UNIVERSIDADE ESTADUAL DE CAMPINAS

Faculdade de Engenharia de Alimentos

CRISTIANO JOSÉ DE ANDRADE

ULTRAFILTRATION OF SURFACTIN AND MANNOSYLERYTHRITOL LIPIDS PRODUCED USING CASSAVA WASTEWATER AS SUBSTRATE

ULTRAFILTRAÇÃO DA SURFACTINA E MANOSILERITRITOL LIPÍDEOS PRODUZIDOS COM MANIPUEIRA COMO SUBSTRATO

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Tese apresentada à Faculdade de Engenharia de Alimentos/Instituto da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Ciência de Alimentos.

Thesis presented to the Faculty of Food Engineering/Institute of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Food Science.

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Este exemplar corresponde à versão final da tese defendida pelo aluno Cristiano José de Andrade, e orientada pela professora Dra. Gláucia Maria Pastore.

CAMPINAS

2016

Agência(s) de fomento e nº(s) de processo(s): FAPESP, 2011/22776-5; FAPESP, 2012/20982-0

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Engenharia de Alimentos
Claudia Aparecida Romano - CRB 8/5816

An24u

Andrade, Cristiano José de, 1984-
Ultrafiltration of surfactin and mannosylerythritol lipids produced using cassava wastewater as substrate / Cristiano José de Andrade. – Campinas, SP : [s.n.], 2016.

Orientador: Glaucia Maria Pastore.
Coorientador: Francisco Fábio Cavalcante Barros.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Engenharia de Alimentos.

1. Ultrafiltração. 2. Surfactina. 3. Manipueira. I. Pastore, Glaucia Maria. II. Barros, Francisco Fábio Cavalcante. III. Universidade Estadual de Campinas. Faculdade de Engenharia de Alimentos. IV. Título.

Informações para Biblioteca Digital

Título em outro idioma: Ultrafiltração da surfactina e manosileritritol lipídeos produzidos com manipueira como substrato
Palavras-chave em inglês:
Ultrafiltration
Surfactin
Cassava wastewater
Área de concentração: Ciência de Alimentos
Titulação: Doutor em Ciência de Alimentos
Banca examinadora:
Glaucia Maria Pastore [Orientador]
Carlos Ricardo Soccol
Helia Harumi Sato
Marcos Rogério Tótola
Marta Cristina Teixeira Duarte
Data de defesa: 09-03-2016
Programa de Pós-Graduação: Ciência de Alimentos

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A Ata da defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

EPÍGRAFE

Compartilho com vocês algumas das mais belas frases e ideias que me deparei durante o curso de doutoramento.

Nada é possível sem homens, nada perdura sem as instituições (Jean Monnet)

Sou uma parte de tudo aquilo que encontrei no meu caminho (Alfred Tennyson)

A Força não provém da capacidade física. Provém de uma vontade indomável (Mahatma Gandhi)

Insanity: Doing the same thing over and over again and expecting different results (Albert Einstein).

A stranger is just a friend you haven't met yet (Irish philosophy).

In the end, we will remember not the words of our enemies, but the silence of our friends. (Martin Luther King, Jr)

The devil can cite scripture for his purpose (William Shakespeare)

You cannot connect the dots looking forward; you can only connect those looking backwards (Steve Jobs).

Resmunga aquele que ao invés de acender uma vela, amaldiçoa a escuridão!

Tudo me é lícito, porém nem tudo me convêm (Paulo aos coríntios).

Nothing in the world is worth having or worth doing unless it means effort, pain, difficulty… I have never in my life envied a human being who led an easy life. I have envied a great many people who led difficult lives and led them well (Theodore Roosevelt)

Deixe que a vastidão do Universo e os tamanhos das estrelas sejam tão grandes quanto você quiser — ainda assim parecerão ínfimos diante do Criador infinito. Ou seja, quanto maior o rei, tão maior será o palácio.

A saudade é o imposto que a vida cobra de quem foi muito feliz por um instante.

I am a seagull Nessun dove. No lido consider my homeland No place, no place to itself binds me:...Today infinity with wings surf of the North Sea
The waves lull me and make me dream...
Now...I see the ruins of the castle
Wrapped in a light veil of silver
Woven from sumptuous rays that the moon of May
Through the doors of the hall spreads (Song of North Sea # 7)

DEDICATÓRIA

À minha família, pelo apoio incondicional. Sem vocês nenhuma conquista valeria a pena.

AGRADECIMENTOS

À Profa. Gláucia Maria Pastore, pela orientação e confiança durante a realização de 2 projetos fomentados pela FAPESP. Ao Dr. Francisco F. C. Barros por co-orientar esta tese. À profa. Paula Jauregi pela acolhida em seu laboratório na University of Reading-UK e empenho nas correções para a publicação dos dados obtidos por esta tese. Aos membros da banca examinadora desta tese pela disponibilidade, auxílio nas correções e críticas. Aos colegas do Laboratório de Bioaromas:

Ana Paula (karaokê inesquecível), Ana Stela, Beth, Débora, Dora, Fabíola (doce Fá), Gustavo, Henrique, Jane, Juliana (amiga do Cielo), Maysa, Mario Mano, Marquinho, Nadeje, Patrícia Sampaio, Renata, Rosângela, Valter, Verônica e tantos outros que por lá passaram. Nadir (Nadirzão, que mesmo após 7 anos de convívio no Laboratório de Bioaromas não apreeendeu nada sobre futebol). Ao Leonardo (gamer, boleiro e parceiro), entre outros.

Em especial à Dra. Ana Paula Resende Simiqueli, por ser um exemplo, no mais amplo dos sentidos.

Ao prof. Alexandre Ponezi pela ajuda com a fermentação (80 litros) e participação na qualificação. Aos colegas pertecentes a outros Laboratórios Beatriz (Bia), Lívia Dias, Paula Speranza (bUnitinha), Fábiano Jares (pela acolhida e companherismo), Joelise (Jô), Maurício, Silvana e Ana (CNPEM), Lidiane (pela função duplicada de irmã e coautora de trabalhos). Aos colegas de Reading (Khairul, Chen, Pornpoin, Gabriel, etc)

Enfim, são tantos....

Resumo

A implantação de processos biotecnológicos incluindo a produção de enzimas, peptídeos, bioaromas, biossurfactantes, entre outros, tem aumentado de forma relevante. De modo geral, o processo de purificação representa ≈ 60% do custo de produção de biossurfactantes, enquanto o meio de cultura ≈ 30%. Este estudo descreve, pela primeira vez, a ultrafiltração de dois biossurfactantes (estudos independentes) que foram produzidos com resíduo agroindustrial como meio de cultura, ou seja, surfactina por *Bacillus subtilis* LB5a e manosileritritol lipídeos por *Pseudozyma tsukubaensis*, ambos usando manipueira como meio de cultura. A surfactina foi produzida por *Bacillus subtilis* LB5a em bioreator (3 litros de volume de trabalho). A espuma (alto teor de surfactina) foi coletada pelo topo do bioreator e utilizada para os cálculos de rendimento do processo e avaliação da purificação por ultrafiltração. Foram produzidos ≈ 336,66 mg de surfactina por litro de meio de cultura. A ultrafiltração da surfactina foi realizada em duas etapas nas quais (i) as micelas de surfactinas foram retidas e, (ii) a adição de solvente orgânico (etanol) provocou a desestabilização das micelas de surfactina, permitindo que as moléculas de surfactina livres (não agregadas) fossem recuperadas no permeado. O processo de ultrafiltração utilizou membranas de polietersulfônica com dois pontos de corte molar, 100 kDa e 50 kDa. Sendo a melhor estratégia à utilização da membrana de 100 kDa na primeira etapa de ultrafiltração e 50 kDa na segunda etapa de ultrafiltração. A ultrafiltração do biossurfactante bruto foi associada com incrustação e/ou polarização por concentração. No entanto, a ultrafiltração do biossurfactante semipurificado resultou em alta recuperação da surfactina (78,25%) com elevada separação das proteínas e redução dos efeitos de incrustação e polarização por concentração. Assim, por um lado o uso de manipueira para a produção de surfactina reduz o custo de produção. Por outro lado, dificulta o processo de purificação. Visto que as etapas de produção, purificação e aplicação devem ser avaliadas sequencialmente, o uso da manipueira como meio de cultura deve ser integrado a um tratamento para a retirada das proteínas da manipueira antes do processo fermentativo, ou anteriormente as etapas de ultrafiltração (teor de proteínas reduzido), como por exemplo a precipitação ácida e extração por solvente orgânico, ou ainda por processos de purificação alternativos a ultrafiltração, como por exemplo a coluna de bolhas. A identificação estrutural química da surfactina foi realizada por duas análises, (i) ionização por dessorção a laser assistida por matriz seguida pela detecção em um analisador

do tipo tempo de vôo e, (ii) espectroscopia de ressônancia nuclear magnética. Atráves destas técnicas foram identificadas 11 isoformas potenciais de surfactina, que por sua vez foram compostas por duas sequências de aminoácidos (Glu1-Leu2-Leu3-Val4-Asp5-Leu6-Leu7) e (Glu1´-Leu2´-Leu3´-Val4´-Asp5´-Leu6´-Val7´). Os manosileritritol lipídeos foram produzidos por *Pseudozyma tsukubaensis* em bioreator (3 litros de volume de trabalho) usando manipueira como meio de cultura. A espuma (alto teor de manosileritritol lipídeos) foi coletada pelo topo do bioreator e utilizada para os cálculos de rendimento do processo e avaliação da purificação por ultrafiltração. Foram produzidos ≈ 1,26 g de manosileritritol lipídeos por litro de meio de cultura, mostrando que a manipueira é um meio de cultura adequado a produção de manosileritritol lipídeos por *Pseudozyma tsukubaensis*. Os experimentos de ultrafiltração com os manosileritritol lipídeos, removeram ≈ 95% de proteínas e retiveram (vesículas) ≈ 80% dos manosileritritol lipídeos. Portanto, uma única etapa de ultrafiltração foi necessária para a purificação dos manosileritritol lipídeos. O processo de ultrafiltração foi escalonado de 20 mL (dispositivo de centrifugação) para 500 mL (equipamento de ultrafiltração de bancada), e os resultados não mostraram disparidade. A produção de manosileritritol lipídeos-B pela linhagem de *Pseudozyma tsukunbaensis* foi confirmada por cromatografia gasosa acoplada à espectrometria de massa, ionização por dessorção a laser assistida por matriz seguida pela detecção em um analisador do tipo tempo de vôo e espectroscopia de ressônancia nuclear magnética, sendo também identificado um segundo estereoisômero (≈ 9%) relacionado ao eritritol. A recuperação de manosileritritol lipídeos-B pela formação e arraste de espuma no bioreator integrada à ultrafiltração é uma notável alternativa de purificação, ao invés da convencional extração com acetato de etila seguido da purificação em coluna de sílica. Após estabelecer a produção e purificação de biossurfactantes, esses compostos foram avaliados quanto ao seu potencial para a recuperação avançada de petróleo. Os experimentos foram realizados com 3 tipos de petróleo, leve, médio e pesado. Baseado nos resultados obtidos nos testes de deslocamento de óleo e índice de emulsão, manosileritritol lipídeos-B são mais eficientes para o processo de recuperação avançada de petróleo do que a surfactina, em particular para o petróleo pesado.

Abstract

The set of biotechnological processes including the production of enzymes, peptides, bioflavours, biosurfactants, among other, is significantly increasing. In general, the purification process represents ≈ 60% of production cost of biosurfactants, whereas the culture medium ≈ 30%. This study describes, for the first time, the ultrafiltration of two biosurfactants (independent studies), which were produced using an industrial waste as culture medium, that is, surfactin by *Bacillus subtilis* LB5a and mannosylerythritol lipids by *Pseudozyma tsukubaensis*. Surfactin was produced by *Bacillus subtilis* LB5a at top-bench bioreactor scale (3 liters of working volume). The foam (high concentration of surfactin) was collected by the top of bioreactor and used for the calculations of yield of process and evaluation of purification by ultrafiltration. The yield was ≈ 366.66 mg of surfactin by liter of culture medium. The ultrafiltration of surfactin was carried out in two-steps (i) the micelles were retained and, (ii) the adition of organic solvent (ethanol) destabilized the surfactin micelles, allowing the free surfactin (unaggregated) be recovered in the permeate. For the process of ultrafiltration, polyethersulfone membranes with two molecular weight cut-off, 100 kDa and 50 kDa, were used. The best strategy was the use of membrane of 100 kDa in the first step of ultrafiltration and 50 kDa in the second step of ultrafiltration. The ultrafiltration of crude biosurfactant was associated with fouling and/or concentration polarization. However, the ultrafiltration of semi-purified biosurfactant resulted in high recovery of surfactin (78.25%), high sepration from proteins and reduced effects of fouling and/or concentration polarization. Thus, on one hand the use of cassava wastewater for the production of surfactin decreases the production costs. On the other hand, makes harder the purification process. Since the steps of production, purification and application should be evaluated sequentially, the use of cassava wastewater has to be integrated to a treatment for remove the proteins before the fermentation process, or before the ultrafiltration steps (lower concentration of proteins), for instance acid precipitation and extraction by organic solvent, or even alternative process of purification, for instance bubble column. The chemical structure identification of surfactin was carried out by two analyses: (i) matrix assisted lazer desorption ionization followed by the detection using analyzer of time of flight and, (ii) nuclear magnetic resonance spectroscopy. By the analyses of these two techniques were identified 11 potential isoforms of surfactin, in which are composed by two sequences of amino acids (Glu1-Leu2-Leu3-Val4-

Asp5-Leu6-Leu7) and (Glu1´-Leu2´-Leu3´-Val4´-Asp5´-Leu6´-Val7´). Mannosylerythritol lipids were produced by *Pseudozyma tsukubaensis* at top-bench bioreactor scale (3 liters of working volume) using cassava wastewater as culture medium. The foam (high concentration of mannosylerythritol lipids) was collected by the top of bioreactor and used for the calculations of yield of process and evaluation of purification by ultrafiltration. The yield was ≈ 1.23 g of mannosylerythritol lipids by liter of culture medium, which demonstrates that cassava wastewater is a good culture medium for the production of mannosylerythritol lipids by *Pseudozyma tsukubaensis*. The experiments of ultrafiltration with mannosylerythritol lipids removed ≈ 95% of proteins and retained (vesicles) ≈ 80% of mannosylerythritol lipids. Therefore, only one step of ultrafiltration was needed for the purification of mannosylerythritol lipids. The process of ultrafiltration was scaled-up from 20 mL (ultrafiltration device) to 500 mL (top-bench ultrafiltration equipment), and the results were similar. The production of mannosylerythritol lipids-B by *Pseudozyma tsukunbaensis* was confirmed by gas chromatography coupled to mass spectrometry, matrix assisted lazer desorption ionization followed by the detection using analyzer of time of flight and nuclear magnetic resonance spectroscopy. It was also identified a second stereoisomer (≈ 9%) related to erythritol. The recovery of mannosylerythritol lipids-B by the foam overflow on the top of bioreactor integrated to ultrafiltration is a remarkable alternative of purification, instead of the traditional extraction using ethyl acetate followed of silica column. After the production and purification of biosurfactants, their potentials for enhanced oil recovery were evaluated. The experiments were carried out with 3 sorts of oils, light, medium and heavy. According to the results obtained of oil displacement and emulsification index tests, mannosylerythritol lipids-B are more efficient on microbial enhanced oil recovery, em particular for heavy oil.

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INTRODUÇÃO GERAL

1. INTRODUÇÃO GERAL

Há uma tendência global no crescimento da aplicação de processos biotecnológicos e seus produtos alinhados com a definição de química verde, que trata do desenvolvimento e aplicação de produtos e processos químicos com o propósito de diminuir e/ou eliminar o uso e a formação de substâncias poluentes, tóxicas para o ambiente.

Nesse contexto, biossurfactantes fornecem oportunidades em substituir seus equivalentes sintéticos, resultando em processos ambientalmente mais amigáveis ou para serem aplicados na recuperação avançada de petróleo. Em relação à indústria de alimentos, os biossurfactantes podem ser aplicados como emulsificadores, estabilizadores de espuma, agentes antimicrobianos, entre outros.

A produção de biossurfactantes com o uso de resíduos agroindustriais como substrato pode diminuir o impacto ambiental e reduzir o custo de produção em até ≈ 30%. Por outro lado, a etapa de purificação pode representar até 60% do custo de produção de biossurfactantes. Portanto, a integração dessa estratégia de produção com um método de purificação eficiente e de baixo custo pode viabilizar a produção em escala industrial.

Surfactina, um lipopeptídeo produzido por *Bacillus subtilis*, é um dos biossurfactantes mais conhecidos. Por outro lado, manosileritritol lipídeos, um glicolipídeos produzido por *Pseudozyma tsukunbaensis*, enquandram-se dentre os biossurfactantes mais promissores.

Embora a produção de surfactina usando resíduos como componentes dos meios de cultura, incluindo manipueira, glicerol (biodiesel), entre outros, já tenha sido amplamente descrita. O estado da arte da produção e purificação (integração) da surfactina, utiliza meio de cultura sintético, atinge rendimentos ≈ 600 mg de surfactina por litro de meio de cultura e aplica técnicas com membranas (microfiltração, ultrafiltração, entre outros) como etapa de purificação.

O estado da arte da produção e purificação dos manosileritritol lipídeos também utiliza meio de cultura sintético com uma fonte de carbono hidrofóbica (por exemplo óleo de oliva), atinge rendimentos > 1000 mg de manosileritritol lipídeos por litro de meio de cultura e aplica extração líquido-líquido, coluna silica de gel e cromatografia de alta performace como etapas de purificação.

Portanto, esta tese descreve os processos de produção de surfactina por *Bacillus subtilis* LB5a e manosileritritol lipídeos por *Pseudozyma tsukubaensis*, usando substrato de baixo custo, e de purificação dos biossurfactantes por ultrafiltração.

1.1. CRONOLOGIA DO DESENVOLVIMENTO DA PESQUISA E DESCRIÇÃO DA ESTRUTURA DA TESE

O Laboratório de Bioaromas da Faculdade de Engenharia de Alimentos da UNICAMP vem desenvolvendo pesquisas com *Bacillus subtilis* utilizando a manipueira como substrato desde o início dos anos 2000. Neste contexto, este trabalho abrange o processo de produção, purificação, identificação química estrutural e aplicação de biossurfactantes.

No capítulo I é descrita uma revisão bibliográfica sobre a produção, características estruturais e, principalmente, sobre a purificação dos biossurfactantes surfactina e manosileritritol lipídeos.

Em 2007, foram publicados artigos de alta relevância sobre a purificação da surfactina por um grupo de pesquisa da University of Reading (Reino Unido). Então, em 2012, foi acordado entre os dois grupos de pesquisa a elaboração de um projeto aplicando a metodologia desenvolvida no Reino Unido com a estabelecida produção de surfactina utilizando a manipueira como substrato, aprimorada no laboratório brasileiro (Capítulo II).

Em 2011, a aluna de doutorado Ana Elizabeth Cavalcante Fai do Laboratório de Bioaromas isolou e identificou a *Pseudozyma tsukubaensis* como potencial produtora de galactooligossacarídeo. Consultando-se a literatura, foi verificado que a essa espécie seria também produtora de um tipo de biossurfactante - manosileritritol lipídeos – que por sua vez é relativamente pouco estudado no ocidente. Logo, testes preliminares foram realizados e indicaram à produção de manosileritritol lipídeos. Em seguida, foi aplicada uma metodologia de produção e purificação semelhante à da surfactina (Capítulo III).

O Capítulo IV, por sua vez, avaliou a aplicação de ambos os biossurfactantes produzidos nos Capítulos II e III no processo de recuperação avançada do petróleo, uma prospecção de integração entre a indústria petroquímica e biotecnológica.

Por fim, no Apêndice I, está a continuidade do trabalho desenvolvido no mestrado sobre a utilização do glicerol oriundo da produção de biodiesel na produção de surfactina, em que processos fermentativos, bem como análises de ionização por dessorção a laser assistida por matriz (do inglês *Matrix Assisted Lazer Desorption Ionization*) seguida pela detecção em um analisador do tipo tempo de vôo (do inglês *Time of Flight)* e, espectroscopia de ressônancia nuclear magnética (do inglês *Nuclear Magnetic Resonance Spectroscopy*), foram incorporadas aos dados originais. Além disso, no Anexo I está o depósito da patente referente ao Capítulo III.

CHAPTER I

ULTRAFILTRATION OF MANNOSYLERYTHRITOL LIPIDS; A PARALLEL WITH SURCTIN

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(Paper submitted to Journal of Surfactants and Detergents)

ULTRAFILTRATION OF MANNOSYLERYTHRITOL LIPIDS; A PARALLEL WITH SURFACTIN

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Abstract

Biosurfactants provide opportunities to replace their synthetic counterparts, resulting in environment-friendly processes. Purification steps can represent around 60% of the production cost of biosurfactants. Ultrafiltration is the most promising technique for purify biosurfactants. This review paper details the ultrafiltration of surfactin, one of the most well-known biosurfactants, and suggests a similar process for mannosylerythritol lipids, one of the most promising biosurfactants. Due to the absence of data on mannosylerythritol lipids purification based on membranes, we speculate that the compilation and discussion of most recent and relevant data on ultrafiltration of surfactin would be helpful to improve further the ultrafiltration of surfactin and also it would put light on (insights) the ultrafiltration of mannosylerythritol lipids. The paper describes interesting aspects of self-assembling properties of surfactin and mannosylerythritol lipids, which may improve the ultrafiltration yields. It also discusses the relationship among homologs of mannosylerythritol lipids to the hydrophobicity of culture media.

Keywords: *Bacillus subtilis*, *Pseudozyma tsukubaensis*, ultrafiltration, surfactin, mannosylerythritol lipids

1. INTRODUCTION

Nomenclature	
PES – Polyethersulfone	BS – Biosurfactants
ST – Surface Tension	DLS – Dynamic Light Scattering
CE – Cellulose Ester	RC – Regenerated Cellulose
UF – Ultrafiltration	CMC – Critical Micelle Concentration
Da – Dalton	L_α – Lamellar phase
MWCO – Molecular Weight Cut Off	MML – Mannosylmannitol
u – Flux Rate	LUV – Large Unilamellar Vesicles
MLV – Multilamellar Vesicles	TMP – Transmembrane Pressure

Worldwide, the application of biotechnological processes is increasing, including the production of enzymes, peptides, bioflavours and biosurfactants (BS). However, the main difficulty for industrial-scale production is basically comprised of culture medium and purification step. The culture medium represents ≈ 30% of production cost, whereas the purification, ≈ 60% [1].

Surfactin, a lipopeptide produced by *Bacillus subtilis*, is among one of the most studied BS. The production of surfactin was already described by Barros et al. [2]; Faria et al. [3] using wastes as culture medium, cassava wastewater and glycerol (biodiesel), respectively. However, on an industrial scale, use a synthetic culture medium is usually used (mineral solution and glucose as carbon source) [4, 5].

Traditional purification steps of surfactin include acid precipitation, solvent extraction, and high-performance liquid chromatography (HPLC) [6, 7, 8, 9]. Also, several attempts of surfactin purification were described, in a unique approach, for instance Dhanarajan et al. [10] reported a strategy of purification composed by adsorption (non-polar resin, HP-20) and dual-gradient elution (purity >91%) or Khondee et al. [11], which detailed the surfactin production from immobilized (chitosan) *Bacillus* sp and purified by foam fractionation unit. On the other hand, mannosylerythritol lipids (MEL) are well-known glycolipids in the East, specifically in Japan, where the culture medium is usually synthetic with hydrophobic carbon source (olive oil), and its purification is carried out by liquid-liquid extraction, silica gel column and HPLC [12, 13].

BS are amphiphilic compounds that inherently self-aggregates above its critical micelle concentration (CMC). The chemical structure of BS has a strong influence on the shape and size of aggregation (Figure 1 and Table 1) [14].

It is worth noting that HLPC is a costly purification step. Thus, alternative methods of purification (cheaper with high recovery and purity) should be explored. Membrane-based techniques and fractionation columns (nitrogen bubbles) are the more promissing techniques for BS purification. Therefore, surfactin and MEL production could be integrated with an ultrafiltration (UF) process that may result in an economical and eco-friendly process.

The literature primarily describes the UF of surfactin, but rarely describes the production of MEL and their recovery. When comparing the chemical structure of surfactin with MEL, they are very differents. Surfactin is a lipopeptide, whereas MEL are glycolipids. However, both are BS; that is, they self-aggregate when at or above the CMC. In addition, solvents may disrupt this sort of aggregation, which is the fundamental property to carry out the UF in two steps. In general, even between compounds of the same group (e.g., surfactin and iturin – both are lipopeptides), significant differences of self-aggregations are observed [15]. Nevertheless, the compilation and discussion of most recent and relevant data on ultrafiltration of surfactin would put light on (insights) the ultrafiltration of mannosylerythritol lipids or even other BS as rammnolipids, iturin, sophorolipids.

2. BIOSURFACTANTS, CONCEPTS AND ASPECTS

Surfactants are an important class of chemicals; they have been used in household and industrial applications at high volume and variety. Most of them are synthesized and derivatized from the oil industry; it was estimated that 10 million tons of surfactants were used in 2007 [8].

BS, on the other hand, are amphiphilic compounds of biological origin. They may be significant on the transport and exchange of compounds through the microbial cellular membrane. Theories explain the reasons for BS synthesis by microorganisms: (i) to inhibit the growth of other microorganisms, (ii) to store energy, (iii) to regulate the cell membrane attachment and detachment, (iv) to solubilize hydrophobic compound, (v) to increase membrane permeability, and (vi) to protect the microorganism against high ionic strength by creating a layer of BS [7, 16].

The industrial interest in BS is in bulk product markets such as laundry detergents and domestic cleaning products. Nevertheless, they have potential applications within various

sorts of industries [4, 17]. Compared with their chemical counterparts, these biomolecules have attracted interest because of theirs versatility as emulsifying agents, surfactants, antimicrobial and functional activities, bioremediation, lower toxicity, biodegradability, ecological acceptability and surface activity at extreme conditions (temperature, pH, salinity) [1, 7, 9, 14, 17]. Even with these properties, large scale production and purification costs make industrial application of BS unfeasible. In this context, purification is the main factor, representing ≈ 60% of production costs [1, 9, 19].

A few papers mentioned the economical factors of BS production. Surfactin, one of the most studied BS, is available from Sigma Chemical Company at 98% purity for US$ 15.3/mg (http://www.sigmaaldrich.com/catalog/product/fluka/86196?lang=pt&region=BR) and recently available at Lipofabrik (http://www.lipofabrik.com/).

Makkar et al. [8] cited the perfect BS price would be US$ 0.011/mg. It is worth noting that chemical surfactants are derived from the petrochemical industry, and the cost of production is US$ 0.002/mg. BS are more expensive; however, cause lower environmental damage, they are also aligned with green chemistry and oil reserves decline projections (chemical surfactants - petrochemical industry. Thus, the decline of oil industry leads to decline the production of chemical surfactant).

This context has led to concentrated studies during the past decade, focused on minimizing production and purification costs of BS; however, researchers often focus only on one of them (production or purification), when it should be studied as an integrated process.

UF is one of the most promising systems of bioproduct purification. An alternative to reduce costs of culture media is using industrial waste such as: cassava wastewater, olive oil and mill effluents, dairy and sugar wastes, lignocellulosic wastes, residues from starch rich substrates (corn, cassava, wheat and potatoes), cashew, apples, orange fruit peels or even industrial and/or municipal waste, which results in cheap substrates that can overcome the yield drawbacks. Moreover, the wastes accumulated in landfills may result in environmental problems as an increase of health issues in the local population and safety hazards associated with gas generation [6, 7, 8, 18].

BS are classified in five groups, based on their chemical structure: (i) lipopeptides and lipoproteins, (ii) glycolipids, (iii) fatty acids, neutral lipids and phospholipids, (iv) polymeric surfactant and (v) particulate BS [16]. Among these, surfactin, a lipopeptide produced by *B. subtilis*, and rhamnolipid, a glycolipid produced by *Pseudomonas aeruginosa*, are well-known for their yields, biotechnological process, chemical structure, among others. Obviously, when comparing with other BS, the production of surfactin and rhamnolipid at

industrial scale are easier due to the information avaliable. However, other BS need to be explored in all steps: production, recovery, purification and application; for example, the mannosylerythritol lipids (MEL), which are glycolipids produced by the member of the genus *Pseudozyma*.

