão da molhabilidade de rochas são utilizados como testes qualitativos iniciais para avaliar o potencial uso de biomoléculas em biorremediação de áreas contaminadas com hidrocarbonetos. Conforme a literatura e os resultados obtidos, os testes foram positivos para uso em biorremediação (Cooper and Goldenberg, 1987; Morikawa et al., 1993; Sen and Swaminathan, 1997; Bodour and Miller-Maier, 1998; Davis et al., 1999; Youssef et al., 2004; Yeh et al., 2005; Abdel-Mawgoud et al., 2008; Pornsunthorntawee et al., 2008; Sriram et al., 2011; Chen et al., 2015; Meena and Kanwar, 2015; Moro et al., 2018). Além disso, os BSFs poderiam ser utilizados em recuperação microbiana avançada de petróleo (MEOR), mostrando assim a versatilidade de se utilizar os BSFs produzidos pelo microrganismo. Assim como os testes anteriores, a concentração micelar crítica (CMC) teve resultados considerados bons para o uso na biorremediação e quanto menor a CMC, menor é a concentração de BSFs necessária para diminuir a tensão superficial entre duas fases. De acordo com a literatura, CMCs dentro da faixa entre 0-40 mg/L são considerados ótimas faixas para um BSF (Zhang and Miller, 1992; Nitschke and Pastore, 2006; Ferhat et al., 2011). Com o resultado dentro de uma faixa de 0-20 mg/L para os BSFs produzidos por *B. amyloliquefaciens* MO13, o uso deste em larga escala é promissor.

A espectrometria de massas confirmou a presença de biossurfatantes que já são reportados na literatura como auxiliadores na biorremediação, sendo produzidos até

mesmo por microrganismos degradadores de hidrocarbonetos (Inakollu et al., 2004; Chen et al., 2008; Ali et al., 2014; Hua and Wang, 2014; Ben Ayed et al., 2015; Gong et al., 2015; Pereira et al., 2018). As surfactinas e iturinas encontradas são produzidas por outros microrganismos em situações de poluição ambiental de forma a auxiliar o processo de captura de hidrocarbonetos como fonte de carbono para o microrganismo. Portanto é de fato verdadeiro que possivelmente esses BSFs podem ser utilizados na biorremediação ambiental. As surfactinas e iturinas identificadas tem ainda o potencial de serem utilizadas em outras áreas como farmacêutica, cosmética e alimentícia, trazendo novas perspectivas de aplicações ao trabalho realizado (Zhang, 2012; Deleu et al., 2013; Mandal et al., 2013; Meena and Kanwar, 2015; Aslam et al., 2019; de Freitas Ferreira et al., 2019; Ding et al., 2019).

5. CONCLUSÕES

- Em biorreatores de menor escala, foram identificadas as melhores condições de temperatura, pH, agitação e aeração para otimizar a produção do mesmo.

- Em larga escala, com o uso dos mesmos valores de parâmetros, foi confirmada uma alta produção de BSFs.

- A glicerina residual pode ser usada como fonte de carbono para a produção de BSFs de *Bacillus amyloliquefaciens* MO13, tendo excelentes resultados na produção.

- A caracterização dos compostos presentes nas amostras demonstrou que as surfactinas e iturinas identificadas estão relacionadas à ação biorremediadora e também podem ser utilizadas nas áreas farmacêutica, cosmética e alimentícia.

- Resultados qualitativos e quantitativos para a produção de *Bacillus amyloliquefaciens* MO13 também corroboram que os BSFs produzidos são promissores para uso em áreas contaminadas com óleo e para aplicação nas áreas farmacêutica, cosmética e da indústria de alimentos.

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