Surfactin and MEL need be better investigated in many subjects. Currently, only sophorolipids are produced at a price that allows their use in commercial formulations, mainly due to the use of resting cells and very high yields, ≈ 422 g/L [8]. Researchers have mainly been working on the downstream improvements of surfactin. On the other hand, few articles illustrate the entire process of the production, purification and application of MEL.

Certainly in the coming years, progress toward MEL technology will result in new products and possibilities; until then, it is required to do screening of producer strains, research for specific applications such as: antibiotic, antifungal, insecticide, antiviral and antitumor agents, as well as to optimize the process using renewable substrates and its recovery and downstream steps [8, 14].

2.1. SURFACTIN

Surfactin (Figure 1), a heptapeptide ($_{L}$-Glu-$_{L}$-Leu-$_{D}$-Leu-$_{L}$-Val-$_{L}$-Asp-$_{D}$-Leu-$_{L}$-Leu) linked to *β*-hydroxy fatty acid, is mainly comprised of 12 to 16 carbon atoms to form a cyclic lactone ring structure, glutamyl and aspartyl residues provide two negative charges (surfactin is anionic) [7, 9, 14, 20]. This remarkable compound can reduce the surface tension (ST) of water from 72 to 27 mN/m at concentration as low as 10 mg/L; it also has bioactive properties including antiviral, antitumor, and antibiotic [1, 6, 9, 14, 15].

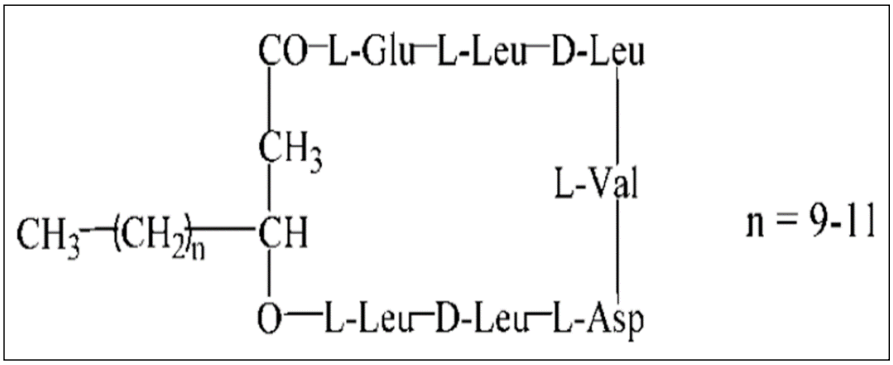


Figure 1. The chemical structure of surfactin [21].

Due to its amphiphilic structure, surfactin has a strong self-assembly ability to form micelles [7, 14]. The structure of the micelle is a core-shell type, when in an aqueous

solution, the hydrocarbon chain and the hydrophobic residues form the core of the micelle. Usually, this supramolecular structure is a non-homogeneous regarding to size distribution with different configurations [7].

Jauregi et al. [15] reported the relationship between volume of micelles and surfactin concentration by dynamic light scattering (DLS) analysis. Micelles repulsed themselves at ≈ 500 mg/L. As a result, a lower volume of micelles was obtained. When the concentration of surfactin was between 50-100 mg/L the volume of micelles were bigger, with unimodal distribution and diameter (d) = 100-200 nm. Finally, when the concentration of surfactin was at 10 mg/L (close to CMC), a bimodal distribution was observed; one with d = 68 nm (micelles) and the second d = 342 nm composed by inter-micellar hydrogen bonds.

It should be clear that surfactin micelles assume different forms such as: spherical, ellipsoidal and/or cylindrical as cited by Seydlová et al. [7] and studied by Knoblich et al. [22]. This is probably due to interaction with other molecules, for instance, proteins and ions, or pH effect.

2.2. MANNOSYLERYTHRITOL LIPIDS - RELATION BETWEEN CULTURE MEDIA AND CHEMICAL STRUCTURE

MEL belong to the glycolipid group. They are extraordinary molecules that have the property to reduce surface tension (ST) of water to less than 30 mM/m; also, their complex structure makes the chemical synthesis impossible.

MEL are synthesized by microorganisms such as *Schizonella melanogramma*, *Candida* sp. (currently known as *Pseudozyma* sp.) as a major component, whereas *Ustilago* sp produces them as a minor component (along with cellobiose lipid); they are also produced by *Kurtzmanomyces* sp. [16]. In this context, the *Pseudozyma tsukubaensis* has received special attention, because it synthesizes only MEL-B, whereas other *Pseudozyma* species such as *P. rugulosa*, *P. antarctica*, *P. parantarctica*, *P. hubeiensis* among others produce a mixture of different MEL homologs [23].

Throughout the past ten years, MEL have regained attention. Arutchelvi et al. [16] suggested a list of research topics that need to be explored including the use of cheaper raw materials as culture media and the optimization of fermentation parameters, purification processing, genetic engineering for hyperproduction, chemical derivatives and identifying enzymes involved in their synthesis. Currently, MEL-B are commercially available from TOYOBO Co., Ltd. (Osaka, Japan). They are synthesized by *P. tsukubaensis* and are added to the product named SurfMellow® as a cosmetic ingredient [24].

The production of surfactin is usually growth associated. On the other hand, the production of MEL is related to the stationary phase. Also, when comparing the yield of production of biosurfactants, the yield of production of MEL is higher (≈ 165 g/L), for instance surfactin (≈ 0.7 g/L) and rhamnolipids (≈ 10 g/L; ≈ 100 g/L hyperproducer) [4, 16, 23].

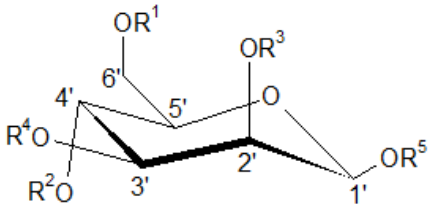
MEL are a mixture of a partially acylated derivative of 4-*O*-*β*-D-mannopyranosyl-D-erythritol. Similarly as surfactin, MEL have homologs (A, -B, -C and –D). The homologs of MEL are classified based only in the presence or absence of acetyl group in C-4´ (R^2) and C-6´ (R^1) (Table 1) [4, 23-24]. Each homolog (Table 1) has none (MEL-D), one (MEL-B or C) or two (MEL-A) acetyl groups at C-4´ and/or C-6´ in the mannose moiety [4, 16, 23].

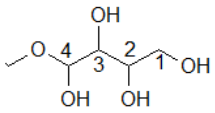
MEL-A, the most hydrophobic forms among the homologs of MEL (A, -B, -C and –D), have low water solubility, which limits their application. On the other hand, MEL-B, -C, -D have higher hydrophilicity and lower CMC value. Fukuoka et al. [26] reported a type of MEL-D (the most hydrophilic forms among the homologs) with only one fatty acyl ester group produced using glucose as sole carbon source.

Confronting data (Table 1), it seems that, there is a relation between the solubility of the culture medium and the production of homologs of MEL. This relation is aligned with one of the theories that explain the reasons of the production of biosurfactants by microorganisms, that is, to solubilize hydrophobic compounds. In this sense, the more hydrophobic culture medium, the more hydrophobic homologs of MEL are synthesized. For instance, a medium composed of olive or soybean oil will favor the strain to acylate the C-4´ and C-6´ or both. Also, an extremely non-polar culture medium (80 g soybean oil/L), as described by Fukuoka et al. [25], will favor the strain to insert a third fatty acid into the MEL and will form the more hydrophobic homolog of MEL already reported. On the other hand, when soluble carbon sources are used, such as sucrose and glucose, the MEL produced are non-acetylated (C-4´ and C-6´), or even the homologs of MEL with only one fatty acid can be produced (usually consisting of 2 fatty acids in C-2´ and C-3´).

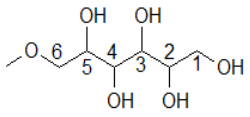
The relation between chemical structure of MEL with their self-aggregation forms (lamella phase, sponge phase, among others) and also with surface activity properties are detailed below. In addition, higher production was obtained using hydrophobic carbon sources; however, hydrophobic culture media results in a more difficult purification process [4, 13, 16].

Table 1. MEL structures and their relation to carbon source, microorganism and yield.

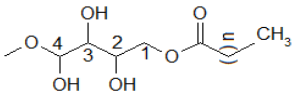




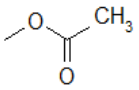
Erythritol



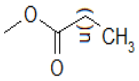
Mannitol



Erythitol + Fatty acid



Acetyl



Fatty acid

MEL-A_i : R^1 = R^2 = Acetyl group, R^3 = R^4 = Fatty acid, R^5 = Erythritol

MEL-A_{ii} : R^1 = R^2 = Acetyl group, R^3 = R^4 = Fatty acid, R^5 = Mannitol

MEL-A_{iii} : R^1 = R^2 = Acetyl group, R^3 = R^4 = Fatty acid, R^5 = Erythitol + Fatty acid

MEL-B : R^1 = Acetyl group, R^2 = H, R^3 = R^4 = Fatty acid, R^5 = Erythritol

MEL-C : R^1 = H, R^2 = Acetyl group, R^3 = R^4 = Fatty acid, R^5 = Erythritol

MEL-D_i : R^1 = R^2 = H, R^3 = R^4 = Fatty acid, R^5 = Erythritol

MEL-D_{ii} : R^1 = R^2 = H, R^3 = H, R^4 = Fatty acid, R^5 = Erythritol

Homolog	Medium	Carbon source	Strain	**Yield (g/L)	Reference
MEL-A_i	*BSM	Olive oil	*P. antarctica*	12.98	[13]
MEL-A_{ii}	*BSM	Olive oil	*P. parantarctica*	18.2	[12]
MEL-A_{iii}	*BSM	Soybean oil	*P. rugulosa*	X	[25]
MEL-B	*BSM	Olive oil	*U. scitaminea*	8.29	[13]
MEL-C	*BSM	Sucrose	*P. siamensis*	1.94	[13]
MEL-D_i	†NM	†NM	Enzymatic synthesis	X	[27]
MEL-D_{ii}	*BSM	Glucose	*P. antarctica*	1.3	[26]

*BSM – basal salt medium; ** - The final concentration was used as yield parameters, † NM - Not mentioned; n - from 8 to 18 (usually)

Some reports strongly suggested that microorganisms use *β*-oxidation residues to synthesize the fatty acids (C-2´ and -3´) of MEL. The non-polar moiety is composed of an even number (carbon); thereby, they are obtained from direct *β*-oxidation intermediates of the fatty acids (C_{16} to C_{18}) (oil). Hence, lipids (as a carbon source) may improve the production. However, Morita et al. [13] reported higher MEL production by *P. siamensis* from sucrose rather than olive oil as a carbon source. In this case, microorganisms probably used fatty acid synthesis to create fatty acids from acetyl-CoA and malonyl-CoA precursors.

Fukuoka et al. [26] and Moritta et al. [13] described the production of MEL using hydrophilic carbon sources, glucose and sucrose, respectively. Both studies reported the predominance of medium-chain acids in MEL (C_8 to $C_{14} \approx 86.6\%$) as fatty acid profile of MEL. Traditionally, hydrophobic carbon sources are used for the production of MEL, in which there is a predominance of medium-chain acids in MEL as fatty acid profile [12, 25, 27]. Thus, it seems that there is no relationship between the sort of carbon source and fatty acid profile of MEL. However, Fukuoka et al. [26] described a substantial increase of MEL production by *P. antarctica* using olive oil rather than glucose and sucrose, in which the low yield of MEL from glucose should be due to the limitation of fatty acid synthesized via acetyl-coenzyme, a glucose derivative.

Therefore, independently of carbon source used (hydrophobic or hydrophilic), MEL will be composed of medium-chain as fatty acids. Although, the yield of production is significantly changed by sort of the carbon source used.

As already mentioned, one of the hypotheses for the production of BS by microorganisms, is to solubilize nutrients in the culture medium, making absorption easier, which is aligned with the data reported by Fukuoka et al. [25]. They described a sort of MEL-A, with a third fatty acid linked to erythritol (tri-acylated MEL-A), the “extra” fatty acid makes the molecule more hydrophobic. It was produced by *P. antarctica* T-34 in a high soybean oil concentration (from 80 to 120 g/L), but not at 40 g/L. Thus, microorganisms may identify how hydrophobic the medium is and as a result synthesize MEL. In this case, 40 g/L was not enough to produce tri-acylated MEL-A.

Furthermore, Fukuoka et al. [27] synthesized the tri-acylated MEL-A from lipase (Novozyme 435), MEL-A and fatty acids. It may be easier to obtain tri-acylated MEL-A from an enzymatic step rather than a very hydrophobic culture medium. The yield reached 40%, and they concluded that, fatty acids are directly introduced into the erythritol moiety.

Fukuoka et al. [26] detailed the MEL production by *P. antarctica* T-34, in this case, using glucose as the sole carbon source. It was found that the strain produced MEL-A, -

B and -C, as well as a “new” MEL-D which was synthesized at rate (20-25%) and had only one fatty acid group (C-3´). They also evaluated an initial content of glucose (4 and 10% w/w) and found that a lower sugar concentration resulted in higher “new” MEL-D production, possibly due to glucose regulation (feedback effect).

Another interesting fact, which verifies the relationship between the medium and sort of MEL, is that the “new” MEL-D was primarily produced from glucose, not from vegetable oils, fatty acid methyl esters, or fatty alcohols. Even with low yields, authors concluded that this molecule is likely to have greater potential for use in oil-in-water-type emulsifiers and laundry detergents because ofits higher water solubility compared to conventional MEL, and therefore, will contribute to facilitating a broader range of applications for environmentally advanced surfactants [26].

It should be taken in consideration that production costs of BS depend on bioprocess feedstock, yield, the cost of downstream processing and the interaction between each of these factors. Thus, the use of hydrophobic substrates rather than hydrophilic becomes more difficult with downstream steps; also, it tends to synthesis hydrophobic MELs (D→A), which may have lower applicability [4].

3. SELF-ASSEMBLY AND CORRELATE PROPERTIES OF SURFACTIN AND MEL; A PROSPECTIVE INFLUENCE IN ULTRAFILTRATION

In the past 20 years, self-assembly of amphiphilic compounds and their potential applications have been the topics of intensive studies. A wide variety of organic molecules form aggregations. Biological structures must be complex in order to be chemically synthesized, due to their chiral centers, functional groups and attractive or repulsive forces between their atoms. Therefore, the application of these unique and sophisticated complex molecules of biological compounds such as biosurfactants may lead to a significant impact on the industry.

Because of high BS production costs, one attractive economical possibility is their combination with synthetic surfactants. However, that could result in a wider variety of micelle sizes and forms; in other words, affecting their expected behavior [4, 15].

3.1. SURFACTIN

In one of the earliest papers about surfactin micelle forms, Knoblich et al. [22] used an ice-embedding technique and transmission electron cryo-microscopy. The micelle forms were studied in different conditions such as pH and salt solutions. The six following

types of micelles were obtained: (i) spherical 4-5 nm (diameter), (ii) spherical 7-8 nm, (iii) small ellipsoidal 9 nm (length) × 6 nm (width), (iv) large globular 9-20 nm, (v) ellipsoidal 19 nm × ll nm, and (vi) cylindrical 40-160 nm (length) × 10-14 nm (width).

Salt solutions showed that $CaCl_2$ (20 mM) and NaCl (100 mM) change surfactin micelles from cylindrical to spherical or ellipsoidal forms [22]. This form may be essential to obtain better yields using membrane-based techniques. Recently Arutchelvi et al. [14] proved that CMC of surfactin is reduced by adding divalent cations, since it reduced the electrostatic repulsive force (polar moiety). The Ni^{+2}, the smallest ionic radius and unstable electronic configuration (Zn^{+2}, Cd^{+2} and Ca^{+2}) had the highest degree of association with surfactin, nevertheless, Ca^{+2} facilitates the formation of large self-aggregated structures due its interaction with more than one surfactin molecules within and between the self-aggregated structures [14].

Therefore, due to the lowest area-volume ratio and geometric symmetry, spherical forms may be the best for UF.

Taking into account that *B. subtilis* needs mineral salts (present in culture medium) to produce surfactin and that concentration of those salts change during the bioprocess, as they are absorbed from culture medium to cytoplasm of microorganism. As a result, the micelles forms may change during the bioprocess (aforementioned).

Han et al. [28] studied the structure of surfactin at pH 7.4 and two concentrations of 103.6 and 310.8 mg/L of surfactin. They reported the distribution of the hydrodynamic radius as bimodal with one peak at 4-6 nm (both concentrations) and another broad peak centered at 85 nm (103.6 mg/L) and ≈ 108 nm (310.8 mg/L). They also confirmed that the secondary structure of surfactin adopts a β-turn at low micelle concentrations of 103.6 and 310.8 mg/L and begins to adopt *β*-sheet conformation at a relatively high micelle concentration of 518 mg/L. It was obtained by using a combination of results from Isothermal Titration Microcalorimetry, DLS, Transmission Electron Microscopy, Atom Force Microscopy and Circular Dichroism measurements. They concluded that surfactin follows the trend to aggregate through inter-micellar hydrogen bonds. Surfactin can display different secondary structures at different concentrations, and the secondary structure of surfactin as a peptide is very sensitive to experimental conditions such as electrolytes and pH.

Therefore, before recovery/purification of surfactin by UF, it is fundamental to begin with experiments aiming to understand the behavior of micelles and then purify it with UF. Also, micelle simulation may result in a better understanding of inter-molecular

interaction (surfactin monomers). This suggestion (micelle simulation) is quite appropriate, since micelle simulations of many surfactants are well-known.

3.2. MANNOSYLERYTHRITOL LIPIDS

Even though non-ionics, MEL are negatively curved lipids. Usually, sugar-based BS can self-assemble into a specific lyotropic liquid crystalline phase, which is stabilized by hydrogen bonds. Chirality of the sugar also affects their lyotropic and thermotropic phase behaviors. All classes of MEL with variations in their hydrophilicity show different self-assembling properties, liquid lyotropic crystals, including liposomes, self-assembled monolayer, lamella phase (L_α), sponge (L_3) phase, and bicontinuous cubic (V_2) phase [16].

A few articles detailed self-assembling properties of MEL and their purification steps. The high diversity in their chemical structure makes this situation complex; for instance, Fukuoka et al. [23] described the diastereomer of the conventional MEL-B from *P. tsukubaensis*. Just above their CMC, this diastereomer self-assembles into the lamellar phases (L_α), which are bilayer sheers separated by layers of water, in turn, these bilayer sheers form large multilamellar vesicle phase (MLV), whereas the conventional MEL-B forms Large Unilamellar Vesicles (LUV) [16]. These differences happen over a remarkably wide range of concentrations and temperature. MEL-A drastically changes into sponge (L_3), which is composed of a network of randomly connected bilayers with a water-channel diameter of 100 nm [16, 23].

As already mentioned, Fukuoka et al. [25] focused on the production of tri-acylated (fatty acids) MEL, and thus, different from conventional homologs of MEL that have only two fatty acids. Thereby, further investigations will probably focus on the self-assembly properties. Obviously, tri-acylated MEL has a higher hydrophilic-lipophilic balance; thus, better emulsion oil-in-water rather than conventional homologs of MEL. Also, the triple-chain amphiphiles highly stabilize bilayer membrane systems, and as a result, the self-assembling structure will be stable, which may make easier the UF process.

Fukuoka et al. [26] described a C-3´mono-acylated MEL, with only one fatty ester and no acetyl groups on the mannose, but in 2011, the same research group used MEL-B and lipase to produce “new” MEL (no acetyl groups on the mannose) and named them MEL-D. Therefore, the C-3´ mono-acylated MEL should also be called MEL-D.

Fukuoka et al. [26] found the surface tension at CMC (γCMC) and CMC of C-3´ mono-acylated MEL-D (Table 2), 33.8 mN/m, 3.6×10^{-4} M, respectively. The CMC is higher in comparison to the C-3´; C-2´ di-acylated MEL-D (1.2×10^{-5} M) report by Fukuoka et al.

[27] – (see Table 2 and compare the C-3´ for both MEL-D). This is the opposite of expected, since the higher hydrophobic, higher is CMC. Additionally, it is worth mentioning that the mono-acylated (MEL-D), compared with MEL-A and -B, showed greater effects on biological activity [26].

A unique approach to modifying BS was reported by Fukuoka et al. [27]; they used MEL-A from *P. antarctica* and MEL-B from *P. tsukubaensis* (supplied by TOYOBO Co., Ltd. Japan). Then, they were deacetylated C-6´ (Table 1) by a lipase-catalysed hydrolysis (Novozym®435), which resulted in MEL-C (from MEL-A) and "new" MEL (from MEL-B). The "new" MEL were named MEL-D, and the catalyst yield was >99% after 7 days. The MEL-D had eliminated the effects of the acetyl groups (C-4´and C-6´), then were determined some self-assembly properties of MEL-D.

MEL-D showed CMC and ST at the CMC (ɣCMC) were 1.2 x 10^{-5} M and 24.6 mN/m, respectively. Thus, higher CMC and hydrophilicity compared to MEL homologs. At low MEL-D concentrations (≤50wt%), they formed two phases composed of white-turbid precipitates and equilibrium water. The sample became one viscous phase, which was translucent and optically anisotropic, and again verified the L_α-phase (white precipitates). In addition, at lower MEL-D concentrations (≤10wt%), relatively large vesicles (ca. 10 μm) were also observed. Therefore, MEL-D are likely to self-assemble into a L_α-phase structure at a remarkably wide concentration range; this behavior is similar to MEL-B, excluding the concentration boundary. Another interesting fact is that d-spacing (inter-layer spacing) was the highest and constant (about 5.1 nm) at low concentration regions (≤50wt%) and linearly decreased with the increase of MEL-D concentration.

The same research group continued to use the lipase, however, in this case in two diastereomers of MEL-B, S-MEL-B (4-*O*-[6´-*O*-acetyl-2´,3´-di-*O*-alka(e)noyl-β-$_D$-mannopyranosyl]-(2S,3R)-erythritol) and R-MEL-B (4-*O*-[6´-*O*-acetyl-2´,3´-di-*O*-alka(e)-noyl-β-$_D$-mannopyranosyl]-(2R,3S)-erythritol), from *U. scitaminea* and *P. tsukubaensis*, respectively [24]. They evaluated the significance of hydrophilic domain in micelles properties, upon appearance, only a slight difference of the sugar portion is likely to give a dramatic effect on the phase behavior. Hence, any self-assembly difference between these molecules originated from the fact they are diastereomers. It was found that carbohydrate configuration effects the interfacial proprieties, in which CMC of the diastereomers R-MEL were higher than S-MELs, possibly due to more hydrophilic R-forms. Optical microscopic observation at 3 mM MEL showed that all homologues efficiently formed vesicles, which is observed at low concentration (≤10wt%). However, data from DLS data demonstrated that

S- and R-MEL vesicles have different sizes. The diastereomer S, for MEL-B and D formed vesicles, 510±230 and 670±290 nm, respectively; whereas the diastereomer R, for MEL-B and -D, formed large vesicles, over the measurement limit (over 1 µm). Hence, subtle molecular differences result in different MEL self-aggregation forms. Since MEL self-aggregates in large vesicles ≈ 500 to 1 000 nm, we strongly recommend the study of the UF of MEL using large MWCO pore size.

In addition, both diastereomers, S and R, formed L_α structures over the following concentration range, from 0 to 80wt%. Interestingly, at low concentrations, two phases appear; one composed of white-turbid precipitates (L_α), and other diluted in water. When the MEL concentration increased, only the L_α phase was apparent [24].

Morita et al. [12] described a novel MEL, in which the erythritol group was replaced by mannitol. High-level MEL producers synthesized a significant amount of mannosylmannitol lipids (MML), when induced by mannitol (4%). MML comprised of di-acetylated mannose showed similar CMC to those from MEL-A and an analogous liquid crystalline structure to those from MEL-B, in other words, the lamellar phase (L_α). These results indicated higher hydrophilicity than MEL-A. Thus, MML and MEL-A, should be used in oil-in-water emulsion, however, their higher hydrophilicity makes them more feasible for industrial applications.

As already mentioned, Morita et al. [13] investigated the use of carbon sources (water-soluble and olive oil). The MEL structures (mannose, erythritol and acetyl group) were similar and dependent on the strain (MEL-A, -B or -C); however, the fatty acid profile showed higher range, when compared with olive oil. The forms of micelles were not described; nevertheless, CMC values did not show significant differences.

Imura et al. [29] studied the self-assembling properties of MEL-A and -B by using the following complementary methods: fluorescence-probe spectroscopy, DLS, freeze–fracture, transmission electron microscopy and synchrotron small/wide-angle X-ray scattering spectroscopy. Interestingly, it illustrated two CMC values for MEL-A, 4 x 10-6 M and 2 x 10-5 M, respectively, CMCi, formed LUV, and CMCii, formed a sponge structure (L3). It is clear that the surfactant concentration had a fundamental significance on the micelle structures, and consequently, influenced UF results. On the other hand, MEL-B had only one CMC, 6 x 10-6 M. Nevertheless, it seems to gradually move from LUV to MLV at 10-5 M to 10-3 M, respectively. Considering this, MLV has good retention in membrane-based techniques, since it is stabilized by multiple layers; nevertheless, it is worth noting that MLV

is two times higher (1-5 µm) than LUV. Also, hydronamic diameters were measured with MEL-A at CMC_i and MEL-B, 179 and 161.9 nm [16].

As already mentioned, MF of MEL is quite a pertinent process, since the vesicles are large enough and it may lead to high flux of filtration. To the best of our knowledge, there is no report using a membrane-based filtration for MEL. The information described above, regarding vesicles, is extremely significant to insights in the field of MEL downstream.

4. METHODS OF PURIFICATION

Normally, in biotechnological processes, the downstream corresponds to around, 60% of total costs; therefore, this step is fundamental to economic viability [1, 9].

In most cases, the method of purification can be classified in 4 steps: (i) clarification, (ii) concentration and/or low resolution purification, (iii) high resolution purification and (iv) procedures to packing and storage. The first step is necessary to withdraw cells and its fragments; second, a concentration method such as precipitation, filtration, is required to remove molecules that are different from the aim compound; third, a high resolution purification that will separate similar chemical structures using chromatography; and fourth, packing and storing is a crucial step to prevent unexpected reactions.

Each step of purification methods may be part of a multi-step strategy; for instance, in the following case of low resolution purification, acid precipitation followed by tangential filtration, or acid precipitation and solvent extraction [30].

Methods of recovery and purification of surfactin include: foam fractionation, liquid-liquid (e.g., n-hexane and ethyl acetate), activated carbon, adsorption or ion exchange resins and acid precipitation [9, 31]. Acid precipitation has been used due to its high recovery yield, but it reaches low purity. For instance, Mullgian and Gibbs [6] reported the collapse of foam from acid precipitation followed by solvent extraction using dichloromethane (1:1, v/v), in which they obtained a purity of 31.6%. Chen et al. [32] described a recovery higher than 97 and a purity of 55% for acid precipitation. It is a simple technique to be used as a low resolution process. Zhang et al. [9] tested a unique approach for surfactin purification by adding inorganic flocculants and polyacrylamide to culture medium, ≈ 90% of surfactin was recovered using $CaCl_2$+Na_2HPO_4. Silva et al. [33] described an interesting approach on production, recovery and purification of surfactin, in which foam fractionation column was integrated to bioreactor of 4.5 L (3 L working volume). The foam reached 4.5 g of surfactin

per liter of foam, which produced 135 mg of surfactin and the foam fractionation method recovered more than 94% of produced surfactin.

Suzuki et al. [34] comprised almost all significant aspects about purification of MEL. They indicate that after the biotechnological process, MEL may be subject to several operations: filtering, extraction and purification. Basically, there is only one methodology for the purification of MEL composed of liquid-liquid extraction (ethyl acetate), silica column (open column) followed by HPLC chromatography (silica-gel column).

Nowadays, more and more high-value bioproducts including surfactin are produced by bioprocess, bringing new challenges to recovery and purification steps [1]. One of the most current and significant subjects in the field of biotechnology industries are the economic aspects; many bioprocesses, in which all parameters are already maximized and the process is largely known, are anxiously waiting for advances in the purification area.

4.1. MEMBRANE-BASED TECHNIQUE

Membrane is defined as a selective barrier between two phases, concentrated (retentate) and permeated, in which the driving force occurs by diffusion or convection and is induced by a physicochemical potential (e.g., pressure, concentration and temperature or electric potential) [20, 30].

The purification of one or more components of solution/suspension through a selective membrane, allowing concentration and fractionation, is an environmentally-friendly method of purification (does not apply harmful compounds). The method of purification through a selective membrane requests also low consumption of energy and usually is easy for scale-up. However, membrane-based techniques of purification are classified as of low resolution purifications, since it does not achieve high level of purity (compound of interesting) [20, 30].

Membrane filtrations are used in many biotechnological industries. Technological advances in this area are mainly related with fouling, concentration polarization [1]. Given these circumstances, some reports described the recovery and purification of surfactin using filtration systems [1, 6, 20, 32, 35].

4.1.1. Ultrafiltration of biosurfactants

Considering that all BS are amphiphilic molecules with an intermediate weight, e.g., surfactin 1036 and rhamnolipids 802 Da, the UF process is pertinent, because porous

sizes are sufficiently large to allow a good flux rate and smaller than micelles, which may allow the recovery of this compound in high yields [6].

Amphiphilic compounds naturally form a supramolecular structure (micelles), which enables the UF in two ways (i) recovering the micelles (retentate) or (ii) as monomers (permeate).

Mulligan and Gibbs [6] tested many membranes for recovery surfactin and rhamnolipids by UF. They worked with collapsed foam and indicated a polyacrylonitrile 50-kDa-MWCO for retention of surfactin and RC 10-kDa-MWCO for rhamnolipids. In the surfactin case, 160-fold purification was achieved.

Arutchelvi et al. [16] cited the self-assembling properties of MEL by using different methodologies such as fluorescence-probe spectroscopy and DLS. It was found that the MEL self-assemble into large unilamellar vesicles (LUV) just above their CMC, which is 2×10^{-5} M (MEL-A), and it drastically changed into a sponge (L_3) like structure, which is composed of a network of randomly connected bilayers with a water-channel diameter of 100 nm, and resembled a multicomponent synthetic surfactant system. This information is quite important in the UF process, because the micelles should be stable enough to be retained by the membrane, and many aspects like size and format have influenced on the yields of ultrafiltration.

Jauregi et al. [15] recently demonstrated that the size of surfactin micelles is affected by its concentration; also, for the presence of other BS (mycosubtilin). They tested a few surfactin concentrations with UF and concluded that when the solution was at a high concentration, the micelles repel themselves due to electric charges but on the other hand, if the surfactin concentration was appropriate (50-100 mg/L), the micelles were larger (volume) and more uniform. This allows the use of a membrane with high MWCO in UF, and consequently, a higher flux rate. This is aligned with the definition of the most important parameters of UF: size and shape of molecules [15].

Rangarajan et al. [18] purified a conditioned Ca^{+2} mixture of surfactin and iturin using PES – 10, 30 and 50 kDa. The cation allowed maximum recovery of lipopeptides (≈ 96%). According to Jauregi et al. [15], the mixture of lipopeptides results in nanoparticle size polimodal distribution (e.g mycosubtilin and surfactin). Thus, probably, the presence of Ca^{+2} reduces the electrostatic repulsive force (polar moiety) and it changed the nanoparticle size distribution in the mixture of surfactin and iturin (compact structure, narrow size distribution and higher stability), which enhanced the recovery.

Therefore, theoretically, UF is a process that can be used for various types of BS due to its inherent ability to self-aggregate. Nevertheless, preliminary experiments should be done in order to understand specific characteristics of each BS (e.g., the relation concentration and volume of self-aggregation structure; solvent and property to disrupt the self-aggregation forms), as well as its interactions with the membrane used that may result in improved recovery yields and purity.

4.1.1.1. Ultrafiltration of surfactin

As already mentioned, UF is a membrane-based technique, which can be used for recovery and purification of surfactin. At or above its CMC, surfactin form micelles can be retained by UF. It separates surfactin from salt and other low-weight molecules since they permeate. However, when a solvent solution is used, micelles are destabilized; in this case, surfactin will permeate. This process purifies surfactin from high-weight molecules that are basically composed of proteins [15].

Purification of surfactin in two steps of UF is convenient, due to advantages usually associated with filtration processes including: simplicity, economical factor, among others, along with the supramolecular structure composed by surfactin micelles. In the first step, surfactin should be in at higher concentration than its CMC. In so doing, these structures will form micelles that are stable enough to be retained by the membrane, different from other non-aggregate molecules (small molecules), such as: alcohols, phthalic acid, amino acid, glycine, serine, threonine, phosphate, alanine and salts [6, 35]. Then, the retentate can be recovered, which is mostly composed of surfactin and other macromolecules that are able to mix with micelles, for instance, proteins. The second step should be based on the retentate obtained in the first step; however, in this case, using a solvent (methanol or ethanol 75%) rather than water. Solvent mostly affects the form of micelles, especially the straight chain. When the solvent is added to surfactant solution, it competes with monomer to occupy the micelle site. In this manner, the solvent disrupts surfactin micelles, which results in a solution composed of surfactin monomers and macromolecules. Afterwards an UF with low MWCO should be used to retain the macromolecules, allowing the surfactin goes as permeate. This process takes advantage of the properties of surfactin: high stability, low chemical reactivity, micelle formation and surfactin size.

Lin and Jiang [35] verified the UF of surfactin using cellulose membranes in a hollow fiber. The strain was grown in a mineral salt medium and glucose as carbon source. Obviously, mineral salt media favors the purification process (compared with complex

media). Good results of recovery and purity, such as 97.9% and 98%, respectively, were reported; however, a low filtration rate was indicated.

Chen et al. [32] described three strategies using filtration. In the first strategy, UF followed nanofiltration; and the second and third strategies followed two steps of UF (Table 2). In the first and second strategies, micelles were dissociated by ethanol (33%) before the step 1 of UF; then, the alcohol was removed from the permeate by acid precipitation and dissolved in NaOH solution (feed solution for step 2); and finally, nanofiltration (first strategy) and UF (second strategy) were carried out, respectively. The third strategy used an alkaline solution for step 1 of UF; then, the retentate micelles were destabilized by ethanol (33%) and used as a feed for step 2 of UF.

Table 2. Approaches for recovery and purification of surfactin based on nano and ultrafiltration techniques [32].

Strategy	1 – Step	2 – Step	Recovery %	Purity %
*First	UF – ethanol 33%	†NF – **alkaline solution	79	86
*Second	UF ethanol 33%	UF – **alkaline solution	72	83
*Third	**UF – alkaline solution	UF – enthanol 33%	87	85

*(Initial concentration) 2054 $mg.L^{-1}$

**pH 11

†NF - nanofiltration

In all cases, the initial feed was composed by treated broth, a pre-treated surfactin solution (acid precipitation). However, according to Jauregi et al. [15], the initial concentration (1,250 mg/L) used by Chen et al. [32] may result in small volume micelles, which can reduce the retentate (micelles). Also, the best solubilization of surfactin is at pH 8.5, but at pH 11, the membrane cleaning should improve [30].

Chen et al. [32] concluded that UF membranes with MWCO less than 100 kDa were found to be suitable for the retention of surfactin micelles; in addition, 87% of recovery yield and more than 85% of purity could be achieved using the second strategy. Furthermore, it resulted in an H-form surfactin, which is more soluble than an Na-form surfactin (first and third strategy).

In a subsequent study, Chen et al. [1] tested a membrane-based process, salting-out (ammonium sulfate) and the hybrid process (see Table 3). They observed that the UF membrane with a MWCO lower than 100 kDa was suitable for the retention of surfactin

micelles, and the nanofiltration membrane with a MWCO lower than 1 kDa for the retention of surfactin monomers. In the salting-out process, they mixed surfactin solution, ammonium sulfate and ethanol. It separated itself into three phases: ethanol-rich (upper layer), third (middle layer, white precipitate), and water-rich phase (lower layer).The surfactin was mainly present in the ethanol-rich phase. When the ethanol (33% v/v) was added before the ammonium sulfate (23% v/v), it favored the separation of surfactin from protein and the process reached recovery yield and purity of surfactin are 84-92 and 68-69%, respectively. The hybrid process enhanced the recovery yield and improved the purity of surfactin. When comparing these results with data previously reported from Chen et al. [32], they do not appear to be significant or require more investigation (Compare Table 2 and 3).

Table 3. Approaches for recovery and purification of surfactin based on nano and ultrafiltration techniques combined to salting-out [1].

Process	**Recovery (%)**	**Purity (%)**	**Characteristics**
UF	68	83	Low recovery yield
Salting-out	93	68	Low purity
Salting-out + UF	81	78	Middle flux
Salting-out + *NF	81	79	Low flux
UF + Salting-out	63	84	Low flux and recovery

*NF - nanofiltration

Isa et al. [36] compared two UF systems, centrifugal devices and stirred cell device. The range of MWCO used was from 10 to 30 kDa. They found that polyethersulfone (PES) membrane was the best UF membrane for the purification of surfactin, especially in the second step of UF. Subsequently, Isa et al. [20] worked with two-step UF of surfactin, recovering that, directly from the broth and tested two sorts of membranes (10 kDa-MWCO), regenerated cellulose (RC) and PES at three transmembrane pressures (TMP) - 1.5, 2.0 and 2.5 bar - in two-step UF. They observed that the TMPs applied have no significant effect in the selectivity of filtration.

As discussed below, cross-flow filtration in two-steps seems to be the best method to recovery and purify surfactin.

4.1.1.1.1. Dead-end filtration

As already mentioned, Lin and Jiang [35] tested the two-steps UF process (30 kDa membrane) for the purification of surfactin, that used mineral salt solutions as the culture medium. The pH in the first UF was according to bioprocess that was perhaps slightly alkaline (from 7 to 8). Good results were obtained (Table 4); primarily due to the initial surfactin concentration used (250 mg/L), which according to Jauregi et al. [15] increases the volume of micelles and allows the UF by 100 kDa membrane. It is worth pointing out that the process did not use a pre-treatment such as acid precipitation; also, all membranes evaluated were cellulose-based, because Isa et al. [36] indicated PES as being better than RC. There is lack of significant data such as the filtration rate and pH.

Chen et al. [32] described the two-stage dead-end UF process using as a feed surfactin solution at pH 11, which was recovered from the culture medium after acid precipitation. They concluded that the micelles were efficiently destabilized by ethanol (33%); additionally, between the solvents tested (methanol and acetone), ethanol showed the lowest retention of surfactin monomers (UF-2). The following membranes: polysulfone, regenerated cellulose (RC), polyethersulfone (PES), polyacrylonitrile, cellulose ester (CE) from 30 to 100 kDa-MWCO, did not show relevant differences for surfactin rejection. Nevertheless, the highest flux obtained was 92.4 and 79.8 L/(h/m^2) for PES (30 kDa-MWCO) and RC (100 kDa-MWCO), respectively. Therefore, it was indicated that PES is more suitable for this purpose. They deduced that the cake formation on the membrane was responsible for dynamic flux decline, which can be minimized by a pre-treatment or a cross-flow UF [32]. Interestingly, they used a stirring system (no higher than 300 rpm) to create more turbulence near the membrane; consequently, it reduced the polarized layer resistance and increased the flux. Finally, they evaluated the pH effect on the rejection yield of surfactin, as a result of that a neutral pH (7) seemed more appropriate. In this context, they suggested that some macromolecular impurities or surfactin precipitate occurs at pH 6.0 and then solutes; and their aggregation are readily blocked on the pores of the membranes, which have a significant impact on the flux rate. As their main result, the UF in two steps with the addition of ethanol after the first UF seems more suitable, with more than 72% of recovery yield and more than 83% of purity was achieved.

Chen et al. [1] observed that the purity of the recovered surfactin was only slightly improved compared with that obtained after acid precipitation; the RC membrane had a good recovery, however, a low flux of 5 L/(h/m^2) was obtained, considered unattractive. A better

strategy is to first add the solvent and then the ammonium sulfate; in that way, micelles will be destabilized, leaving free proteins and other compound; after that, the salting out effect will precipitate those molecules. They concluded that the optimal condition is 33% (v/v) ethanol; 23% (w/v) salt, reaching the recovery yield and purity of 94–96 and 67–69%, respectively. However, this method requires extra steps of pre-treatment of bioprocess as acid precipitation to recover surfactin and re-dissolution of precipitate in alkaline solution (pH 11), followed by filtration by the UF process. This process offers high recovery and relatively high purity of surfactin; however, the extra steps taken would add to the complexity of the process and could have an effect on the final cost of surfactin production.

4.1.1.1.2. Cross-flow filtration

Chen et al. [30] reported on UF cross-flow for recovery and purification of surfactin from a solution of BS (obtained from the broth treated by acid precipitation) dissolved in alkaline solution. They tested with a range of TMP, cross-flow velocities and initial concentration of surfactin. Before the UF process, the synthetic culture medium was acidified. Then, the precipitate was recovered after centrifugation, that is, two steps of low resolution purification method. The type of critical flux indicated that some soluble molecules metabolized by *B. subtilis* were small enough to go into the pores of the membrane and be absorbed onto the pore walls, which is favored by attractive electrostatic forces and high solute concentrations.

Chen et al. [30] worked with two membranes, PES and CE. They considered the hydrophobicity factor and its effects on the purification of surfactin. The hydrophobic groups may adsorb the hydrophobic surfactant tails, which improves surface wettability; whereas on hydrophilic membrane groups, the peptides may adsorb but the wettability is reduced, as a result of that, the flux with PES membrane was higher than CE at a surfactin concentration of 1,480 mg/L; however, CE showed better results of rejection of surfactin. Also, both membranes showed that the flux was TMP-dependent below 2.9 psi and became TMP-independent at 2.9 psi or higher.

Comparing dead-end UF with PES (100 kDa-MWCO), the cross-flow resulted in equivalent recovery and slightly higher purity in the retentate (83 and 79%, respectively) under comparable conditions [1, 30].

Isa et al. [36] indicated that 98% (+/-4.1) of surfactin was recovered (retentate) in the UF-1 by using RC 10kDa with stirred cell. In the UF-2, the recovery and purity of surfactin were significantly high, 96 and 94%, respectively. Although in the second step of

purification, the flux of PES membrane decreased significantly over time, 130 to 30 ($L(h.m^2)$). In this matter, they showed that PES membrane was affected more by concentration polarization than RC. They draw additional attention to solvent effects, which may result in pore constriction or dilation of UF membranes; RC-methanol resulted in dilatation and PES-methanol in constriction. Finally, they measured particle sizes, by DLS, in the broth, the retentate solution generated in UF-1 (aqueous) and the solvent solution (feed solution of UF-2). The first two showed similar particle sizes, approximately 9 nm, which is aligned to data of commercial surfactin aqueous solution; also, broth conditions seemed to slightly increase the micelle size, which depends on factors such as ionic strength and presence of organic compounds that may coexist in the micelle and thus increase its size. On the other hand, after the addition of 50% (v/v) methanol solution to this retentate, particles with a mean diameter of 9 nm corresponding to surfactin micelles were no longer present in the solution, indicating the rupture of such structures. Moreover, the presence of larger particles was detected, with a mean diameter of 100 nm. Such particles could be protein aggregates induced by methanol.

Tables 4 and 5 illustrate a compiling of fundamental parameters of UF such as: initial concentration, sort of membrane, pH and TMP. The first one shows that the aim is retentate surfactin micelles. Conversely, Table 5 indicates the results for experiments using a micelle-destabilizing; thereby, the surfactin can be recovery into permeate.

As in Table 4, it is possible to recover up to 98% of surfactin [36]. However, it is also necessary to achieve high flux, which may allow the industrial scale. In this case, the highest flux ($175L/(h.m^2)$) was obtained by Chen et al. [32] using a PES (100) with surfactant content up to 400 mg/L. Obviously, the flux increases with the porous size. The first UF basically removes salts. Hence, there is no significant impact on purity. Also, the following parameters, flux and rejection of surfactin should be evaluated to improve the process. Some parameters are significant in this step, for instance, pH of feed solution that may precipitate proteins with subsequent decrease of flux; or as already mentioned the surfactin content of feed and its effects under micelle interactions.

Table 4. Recovery of surfactin in the retentate.

References	**C_0 (mg/L)	Membrane	TMP (psi)	Rejection of surfactin (%)	pH	Flux (L/(h.m^2))
[35]	250	RC (30)	30.45	97.9	*NM	*NM
[32]	400	PES (100)	12.5	93	7	175
[36]	583	RC (10)	29	98	**7**	*U
[30]	4 020	CE (100)	8.7	97	**7**	120
[1]	1 530	PES (100)	12.5	87	11	25
[20]	596	PES (10)	29	83	*NM	83

* NM - Not mentioned

Table 5. Recovery of surfactin by UF in the permeate.

Authors	†C_0 (mg/L)	Membrane	TMP (psi)	Recovery (%)	Purity (%)	Flux (L/(h.m^2))	Micelle-destabilizing
[35]	5 000	RC (30)	30.45	95	98	*NM	methanol (50 %)
[32]	2 054	PES (100)	12.5	87	85	*NM	ethanol (33 %)
[36]	571	RC (10)	29	96	93	30	methanol (50 %)
[30]	2 550	CE (100)	8.7	80	74	220	ethanol (50%)
[1]	2 054	PES (100)	12.5	81	78	5	ethanol (33%) and ammonium sulfate (23%)
[20]	560	PES (10)	36.5	94	96	118	methanol (50%)

* Not mentioned

† Initial concentration

In Table 5, methanol and ethanol are used as micelle-destabilizing. Ethanol has commercial advantages compared with methanol. For instance, it is cheaper and larger scale, has lower toxicity and mainly, it is a product from a sustainable process. It is worth noting that in this step, higher surfactin concentration may be used, since it will be as monomers. The purity should be a more significant parameter, as well as flux and recovery.

Considering all aspects, Isa et al. [20] obtained the best condition for UF-2, mainly due to the high recovery and purity, 94 and 96%, respectively and good flux ($118 L/(h.m^2)$).

Therefore, there are strong evidences that good results can be achieved using Chen et al. [32] parameters in UF-1, and Isa et al. [20] in UF-2, and the later may use ethanol as micelle-destabilizing.

5. CONCLUSION AND PERSPECTIVE

Among all the purification techniques on BS, UF seems to be the most prominent. Recent reports have proposed a two-stage UF process for recovery and purification of surfactin, reaching 94 and 96%, respectively. Concentration of surfactin solution between 50-100 mg/L of surfactin appears to be a more convenient initial concentration to UF, since large (volume) and uniform micelles are formed. Therefore, the UF in two steps, if aligned with other techniques, such as the production using industrial wastes as culture medium and the recovery by the foam overflow during the bioprocess, would significantly decrease the surfactin production cost and thus, allowed the industrial scale production and application. On the other hand, there is no report about membrane-based filtration of MEL. Theoretically, MEL can be recovered and purified by UF process in two steps – in the same way as surfactin – which may lead to a significant impact on production cost.

As perspective, a deep study on ultrafiltration of surfactin by adding divalent cations, which may improve the yields. A scale-up of surfactin using UF in two steps as downstream method and reach high yields. The production of surfactin and MEL using agroindustrial wastes as culture medium integrated to UF in two steps as downstream method, which would reduce the production cost of these biosurfactants.

Acknowledgments

This project was financially supported by FAPESP-Brazil, which purpose is to fund technological projects.

Declaration of interest statement

The authors report no declaration of interest.

6. REFERENCES

[1] H-L. Chen, Y-S Chen and R-S. Juang, "Recovery of surfactin from fermentation broths by a hybrid salting-out and membrane filtration process," *Separation and Purification Technology*, vol. 59, no 3, pp. 244-252, 2008b.

[2] F. F. C. Barros, A. N. Ponezi and G. M. Pastore, "Production of biosurfactant by *Bacillus subtilis* LB5a on a pilot scale using cassava wastewater as substrate," *Journal of Industrial Microbiology & Biotechnology*, vol. 35, no 9, pp. 1071-1078, 2008.

[3] A. F. Faria, D. S. Teodoro-Martinez, G. N. O. Barbosa, B. G. Vaz, I. S. Silva, J. S. Garcia, M. R. Tótola, M. N. Eberlin, M. Grossman, O. L. Alves and L. R. Durrant, "Production and structural characterization of surfactin (C14 /Leu7) produced by *Bacillus subtilis* isolate LSFM-05 grown on raw glycerol from the biodiesel industry," *Process Biochemistry,* vol. 46, no 10, pp. 1951-1957, 2011.

[4] R. Marchant and I. M. Banat, "Biosurfactants: a sustainable replacement for chemical surfactants?," *Biotechnology Letters*, vol. 34, no 9, pp. 1597-1605, 2012.

[5] F. Coutte, P. Jacques, D. Lecouturier, J-S. Guez, P. Dhulster, V. Leclère and M. Béchet, WO 2013050700 A2, "*Bacillus* sp. biosurfactants, composition including same, method for obtaining same, and use thereof," 2013.

[6] C. N. Mulligan and B. F. Gibbs, "Recovery of biosurfactants by ultrafiltration," *Journal of Chemical Technology and Biotechnology*, vol. 47, no. 1, pp. 23-29, 1990.

[7] G. Seydlová, R. Čabala and J. Svobodová, "Surfactin: novel solutions for global issues": In: M. A. Komorowska, S. Olsztynska-Janus (eds): Biochemical Engineering, Trends, Research and Technologies. InTech, Rijeka, pp. 305-330, 2011.

[8] R. S. Makkar, S. S. Cameotra and I. M. Banat, "Advances in utilization of renewable substrates for biosurfactant production," *AMB Express*, vol. 1, no 1, pp. 1-19, 2011.

[9] D. Zhang, Y. Whang, Y. Lu, C. Zhang and Z. Lu, "An efficient method for separation of surfactin from *Bacillus amyloliquefaciens* fmb50 broth by flocculation," *Process Biochemistry*, vol. 49, no 7, pp. 1182–1188, 2014.

[10] G. Dhanarajan, V. Rangarajan and R. Sen, "Dual gradient macroporous resin column chromatography for concurrent separation and purification of three families of marine bacterial lipopeptides from cell free broth," *Separation and Purification Technology*, vol. 143, pp. 72-79, 2015.

[11] N. Khondee, S. Tathong, O. Pinyakong, R. Müller, S. Soonglerdsongpha, C. Ruangchainikom, C. Tongcumpou and E. Luepromchai, "Lipopeptide biosurfactants production by chitosan-immobilized *Bacillus* sp. GY19 and their recovery by foam fractionation," *Biochemical Engineering Journal*, vol. 93, pp. 47-54, 2015.

[12] T. Morita, T. Fukuoka, M. Konishi, T. Imura, S. Yamamoto, M. Kitagawa, Y. Sogabe and D. Kitamoto, "Production of a novel glycolipid biosurfactant, mannosylmannitol lipids, by *Pseudozyma parantarctica* and its interfacial properties," *Applied Microbiology and Biotechnology*, vol. 83, no 6, pp. 1017-1025, 2009a

[13] T. Morita, Y. Ishibashi, T. Fukuoka, T. Imura, H. Sakai, M. Abe and D. Kitamoto, "Production of glycolipid biosurfactant, mannosylerythritol lipids, using sucrose by fungal and yeast strains, and their interfacial properties," *Bioscience, Biotechnology, and Biochemistry*, vol. 73, no 10, pp. 2352-2355, 2009b.

[14] J. I. Arutchelvi, J. Sangeetha, J. Philip and M. Doble, "Self-assembly of surfactin in aqueous solution: Role of divalent counterions," *Colloids and Surfaces B: Biointerfaces*, vol 116, pp. 396-402, 2014.

[15] P. Jauregi, F. Coutte, L. Catiau, D. Lecouturier and P. Jacques, "Micelle size characterization of lipopeptides produced by *B. subtilis* and their recovery by the two-step ultrafiltration process," *Separation and Purification Technology*, vol 104, pp. 175-182, 2013.

[16] J. I. Arutchelvi, S. Bhaduri, P. V. Uppara and M. Doble, "Mannosylerythritol lipids: a review," *Journal of Industrial Microbiology & Biotechnology*, vol. 35, no 12, pp. 1559-1570, 2008.

[17] M. M. Müller, J. H Kügler, M. Henkel, M. Gerlitzki, B. Hörmann, M. Pöhnlein, C. Syldatk and R. Hausmann, "Rhamnolipids - Next generation surfactants?," *Journal of Biotechnoogyl*, vol. 162, no 4, pp. 366-380, 2012.

[18] V. Rangarajan, G. Dhanarajan and R. Sen, "Improved performance of cross-flow ultrafiltration for the recovery and purification of Ca^{2+} conditioned lipopeptides in diafiltration mode of operation," *Journal of Membrane Science*, vol. 454, pp. 436-443, 2014.

[19] B. S. Saharan, R. K. Sahu and D. Sharma, "A review on biosurfactants: fermentation, current developments and perspectives," *Genetic Engineering and Biotechnology Journal*, vol. 2011, pp. 1-14, 2012.

[20] M. H. M. Isa, R. A. Frazier and P. Jauregi, "A further study of the recovery and purification of surfactin from fermentation broth by membrane filtration," *Separation and Purification Technology*, vol. 64, no 2, pp. 176-182, 2008.

[21] H-Z. Gang, J-F. Liu and B-Z. Mu, "Molecular dynamics study of surfactin monolayer at the air/water interface," *The Journal of Physical Chemistry B*, vol. 115, no 44, pp. 12770–12777, 2011.

[22] A. Knoblich, M. Matsumoto, R. Ishiguro, K. Murata, Y. Fujiyoshi, Y. Ishigami and M. Osman, "Electron cryo-microscopic studies on micellar shape and size of surfactin, an anionic lipopeptide," *Colloids and Surfaces B: Biointerfaces*, vol. 5, no 1-2, pp. 43-48, 1995.

[23] F. Fukuoka, T. Morita, M. Konishi, T. Imura and D. Kitamoto, "A *basidiomycetous* yeast, *Pseudozyma tsukubaensis*, efficiently produces a novel glycolipid biosurfactant. The identification of a new diastereomer of mannosylerythritol lipid-B," *Carbohydrate Research*, vol. 343, no 3, pp. 555-560, 2008.

[24] T. Fukuoka, T. Yanagihara, T. Imura, T. Morita, H. Sakai, M. Abe and D. Kitamoto, "The diastereomers of mannosylerythritol lipids have different interfacial properties and aqueous phase behavior, reflecting the erythritol configuration," *Carbohydrate Research*, vol. 351, pp. 81-86, 2012.

[25] T. Fukuoka, T. Morita, M. Konishi, T. Imura, H. Sakai and D. Kitamoto, "Characterization of new glycolipid biosurfactants, tri-acylated mannosylerythritol lipids, produced by *Pseudozyma* yeasts," *Biotechnology Letters*, vol. 29, no 7, pp. 1111-1118, 2007a.

[26] T. Fukuoka, T. Morita, M. Konishi, T. Imura, H. Sakai and D. Kitamoto, "Structural characterisation and surface-active properties of a new glycolipid biosurfactant, mono-acylated mannosylerythritol lipid, produced from glucose by *Pseudozyma antarctica*," *Biotechnological Products and Process Engineering*, vol. 76, no 4, pp. 801–810, 2007b.

[27] T. Fukuoka, T. Yanagihara, T. Imura, T. Morita, H. Sakai, M. Abe and D. Kitamoto, "Enzymatic synthesis of a novel glycolipid biosurfactant, mannosylerythritol lipid-D and its aqueous phase behavior," *Carbohydrate Research*, vol. 346, no 2, pp. 266–271, 2011.

[28] Y. Han, X. Huang, M. Cao and Y. Wang, "Micellization of surfactin and its effect on the aggregate conformation of amyloid β (1-40)," *The Journal of Physical Chemistry B*, vol. 112, no 47, pp. 15195-15201, 2008.

[29] T. Imura, N. Ohta, K. Inoue, N. Yagi, H. Negishi, H. Yanagishita and D. Kitamoto, "Naturally engineered glycolipid biosurfactants leading to distinctive self-assembled structures,"*Chemistry - A European Journal*, vol. 12, no 9, pp. 2434-2440, 2006.

[30] H-L. Chen, Y-S. Chen and R-S. Juang, "Flux decline and membrane cleaning in cross-flow ultrafiltration of treated fermentation broths for surfactin recovery," *Separation and Purification Technology*, vol. 62, no 1, pp. 47-55, 2008a.

[31] N. S. Shaligram, R. S. Singhal, "Surfactin – A review on biosynthesis, fermentation, purification and applications," *Food Technology & Biotechnology*, vol. 48, no 2, pp. 119–134, 2010.

[32] H-L. Chen, Y-S. Chen, R-S. Juang, "Separation of surfactin from fermentation broths by acid precipitation and two-stage dead-end ultrafiltration processes," Journal *of Membrane Science*, vol. 299, no 1-2, pp. 114-121, 2007.

[33] M. T, S. Silva, C. M. F. Soares, A. S. Lima, C. C. Santana, "Integral production and concentration of surfactin from *Bacillus* sp.ITP-001 by semi-batch foam fractionation," Biochemical Engineering Journal, In Press, Corrected Proof, doi:10.1016/j.bej.2015.04.010

[34] M. Suzuki, M. Kitagawa, S. Yamamoto, A. Sogabe, D. Kitamoto, T. Morita, T. Fukuoka, T. Imura, US 2010/0228013 A1, "Activator including biosurfactant as active ingredient, mannosylerythritol lipid, and production method thereof," 2010.

[35] S-C. Lin, H-J. Jiang, "Recovery and purification of the lipopeptide biosurfactant of *Bacillus subtilis* by ultrafiltration," *Biotechnology Techniques*, vol. 11, no 6, pp. 413-416, 1997.

[36] M. H. M. Isa, D. E. Coraglia, R. A. Frazier, P. Jauregi, "Recovery and purification of surfactin from fermentation broth by a two step ultrafiltration process," *Journal of Membrane Science*, vol. 296, no 1-2, pp. 51-57, 2007.

CHAPTER II

ULTRAFILTRATION IN TWO STEPS OF SURFACTIN PRODUCED BY *Bacillus subtilis* LB5a USING CASSAVA WASTEWATER AS SUBSTRATE AND ETHANOL AS MICELLE-DESTABILIZING

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(Paper submitted to Journal of Chemical Engineering and Biotechnology)

ULTRAFILTRATION IN TWO STEPS OF SURFACTIN PRODUCED BY *Bacillus subtilis* LB5A USING CASSAVA WASTEWATER AS SUBSTRATE AND ETHANOL AS MICELLE-DESTABILIZING

Ultrafiltration of surfactin using cassava wastewater as substrate

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Abstract

BACKGROUND

Bacillus subtilis synthesizes surfactin, a powerful surface-active agent. It has interesting potential applications, however, due to its high cost of production, commercial use is impracticable. The downstream processing represents ≈ 60% of production costs and the culture medium ≈ 30%. Many reports focused, separately, on production of surfactin using by-products (reduce cost) or the purification using synthetic medium. Therefore, the aim of this work was to evaluate, for the first time, the surfactin production using a low-cost substrate, integrated to the ultrafiltration in two steps.

RESULTS

Membranes of polyethersulfone-100-kDa efficiently retained surfactin micelles - the first step of ultrafiltration, whereas, the second step required membranes of 50-kDa to separate surfactin monomers from proteins. On one hand, the ultrafiltration of crude biosurfactant was associated with fouling and/or concentration polarization. On the other hand, the ultrafiltration of semi-purified biosurfactant was adequate, resulting in high total recovery of surfactin (78.25%) and minimal problems with fouling and/or concentration polarization.

CONCLUSION

Therefore, ultrafiltration in two steps using cassava wastewater as a low-cost culture medium is feasible, nevertheless, making the previous ultrafiltration treatment by solvent extraction essential. The NMR and MALDI-TOFMS analyses identified 11 potential surfactin homologous composed by two amino acid sequences.

Keywords: Fermentation, purification, residues, ultrafiltration, waste-water

1. INTRODUCTION

A wide variety of microorganisms produce biosurfactants including *B. subtilis* that synthesizes lipopeptides such as surfactin, iturin, fengycin, etc. These compounds have high surface activity and resistance to extreme conditions. [1-3] They have raised a lot of interest due to their remarkable properties such as: high emulsification index in a wide range of hydrophobic substrates, and maintenance of surface activity under extreme conditions of temperatures, pH and ionic strenght. [2-3]

Biosurfactants can be produced using industrial wastes and by-products as culture medium. In the production of surfactin from *B. subtilis*, the use of cassava wastewater is well-known; this waste seems to be an ideal match, since it is available in large amounts throughout the year and in all regions of Brazil. [1] However there is a lack of knowledge about technical feasibility of the downstream process of surfactin that was produced using industrial wastes as culture medium. Downstream, is also the most important economical factor, since it represents about ≈ 60% of the total production cost. [4-5]

Conventional methods for purification of surfactin produced by *B. subtilis* include acid precipitation followed by extraction from organic solvents, and techniques of adsorption. [4-5]

In the past ten years the ultrafiltration (UF) based downstream processing and, specifically, the two-step UF[6] has shown to be the most promising both in terms of the yields and purity and its scalability and it is currently being applied in the manufacturing of lipopeptides. In the first step, surfactin micelles are recovered as retentate. An organic solvent is added to that retentate in order to disrupt the micelles. Then, a second step of UF is carried out to obtain monomers of surfactin as permeate.

Table 1 compiles the parameters and yields of surfactin UF.

In most cases, the fermentation process is carried out using a synthetic culture medium. However, there have been no reports about the UF of surfactin produced using cassava wastewater as a culture medium.

Table 1. Parameters and yields of ultrafiltration – surfactin – in two steps.

First step of ultrafiltration – retentate						
Flux ($L(h.m^2)^{-1}$)	**$^{\dagger}C_o$ ($mg.L^{-1}$)**	**Membrane**	**††TMP (psi)**	**Rejection of surfactin (%)**	**pH**	**References**
25	1530	**PES	12.5	87	11	4
175	400	**PES	12.5	93	7	7
120	4020	**CE	8.7	97	7	8
*NM	583	**RC (10)	29	98	7	9
83	596	**PES (10)	29	83	*NM	10
*NM	250	**RC (30)	30.45	97.9	*NM	11

Second step of ultrafiltration – permeate							
Micelle-destabilizing conditions	**$^{\dagger}C_o$ ($mg.L^{-1}$)**	**Membrane**	**††TMP (psi)**	**Recovery (%)**	**Purity (%)**	**Flux ($L(h.m^2)^{-1}$)**	**References**
EtOH (33%) and $(NH4)_2SO_4$ (23%)	2,054	**PES (100)	12.5	81	78	5	4
MeOH (33%)	2,054	**PES (100)	12.5	87	85	*NM	7
EtOH (50%)	2,550	**CE (100)	8.7	80	74	220	8
MeOH (50%)	571	**RC (10)	29	96	93	30	9
MeOH (50%)	560	**PES (10)	36.5	94	96	118	10
MeOH (50%)	5,000	**RC (30)	30.45	95	98	*NM	11

* Not mentioned

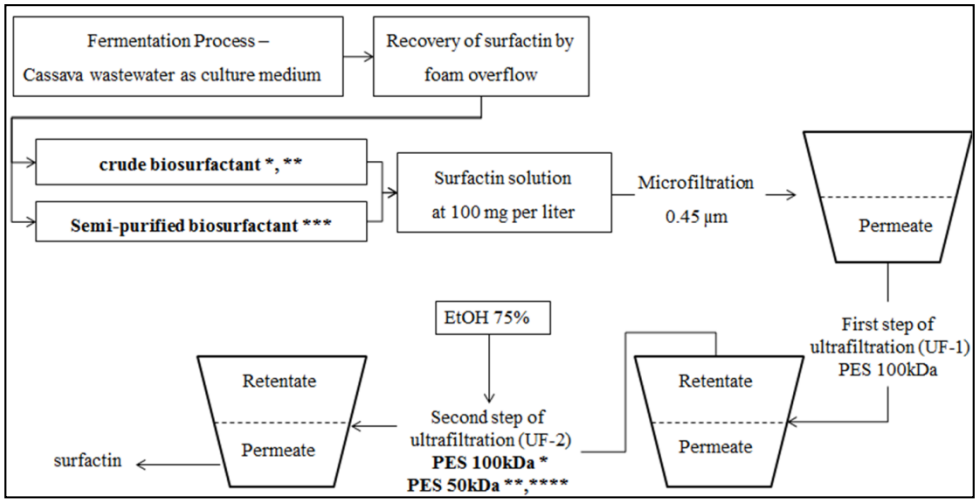
** Membranes – (PES-polyethersulfone; CE-cellulose ester; RC-regenerated cellulose)

†Co Initial concentration

†† Transmembrane pressure

Thus, we speculate that the production of surfactin using cassava wastewater as culture medium combined with the UF process in two steps would lead to a significant reduction in the cost of production of surfactin. Therefore, the aim of this work was to evaluate the technical feasibility of the purification of surfactin produced using cassava wastewater as culture medium (Fig 1.). Thus, to the best of our

knowledge, this is the first report that produced surfactin using cassava wastewater collected by foam overflow and further UF (two-steps method). In addition, the dissolved oxygen (DO) and viable cell count in the foam (foam has the highest concentration of biosurfactant) were analyzed, which gave an indication of the progress of the fermentative process.



*** stategy i; ** strategy ii; ***strategy iii**

Figure 1. Overview of ultrafiltration of surfactin produced from *B. subtilis* using cassava wastewater as culture medium.

2. MATERIAL AND METHODS

2.1. CHEMICALS

The chemicals used included: acetonitrile (Sigma-Aldrich ≥99.8%), bicinchoninic acid kit (Sigma-Aldrich), ethanol (Sigma-Aldrich ≥99.5%), bovine serum albumin (Sigma-Aldrich ≥98%), deuterated dimethyl sulfoxide (Cambridge Isotope Laboratories Inc. >99.9%), trifluoroacetic acid (Sigma-Aldrich ≥99%), and surfactin (Sigma-Aldrich ≥98%).

2.2. SURFACTIN PRODUCTION - BIOPROCESS

2.2.1. Culture medium

The cassava wastewater (variety IAC-13) was collected from a flour industry and transported to the laboratory at room temperature. Next, it was boiled (3

min at 100 ^{o}C), centrifuged (10,000 x *g* during 10 min at 5 ºC (Beckman Coulter, Alegra X-22r), and the supernatant was stored (-18 ^{o}C).

2.2.2. Microorganism and *inoculum*

B. subtilis LB5a was used as a surfactin producer. The inoculum was standardized according to Barros et al. (2008). [1]

2.2.3. Fermentation parameters and sampling

Cassava wastewater (3.0 liters working volume) was placed in a bioreactor (Bioflo® & Celligen® 310 - New Brunswick Scientific). The culture medium was sterilized at 121 ºC for 20 min. Fermentation parameters used included: 100 rpm and aeration rate of 0.4 vvm (vessel volume per minute) maintained in the first 24 h, and then 150 rpm and 0.8 vvm from 24 to 72 h. [1] The sensor (Mettler Toledo - INPRO 6830/12/320) of DO was programmed to measure every 30 sec during the entire fermentation processes; it was also calibrated by disconnecting the cable from the bioreactor (0%) and by 50 rpm and 4 L.min^{-1} min (100%). Samples of the culture medium and foam were collected on a 12 h basis to analyze viable cell count, content of glucose, volume of foam and surface tension (ST). In order to obtain enough surfactin for the purification experiments, seven fermentations were carried out.

2.2.4. Volumetric oxygen transfer coefficient

Volumetric oxygen transfer coefficient (K*la*) was measured by dynamic methods. Measurements of DO were carried out using a probe (INPRO 6830/12/320). The medium (3 L of cassava wastewater) was bubbled with nitrogen to remove oxygen. Then, aeration was started (2 L.min^{-1}) and DO values were used to calculate the K*la*. [12]

2.2.5. Biosurfactant recovery

The foam was collected from the top of the bioreactor during its production, as described by Barros et al. (2008). [1] The foam was collapsed and its volume was measured, and then centrifuged at 10,000 x *g* for 20 min. Afterwards, the ST was measured in the supernatant phase using a tensiometer (Krüss GmbH K-12) by plate method. [1]

2.2.6. Pre-purification (ultrafiltration) – crude and semi-purified biosurfactant

The collapsed foam was acidified with HCl solution (2 and 0.1 N) to pH = 2, and the solution remained for 24 h at room temperature; then it was centrifuged at 10,000 x *g* for 20 min. The precipitate was collected, neutralized with NaOH solution (2 and 0.1 N) and dried at 50 ^{o}C; the powder was named crude biosurfactant.

The crude biosurfactant (obtained from the all seven fermentation) was dissolved in chloroform: methanol 65:15 (v:v) and filtered through a membrane with pore size of 0.22 µm. The filtrate was recovered and dried at room temperature. [1] The resulting powder was classified as semi-purified biosurfactant. Yields were calculated by dividing total mass obtained of crude or semi-purified biosurfactant by the volume of culture medium (3 L). Yields were also calculated dividing total mass obtained of crude or semi-purified biosurfactant by the volume of colapsed foam (foam overflow).

2.3. ANALYTICAL PROCEDURES - PRODUCTION STAGE

2.3.1. Measurement of surface activity

Critical micelle dilutions (CMDs) are the ST values of the sample diluted at 10-times (CMD-1) and 100-times (CMD-2). The ST measurements (CMDs) were carried out on the centrifuged culture medium and foam samples (12 h basis) by using the plate method at room temperature in a Krüss GmbH K-12 tensiometer (K-12 model, Krüss GmbH). [1]

2.4. PURIFICATION OF SURFACTIN BY TWO-STEP ULTRAFILTRATION PROCESS

2.4.1. Process overview

First, the purity of surfactin in crude and semi-purified biosurfactant (see surfactin concentration analysis) was measured. Then, an aqueous solutions of crude biosurfactant and semi-purified biosurfactant (Tris-buffer pH 8.5 - optimum solubilization of surfactin [4,5,9]) were made with the concentration of surfactin at 100 mg.L^{-1}, filtered (0.45 µm) and used as a feed in the first UF step (UF-1). [6] UF-1 retained the surfactin micelles and proteins (retentate), while salt and small molecules were recovered as permeate. From the retentate of UF-1, a solvent solution was prepared (ethanol 75%), followed by the second UF step (UF-2). Since ethanol disrupts surfactin

micelles to monomers, this process aimed to retain proteins, so the surfactin can be recovered as permeate (Fig 1). After these two UF steps, high recovery and purity are expected as shows the Table 1. Basically, three analyses were carried out in all samples feed, permeate and retentate of UF-1, and permeate and retentate of UF-2 to evaluate the UF processes including: nanoparticle size (Dynamic Light Scattering - DLS), concentration of surfactin (High Performance Liquid Chromarography - HPLC) and protein (Bicinchininic Acid Method - BCA).

The two-step ultrafiltration process was applied following three different strategies (i, ii and iii) (Fig. 1). The first two strategies used a crude biosurfactant, and the third strategy used a semi-purified biosurfactant. In all strategies PES membranes were used with different molecular weight cut-off (MWCO).

2.4.2. Centrifugal device of ultrafiltration in two steps

The experiments of ultrafiltration were carried out using Vivaspin 20 with PES – 50 and 100 kDa, containing membrane of 6 cm^2 of active area. For UF-1, biosurfactant solution (crude or semi-purified) at 100 $mg.L^{-1}$ of pure surfactin (see surfactin concentration analysis) was used as feed, in which 20-15 mL was added to the filter unit (100 kDa), centrifuged at 2205 x *g* (10 or 20 min) and 20 ^oC. Next, the retentate (from UF-1 $\approx$ 0.7 mL) was dissolved in 20-15 mL of ethanol (75%) and centrifuged once again (10 or 20 min). The retentate (UF-2) was dissolved in 15-20 mL of tris-buffer (8.5). Finally, all solutions (retentate and permeate of UF-1, -2) were analyzed for concentration of surfactin by HPLC, nanoparticle size by DLS and concentration of protein by BCA.

The rejection coefficient (R) by a membrane was defined as shown the Equation 1. [6] Two rejection coefficients were calculated (i) for surfactin (R_s) and (ii) for protein (R_p).

Equation 1. $R = (C_f\text{-}C_p/C_f)$

Where C_F and C_P are the concentration of surfactin (C_s) or protein (C_p) in the feed and permeate, respectively.

It was also calculated the purity in terms of protein as mass fraction of surfactin in relation to the sum of mass of surfactin and protein (P_p) in the UF-1 and UF-2 as shown below: [6]

Equation 2. $P_p = [(C_s/C_s + C_p)] \times 100$

The equation 2 was applied to calculate the purity in the feed, retentate and permeate.

Finally, it was calculated the total recovery of surfactin (TRS) by the equation 3, in which M_s is the mass of surfactin.

Equation 3. $TRS = [(M_{si}/M_{sii})] \times 100$

For the UF-1 (TRS_i), M_{si} is the mass of surfactin in the retentate whereas M_{sii} is the mass of surfactin in the feed. For the UF-2 (TRS_{ii}), M_{si} is the mass of surfactin in the permeate whereas M_{sii} is the mass of surfactin in the feed. It was also calculated the TRS_t in the UF-1 and UF-2, where M_{si} is the mass of surfactin in the initial feed (UF-1) and M_{sii} is the mass of surfactin in the permeate (UF-2). The M_s was obtained multipling C_s by the volume of solution.

2.4.3. Analytical procedures - purification

2.4.3.1. Protein concentration

The total amount of protein present at each stage of the purification procedure was determined by the BCA. A calibration curve was produced, using bovine serum albumin the protein standard solution. [9]

2.4.3.2. Surfactin concentration analysis

Surfactin concentration was determined by reverse phase HPLC from a filtered (0.45 µm) solution (tris buffer pH 8.5 – 10 mM) of crude biosurfactant (≈ 1200 $mg.L^{-1}$). The system used was a Gilson 306 (Rockford, IL, USA) with a C-18 column of dimensions 250 mm × 4.6 mm, and a particle size of 5 µm. The flow rate of the mobile phase was 1.1 $mL.min^{-1}$ with the initial gradient starting from 50 to 80% acetonitrile in

0.1% trifluoroacetic acid in the first 15 min. The gradient remained at 80% for 20 min before increasing to 100% for 5 min as a washing step, returning to 50% once again. A 50 µL sample was injected into each run, which lasted 60 min, and eluent absorbance monitored at 214 nm. The system was calibrated using pure surfactin (≥98%) obtained from Sigma-Aldrich. The area of the peaks (samples) eluting at 23.18 and 27 min were identified as having the same retention times as those peaks eluting from the standard, which were added to give the total surfactin peak area. [9]

2.4.3.3. Particle size measurements - micelles

The nanoparticle sizes were evaluated by DLS, using a Zetasizer Nano ZS system (Malvern, UK). [9] All samples (feed; permeate UF-1; UF-2 and retentate UF-1; UF-2) were analyzed at least two times, and information about the size distribution by volume was used as a parameter.

2.5. CHEMICAL STRUCTURE IDENTIFICATION OF PRODUCED SURFACTIN (STRATEGY III)

Three different approaches, Infrared Spectroscopy (IR), Matrix Assisted Laser Ionization Time-of-flight (MALDI-TOFMS) and Nuclear Magnetic Resonance (NMR), were used in order to investigate the chemical structure of the produced surfactin (strategy iii). The sample was prepared for infrared analysis (FTLA2000) by mixing approximately 1 mg of produced surfactin (strategy iii) with 100 mg of KBr and pressing the mixture into the form of a pellet at 134 MPa for 2–3 min to obtain transparent pellets. The IR spectrum of the pellet was collected from 400 to 4000 wavelengh (cm^{-1}). [13] MALDI-TOFMS spectra were performed using an UltrafleXtreme MALDI-TOF mass spectrometer (Bruker) operating in the refraction mode at an accelerating voltage of 22.5 kV. Mass spectra were acquired in m/z range of 700-3500 with ions generated from Smartbeam™ laser irradiation using a frequency of 2000 Hz, a lens 7 kV and the delay time was 110ns. Matrix-suppression was set to 500 Da. External calibration was performed by using the peptide calibration standard (Bruker Daltonics). [14]NMR experiments were performed at 298 K using an Agilent DD2 500 MHz spectrometer equipped with a 5 mm triple resonance probe. After lyophilization, 8 mg of the produced surfactin (strategy iii) was dissolved in 600 µL of deutered dimethyl

sulfoxide ($^{2}H_6$-DMSO CIL-Cambridge Isotope Laboratories Inc.). Resonance peaks were assigned using standard methods including correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY) and nuclear Overhauser enhancement spectroscopy (NOESY). The TOCSY spectra were acquired using a mixing time of 100 ms. NOESY spectra were recorded with a mixing time of 250 and 350 ms. All two-dimensional experiments were acquired using a spectral width of 6983 Hz, a matrix size of 4096 X 512 points and relaxation delay of 1.5 s.

Data were processed using the NMRPipe/NMRVIEW software.[15-16] Prior to Fourier transform, the time domain data were zero-filled in both dimensions to yield a 4K X 2K data matrix. When necessary, a fifth-order polynomial baseline correction was applied after transformation and phasing.

3. Results and discussion

3.1. SURFACTIN PRODUCTION - FERMENTATION

3.1.1. Fermentation process and recovery of surfactin

The production of surfactin from *B. subtilis* LB5a using cassava wastewater as culture medium was already reported at the following scales, Erlenmeyer flask (250 mL) and pilot bioreactor (80 L) [1] however, its purification by the two-step UF process has not been reported before. Even with the subtle changes that were implemented, such as the increase of aeration after 24 h rather than 12 h, working volume, bioreactor, etc., similar process parameters were observed with those previously reported by Barros et al. (2008). [1] In addition, the DO (culture medium) and viable cells in the foam were evaluated for the first time, which enable a more accurate description of the fermentation process.

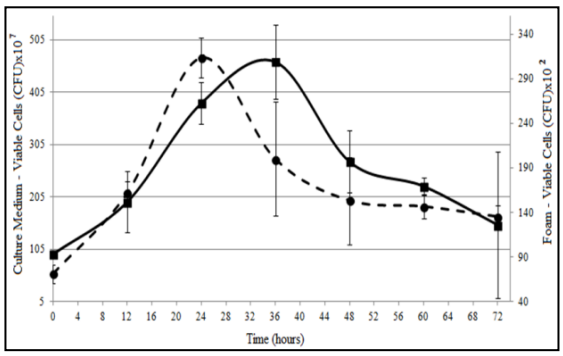


Figure 2. Viable cell counts, (–●–) culture medium, (–■–) recovered foam.

As expected a similar profile between viable cell count in culture medium and foam was found since higher cell concentration in the medium favors carrying cells in foam. The analysis of cells in the foam enabled to establish that a significant number of cells was removed during the process, for instance, at 36 h ≈ $4x10^4$ viable cells per mL of foam; thus, from 330 mL (volume of foam produced at 36 h) ≈ 10^6 cells were removed from the bioreactor. This data supports a more accurate understanding of microbial growth. Nevertheless, it only relates to viable cells, and the high surfactin and low nutrient concentration (foam) will most likely lyse some cells; therefore, we speculate that the results were underestimated. Finally, the high viable cell count in the culture medium reached ≈ 10^8 CFU when the stationary phase was between 24 and 48 h.

The ST of culture medium showed a decrease in the first 24 h, in other words, the biosurfactant content increased. As already expected, the recovery of surfactin increased due to the change in the aeration rate from 0.4 to 0.8 vvm (at 24 h). As a result, the ST values in the beginning and at the end of fermentation were similar, which indicates a high recovery of surfactin.

The ST activity of the foam is remarkable, from basically 12 h until the end of fermentation, the ST and its CMD-1 remained around 27 $mN.m^{-1}$. Taking into account that the critical micellar concentration (CMC) of surfactin is ≈ 10 $mg.L^{-1}$ and CMD-1 values remained at 27 $mN.m^{-1}$, it is easy to conclude that the surfactin concentration was at least 100 $mg.L^{-1}$. In addition, CMD-2 data showed ST around the CMC ≈ 30 $mN.m^{-1}$. A more accurate determination of the concentration obtained by HPLC analysis indicated the exact concentration of surfactin in the crude biosurfactant (see purification of surfactin by two-step UF).

We believe that the recovery by foam overflow is a good strategy, when it is used in a particularly complex culture medium such as cassava wastewater. This technique is advantageous since it primarily separates surfactin and proteins (both contain the property to make foam) from culture medium. In addition, the high concentration of surfactin in the culture medium may act as an inhibitor on the *B. subtilis* LB5a itself, leading to reduced growth and yields of surfactin.

The recovery of surfactin by foam overflow results is a bias. Relatively high aeration rate is necessary for recovery by foam overflow. Nevertheless, depleted oxygen condition [9] and micro-aeration conditions, ≈ 30% of DO [17] resulted in better yields of

surfactin production. During the fermentation using cassava wastewater, the DO remained at 0% (Fig 3.) and it was found the *Kla* 102.02 h^{-1}. In this context, Fahim et al. (2012) [12] described that the optimum *Kla* for the production of surfactin was 216 h^{-1} (0.04-0.08 s^{-1}). Hence, the fermentations were operated in good conditions, since it was obtained high surfactin recovery from culture medium (high ST measurement values in the culture medium, that is, low concentration of BS), high volume of foam collected ≈ 1000 mL (+/- 84) and DO around 0% most of the times, that is, with restriction of oxygen, which improves the production of surfactin. [18] However, based on the results obtained (DO and *Kla*) and in order to obtain better productivity, higher aeration could be applied, which will lead to higher DO (it should stay below 30% [17]) and higher *Kla* (closer to optimum value described by Fahim et al. (2012) [12]).

The profile of DO and dextrose content during the fermentation of *Bacillus subtilis* Lb5a and production of surfactin are shown in Figure 3.

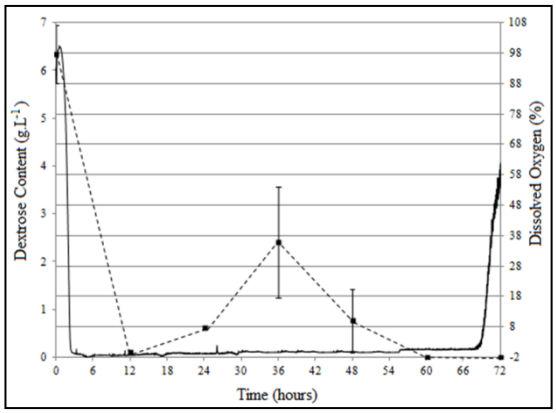


Figure 3. Dissolved Oxygen, (——), Dextrose Content (▬■▬) during the fermentation of *Bacillus subtilis* LB 5a and production of surfactin.

Initially (0 h), the dextrose concentration ($g.L^{-1}$) was at 6.5 and decreased to 0 at 12 h. Then, higher concentrations of dextrose were observed 0.5 at 12 h, 2.5 at 36 h and decreased again until the end of the bioprocess. This trend was already reported [1] and indicates that *Bacillus subtilis* LB5a produces amylases, and these amylases are produced to hydrolyze starch remains in the culture medium when dextrose is at low concentration.

The DO profile indicates that microorganisms hardly sense the change of culture medium (due to inoculation) from nutrient broth to cassava wastewater, and

based on DO, the lag phase took place within the first two hours. Then, it abruptly decreased to 0% and remained so for most of the time (from ≈ 3 to 68 h). This behavior is extremely good because the microorganism growth at aerobic and anaerobic conditions (0%) and the fermentation happened mainly at oxygen depleted conditions which favors the production of surfactin. [9,12,18,19] Also, as mentioned above, the aeration rate was enough to generate foam (which is proportional to the production fo biosurfaction) and in this way facilitated the recovery of surfactin in the foam. Finally, at 68 h, the DO increased, indicating the death phase.

It was found that the highest volume of foam was reached at 36 h, which is aligned with the viable cell profile. It shows that surfactin production was growth-associated. On the other hand the pH increased from ≈ 5.5 to 7.5, this sort of fermentation (alkaline) is characteristic of *B. subtilis*. [1]

All seven bioprocesses showed a low relative standard deviation. In each fermentation and its collected foam, 2.80 (+/- 0.6 g) of crude biosurfactant was obtained, in other words, 0.93 g per liter of culture medium.

3.1.2. Purification of surfactin by a two-step ultrafiltration process

The HPLC analysis showed that crude biosurfactant had 36.14 (+/- 9.05% $w.w^{-1}$) pure surfactin; thereby, ≈ 1010 $mg.L^{-1}$ of surfactin was in the foam, and a total of 1.01 g of pure surfactin was produced from each batch (3 liters of culture medium) or 336.66 $mg.L^{-1}$. This yield was lower than that reported by Isa et al. (2007) [9], which achieved 583 mg of surfactin per liter of culture medium and recovered surfactin directly from the culture medium. It is worth noting that the optimization of the production of surfactin was not the focus of this study, and it was also underestimated because it was considered that 100% of surfactin was recovered in the foam (remnants of surfactin were in the culture medium, bioreactor walls, etc.).

3.1.2.1. Strategy i

A feed solution (312 mg of crude biosurfactant.L^{-1}) was elaborated based on the results of purity of surfactin (36.14%). This solution was analyzed by HLPC, and surfactin concentration was determined as 105.85 $mg.L^{-1}$. As reported by Jauregi et al. (2013) [6] surfactin can be retained by PES 100 kDa at ≈ 100 $mg.L^{-1}$. The DLS analysis

indicated that surfactin micelles had a diameter (d) of 71.4 nm. In addition, micelles showed cylindrical form (d=71.4 and length of 30.3 nm).

As in the feed (nanoparticles ≈ 100 nm), the permeate of UF-1 showed micelles of d=129 nm. This configuration is most likely due to interaction between surfactin micelles (Table 2, 19.21 $mg.L^{-1}$ of surfactin), proteins and salts. On the other hand, the surfactin was at 70.12 $mg.L^{-1}$ in the retentate (see retentate of UF-1 in Table 2) and nanoparticles with larger diameter, 466 nm (see retentate of UF-1 in Table 3). The denaturation of proteins may explain the formation of nanoparticles with larger diameter.

The coefficient of surfactin rejection (R_s) indicated that 82% of surfactin was rejected by the membrane, whereas the coefficient of proteins rejection (R_p) was 68% (UF-1 in Table 3). Thus, the UF-1 probably separated most of the surfactin micelles from small molecules (e.g., peptides, organic acids, etc.) and 32% of proteins, which is quite advantageous.

Regarding the UF-2, the retentate from UF-1 (solubilized in ethanol 75%) was utilized as a feed solution; a solution mainly comprised by surfactin (monomers) and proteins.

Nanoparticles with d=466 nm were observed in the retentate of UF-1 (feed UF-2). Since ethanol 75% efficiently disrupted surfactin micelles [6] the presence of these nanoparticles is explained by the addition of solvent, which may denature proteins – resulting in large nanoparticles (may be aggregations of proteins).

The permeate of the UF-2 had nanoparticles of d=0.739 nm and concentration of surfactin at 65.66 $mg.L^{-1}$ that resulted in low retention of surfactin R_s=6% and therefore good recovery of surfactin in the permeate. However, the protein followed the same trend (R_p=5%) and was also recovered in the permeate which resulted in low purity ≈ 44 g of surfactin/ 100 g surfactin and proteins. Also, the total recovered of surfactin (TRS_t) was 62%.

The total recovered of surfactin in the UF-1 (TRS_i) reached 66.78%, whereas the total recovered of surfactin in the UF-2 (TRS_{ii}) was 93.64% (Table 3). Thus, ≈ 33% of surfactin was lost in UF-1, on the other hand only ≈ 6% of surfactin was lost in the UF-2. Isa et al. (2007) [9] demonstrated that surfactin micelles can be effectively recovered in centrifugal device of ultrafiltration by using either 10 kDa RC

or PES membranes. The authors obtained higher TRS_i (90%) using a regenerated cellulose membrane of 30 KDa and lower TRS_{ii} (91%) using a regenerated cellulose membrane of 10 KDa. It should be mentioned that they used a synthetic culture medium, which favors the UF due to the presence of only one source of proteins – the microorganisms. However, we produced surfactin using food industry waste, a complex medium and consequently the UF was carried out with a solution composed by proteins coming from two different sources - cassava wastewater and *Bacillus subtilis* LB5a. Also, the membranes of 30 and 10 kDa used by Isa et al. (2007) [9] have low flux (small pore size), resulting in low productivity (long time) at industrial scale.

The size of micelles, relatively, followed the same trend as reported by Jauregi et al. (2013) [6] in which concentrations between 50-100 $mg.L^{-1}$ of surfactin resulted in the largest micelles with d between 100-200 nm. Also, according to Knoblich et al. (1995) [20] surfactin micelles adopt cylindrical form due to the presence of salts ($CaCl_2$ and NaCl) or the pH of solution. As a result, proteins, salts, etc., from the cassava wastewater and/or synthesized from *B. subtilis* may have some influence on the shape of surfactin micelles.

In conclusion, the size, forms and the rejection of surfactin, produced using cassava wastewater as culture medium, by the membrane of 100 KDa in the UF-1 were in agreement with previous findings that were described in the literature. [10,6] Therefore, even when using a membrane with large MWCO (PES 100 kDa), high R_s 82% (Table 3) was observed. Consequently, UF-1 was an adequate process. However in UF-2, due to the high MWCO of the membrane (PES-100 kDa), proteins were also permeated, which led to no purification. Therefore, strategy (ii) was applied where all parameters of UF-1 were maintained, and the MWCO of membrane in the UF-2 was reduced from 100 to 50 kDa.

3.1.2.2. Strategy ii

As shown in Table 2, the feed solution for strategy (ii) (180.17 $mg.L^{-1}$ of crude biosurfactant) had nanoparticles (micelles) of similar size to those in the feed solution of strategy i (d=72.3 nm and 81.13 $mg.L^{-1}$ of surfactin). Samples of permeate and retentate (UF-1) and permeate (UF-2) showed similar size of nanoparticles, R_s and R_p to those described in strategy (i) (Table 3). This data indicated good reproducibility

of the UF-1 process. However, in the retentate of UF-2, contrary to that was obtained in strategy (i), a high R_p of 49% and a low R_s of 1% was observed. Also, comparing with strategy (i) the permeate of UF-2 (strategy ii) showed higher purity (≈ 59 g of surfactin/ 100 g surfactin and proteins) and higher TRS_t 86.23%.

Thus, the use of membrane with smaller (50 kDa) MWCO in UF-2 (instead of 100 kDa – strategy i) improved the separation of surfactin from proteins. However, it was observed the longer time of ultrafiltration (20 minutes rather than 10 minutes – strategy i). Obviously, the longer time is a significant problem (productivity).

Therefore, even with interesting results obtained by strategy ii (good recovering of surfactin in UF-1 and good separation of surfactin from proteins in the UF-2), the strategy iii (UF-1 with 100 kDa and UF-2 with 50 kDa) was carried out to using a solution of surfactin with higher purity, in order to achieve the ultrafiltration in 10 minutes.

The feed solution of UF (strategy iii) was composed of semi-purified biosurfactant (see the item in material and methods "pre-purification (ultrafiltration) – crude and semi-purified biosurfactant"), rather than crude biosurfactant (strategies i and ii). We speculate that the reduction of proteins concentration would eliminate the problems with fouling and/or concentration polarization, improve the yields of surfactin recovery and reduce the time of ultrafiltration.

3.1.2.3. Strategy iii

The feed solution (188.17 $mg.L^{-1}$ of semi-purified biosurfactant) had 94.24 $mg.L^{-1}$ of surfactin at 50.08% purity. Thus, the extraction step increased the purity of surfactin from 36.14% (crude biosurfactant) to 50.08% (semi-purified biosurfactant). It is expected that this reduction of impurities (basically proteins) would make the UF process easier.

Concerning the UF-1, R_s = 0.87 indicated the same trends as strategies (i) and (ii). However the rejection of proteins was lower, R_p= 0.39.

The UF-2 had a R_s = 0.02, which also followed the same trend as strategies (i) and (ii), indicating that ethanol 75% efficiently disrupted surfactin micelles (crude and semi-purified biosurfactant), whereas R_p = 0.05 followed the same trend as strategy (ii); however, this process took only 10 min, indicating that fouling and/or concentration

polarization was minimized. Also, comparing with the strategy (i) and (ii), the permeate of UF-2 (strategy iii) showed higher purity (≈ 80 g of surfactin/ 100 g surfactin plus proteins). In this context, Chen et al. (2008) [8] detailed that the flux decline during cross-flow UF with PES 100 membranes was predominantly caused by the concentration polarization, as well as weak adsorption of small amino acids and the formation of a gel layer on the membrane surface. Therefore, the extraction by solvent seems to be a fundamental step for two-step UF of surfactin produced using cassava wastewater as culture medium.

3.1.2.4. Comparison and evaluation of strategies i, ii and iii

Comparing the three strategies of ultrafiltration (Table 2), the P_{pi} feed of strategy (iii) showed the highest value. Also, the retention of surfactin (R) increased from strategy (i) and (ii) to (iii) (44, 43, 67 respectively). Regarding purity in terms of protein (P_{pi}), no improvements were observed for strategy (i) (44% in the feed and in permeate), whereas it increased significantly for strategies (ii) and (iii). In the latter, the purity of 80% was reached.

The best results of purification were obtained with strategy (iii) (P_{pi} 67% and P_{pii} 80%). The strategy (ii) showed also good results (P_{pi} 43% and P_{pii} 59%). Jauregi et al. (2013) [6] described the ultrafiltration of surfactin after the production using synthetic culture medium. The authors reported that the P_{pi} was ≈ 92% using a PES 100 kDa in and P_{pii} was ≈ 94%, whereas Isa et al. (2008) [10] obtained P_{pi} ≈ 88% and P_{pii} ≈ 96% using a PES 10 kDa. Better results of P_p were obtained with the synthetic culture medium [6,10] than with the cassava water (this study) may be due to lower protein content in the feed (ultrafiltration).

Table 2. Concentration of protein (PC) and surfactin (SC) in the feed, retentate (R) and permeate (P) of the first and second ultrafiltration steps (UF1 and UF2) for strategies i, ii and iii.

Ultrafiltration – First Step (UF-1)									
	PES - 100 kDa			PES - 100 kDa			PES - 100 kDa		
	Strategy (i)			**Strategy (ii)**			**Strategy (iii)**		
	Feed	R	P	Feed	R	P	Feed	R	P
SC	105.	70.12	19.21	81.13	70.73	7.02	94.24	75.54	12.35
PC	194.	87.41	62.85	112.7	93.65	28.66	83.14	36.31	50.64
P_{pi}	35	44	23	41	43	19	53	67	19
Ultrafiltration – Second Step (UF-2)									
	PES - 100 kDa			PES - 50 kDa			PES - 50 kDa		
	Strategy (i)			**Strategy (ii)**			**Strategy (iii)**		
	Feed	R	P	Feed	R	P	Feed	R	P
SC	70.1	8.57	65.66	70.73	12.94	69.96	75.54	0.94	73.74
PC	87.4	0	83.41	93.65	35.35	47.78	36.31	16.24	18.15
P_{pii}	44	100	44	43	26	59	67	5	80

SC – surfactin concentration ($mg.L^{-1}$); PC – protein concentration ($mg.L^{-1}$).

$^{\dagger}P_p$ – purity of surfactin as mass fraction of surfactin in relation to sum of mass of surfactin and protein (% $w.w^{-1}$) – P_{pi} (UF-1) and P_{pii} (UF-2).

The proteins from cassava wastewater and *B. subtilis* LB5a are capable of forming foam or be incorporated into the biosurfactant foam, and consequently will be recovered in the foam overflow (see item 2.2.5. - biosurfactant recovery). The production of surfactin using cassava wastewater (or any other waste) followed by the UF, perhaps is a feasible process only when associated with recovery of surfactin by the foam overflow (as a pre-purification process, previous to UF), that is, industrial wastes as cassava wastewater have so many impurities that will become very hard to use them as culture medium and after that apply UF directly in the culture medium (without pre-purification process), in which very likely the membrane fouling will be the main problem. However foam overflow will facilitate the UF by first separating in the foam overflow the foam-forming compounds, such as surfactin and some proteins.

Table 3. UF in two steps; coefficient of rejection and nanoparticle size – strategies i, ii and iii.

Ultrafiltration – First Step (UF-1)									
	PES - 100 kDa			PES - 100 kDa			PES - 100 kDa		
	Strategy (i)			**Strategy (ii)**			**Strategy (iii)**		
	Feed	R	P	Feed	R	P	Feed	R	P
d	71.4	466	129	72.3	428	123	78	441	60.3
R_s		0.82			0.91			0.87	
R_p		0.68			0.75			0.39	
TRS_i		66.78			87.18			80.16	
Ultrafiltration – Second Step (UF-2)									
	PES - 100 kDa			PES - 50 kDa			PES - 50 kDa		
	Strategy (i)			**Strategy (ii)**			**Strategy (iii)**		
	Feed	R	P	Feed	R	P	Feed	R	P
D	466	60.3	0.74	428	20.9	20.9	441	35.8	22.5
R_s		0.06			0.01			0.02	
R_p		0.05			0.49			0.50	
TRS_{ii}		93.64			98.91			97.62	
TRS_t		62.53			86.23			78.25	

R – retentate; P – permeate

*R_s or R_p - Rejection coefficient – equation 1; d – diameter of nanoparticle size (nm)

†TRS – Total recovery of surfactin – equation 3. – TRS_i (UF-1), TRS_{ii} (UF-2) and TRS_t (UF-1 and UF-2).

As shown in Table 3, the strategy (i), (ii) and (iii) showed high values of R_s (>0.82) – UF-1. This means that, more than 82% of surfactin (in micellar form) was rejected in the first step of UF.

Concerning the entire process (UF-1 and UF-2), high TRS_t was observed for the three strategies, i (62%), ii (86%) and iii (78). For strategy i, the UF-2 was useless, since there was no separation, both protein and surfactin obtained low (≤0.06) R_s and R_p, respectively. Whereas in the UF2 of strategies (ii) and (iii), high values of R_p (≈ 0.5) and low values of R_s (≤0.06) were obtained, that is, in the second step (UF-2) selective separation of surfactin from proteins was achieved as almost all surfactin was recovered in the permeate (TRS_{ii} 98.91% and 96.92) and a large proportion of protein was retained

($R_p \approx 0.5$). This is also shown by the increase in purity from strategy i (44) to ii (59) and iii (80).

It is worth noting that only with the strategy (iii), where acid precipitation followed by solvent extraction (semi-purified biosurfactant) were applied prior to UF, led to both, good surfactin recovery (TRS_t 78.25%), and effective separation from proteins and at high flux (Table 2 and Table 3). Thus, the strategy (iii) is a remarkable process since it removed 78.16% of proteins (concentration of proteins in the permeate UF-2/concentration of protein in the feed UF-1) and recovered 78.25% of surfactin. However, the strategy (iii) added an extra purification step (solvent extraction), which would increase the cost of production.

Cassava wastewater is a low-cost culture medium comprised of carbohydrates, minerals, proteins, etc. Thus, on the other hand, considering the two-step UF of surfactin, the proteins from cassava wastewater make the purification harder, requiring solvent extraction (crude biosurfactant → semi-purified biosurfactant). The removal of proteins (e.g. precipitation) in the cassava wastewater - as previous treatment (before fermentation) – may be considered a feasible option to improve the process, eliminating the need of the prepurification step. However, the protein is a valuable nitrogen source which has a significant effect on the production of surfactin from *B. subtilis* (preferably organic nitrogen); the lower the nitrogen source - the lower the surfactin production. [21]

Results above bring news about some interesting issues concerning production of surfactin using cassava wastewater and other biotechnological processes, which use industrial waste as culture medium. Since, on one hand the use of industrial waste as culture medium does reduce the cost of production, but on the other hand makes the separation and purification of the products more complicated, as a larger number of steps will need to be applied in order to to achieve the desirable level of purity. Thus, the extra effort to purify the products obtained from biotechnological processes that used industrial waste as culture medium, will need to be taken into consideration in the costing of the process.

3.2. CHEMICAL STRUCTURE IDENTIFICATION OF PRODUCED SURFACTIN (STRATEGY III)

Bacillus produces lipopeptides, which are classified in three families: surfactin, iturin and fengycin. Each family has a specific number of aminoacids, but with different residues at specific position. It also has different length and isomery of *β*-hydroxyl fatty acid, that is, lipopeptides have a remarkable heterogeneity of molecular weight. The analysis of MALDI-TOFMS data showed the presence of compounds within/near the range of surfactin homologous (1045-1080 m/z): (i) 1043.53; (ii) 1049.57; (iv) 1065.57; (v) 1066.58; (vi) 1068.58; (vii) 1079.60; (viii) 1082.57; (ix) 1093.55; (x) 1096.62 and (xi) 1109.60 (m/z). These molecules were clearly separated in three groups (≈ 1066, 1079 and 1093 m/z). These groups probably are related to length of *β*-fatty acids. [14] Thus, potentially, at least 11 surfactin homologous were produced by *B.subtilis* LB5a using cassava wastewater as culture medium.

The IR analysis of produced surfactin (strategy iii) was similar to reported by Faria et al. (2011) [13], that is, strongly absorbing band at 1639 cm^{-1}, which correspond to peptide.

The NMR analysis identified three sequences of amino acids. One of them was not considered due to the very low signal intensity. Thus, 14 strong NH-signals correlations were detected between 7.207 and 9.681 ppm, in which they correspond to the two sequences of amino acids, defined in this study as S and S´- Glu1-Leu2-Leu3-Val4-Asp5-Leu6-Leu7 and Glu1´-Leu2´-Leu3´-Val4´-Asp5´-Leu6´-Val7´ (Figures 4 and 5, Table 4). All protons from leucine residues (4 in S and 3 in S´) were identified by βCH_2 ($\omega 1$ ≈ 1.66 to 1.33 ppm), γCH ($\omega 1$ ≈ 1.47 to 1.33 ppm) and δCH_3 ($\omega 1$ ≈ 0.8 ppm). Aspartic acids (S and S´) were identified by two βCH_2 crosspeaks (S - 2.62 and 2.17 ppm; S´ - 2.66 and 2.11). Glutamic acid (S and S´) was identified by a single pattern with two βCH_2 signals ($\omega 1$ ≈ 1.95 to 1.75 ppm) and two for γCH_2 ($\omega 1$ ≈ 2.04 to 1.98 ppm). All valines residues showed common pattern with a single βCH ($\omega 1$ ≈ 2.0 ppm) and γCH_3 ($\omega 1$ ≈ 0.8 ppm) which sometimes were superposed to the δCH_3 of the leucines. The identification of proton ressonances of C3H C2H C2H´ C4H $(CH_2)_n$ CH_3, were found to be similar in S and S´; and indicated (overlapping signals) that length of *β*-fatty acid (from 13 to 15 – expected), which is bonded to the amino acids. It also confirmed the presence of glutamic acid.

Table 4. 'H chemical shifts of two sequence of produced surfactin (strategy iii) - ($^2H_6$-DMSO at 25°C). For the non-peptide moiety, carbon atoms are numbered as in Fig. 5.

		HN	αH	βH		γH		γCH₃		δCH₃	
S	Glu1	9.491	4.271	1.956	1.818	2.044	1.985				
	Leu2	9.567	4.218	1.500	1.472	1.472				0.828	0.787
	Leu3	7.457	4.351	1.441	1.337	1.337				0.865	0.798
	Val4	8.439	4.057	2.155				0.891	0.829		
	Asp5	8.305	4.287	2.622	2.172						
	Leu6	7.291	4.177	1.556	1.474	1.474				0.860	0.816
	Leu7	8.421	4.339	1.663	1.521	1.429				0.821	0.804
S´	Glu1'	9.681	4.261	1.944	1.756	2.038	2.006				
	Leu2'	9.616	4.218	1.500	1.472	1.472				0.828	0.787
	Leu3'	7.442	4.351	1.441	1.337	1.337				0.865	0.798
	Val4'	8.329	4.050	2.161				0.892	0.818		
	Asp5'	8.453	4.290	2.669	2.116						
	Leu6'	7.207	4.295	1.532	1.432	1.432				0.867	0.817
	Val7'	8.275	4.039	2.021				0.845	0.808		

	Lipid chain	C3H	C2H	C2H´	C4H	$(CH2)_n$	CH3
S	H	4.933	2.801	2.292	1.557	1.213	0.833
S´	H	4.918	2.824	2.292	1.577	1.213	0.833

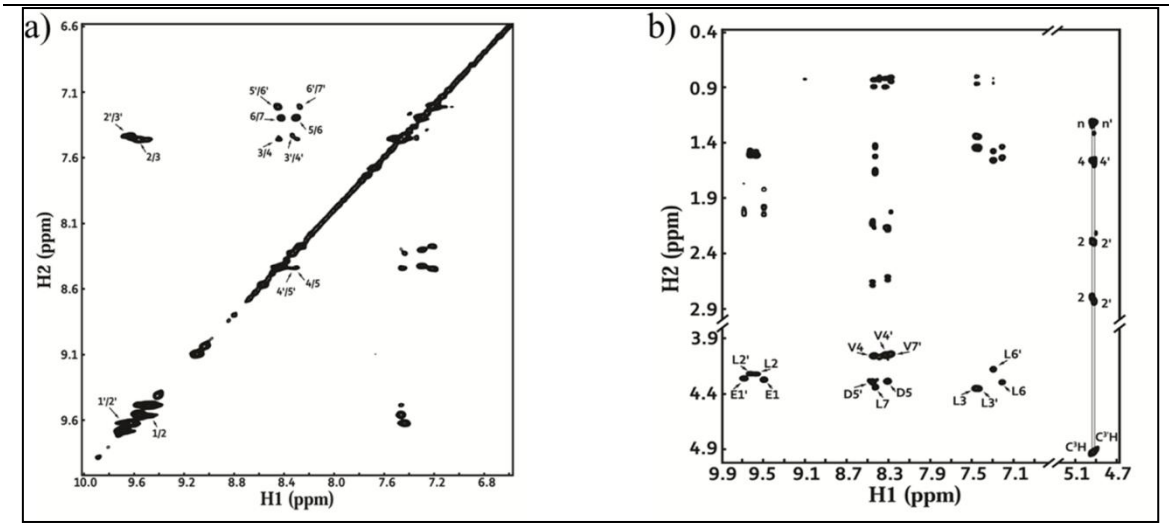


Figure 4. 2 D-NMR spectra of purified surfactin (strategy iii) ≈ 8 mg in 600 µL of $^2H_6$-DMSO. (a) NH-NH region of NOESY spectra (25 °C and 350 ms), showing sequential

connectivities labelled. The progressive ordering of numbers indicates the two sequential pathways, represented by S ans S´, (b) NH-αH and NH side-chain regions of TOCSY spectra (25 °C and 100 ms). Residues assignments are displayed and related to each sequence S and S´. Scalar connectivities observed between C3H and protons of the non-peptide moiety; crosspeaks involving C2H, C2H', C4H, $(CH2)_n$.

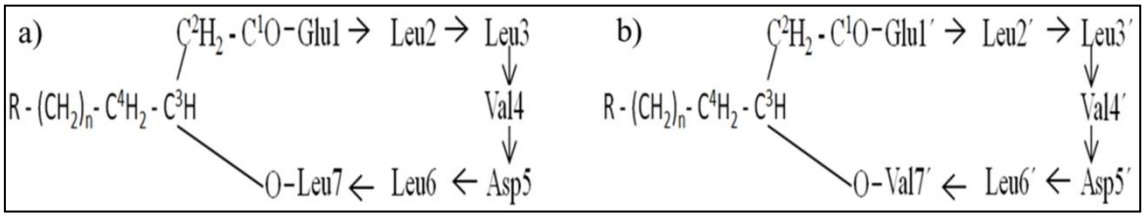


Figure 5. Primary structures of produced surfactin (strategy iii) – a) S – sequence; b) S´ - sequence.

It was already reported that the 3rd and 6th amino acids show D stereo configuration. [22-23] On natural abundance basis, L stereo configuration is significantly higher than D stereo one. The D stereo configuration of surfactin is one of key surfactin properties such as antimicrobial.

As already mentioned surfactin is composed by 7 aminoacids. Comparing the sequences of amino acids, previously reported, there is a trend that only the 2nd, 4th and 7th amino acids are changeable, while the 1st (Glu), 3rd (Leu), 5th (Asp) and 6th (Leu) are unchangeable (Table 5).

Table 5. Amino acid sequence of surfactin.

References	Amino acid position						
	1st	*2nd	3rd	*4th	5th	6th	*7th
Grangemard et al. (1997) [22]	Glu	Leu	Leu	Ile	Asp	Leu	Ile
Grangemard et al. (1997) [22]	Glu	Ile	Leu	Ile	Asp	Leu	Ile
Korenblum et al (2012) [24]	Glu	Leu	Leu	Val	Asp	Leu	Leu
This work - S	Glu	Leu	Leu	Val	Asp	Leu	Leu
This work - S´	Glu	Leu	Leu	Val	Asp	Leu	Val

* amino acid positions that more than one sort of amino acid can be found

Cassava wastewater was already explored in many biotechnological processes, for instance biotransformation. [25] In this study we evaluated the biosurfactant production, which based on MALDI-TOFMS and NMR analysis indicated that there are at least 11 surfactin homologous, with two main amino acid sequences, resulting in a remarkable heterogeneity of molecular structure, which will potentially have different properties (surface activity, antimicrobial, etc.).

4. CONCLUSION

For the first time, the UF process was applied to recovery surfactin that was produced by *Bacillus subtilis* LB5a using cassava wastewater as substrate. Solutions of crude and semi-purified biosurfactant at 100 mg L^{-1} of surfactin result in larger surfactin micelles, which can be retained in UF-1. In UF-2, the 100 kDA membrane led to poor purification whereas high purity was achieved with the 50 kDa membrane. Therefore the best results were obtained with strategies (ii) and (iii) however the highest purity in terms of protein was obtained with strategy (iii). These results and also the comparison with our previous results obtained with production of surfactin in synthetic medium show that the higher the protein content in the culture (feed) the more complicated the purification and therefore a larger number of steps will need to be added if a high purity product is required. Thus, on one hand the use of cassava as medium for production of surfactin could reduce the cost of production but on the other hand it could complicate the purification with the subsequent increase in production cost. Furthermore the NMR and MALDI-TOFMS analyses identified 11 potential surfactin homologous, which are composed by different β-fatty acids and two amino acid sequences – S and S´.

Acknowledgments

The authors are grateful to the São Paulo State Research Foundation (Fapesp) for their financial support, Brazilian Bioscience National Laboratory (institution of CNPEM) for NMR analysis and Polytechnic School of University of São Paulo for MALDI-TOFMS analysis.

5. REFERENCES

1. Barros FFC, Ponesi AN and Pastore GM, Production of biosurfactant by *Bacillus subtilis* LB5a on a pilot scale using cassava wastewater as substrate. *J Ind Microbiol Biotechnol* **35**:1071-1078 (2008). DOI: 10.1007/s10295-008-0385-y

2. Makkar RS, Cameotra SS and Banat IM, Advances in utilization of renewable substrates for biosurfactant production. *AMB Express* **1**:1-19 (2011). DOI: 10.1186/2191-0855-1-5

3. Pacwa-Płociniczak M, Płaza GA, Piotrowska-Seget Z and Cameotra SS, Environmental applications of biosurfactants: Recent Advances. *Int J Mol Sci* **12**:633-654. (2011). DOI: 10.3390/ijms12010633

4. Chen HL, Chen YS and Juang, RS, Recovery of surfactin from fermentation broths by a hybrid salting-out and membrane filtration process. *Sep Purif Technol* **59**:244-252. (2008). DOI: 10.1016/j.seppur.2007.06.010

5. Saharan BS, Sahu RK and Sharma D, A review on biosurfactants: fermentation, current developments and perspectives. *Genet Eng Biotechnol J* **2011**:1-14. (2012).

6. Jauregi P, Coutte F, Catiau L, Lecouturier D and Jacques P, Micelle size characterization of lipopeptides produced by *B. subtilis* and their recovery by the two-step ultrafiltration process *Sep Purif Technol* **104**:175-182. (2013). DOI:10.1016/j.seppur.2012.11.017

7. Chen HL, Chen YS and Juang, RS, Separation of surfactin from fermentation broths by acid precipitation and two-stage dead-end ultrafiltration processes. *J Membr Sci* **299**:114-121. (2007). DOI: 10.1016/j.memsci.2007.04.031

8. Chen HL, Chen YS and Juang RS, Flux decline and membrane cleaning in cross-flow ultrafiltration of treated fermentation broths for surfactin recovery. *Sep Purif Technol* **62**:47-55. (2008). DOI:10.1016/j.seppur.2007.12.015

9. Isa MHM, Coraglia DE, Frazier RA and Jauregi P, Recovery and purification of surfactin from fermentation broth by a two step ultrafiltration process. *J Membr Sci* **296**:51-57. (2007). DOI:10.1016/j.memsci.2007.03.023

10. Isa MHM, Frazier RA and Jauregi P. A further study of the recovery and purification of surfactin from fermentation broth by membrane filtration. *Sep Purif Technol* **64**:176-182. (2008). DOI:10.1016/j.seppur.2008.09.008

11. Lin S-C and Jiang H-J, Recovery and purification of the lipopeptide biosurfactant of *Bacillus subtilis* by ultrafiltration. *Biotechnol Tech* **11**:413-416. (1997). DOI: 10.1023/A:1018468723132

12. Fahim S, Dimitrov K, Gancel F, Vauchel P, Jacques P and Nikov I, Impact of energy supply and oxygen transfer on selective lipopeptide production by *Bacillus subtilis* BBG21. *Bioresour Technol* **126**:1-6. (2012). DOI: 10.1016/j.biortech.2012.09.019

13. Faria AF, Teodoro-Martinez DS, Barbosa GNO, Vaz BG, Silva IS, Garcia JS, Tótola MR, Eberlin MN, Grossman M, Alves OL and Durrant LR, Production and structural characterization of surfactin (C14 /Leu7) produced by *Bacillus subtilis* isolate LSFM-05 grown on raw glycerol from the biodiesel industry. *Process Biochem* **46**:1951-1957. (2011). DOI: 10.1016/j.procbio.2011.07.001

14. Ayed HB, Hmidet N, Béchet M, Chollet M, Chataigné G, Leclère V and Jacques P, Identification and biochemical characteristics of lipopeptides from *Bacillus mojavensis* A21. *Process Biochem* **49**:1699-1707. (2014). DOI: 10.1016/j.procbio.2014.07.001

15. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J and Bax A, NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* **6**:277–293. (1995). DOI: 10.1007/BF00197809

16. Johnson BA and Blevins RA, NMR View: A computer program for the visualization and analysis of NMR data. *J Biomol NMR* **4**:603–614. (1994). DOI: 10.1007/BF00404272.

17. Ghribi D and Ellouze-Chaabouni S, Enhancement of *Bacillus subtilis* lipopeptide biosurfactants production through optimization of medium composition and adequate control of aeration. *Biotechnol Res Int* **2011**:1-6. (2011). DOI: 10.4061/2011/653654

18. Zimmermann HF, Anderlei T, Buchs J and Binder M, Oxygen limitation is a pitfall during screening for industrial strains. *Appl Microbiol Biotechnol* **72**:1157-1160. (2006).

19. Abdel-Wawgoud AM, Aboulwafa MM and Hassouna NAH, Characterization of surfactin produced by *Bacillus subtilis* isolate BS5. *Appl Biochem Biotechnol* **150**:289-303. (2008). DOI: 10.1007/s12010-008-8153-z

20. Knoblich A, Matsumoto M, Ishiguro R, Murata K, Fujiyoshi Y, Ishigami Y and Osman M, Electron cryo-microscopic studies on micellar shape and size of surfactin, an anionic lipopeptide. *Colloid Surfaces B* **5**:43-48. (1995). DOI:10.1016/0927-7765(95)01207-Y

21. Davis DA, Lynch HC and Varley J, The production of surfactin in batch culture by *Bacillus subtilis* ATCC 21332 is strongly influenced by the conditions of nitrogen

metabolism. *Enzyme Microb Tech* **25**:322-329. (1999). DOI:10.1016/S0141-0229(99)00048-4

22. Grangemard I, Peypoux F, Wallach J, Das BC, Labbe H, Caille A, Genest M, Marger-Dana R, Ptak M and Bonmatin J-M, Lipopetides with improved properties: Structure by NMR, purification by HPLC and structure-activity relationship of new isoleucyl-rich surfactin. *J Pept Sci* **2**:1-10. (1997). DOI: 10.1002/(SICI)1099-1387(199703)3:2<145::AID-PSC96>3.0.CO;2-Y

23. Peypoux F, Bonmatin J-M, Labbe H, Grangemard I, Das BC, Ptak M, Wallach J and Michel G, [Ala4]Surfactin, a novel isoform from *Bacillus subtilis* studied by mass and NMR spectroscopies. *Eur J Biochem* **224**:89-96. (1994). DOI: 10.1111/j.1432-1033.1994.tb19998.x

24. Korenblum E, Araujo LV, Guimarães CR, Souza LM, Sassaki G, Abreu F, Nitscke M, Lins U, Freire DMG, Barreto-Bergter E and Seldin L, Purification and characterization of a surfactin-like molecule produced by *Bacillus* sp. H2O-1 and its antagonistic effect against sulfate reducing bacteria. *BMC Microbiol* **12**:1-13. (2012). DOI: 10.1186/1471-2180-12-252

25. Maróstica Jr, MR and Pastore, GM, Production of R-(+)-α-terpineol by the biotransformation of limonene from orange essential oil, using cassava waste water as medium. *Food Chem* **101**:345-350. (2007). DOI:10.1016/j.foodchem.2005.12.056

CHAPTER III

PRODUCTION, PURIFICATION AND IDENTIFICATION OF MANNOSERYLTHRITOL LIPIDS PRODUCED BY *Pseudozyma tsukunbaensis* USING CASSAVA WASTEWATER AS SUBSTRATE – ULTRAFILTRATION AND SCALE UP

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(Paper submitted to Bioresource Technology)

Production, purification and identification of mannosylerylthritol lipids produced by *Pseudozyma tsukubaensis* using cassava wastewater as substrate - ultrafiltration and scale-up

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Abstract

Pseudozyma tsukubaensis is mannosylerythritol lipids-B producer. Purification of biosurfactants represents ≈ 60% of production costs, whereas culture medium ≈ 30%. Therefore, the aim of this work was to evaluate the mannosylerythritol lipids production using cassava wastewater - a low-cost substrate - integrated to ultrafiltration. Cassava wastewater was a feasible culture medium to *P. tsukubaensis*. The experiments at small-scale of ultrafiltration (20 mL) indicated that ≈ 80% of the mannosylerythritol lipids was retained by the membrane, while, more than 95% of proteins were permeated. The purification process was scaled up (from 20 mL to up to 500 mL) and followed the same trend of the experiments at small scale. The chemical structure identification proved the production of mannosylerythritol lipid-B homolog and also the production of a second stereoisomer (≈ 9%) which is related to moiety of erythritol. The recovery of mannosylerythritol lipid-B by foam overflow integrated to ultrafiltration is a remarkable alternative for purification, rather than the traditional ethyl acetate extraction integrated to silica column, which very likely represents cost-effective production).

Keywords: *Pseudozyma tsukubaensis*; cassava wastewater; mannosylerythritol lipids-B; ultrafiltration

1. INTRODUCTION

Biosurfactants are compounds produced by living cells, for instance, microorganisms. Their chemical structure consists in two parts, a polar (hydrophilic) moiety and non-polar one (hydrophobic).

Rhamnolipids, surfactin, sophorolipids are the most well-known biosurfactant, however, others surface active agents have been receiving attention, for instance, mannosylerylthritol lipids (MEL), which have remarkable chemical structure. MEL consist of a mixture of a partially acylated derivative of 4-*O*-*β*-D-mannopyranosyl-D-erythritol (Morita et al. 2015a, Yu et al. 2015, Faria et al. 2014, Fan et al. 2014, Sajna et al. 2013, Arutchelvi et al. 2008, Hubert et al. 2012, Konishi et al. 2011, Fukuoka et al. 2008, 2011, 2012). In this sense, there are 4 MEL homologs -A, -B, -C and -D, which are classified only based on the acetylation of C-4´and C-6´ (mannose) (Arutchelvi et al. 2008, Hubert et al. 2012, Konishi et al. 2011, Fukuoka et al. 2008, 2011, 2012, Marchant and Banat, 2012). MEL are synthesized by several microorganisms. Morita et al. (2015a) detailed the relation between the production of MEL homolog and the molecular phylogenic tree of *Pseudozyma* and *Ustilago*. *P. rugulosa*, *P. aphidis*, *P. antarctica* and *P. crassa* are high producer of MEL-A and *P. siamensis*, *P. hubeinsis*, *U. cynodontis* are high producer of MEL-C, and *P. tsukunbaensis* is producer of MEL-B.

There is an increasing interest in MEL due to their potential applications such as (i) pharmaceutical drug, for instance in the treatment of schizophrenia (Hubert et al. 2012, Yu et al. 2015, Sajna et al. 2013), antitumor against human leukaemia and mouse melanoma cells (Faria et al. 2014, Sajna et al. 2013, Yu et al. 2015), (ii) agent of bioremediation of petroleum contaminants, (iii) cosmetic formulations (Recke et al. 2013) and (iv) laundry detergent formulations (Sajna et al. 2013).

All MEL homologs have relative low water solubility. This property restricts many potential applications. Thus, Morita et al. (2015b) described the production of mono-acetylated mannosyl-L-arabitol lipid, which showed higher water solubility than MEL-B. Mono-acetylated mannosyl-L-arabitol lipid was synthesized due to elongation of erythritol moiety (hydrophilic) using a culture medium supplemented with L-arabitol.

Cassava wastewater is the main residue of cassava starch industry. Cassava wastewater corresponds ≈ 30% – cassava wastewater generated per cassava processed (w:w). This waste has high content of both macro and micronutrients (dextrose, fructose, saccharose, magnesium [Mg^{+2}], calcium [Ca^{+2}], manganese [Mn^{+2}], iron [Fe^{+2}], zinc [Zn^{2+}] and nitrogen compounds), which can be used in many biotechnological processes, including the production of biosurfactant (Barros et al. 2008).

Regarding to biosurfactant production costs, the purification process is the most significant step – represents 60% (Chen et al. 2008b; Saharan et al. 2012). In this context, Isa et al. (2007) and Chen et al. (2007) have applied an interesting strategy of purification: two-stage ultrafiltration (UF) for the separation and recovery of surfactin and they indicated good recovery and purity. Rangarajan et al. (2014) showed that divalent ions (Ca^{+2}) increased the recovery of surfactin (anionic biosurfactant) by ultrafiltration. All these methodologies take advantage of self-aggregation forms – which can be extrapolated for all biosurfactants, for instance MEL.

As highlighted by Hubert et al. (2012), intense researches have focused on the reducing of production costs of glycolipids synthesized by microorganisms and also on downstream processes.

We speculate that cassava wastewater could be a good culture medium to *P. tsukubaensis* growth and production of MEL. In addition, the UF could be integrated to the bioprocess, which would, significantly, reduce the cost of production. Thus, the aim of this work was to evaluate the technical feasibility of UF in two steps of MEL produced using cassava wastewater as culture medium (Fig. 1). Additionally, as suggested by Morita et al. (2015a), the production process is based on water-soluble nutrients, thus an easier downstream is expected.

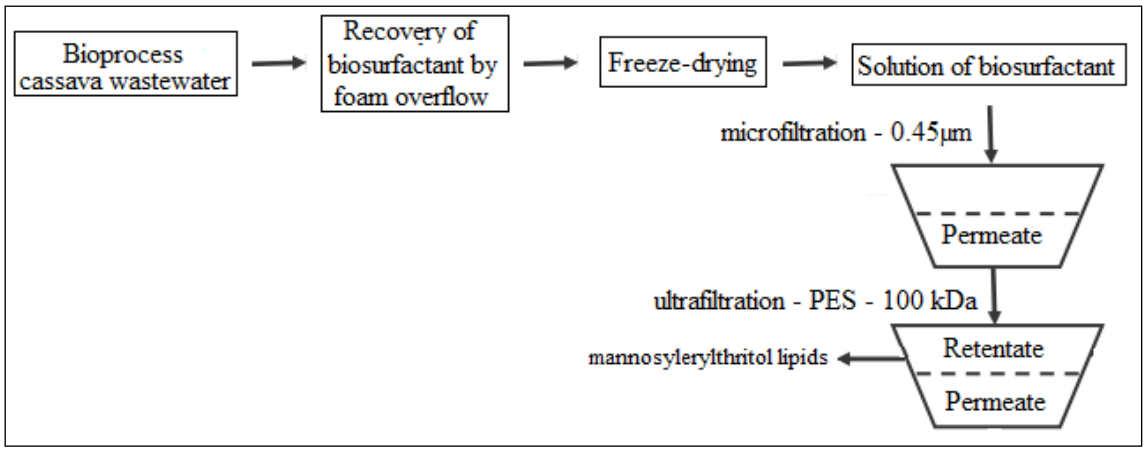


Figure 1. Overview of ultrafiltration of MEL produced from *P. tsukubaensis* using cassava wastewater as culture medium.

2. MATERIAL AND METHODS

2.1. CHEMICALS

The chemicals used: acetonitrile (Synth ≈ 99.8%), bicinchoninic acid kit (Sigma-Aldrich), bovine serum albumin (Sigma-Aldrich ≥ 98%), chloroform (Synth ≈ 99.8%), deuterated chloroform (Sigma-Aldrich > 99.8%), methanol (Sigma-Aldrich ≥ 99.6%), tetramethylsilane (Sigma-Aldrich > 99%), trifluoroacetic acid (Sigma-Aldrich ≥ 99%), trypan blue 0.4% (Thermo Fisher) and *α*-cyano-4-hydroxycinnamic acid (Sigma-Aldrich ≥ 98%),

2.2. PRODUCTION OF MANNOSYLERYLTHRITOL LIPIDS

2.2.1. Microorganism and *inoculum*

A loop of *P. tsukubaensis* culture growth pertaining to the culture collection of the BioFlavors Laboratory of DCA/FEA/UNICAMP was transferred to medium composed by ($g.L^{-1}$) 0.1 sucrose, 0.1 glucose, 0.2% peptone, 0.1% yeast extract, 0.004% $MgSO_4$ and 00.5% K_2HPO_4 (YEPD) and maintained in incubator (48 h, 30 ^oC). It was mixed with sterilized glycerol 90 and 10% ($v.v^{-1}$) respectively, placed in microcentrifuge tubes (1 mL) and stored (-18 ^oC). Then, one microcentrifuge tube was placed in a conical flask containing supplemented YEPD broth and maintained at 30 °C for 48 h in a rotary shaker incubator at a speed of 150 rpm. The medium was standardized at 0.5 by measuring the optical density at λ= 600 nm for a viable cell (which according to calibration curve represents in wet weight basis, 0.02155 g of cells

per 100 mL of YEPD) and a volume 7% (volume of inoculums per volume of culture medium – v:v) was used as *inoculum*.

2.2.2. Culture medium

Cassava wastewater (variety IAC-13) was collect from a flour industry and transported to laboratory at room temperature. After that, the residue was boiled, centrifuged at 10,000 x *g* during 10 minutes and 5 ^{o}C (Beckman Coulter, AlegraX-22r). The supernatant was stored (-18 ^{o}C) and unfrozen before the bioprocess (Barros et al. 2008).

2.2.3. Bioprocess parameters and sampling

Culture medium, cassava wastewater, was sterilized at 121 °C for 20 minutes. Then it was placed in a bioreactor - Bioflo® & Celligen® 310 - New Brunswick Scientific (3.0 liters working volume). The conditions at fermentation were 100 rpm and aeration rate of 0.4 vvm (vessel volume per minute) in the firsts 24 h, then 150 rpm and 0.8 vvm from 24 to 84 h, for all 7 bioprocess (F-1…F-7). Samples of the culture medium were collected at 12 hour-basis until 84 h (bioreactor) and used to measure viable cell count, content of glucose, volume of foam and surface tension measurements (ST).

2.2.4. Analytical methods used for the evaluation of fermentation process – production of MEL

2.2.4.1. Cell growth

In order to color the cells and consequent easier visualization of cells of *P. tsukubaensis* by microscopy using a Neubauer chamber. One drop of trypan blue (0.2%) was mixed with 1 mL of each sample (culture medium). It was used serial dilution (NaCl 0.7%) when the concentration was higher than 2 x 10^6 cells per mL.

2.2.4.2. Content of glucose

Content of dextrose was analyzed by enzymatic/colorimetric technique (Laborlab).

2.2.4.3. Measureaments of surface activity

Approximately 20 mL of each sample, culture medium and centrifuged foam, was centrifuged at 10,000 x *g* for 20 minutes. Then, the ST and its dilutions (CMDs) were measured in the supernatant phase using the plate method in a Krüss GmbH K-12 tensiometer (Hamburg, Germany) (Barros et al. 2008). Critical micelle dilutions (CMDs) are the surface tension values of the sample diluted at 10-times (CMD-1), 100-times (CMD-2) and 1000-times (CMD-3).

2.2.5. Mannosylerythritol lipids recovery

Foam from bioreactor was collected during its production at the top of the bioreactor (Barros et al. 2008). At the end of the bioprocess the collapsed foam (liquefied) volume was measured, centrifuged at 10,000 x *g* for 20 minutes. Then, the ST and its CMDs measured using the supernatant phase - plate method (Barros et al. 2008). Finally, the collapsed foam was lyophilized (LS 3000 TERRONI), stored at -18 ^{o}C. The foam of first bioprocess (F-1) was collected and dried in 12-hour basis, separately.

2.3. PURIFICATION OF MANNOSYLERYLTHRITOL LIPIDS BY ULTRAFILTRATION PROCESS

2.3.1. Process overview

Samples of foam powder (lyophilized) – bioprocesses F-1 (12-hour basis), F-2, F-3 and F-4 - were solubilized in Tris-buffer pH 8.5 – 10 mM, filtered (0.45 µm) and used for estimate the MEL concentration (HPLC). Then, a volume of 15 mL was placed in the centrifugal device polyethersulfone (PES) 100 kDa (Vivaspin) and centrifuged at 2205 x *g* during 10 min. To the retentate of (≈ 0.8 mL) was added ≈ 14.2 mL of buffer (Isa et al. 2008). All samples feed, retentate and permeate had its concentration of protein, MEL concentration and nanoparticle size measured.

Finally, it was carried out the scale up, with a volume of 250 mL. In the feed and permeate was measured concentration of protein, MEL concentration and nanoparticle size, also the flow rate of UF.

2.3.2. Analytical methods of purification

2.3.2.1. Mannosylerylthritol lipids concentration analysis

MEL concentration was determined by reverse phase HPLC. The system used was a Gilson 306 (Rockford, IL, USA), with a C-18 column of dimensions 250 mm × 4.6 mm and a particle size of 5 µm. The flow rate of the mobile phase was 1.0 $mL.min^{-1}$ - isocratic chromatography - with 70% acetonitrile in 0.1% trifluoroacetic acid and 30% HPLC-grade water in 0.1% trifluoroacetic acid. A 50 µL sample was injected in each run which lasted for 65 minutes (55 minutes with detector on and 10 minutes as column cleaning step). The eluent absorbance was monitored at 206 nm. The system was calibrated using MEL-B standard obtained from Toyobo-Japan. The area of the peaks eluting between 11, 16, 23 and 25 minutes, which were identified as having the same retention times as those peaks eluting from the standard, were added to give the total MEL peak area. This value was used to determine the MEL concentration in the samples, as well as samples from the purification procedures.

2.3.2.2. Kinetics of production – MEL

Only the fermentation (F-1) was used to evaluate the kinects of production of MEL. The samples of foam were taken at 12 h basis, lyophilized separately and solubilized (≈ 700 $mg.L^{-1}$) in Tris-buffer 10 mM pH 8.5. Finally, the solutions (lyophilized foam in buffer) were analyzed by HPLC. The HPLC data (concentration of MEL) were correlated with sample collection interval.

2.3.2.3. Protein concentration

The total amount of protein present at each stage of the purification procedures was determined by the bicinchoninic acid method (BCA). A calibration curve was produced using bovine serum albumin (BSA) as the protein standard solution (Isa et al. 2007).

2.3.2.4. Self-assembly size of MEL and its relation with the concentration

The nanoparticle size of all samples of ultrafiltration process was analyzed by dynamic light scattering (DLS), using a Zetasizer Nano ZS system (Malvern, UK). This system is able to detect particles ranging from 0.6 nm to 6 µm (Isa et al. 2007).

It was also investigated the relation between the nanoparticle size of self-aggregated forms and the concentration of MEL standard (tris-buffer 10 mM pH 8.5; from 12.5 to 500 $mg.L^{-1}$).

2.3.2.5. Centrifugal device of ultrafiltration

The experiments of ultrafiltration were carried out with two repetitions using brand news Vivaspin 20 with PES – 100 kDa, containing membrane of 6 cm^2 of active area. For the repetitions 1 and 2, were elaborated solutions containing 1836.32 and 1407.75 $mg.L^{-1}$ of biosurfactant (powder – lyophilized foam of fermentation), respectively. The data of HLPC indicated the concentration of MEL (see MEL concentration analysis) in the repetitions 1 and 2 were 610.74 and 502.71 $mg.L^{-1}$, respectively. Then, 15 mL (feed) of each solution (repetitions 1 and 2) were placed in the ultrafiltration unit (100 kDa), centrifuged at 2205 x *g*, 10 minutes and 20 ^{o}C. Finally, all solutions (retentates and permeates of UF) were analyzed: concentration of MEL, DLS and concentration of protein.

The rejection of MEL or protein by a membrane was defined as rejection coefficient (R) as below (Jauregi et al. 2013):

Equation 1. $R = C_f\text{-}C_p/C_f$

Where C_F and C_P are the concentration of MEL (C_m) or protein (C_p) in the feed and permeate, respectively.

2.3.2.6. Top-bench ultrafiltration – scale up

Lab scale UF of the fermentation broth was performed with a magnetically stirred Labscale TFF system (Millipore) with PES 100 kDa (Pellicon® XL) of an effective filtration area of 50 cm^2. The stirrer speed and pump speed were kept at 3.0 and 2.5, respectively. The feed pressure gauge and retentate pressure gauge were kept at between 10-30 psi and 10 psi, respectively.

The system was cleaned, before and after the experiments and stored at 4 ^{o}C, according to the manufacture's protocol.

The UF was carried out twice with 250 mL of feed, MEL solution 1091.59 $mg.L^{-1}$ of foam (powder), that is, at 294.73 $mg.L^{-1}$ of pure MEL (see MEL concentration analysis). The flow rate was monitored during the course of UF.

Equation 2 = flow rate [(volume/membrane area)/time] = (LMH or $L.m^{-2}.h^{-1}$) = ($mL.cm^{-2}.min^{-1}$) x 600*

* (the conversion of liter to mL; square meters to square centimeters; hours to minutes).

After the reduction of feed/retentate (feedback system) to 25 mL, samples of permeate and feed/retentate were taken and the concentration of MEL (HPLC), nanoparticle size (DSLS) and proteins were measured.

The rejection of MEL or protein by a membrane was defined as the same centrifugal device of UF (equation 1).

2.4. MOLECULAR IDENTIFICATION OF MEL

2.4.1. Infrared

Infrared spectra were measured with an IRA-3 spectrophotometer (JASCO) (Kitamoto et al. 1990).

2.4.2. Fatty acids

The fatty acids of the purified product were examined by gas chromatography–mass spectrometry (GC–MS). The methyl ester derivatives of fatty acids were prepared by mixing the purified MEL-B (10 mg) with 5% HCl–MeOH reagent (1 mL) at 80 ^{o}C for 20 min. After the reaction mixture was quenched by the addition of water (1 mL), the methyl ester derivatives were extracted with n-hexane (2 mL) and then analyzed by GC–MS with a HP-5 with the temperature programed from 90 ^{o}C (held for 3 min) to 240 ^{o}C at 5 $^{o}C.min^{-1}$ (Fukuoka et al. 2008).

2.4.3. MALDI-TOFMS

Solutions of semi-purified biosurfactant were analyzed using the dried-droplet sample preparation technique directly spotting 1 µL of samples directly onto a

polished steel MALDI Target, model MTP 384 (Bruker Daltonics, Bremen, Germany). After drying the sample, 1 µL of matrix solution (alpha-hydroxycinnaminic acid saturated solution in acetonitrile-methanol-water, 1:1:1) was added and allowed to air dry at room temperature.

MALDI-TOFMS spectra were performed using an UltrafleXtreme MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) operating in the refraction mode at an accelerating voltage of 22.5 kV. Mass spectra were acquired in m/z range of 700-3500 with ions generated from Smartbeam^{TM} laser irradiation using a frequency of 2000 Hz, a lens 7 kV and the delay time was 110 ns. Matrix-suppression was set to 500 Da, and the mass spectra were generated by averaging 1,500 laser shots. The laser intensity was set just above the threshold for ion production. External calibration was performed by using the [M+H]+ signals of Angiostin II, Angiostin I, Substance P, Bombesin, ACTH_clip(1-17), ACTH_clip(18-39), Somatostin(28) (Peptide calibration standard – Bruker Daltonics, Bremen, Germany). The peptide mixture was dissolved in TA50 solvent (mixture of acetonitrile and 0.1% trifluoro acetic acid - volume ratio 1:2) (Fukuoka et al. 2008)

2.4.4. NMR

NMR experiments were carried out in $CDCl_3$ using an Agilent DD2 spectrometer at the Brazilian National Biosciences Laboratory (LNBio/CNPEM), operating at a ^{1}H Larmor frequency of 499.726 MHz. The coupling constants were measured in hertz (Hz) and the chemical shifts (δ ^{1}H, δ ^{13}C) ascribed in ppm, which were related to tetramethylsilane (TMS, δ-0). The purified MEL was lyophilized. Then $\approx$ 30 mg was diluted in 700 µL of deuterated chloroform ($CDCl_3$) for data acquisition. 2D homo- and heteronuclear spectra such as COSY ($^{n}J_{H-H}$, scalar), NOESY ($^{n}J_{H-H}$, dipolar), HSQC ($^{1}J_{H}$-C, scalar) e HMBC ($^{n}J_{H}$-C, scalar) were also performed (Fukuoka et al. 2007a, 2007b, 2008).

3. RESULTS AND DISCUSSION

3.1. PROCESS OVERVIEW

MEL are synthesized by microorganisms such as *Schizonella melanogramma*, *Candida* sp. (currently known as *Pseudozyma* sp.) as a major

component, whereas *Ustilago* sp. produces them as a minor component. MEL are also produced by *Kurtzmanomyces* sp (Arutchelvi et al. 2008, Morita et al. 2015a). In this context, the strain of *P. tsukubaensis* has received special attention, mainly due to production of only one MEL homolog (MEL-B), since other *Pseudozyma* species produce a mixture of different MEL homologs (Fukuoka et al. 2008, Konishi et al. 2011).

The production of MEL at flask fermentation scale is relatively well-reported whereas a few attempts have been made to produce MEL at bench-top bioreactor scale and/or using water-soluble carbohydrates instead of the traditional hydrophobic carbohydrates such as olive oil (Arutchelvi et al. 2008, Morita 2009, Morita et al. 2015a).

Regarding the purification of MEL, traditionally is used a methodology composed by two steps (i) extraction of MEL by applying ethyl acetate directly in the culture medium followed by (ii) silica column (Morita et al. 2015, Faria et al. 2014, Fan et al. 2014, Sajna et al. 2013, Recke et al. 2013, Konishi et al. 2011, Hubert et al. 2012). However, according to Isa et al. (2007), UF is the most promising technique to purify surfactin and could be extrapolated to others biosurfactants, such as MEL.

Therefore, this work has a unique approach, which assembled 3 subject-matter on production of MEL, (i) producer - one of the best MEL producers (*P. tsukubaensis*), (ii) low cost substrate - an agro-industrial waste as culture medium and (iii) purification process - the UF and its scale-up.

3.2. ANALYTICAL TECHNIQUE TO MEASURE MEL CONCENTRATION - HPLC

MEL have 4 homologues -A, -B, -C and -D. They are classified based only in the acetylation of C-4´and C-6´ (mannose) (Arutchelvi et al. 2008; Fukuoka et al. 2012, Marchant and Banat, 2012, Hubert et al. 2012). MEL have two fatty acids in their chemical structure. These fatty acids vary from C_8 to C_{14} ≈ 86.6 % (Fukuoka et al. 2007b; Morita et al. 2009). Thus, molecules with different molecular weight are classified as the same homologues, due to the number and position of the acetyl groups on mannose or erythritol and to the fatty acid chain (Hubert et al. 20120. For instance, two molecules (i and ii), in which both are MEL-A, that is, C-4´and C-6´ are acetylated. The first one (i) has C_8 (fatty acid chain) and C_{10}, the second one has two $C_{14.}$ Thereby,

obvious they have different molecular weights. As a result, an ideal chromatograph should separate these homologues.

To the best of our knowledge, only normal phase - silica column (Sajna et al. 2013, Recke et al. 2013, Konishi et al. 2011, Faria et al. 2014, Morita et al. 2015b) or droplet counter-current chromatography (Hubert et al. 2012) were used as a separation and further identification of MEL.

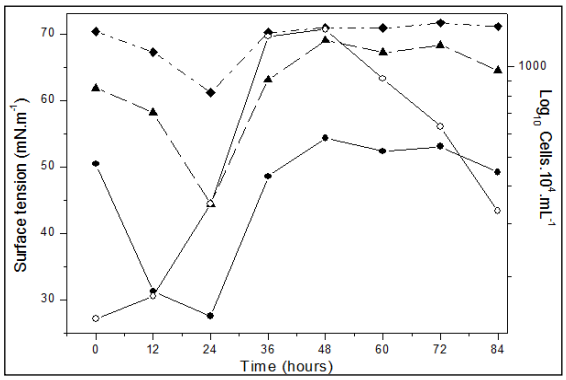
MEL are hydrophobic compounds with high hydrophilic/lipophilic balance (HLB) 8.8. Thus, the chromatography using silica column (to measure the concentration of MEL) is inadequate, because there is no separation of homologues that have differents fatty acids (poor separation due the combination of hydrophilic column with hydrophobic compound of interest) and mainly due to the restriction of based-water samples (water damages the silica columns), for instance samples of culture medium. In other words, for analyse the concentration of MEL in samples obtained from a culture medium, it is fundamental carried out the liquid-liquid extraction with organic solvent (remove water) and the liquid-liquid extraction does not guarantee that 100% of MEL migrated from the culture medium to organic solvent (Kim et al. 2002).

We described for the first time the analysis of MEL using a reverse column (C-18). The chromatogram identified 4 peaks of MEL-B ≈ 11, 16, 23 and 25 minutes. Each peak correlates to one MEL-B homologues with different fatty acids.

3.3. BIOREACTOR BIOPROCESS

The Figure 2 shows the ST measurements and cell counting for the culture medium.

The cell counting indicated that the highest rate of log phase took place between 24 to 36 h. This was expected, since at 24 h, the aeration and agitation changed from 0.04 vvm and 100 rpm to 0.08 vvm and 150 rpm. The stationary phase was reached at 36 h, which is 12 h early than in the flask bioprocesses. This difference is associated to better conditions provided by bioreactor (transfer of oxygen, temperature control, agitation, etc). The cell counting data was subtly lower (bioreactor), probably due to the recovery of biosurfactant by foam overflow, which withdraw microbial cells out of the system.



*Error bars were deliberately hidden

Figure 2. Culture medium - bioreactor experiments: ST (—●—), CMD-1 (- -▲- -), CMD-2 (-···◆-···), Cell counting (—○—).

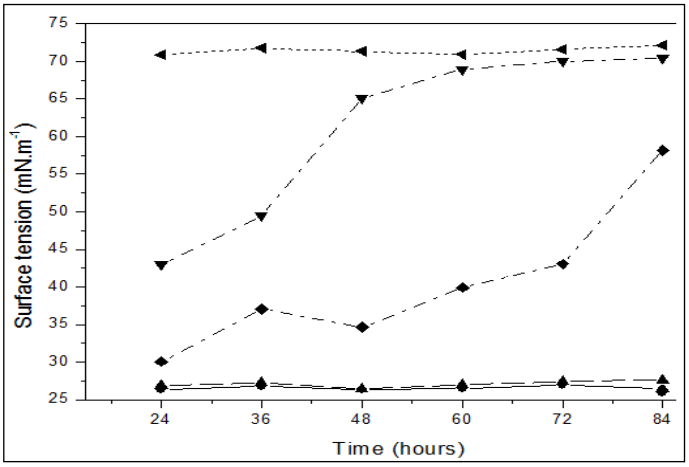
In the firsts 24 h of fermentation, the ST dropped from ≈ 50 to 26 $mN.m^{-1}$. Then, it increased to ≈ 52 $mN.m^{-1}$ at 48 h. In fact, this behavior is associated to biosurfactant recovery by foam overflow from the culture medium, in which the change on aeration and agitation at 24 h, increased the foam formation (in the bioreactor), and consequent the higher recovery of biosurfactants.

For avoid the foam recovery composed by proteins coming from cassava wastewater (the agitation and aeration of bioreactor produces foam only with cassava wastewater). It was considered only the foam collected from 24 to 84 h. The highest volumes were obtained at 24 h (256 mL), 36 h (258 mL) and 48 h (283 mL) and dropped to 160 mL at 60 h, 73 mL at 72 h and 26 mL at 84 h. Thus, the total collapsed foam ≈ 1000 mL was recovery per batch. Since it was used 3 L of culture medium, the foam recovered represents around 33%, that is, an excellent evidence of good biosurfactant production (the higher volume of foam, the better production of biosurfactant).

During the bioprocess, pH ranged from ≈ 5 to 8. Initially (0 h), the dextrose concentration ($mg.L^{-1}$) was at 6850 and decreased to 1960 at 36 h. Then, a higher concentration of dextrose (3430) was observed at 48 h and decreased again until the end of the bioprocess. This trend indicates that *P. tsukubaensis* produces amylases, and these amylases are produced to hydrolyze starch remains in the culture medium when dextrose is at low concentration. Konishi et al. (2011) described the glucose consumption during the production of MEL, which reached 0 $g.L^{-1}$, although the culture

medium was composed of a consortium of carbon sources, olive oil and yeast extract. It is worth nothing that yeast extract has peptone and amino acids that can be used as carbon source (Yan et al. 2012).

The analysis of Figure 3 shows the ST measurements of the collapsed foam.



*Error bars were deliberately hidden

Figure 3. Collapsed foam, bioreactor, ST (—●—), CMD-1 (- -▲- -), CMD-2 (-··◆-··), CMD-3 (- - ▼- - -), CMD-4 (···◀···).

From 24 to 84 h, the ST and CMD-1 and CMD-4 data were constant. The first two due to the higher concentration of collapsed foam than the CMC – which result in constant ST measurements, whereas the CMD-4 (dilution of 10,000 times) due to the low concentration or absence of biosurfactant, that is, ST values were only related to the distillated water ≈ 72 $mN.m^{-1}$.

On the other hand, the CMD-2 and CMD-3 values changed during the bioprocess. Both analyses followed the same trend, the lowest ST measurements, that is highest concentrations of biosurfactant, were obtained from 24 to 48 h.

As detailed by Arutchelvi et al. (2008) and Yu et al. (2015), the ST at the CMC (γ-$_{CMC}$) of MEL homologs are: MEL-A 28.4 $mN.m^{-1}$; MEL-B 28.2 $mN.m^{-1}$; MEL-C 25.1, 24.2, 30.7 $mN.m^{-1}$, whereas Sajna et al. (2013) reported that γ-$_{CMC}$ of MEL-C by *P. siamensis* is 33.$mN.m^{-1}$.

Thus, the obtained values, in particular ST (Fig. 3) are very similar to previously reported data, as described above (Arutchelvi et al. 2008, Sajna et al. 2013,

Yu et al. 2015). These data (Fig. 3) indicate the production of MEL, nevertheless do not confirm the production of MEL. It is worth noting that the foam may be composed mostly of MEL and proteins. Proteins have ST properties, which can slightly influence the ST measurements (comparison between obtained data with previously reported data).

Therefore, very likely cassava wastewater was a good culture medium for biosurfactant production from *P. tsukubaensis*, due to high production of foam. There are, also, strong evidences that foam was composed by MEL (ST values).

3.3.1. Production of MEL – kinetics and yield

The concentration of MEL in the foam (HPLC) followed the same trend as surface activity measurements and volume of recovered foam, that is, the higher was the biosurfactant concentration in the recovered foam, the higher was the volume of recovered foam. The purity levels of MEL (foam) were higher in the beginning of fermentation: 24 h - 38% (256 mL of foam), 36 h - 45% (258 mL) and 48 h - 51% (283 mL). Then, the concentration of MEL decreased at 60 h - 33% (161 mL), 72 h - 27% (73 mL) and 84 h - 25% (40 mL). Thus, confronting these data with cell counting, the biosurfactant production was mostly on log phase.

After each fermentation (excepting that used for evaluated the kinect as described above) the foam collected in 12-hour basis was blended, resulting in total foam produced by fermentation. Then, it was lyophilized. The mass of powder obtained by fermentation was ≈ 14.01 g and it showed ≈ 27% of MEL (w:w), that is, it was produced 1.26 g of MEL.L^{-1} of culture medium.

To the best of our knowledge, Morita et al. (2009) were the firsts to describe the MEL production using water-soluble traditional fermentable carbohydrates. They reported the production of MEL-A from *P. antarctica* JCM 10317 using glucose and sucrose, 1.61g.L^{-1} and 1.94 g.L^{-1}, respectively, also the production of MEL-C from *P. siamensis* CBS 9960 1.08 g.L^{-1} and 1.94 g.L^{-1} using glucose and sucrose, respectively. Later, Faria et al. (2014) studied the production of MEL from *P. antarctica* PYCC 5048^{T}, *P. aphidis* PYCC 5535^{T} and *P. rugulosa* PYCC 5537^{T} by the use of three different carbon sources, glucose, xylose or arabinose, separetely. They described similars maximum specific growth rates, although a lag phase was observed only for

xylose and arabinose. Even with lower yield, when comparing with vegetable oils, the purification process would be easier (Morita et al. 2015a).

Arutchelvi et al. (2008) described the production of MEL a non-growth associated bioprocess. Faria et al. (2014) observed that the production of MEL, using water-soluble carbon source occurred mainly in stationary phase. However, the production of MEL was relatively growth-associated, maybe due to the use of either *P. tsukunbaensis* and soluble carbon source rather than the hydrophobic carbon source.

Sophorolipids and mannosylerythritol lipids are the only biosurfactants largely produced by microorganisms (> 100 $g.L^{-1}$ for MEL and 300 $g.L^{-1}$ for sophorolipids) (Hubert et al. 2012, Sajna et al. 2013). For instance, Konishi et al. (2011) obtained 49.2 g of $MEL.L^{-1}$ of culture medium in a batch bioprocess using a culture medium with a consortium of carbon sources (10 $g.L^{-1}$ yeast extract, 100 $g.L^{-1}$ glucose, and 100 $g.L^{-1}$ olive). Then, in a subsequent study they changed the fermentation process, from batch to feed batch and reached 129 g of $MEL.L^{-1}$ of culture medium, with a volumetric productivity of 18.4 $g.L^{-1}.day^{-1}$ (Konishi et al. 2011), whereas Sajna et al. (2013), obtained 34 g of $MEL.L^{-1}$ of culture medium with a volumetric productivity of 3.7 $g.L^{-1}.day^{-1}$ using soybean oil (8% $w.v^{-1}$), yeast extract and minerals.

Yu et al. (2015) and Morita et al. (2015) compiled the production ($g.L^{-1}$) of MEL (Table 1).

Table 1. The production of MEL by *Pseudozyma* species.

Reference	**MEL producer**	***g.L^{-1}**	***g.L^{-1}**	***g.L^{-1}**
Yu et al. 2015	*Pseudozyma aphilis*	165		
	P. rugulosa	142		
	P. antarctica	140	26	
	P. parantarctica	106.7		
	P. hubeiensis	76.3		
	P. tsukunbaensis	73.1		
	P. siamensis	18.5		
	P. graminicola	10		
Morita et al. 2015	*P. antarctica*	40	10	1.3
	P. parantarctica	1.2	22.7	18.2
	P. schanxiensis	2.72		
	P. churashimaensis	3.8		
	P. crassa	4.6		
	P. fujiformata	4		

* The highest reached concentration was used as a parameter of yield.

Therefore, the yield (g of MEL.L^{-1} of culture medium), obtained by this study (1.26) was lower, when comparing with previously reported data (Table 1). However, it is similar to that reported by Morita et al. (2009), 1.61 and 1.94, that also used water-soluble traditional fermentable carbohydrates.

3.4. PURIFICATION OF MANNOSYLERYLTHRITOL LIPIDS BY ULTRAFILTRATION PROCESS

MEL are one of the most promising biosurfactant. Currently, there is lack of knowledge in all topics of MEL, production, purification and application.

Regarding any biotechnological process, obviously, the interactions between production, purification and application should be carefully taken into account. Traditionally, for the production of MEL is used a synthetic culture medium and ethyl acetate extraction from the culture medium followed by silica column as a purification process (Morita et al. 2015, Faria et al. 2014, Fan et al. 2014, Sajna et al. 2013, Recke et al. 2013, Konishi et al. 2011, Hubert et al. 2012)

3.4.1. Purity of MEL - lyophilized foam

The foam collected from each fermentation process, after centrifugation (to remove biomass) and lyophilization (powder) showed a purity of ≈ 30%. It is worth noting that the main impurities were proteins (see ultrafiltration process), in which most likely, came from two sources cassava wastewater and *P. tsukubaensis*. In other words, if used a synthetic culture medium for the production of MEL, the microorganism will be the only one source of proteins.

3.4.2. Centrifugal device of ultrafiltration

The relation between concentration of MEL-B and nanoparticle size showed that at high concentration (500 and 300 mg.L^{-1}) there is a unimodal distribution with small nanoparticle size ≈ 10 nm (diameter), whereas at 150 to 12.5 mg.L^{-1}, there is a trend to form a bimodal distribution with large nanoparticles of 100 and 800 nm. Thus, based on the self-assembly properties of MEL-B, an ideal initial concentration of feed should be between 150 and 12.5.L^{-1} mg of MEL, which result in large structure and consequently better recovery as rententate. However, the feed solutions showed a

unimodal form: d =1220 nm, at 610.74 mg.L^{-1} of MEL (experiment 1) and d=1754 nm, at 502.71 mg.L^{-1} of MEL (experiment 2). The difference between standard and sample results of nanoparticle distribution, probably, is related to the interactions between MEL-B, proteins and ions. Thus since, large nanoparticles were observed, it was decide to carry out the UF with the feed at 610.74 mg.L^{-1} of MEL and 502.71 mg.L^{-1} of MEL.

The experiments of ultrafiltration retained around 80% of the MEL and more than 95% of proteins were permeated. Thus, UF of MEL is a remarkable purification process due to the following considerations (i) high purification from proteins and high recovering of MEL, (ii) the use of membrane of large pores (100 kDa). Usually, the use of membranes of large pores results in high flux of UF and minimizes problems with fouling (easier scale up) and, (iii) only one step is required to purify MEL from proteins and also low molecular weight compounds (amino acids, organic acids, salts, among others).

Isa et al. (2007) described the purification of surfactin by two steps of ultrafiltration. The first step separates surfactin from low molecular weight compounds, whereas the second step separates surfactin from proteins. The diference between the ultrafiltration of MEL and surfactin, that is, the need of a second step of ultrafiltration, probably is due to volume of nanoparticles of MEL (self-aggregation), which are bigger than surfactin. Also, MEL are nonionic biosurfactant whereas surfactin is an anionic biosurfactant, that is, surfactin interacts electronically with proteins making the purification process (surfactin-proteins) harder.

Therefore, due to the noteworthy outcomes of MEL ultrafiltration (high recovery of nanoparticles of MEL and good purification of MEL from proteins) using centrifugal device of ultrafiltration (20 mL), the process was scaled up at top-bench volume, 500 mL (250 mL working volume).

3.4.3. Bench-top ultrafiltration – scale up

The ultrafiltration at bench-top scale took 45 minutes and reduced the initial volume of feed (250 mL) to 25 mL using a recirculation process. During the first 25 minutes, the flux significantly decreased from 90 to 55 L.m^{-2}.h^{-1}. Then, in the last 20 minutes, the flux subtle reduced from 55 to 45 L.m^{-2}.h^{-1} (Fig. 4).

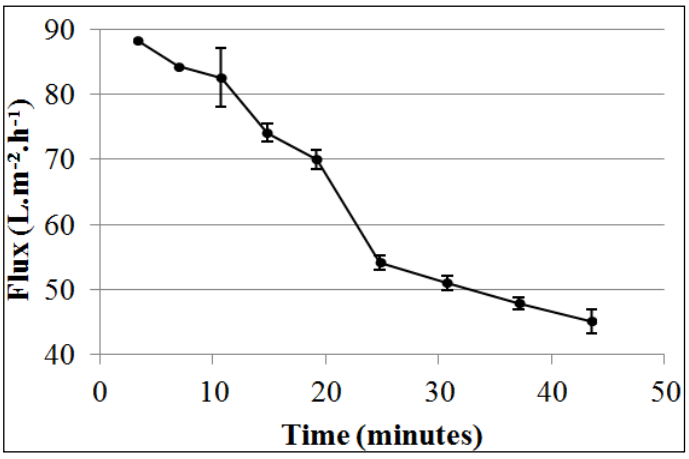


Figure 4. Flux of ultrafiltration (—).

Probably the main factor for the reduction of flux was the fouling due to the proteins and nanoparticles of MEL. It is worth noting that there are two sources of proteins *P. tsukubaensis* and cassava wastewater. This implies there was a wide range of proteins (large, small, etc) in the ultrafiltration system, which may interact with membrane in different ways.

The experiments of ultrafiltration at bench-top scale were carried out on recirculation mode (the retentate returns as feed), the initial volume of feed/retentate decreased (from 250 mL to 25 mL). On the other hand, the volume of permeate increased, that is, the volume of feed/retentate and permeate are inversely proportional. The analysis of Figure 5 indicates that the concentration of MEL (feed/retentate) increased (from 294.7 $mg.L^{-1}$ to 859.52 $mg.L^{-1}$) which proves that PES-100 membrane retained the nanoparticles of MEL, whereas the concentration of protein in the feed/retentate significantly decreased, which indicates that proteins were permeated.

The UF of nanoparticles of MEL at bench-top scale was suitable process. The experiments of UF were carried out with ≈ 272 mg of lyophilized foam dissolved in 250 mL of tris buffer (1091.59 $mg.L^{-1}$). After the UF, the finest product (25 mL of feed/retentate) was at ≈ 860 $mg.L^{-1}$ of MEL, that is, 21.5 mg of MEL (25 mL x 860 $mg.1000\ mL^{-1}$). Therefore, ≈ 272 mg of lyophilized foam resulted in 21.5 mg of MEL. Since, each fermentation produced ≈ 14.01 g of lyophilized foam. Theoretically, ≈ 1.1 g of purified MEL (finest product) could be produced by the integration between fermentation and UF.

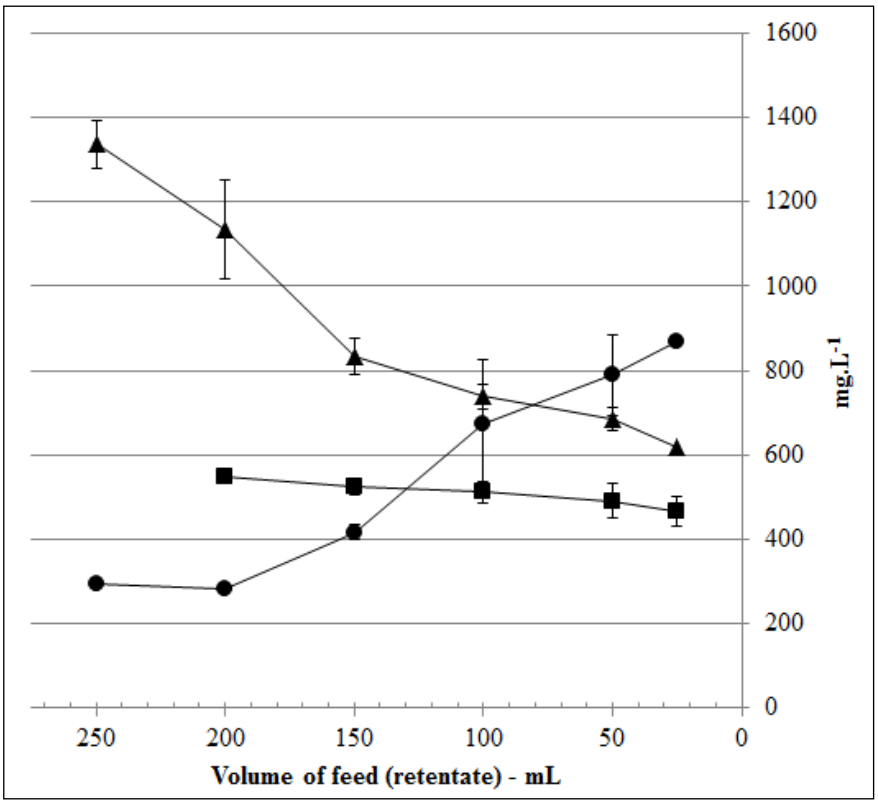


Figure 5. Concentration of MEL - feed/retentate (●), Concentration of protein - feed/retentate (▲); Concentration of protein - permeate (■).

3.5. CHEMICAL IDENTIFICATION OF PURIFIED MEL – FATTY ACID PROFILE, MALDI-TOFMS, NMR AND INFRARED.

The CG-MS analysis showed the presence of fatty acids C8:0; C10:0; C12:1; C12:0; C14:1 and C18:1, in which C8:0, C12:1 and C14:1 were the main peaks, which is relatively simitar to that described by Sajna et al. (2013), C14:1, C16:0, C16:1 and also to Fukuoka et al. (2008) C12 and C14 molecules. Later, Fukuoka et al. (2011) identified the presence of C8:0, C10:0; C12:0, C12:1, C14:0, C14:1 and C14:2. Although, Fan et al. (2014) described the presence, mainly, of longer fatty acid chains C18:0, C18:1 and C20:0. Finally, Fan et al. (2014) detailed that the main fatty acids were C8:0, C18:0, C18:1 and C20:0, that is, a wide range from short to long chains.

According to Faria et al. (2014), usually MEL are composed by two short-chain fatty acids, C8-C12. However, as mentioned above, it is very difficult to define/predict the fatty acid profile, it may depend of MEL producer, culture medium,

temperature, pH, ionic strength, etc., (bioprocess conditions), lag, log, stationary or death-phase (stage of bioprocess), etc. Also, the analysis of fatty acid profile by CG-MS required the esterification (separation of fatty acids from mannose - free fatty acids, then the fatty acids are esterified by methanol), which means that only a broad profile of fatty acids is known, in other words, is impossible relates exactly each fatty acids to its position in mannose. In this sense, considering the hydrophobicity of culture medium, one of the best comparisons is described by Morita et al. (2009). They compared the fatty acid profile of 4 microorganisms, using two source of carbon - separately; vegetable oil and sucrose. The profile of fatty acid significantly changed, in which, when was used sucrose, a broader profile was found.

It is worth to point out, that the shorter-chain fatty acids and also the lower number of acetylation of C-4´and C-6´ (mannose), the higher solubility of MEL in water. Thus, either, the fatty acids chain and number of acetylation of C-4´and C-6´ (mannose), should be minimized in order to improve the applications of MEL in water-based process. In this paper, was used a hydrophilic carbon source (cassava wastewater), which indicates the use of fatty acid synthesis to create fatty acids from acetyl-CoA and malonyl-CoA precursors (Yu et al. 2015). The most of papers about MEL used hydrophobic carbon sources to produced MEL and strongly suggested that the microorganisms use β-oxidation residues to synthesize the fatty acids (Yu et al. 2015, Morita 2015a, Arutchelvi et a. 2008).

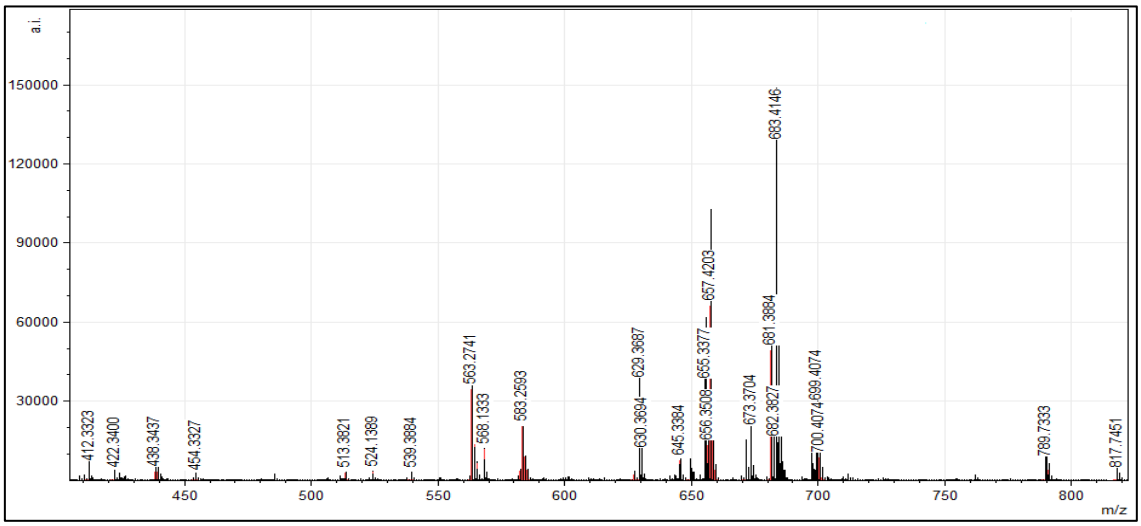


Figure 6. MALDI-TOFMS spectrum of MEL produced from *P. tsukubaensis.*

P. tsukubaensis produced many homologues of MEL, in which the two highest peaks were 683.41 and 657.42 m/z (Fig. 6). That diversity should be mainly related to chain length of fatty acids C-2´ and C-3´ of mannose as demonstrated by analysis of fatty acids. Fukuoka, et al. (2008) identified (main peaks) 683.8 and 657.8 m/z. In theory and disregarding fatty acids and erythritol, the m/z of MEL-B and MEL-C are the identical. Sajna et al. (2013) analyzed by MALDI-TOFMS the production of MEL-C, which were observed 3 main peaks at 607.42, 634.57 and 660.57 m/z. Thus, MALDI-TOFMS analysis showed very high similarity to previously reports, which strongly indicates the production of MEL-B or MEL-C.

The analysis of infrared data indicate high absorption on 3400 (O-H), 1730 (C=O), 1240 (C-O) and 1075 (-O-), which is very similar to results obtained by Kitamoto et al. (1990).

Structure determination of MEL was performed by ^{1}H, ^{13}C nuclear magnetic resonance (NMR) and two-dimensional NMR analysis, such as COSY (^{1}H-^{1}H correlation spectroscopy), HSQC-^{13}C-DEPT (heteronuclear single quantum coherence with DEPT, ^{1}JC-H), HMBC (heteronuclear multiple bond correlation, ^{n}JC-H), and the nuclear effect overhauser (NOE). The ^{1}H NMR chemical shifts, multiplicities and coupling constants are shown in Table 2, whereas the ^{13}C NMR data are in Table 3.

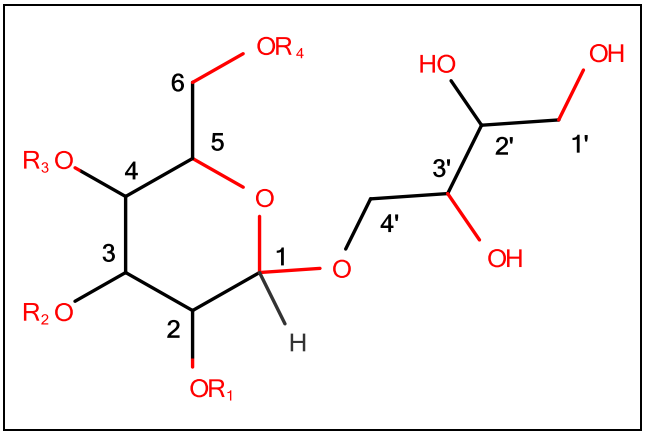
The ^{1}H NMR data showed similar pattern to those already reported (Morita et al. 2015b, Fukuoka et al. 2007a, 2007b, 2008, Recke et al. 2013, Sajna et al. 2013, Fan et al. 2014, Faria et al. 2014), although significant differences were observed. The signal at 4.76 ppm was assigned to anomeric hydrogen H-1, whereas, doublet at 5.49 ppm and doublet of double doublets at 4.95 ppm were assigned to H-2 and H-3, respectively and estimated as hydrogens bonded to esterified carbons C-2 and C-3 of the mannose. Additionally, it was observed two doublets of doublets, one at 4.41 ppm (J=12.13 and 5.22 Hz) and the second one at 4.46 ppm (J=12.41 and 2.54 Hz), which were assigned to diastereotopic protons H-6a and H-6b. Moreover, a singlet with integral for three hydrogens was observed at 2.14 ppm and was assigned as the methyl bonded to acetyl group.

The triplets (6.03 Hz) at 0.88 ppm and with integral value to six hydrogens were assigned to two methyl-end carbon chain lipid. The results strongly indicate the presence of two acyl groups of fatty acids and an acetyl group. The coupling constants

and the correlations observed in the COSY corroborated to the correct assignments of the protons and the stereochemistry of the chiral centers.

A shift of C-1 of the D-mannose unit to 99.10 ppm indicates that the O-glycosidic bond was between C-1 of D-mannose to meso-erythritol unit, which was confirmed by the HMBC correlations (Tables 2 and 3). On the ^{13}C NMR spectrum, three peaks derived from carbonyl groups were assigned at 171.64, 173.59 and 173.40 ppm (Table 3). HMBC analysis showed that each of these carbonyl carbons was correlated with one of the protons of *D*-mannose: H-6, H-2, and H-3, respectively. Moreover, the methyl protons at 2.14 ppm showed correlation to carbonyl carbon at 171.64 ppm.

Therefore, the NMR spectra analysis indicates that the purified sample has the structure of MEL-B, in which R_1 (C-2) and R_2 (C-3) are acyl groups, R_3 is a hydroxyl and R_4 is acetyl group (Fig. 7). It was also observed a minority second stereoisomer, between 8 to 10% by 1H-NMR spectrum (Fig. 8).



R_1 and R_2= Fatty acids; R_3= H; R_4= -C(O)CH_3

Figura 7. Chemical structure of purified sample (MEL-B).

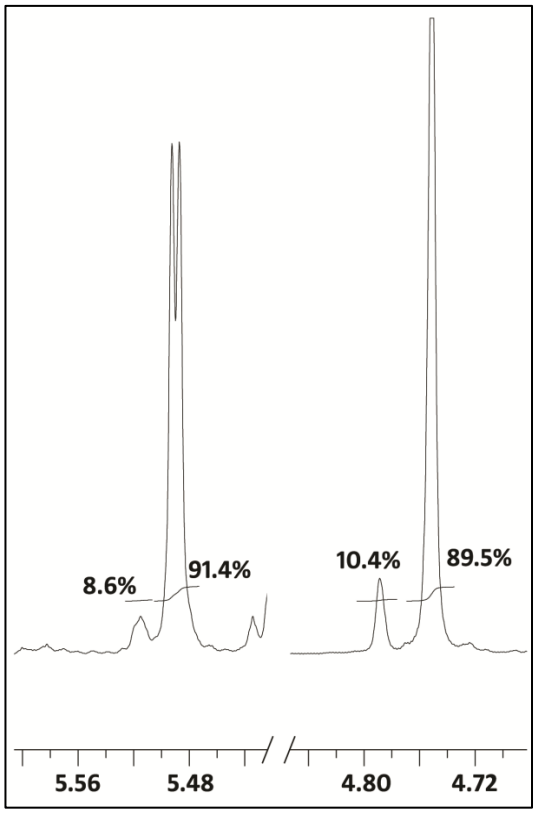


Figura 8. ^{1}H NMR data in $CDCl_3$ of purified sample and the presence of a second stereoisomer between 8% and 10%, which was based on signals of protons H-2 (5.49 ppm) and H-1 (4.76 ppm).

Table 2. ^{1}H NMR data in $CDCl_3$ of purified sample (s: singlet, d: doublet, dd: doublet of doublet , ddd: doublet of double doublets; t: triplet, m: multiplet, brs: broad signal. R_1 and R_2 are fatty acids, R_3 is hydroxyl and R_4 is acetyl group.

Functional groups		δ^1H (ppm) and multiplicities	Coupling constants (J in Hz)	COSY correlations	HMBC correlations
Sugar *D*-mannose					
H-1		4.76 (brs)	<2,0	H-2, H-3, H-4'a, H-4'b	C-2, C-3, C-4'
H-2		5.49 (d)	3.31	H-1, H-3	C-1, C-3, C-4, 173.59 (R_1)
H-3		4.95 (ddd)	10.04, 3.35 and 1.46	H-1, H-2, H-4	C-1, C-2, C-4, 173.40 (R_2)
H-4		3.78 (m)		H-3, H-5	C-3, C-5, C-6
H-5		3.59 (m)		H-4, H-6a, H-6b	C-4, C-6
H-6a		4.41(dd)	12.13, 5.22	H-5, H-6b	C-4, C-5, 171,64 (R_4)
H-6b		4.46 (dd)	12.41, 2.54	H-5, H-6a	C-4, C-5, 171,64 (R_4)
Hydroxyls	**R_3**	2.82 – 3.49 (brs)			
***meso*-Erythritol**					
H-1'a		3.66 – 3.73 (m)		H-1'b, H-2'	C-2', C-3'
H-1'b		3.56 – 3.62 (m)		H-1'a, H-2'	C-2', C-3'
H-2'		3.56 – 3.62 (m)		H-1'a, H-1'b, H-3´	C-1', C-3', C-4'
H-3'		3.69 – 3.75 (m)		H-2', H-4'a, H-4'b	C-1', C-2', C-4'
H-4'a		3.88 (dd)	11.17, 5.34	H-1, H-3', H-4'b	C-1, C-2', C-3'
H-4'b		3.93 (dd)	11.17, 3.40	H-1, H-3', H-4'a	C-1, C-2', C-3'
Hydroxyls		2.82 – 3.49 (brs)			
Acetyl	**Chain**				
-CH_3	**R_4**	2.14 (s)			171.64
Fatty acids					
-CH_3	**R_1, R_2**	0.88 (x2) (t)	6.07		
-CO-CH_2-	**R_1, R_2**	2.30 (m)			173.40; 173.59
		2.40 (t)	7.65		173.40; 173.59
-CO-CH_2CH_2-	**R_1, R_2**	1.57 – 1.70 (m)			173.40; 173.59
-$(CH_2)_n$-	**R_1, R_2**	1.22 – 1.39 (m)			
-CH=CH-	**R_1, R_2**	5.20 – 5.44 (m)			
-CH=CH-CH_2-	**R_1, R_2**	1.96 – 2.10 (m)			

δ^1H: Chemical shift in ppm; Coupling constant (^{n}J) in Hz.

Table 3. ^{13}C NMR data in $CDCl_3$ (at 125 MHz) of purified sample. R_1 and R_2 are fatty acids, R_3 is hydroxyl hydrogen and R_4 is acetyl group.

Functional		$\delta^{13}C$ (ppm)
Sugar		
***D*-mannose**		
C-1		99.10
C-2		68.81
C-3		73.18
C-4		65.65
C-5		74.47
C-6		63.19
***Meso*-erythritol**		
C-1'		63.65
C-2'		71.87
C-3'		71.30
C-4'		72.19
Acetyl group	**Chain**	
-CH_3	**R_4**	21.07
-C=O	**R_4**	171.64
Fatty acids		
-C=O (in C-2)		173.59
-C=O (in C-3)		173.40
-CH_3	**R_1, R_2**	14.33
-CO-CH_2-	**R_1, R_2**	34.19
		34.02
-CO-CH_2CH_2-	**R_1, R_2**	25.04
-$(CH_2)_n$-	**R_1, R_2**	22.62 – 32.12
-CH=CH-	**R_1 or R_2**	127.50 – 131.33
-CH=CH-CH_2-	**R_1 or R_2**	26.25

$\delta^{13}C$: Chemical shift in ppm; Multiplicities of the carbons were defined by HSQC-DEPT spectrum.

4. CONCLUSION AND PERSPECTIVE

Cassava wastewater is a feasible alternative culture medium to the production of MEL-B from *P. tsukubaensis*. Comparing with the traditional purification steps with ethyl acetate extraction integrated to silica column, the recovery of MEL-B by foam overflow integrated to ultrafiltration is a remarkable strategy, since it does not apply any organic solvent, which is aligned to green chemistry concept, and it is also, theoretically, cheaper. The

chemical identification of MEL-B indicated the minority second stereoisomer, between 8 to 10%. As prospection, we hope to conduct a research to evaluate the production of MEL using cassava wastewater supplemented to hydrophobic compounds in order to improve the yields and its effects on the ultrafiltration.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The authors are grateful to the São Paulo State Research Foundation (Fapesp) for their financial support, Brazilian Bioscience National Laboratory (institution of CNPEM) for NMR analysis and Chemical Engineering Department of the Polytechnic School of the University of São Paulo for MALDI-TOFMS analysis.

5. REFERENCES

Arutchelvi, J.I., Bhaduri, S., Uppara., P.V., Doble, M., 2008. Mannosylerythritol lipids: a review. J. Ind. Microbiol. Biotechnol. 35, 1559-1570.

Barros, F.F.C., Ponezi, A.N., Pastore, G.M., 2008. Production of biosurfactant by *Bacillus subtilis* LB5a on a pilot scale using cassava wastewater as substrate. J. Ind. Microbiol. Biotechnol. 35, 1071-1078.

Chen, H-L., Chen, Y-S., Juang, R-S., 2007. Separation of surfactin from fermentation broths by acid precipitation and two-stage dead-end ultrafiltration processes. J. Membrane Sci. 299, 114-121.

Chen, H-L., Chen, Y-S., Juang, R-S., 2008. Recovery of surfactin from fermentation broths by a hybrid salting-out and membrane filtration process. Sep. Purif. Technol. 59, 244-252.

Fan, L-L., Dong, Y-C., Fan, Y-F., Zhang, J., Chen, Q-H., 2014. Production and identification of mannosylerythritol lipid-A homologs from the *ustilaginomycetous* yeast *Pseudozyma aphidis* ZJUDM34. Carbohy. Res. 392, 1-6.

Faria, N.T., Santos, M.V., Fernandes, P., Fonseca, L.L., Fonseca, C., Ferreira, F.C., 2014. Production of glycolipid biosurfactants, mannosylerythritol lipids, from pentoses and d-glucose/d-xylose mixtures by *Pseudozyma* yeast strains. Process Biochem. 49, 1790-1799.

Fukuoka, T., Morita, T., Konishi, M., Imura, T., Sakai, H., Kitamoto, D., 2007a. Characterization of new glycolipid biosurfactants, tri-acylated mannosylerythritol lipids, produced by *Pseudozyma* yeasts. Biotechnol. Lett. 29, 1111-1118.

Fukuoka, T., Morita, T., Konishi, M., Imura, T., Sakai, H., Kitamoto, D., 2007b. Structural characterisation and surface-active properties of a new glycolipid biosurfactant, mono-acylated mannosylerythritol lipid, produced from glucose by *Pseudozyma antarctica*. Appl Microbiol Biotechnol. 76, 801–810.

Fukuoka, F., Morita, T., Konishi, M., Imura, T., Kitamoto, D., 2008. A *basidiomycetous* yeast, *Pseudozyma tsukubaensis*, efficiently produces a novel glycolipid biosurfactant. The identification of a new diastereomer of mannosylerythritol lipid-B. Carbohy. Res. 343, 555-560.

Fukuoka, T., Yanagihara, T., Imura, T., Morita, T., Sakai, H., Abe, M., Kitamoto, D., 2011. Enzymatic synthesis of a novel glycolipid biosurfactant, mannosylerythritol lipid-D and its aqueous phase behavior. Carbohy. Res. 346, 266–271.

Fukuoka, T., Yanagihara, T., Imura, T., Morita, T., Sakai, H., Abe, M., Kitamoto, D., 2012. The diastereomers of mannosylerythritol lipids have different interfacial properties and aqueous phase behavior, reflecting the erythritol configuration, Carbohyd. Res. 351, 81-86.

Hubert, J., Ple´, K., Hamzaoui, M., Nuissier, G., Hadef, I., Reynaud, R., Guilleret, A., Renault, J-H., 2012. New perspectives for microbial glycolipid fractionation and purification processes. Comptes. Rendus. Chimie. 15, 18-28.

Isa, M.H.M., Coraglia, D.E., Frazier, R.A., Jauregi, P., 2007. Recovery and purification of surfactin from fermentation broth by a two step ultrafiltration process. J. Membrane. Sci. 296, 51-57.

Isa, M.H.M., Frazier, R.A., Jauregi, P., 2008. A further study of the recovery and purification of surfactin from fermentation broth by membrane filtration. Sep. Purif. Technol. 64, 76-182.

Jauregi, P., Coutte, F., Catiau, L., Lecouturier, D., Jacques, P., 2013. Micelle size characterization of lipopeptides produced by *B. subtilis* and their recovery by the two-step ultrafiltration process. Sep. Purif. Technol. 104, 75-182.

Kim, H-S., Jeon, J-W., Kim, S-B., Oh, H-M., Know, T-J., Yoon, B-D., 2002. Surface and physico-chemical properties of a glycolipid bisurfactant, mannosylerythritol lipid from *Candida antartica*. Biotechnol. Lett. 24, 1637-1641.

Kitamoto, D., Akiba, S., Hioki, C., Tabuchi, T., 1990. Extracellular accumulation of mannosylerythritol lipids by a strain of *Candida antarctica*. Agric. Biol. Chem, 54, 31-36.

Konishi, M., Nagahama, T., Fukuoka, T., Morita, T., Imura, T., Kitamoto, D., Hatada Y., 2011. Yeast extract stimulates production of glycolipid biosurfactants, mannosylerythritol lipids, by *Pseudozyma hubeiensis* SY62. J. Biosci. Bioeng. 111, 702-705.

Marchant, R., Banat, I.M., 2012. Biosurfactants: a sustainable replacement for chemical surfactants? Biotechnol. Lett. 34, 1597-1605.

Morita, T., Ishibashi, Y., Fukuoka, T., Imura, T., Sakai, H., Abe, M., Kitamoto, D., 2009. Production of glycolipid biosurfactant, mannosylerythritol lipids, using sucrose by fungal and yeast strains, and their interfacial properties. Biosci. Biotech. Bioch. 73, 2352-2355.

Morita, T., Fukuoka, T., Imura, T., Kitamoto, D., 2015a. Mannosylerythritol lipids: Production and applications. J. Oleo Sci. 64, 133-141.

Morita, T., Fukuoka , T., Kosaka, A., Imura, T., Sakai, H., Abe, M., Kitamoto, D., 2015b. Selective formation of mannosyl-L-arabitol lipid by *Pseudozyma tsukubaensis* JCM16987. Appl. Microbiol. Biotechnol. 99, 5833-5841.

Rangarajan, V., Dhanarajan, G., Sen, R., 2014. Improved performance of cross-flow ultrafiltration for the recovery and purification of Ca^{2+} conditioned lipopeptides in diafiltration mode of operation. J. Membrane. Sci. 454, 436-443.

Recke, V.K., Beyrle, C., Gerlitzki, M., Hausmann, R., Syldatk, C., Wray, V., Tokuda, H., Suzuki, N., Lang, S., 2013. Lipase-catalyzed acylation of microbial mannosylerythritol lipids (biosurfactants) and their characterization. Carbohy. Res. 373, 82-88.

Saharan, B.S., Sahu, R.K., Sharma, D., 2012. A review on biosurfactants: fermentation, current developments and perspectives. Genet. Eng. Biotechnol. J. 2011, 1-14.

Sajna, K.V., Sukumaran, R.K., Jayamurthy, H., Reddy, K.K., Kanjilal, S., Prasad, R.B.N., Pandeya, A., 2013. Studies on biosurfactants from *Pseudozyma* sp. NII 08165 and their potential application as laundry detergent additives. Biochem. Eng. J. 78, 85-92.

Yan, S., Liang, Y., Zhang, J., Liu, C-M., 2012. *Aspergillus flavus* grown in peptone as the carbon source exhibits spore density- and peptone concentration-dependent aflatoxin biosynthesis. BMC Microbiol. 12, 106-119.

Yu, M., Liu, Z., Zeng, G., Zhong, H., Liu, Y., Jiang, Y., Li, M., He,X., He, Y., 2015. Characteristics of mannosylerythritol lipids and their environmental potential. Carbohy. Res. 407, 63-72.

CHAPTER IV

COMPARATIVE STUDY ON MICROBIAL ENHANCED OIL RECOVERY USING MANNOSYERYTHRITOL LIPIDS OR SURFACTIN AND THEIR EMULSIFICATION PROPERTIES

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(Paper submitted to Journal of Surfactants and Detergents)

Comparative study on microbial enhanced oil recovery using mannosylerithritol lipids or surfactin and their emulsification properties.

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Abstract

Worldwide oil production has been declining. Microbial enhanced oil recovery is one of the most important tertiary recovery processes. The aim of this work was to evaluate the surface activity properties of surfactin and mannosylerithritol lipids, in particular, on microbial enhanced oil recovery. Solutions of both surfactin and mannosylerithritol lipids standards were compared to the produced surfactin and mannosylerithritol lipids that were produced using cassava wastewater as substrate and purified by ultrafiltration, on experiments related to oil recovery: surface activity at extreme conditions one a time and their interactions, oil displacement, removal of oil from sand and emulsification index. The experiments were carried out with light, medium and heavy oils. Central composite design rotational experiments indicated that ionic strength was significant for the surface activity of surfactin, whereas ionic strength, temperature and pH were significant for the surface activity of mannosylerithritol lipids. Regarding to the oil displacement test, the produced biosurfactants followed the same trend that standards, that is, mannosylerithritol lipids obtained higher clear zone than surfactin. Also, for both biosurfactants, surfactin and mannosylerithritol lipids, obtained higher clear zones in the experiments with heavy oil rather than medium and light oils. These results are aligned to the data of removal oil from sand, indicating a good prospecting on microbial enhanced oil recovery, in particular for applying mannosylerythritol lipids in wells with heavy oils.

Key words: microbial enhanced oil recovery; surfactin; mannosylerythritol lipids.

1. INTRODUCTION

Nomenclature	
American Petroleum Institute (API)	Central composite rotational design (CCRD)
Microbial-enhanced oil recovery (MEOR)	Critical micelle dilution (CMD)
Mannosylerithritol lipids (MEL)	Sodiumdodecyl sulfate (SDS)
Surface tension (ST)	

Currently and in the years to come, petroleum has been playing the most important role in energy sectors and also supplies the many basic industries (rubber, chemicals, etc). More than 90% of petroleum production is related to conventional oil, that is, light and medium oils. However, the explorations of heavy and extra heavy oils are growing rapidly [1].

Worldwide oil production has been declining due to the higher and higher demand of energy by the increasing population, physical limit of oil wells, difficult in find and explored new oil fields, in particular conventional oils [2]. In this context, regarding to the total energy uses, fossil fuels represents from 80-90%, in which oil and gas together are about 60% of fossil fuels, that is, 48-54% of total [1-2].

Petroleum, known as crude, is a mixture of hundreds of organic compounds and trace amounts of inorganic compounds. Although each organic compound has unique physical and chemical properties, collectively they are often divided into the paraffins, naphthenes and aromatic hydrocarbons [3]. Crude oils are complex mixtures. According to American Petroleum Institute (API), they are classified by relative density classified in 4 groups light (API > 31.1), medium (API from 22.3 to 31.1), heavy (API< 22.3) and extra heavy (API < 10.0). Thus, due to the chemical complexity of each oil, this study generalized and considered the lower API, the more hydrophobic is the oil.

The primary technique of oil recovery uses stored energy of wells – pressure and temperature – and recoveries ≈ 35% of total oil in the well. The secondary technique uses external energy source, for instance injection of water and recoveries ≈ 20%, that is, each already explored wells has about 35–55% of the initial oil volume [1-2]. Thus, the aim of enhanced oil recovery technologies is mainly the remaining oil in the wells – after the primary and secondary techniques - which is ≈ 3 trillion barrels [2].

Microbial enhanced oil recovery (MEOR) is one of the most important tertiary recovery processes. Preliminary studies were carried out in the 1960s. The application of MEOR can be classified in underground (*in situ*) or aboveground (*ex situ*). The underground

methodology aims to increase the biomass in the wells, that is, the fermentation takes place in the wells – *in situ*, whereas, the aboveground applies compounds produced by the fermentation. MEOR rise up the oil recovery ≈ 3%, however laboratory scale experiments predict up to 10% [4]. The presence of biosurfactant in wells reduces the surface tension of oil in wells, resulting in an easier process of oil recovery.

We speculate that MEOR is an interesting and promising technique as a tertiary recovery process of petroleum, in particular for non-conventional oils - heavy and extra heavy oils. Probably, MEOR will be a significant technology. In this context, *ex situ*, that is, the production, purification and subsequent application of biosurfactants in the wells seem a better strategy rather than the production *in situ*. Since, many uncontrollable and complex situations are involved on the *in situ* application such as diversity of chemical in the wells and compounds from microorganisms, time of operation, variation of temperature, pH, ionic strength, and reproduction of endogenous microorganisms in the laboratory, etc.

This study describes for the first time, the surface activity measurements of two biosurfactants, mannosylerithritol lipids (MEL) and surfactin at extreme conditions of temperature, ionic strength and pH and its interaction – similar conditions to the oil wells. Then, it was evaluated the MEOR of 3 types oils - heavy, medium and light - using standard biosurfactants solution and produced biosurfactant solution.

2. MATERIAL AND METHODS

2.1. MEASUREMENT OF SURFACE ACTIVITY

The surface tension (ST) measurements were carried out by using the plate method at room temperature in a Krüss GmbH K-12 tensiometer.

The surface activity of produced MEL and produced surfactin was measured by central composite rotational design (CCRD) experiments. The critical micelle dilution (CMD) corresponds the ST value of a sample diluted 10 times (CMD-1) and 100 times (CMD-2) [5].

2.2. STABILITY OF SURFACE ACTIVITY OF MANNOSYLERITHRITOL LIPIDS AND SURFACTIN IN EXTREME CONDITIONS: pH, TEMPERATURE AND IONIC STRENGTH

Sodium dodecyl sulfate (SDS), standard surfactin (Lipofabrik) and MEL (Toyobo) standard solutions were prepared separately at 100 $mg.L^{-1}$. Produced MEL and surfactin solutions were at 869.52 and 75.74 $mg.L^{-1}$, respectively. The effect of ionic strength

on surface tension activity was tested using synthesized brine with composition of $g.L^{-1}$: Na_2SO_4: 1.26, $NaHCO_3$: 0.051, NaCl: 0.75, $CaCl_2$: 9.2, $MgCl_2$: 7.6, KCl: 0.61 [6].

The stability of surface activity of biossurfactants in extreme conditions of pH, temperature and ionic strength were first investigated one at a time, in which the pH was evaluated at 2 unit basis, from 2 to 12, whereas 3 temperatures were tested during 60 minutes, 79, 100 and 121 ^{o}C and finally the ionic strength 2.5, 5, 10 and 20 $g.L^{-1}$ of synthesized brine. Then, surface tension and its critical micelle dilutions were measured.

Thereafter, as shows the Table 1, the CCRD evaluated the effect of the interactions among temperature, pH and ionic strength on surface tension activity.

Ideally none of the factors (temperature, ionic strength and pH) should increase the values surface activity, that is, the lower values, the better responses.

Table 1. Central composite rotational design.

Experiment	Coded levels			Experimental Levels (Factors)			Produced surfactin (Response 1)		Produced MEL (Response 2)		
	x_1	x_2	x_3	*Temperature (^oC)	†pH	**Ionic strenght ($g.L^{-1}$)	ST ($mN.m^{-1}$)	CMD-1 ($mN.m^{-1}$)	ST ($mN.m^{-1}$)	CMD-1 ($mN.m^{-1}$)	CMD-2 ($mN.m^{-1}$)
1	-1	-1	-1	87.5	4.02	6.04	33.38	48.91	28.25	29.85	59.9
2	+1	-1	-1	112.5	4.02	6.04	37.53	62.44	30.18	30.62	72.09
3	-1	+1	-1	87.5	9.97	6.04	37.67	48.29	27.87	31.32	57.1
4	+1	+1	-1	112.5	9.97	6.04	35.02	51.23	29.42	33.14	69.07
5	-1	-1	+1	87.5	4.02	16.45	41.27	72.34	27.5	34.35	61.28
6	+1	-1	+1	112.5	4.02	16.45	43.95	72.54	27.23	32.82	72.46
7	-1	+1	+1	87.5	9.97	16.45	45.42	72.54	27	29.24	50.69
8	+1	+1	+1	112.5	9.97	16.45	43.15	72.71	27.06	33.85	67.59
9	-1.68	0	0	80	7	11.25	38.36	72.5	27.4	34.85	49.44
10	+1.68	0	0	121	7	11.25	46.65	60.95	27.43	31.25	70.93
11	0	-1.68	0	100	2	11.25	47.53	72.74	27.99	30.69	72.8
12	0	+1.68	0	100	12	11.25	40.94	63.6	31.63	44.67	72.31
13	0	0	-1.68	100	7	2.5	29.95	49.87	28.32	32.75	37.01
14	0	0	+1.68	100	7	20	42.07	72.21	26.93	34.58	47.77
15	0	0	0	100	7	11.25	38.71	71.99	28.58	39.63	52.35
16	0	0	0	100	7	11.25	37.36	70.28	27.72	34.68	43.83
17	0	0	0	100	7	11.25	37.76	67.36	28.72	32.51	48.02

***** The temperature range defined in the study was based in boiling point of water (under, at and above).

† The pH range defined in this study was very wide (from 2 to 12) in order to cover all pH that are found in oil wells.

** Ionic strenght range defined in this study was based on previous studies [5-6].

Then based on the analysis of CCRD data, the validation experiments were carried out.

2.3. EMULSIFICATION INDEX

In order to obtain the highest solubility, surfactin was solubilized in buffer pH 8.5 at 100 $mg.L^{-1}$. The emulsification index was measured using the method described by Cooper and Goldenberg [7], whereby 6 mL of each hydrocarbon was added to 4 mL of each the biosurfactant solutions: surfactin standard, MEL standard; produced surfactin and produced MEL. Then, each screwcap test tube was vortexed for 2 minutes. The emulsion stability was determined after 24 h (E_{24}) and 120 h (E_{120}) and the emulsification index was calculated by dividing the measured height of the emulsion layer by the total height of mixture and multiplying it by 100. SDS was used as a standard of emulsifier at 1 $mg.mL^{-1}$. Benzene (Sigma-Aldrich >99%), toluene (Sigma-Aldrich >99.3%) and xylene mixture (Sigma-Aldrich ≥98.5 %), light oil, medium oil and heavy oil, also, edible vegetable oils from canola (Bunge), sunflower (Bunge), corn (Bunge), sunflower with Brazil nut (Bunge) and soybean (Cargill) were evaluated. Each experiment was carried out in duplicate to determine the height of the emulsion by the software ImageJ (1.48v - version).

2.4. TRIALS OF MEOR

2.4.1. Removal of crude oil from sand

Surfactin, MEL and SDS as agent of oil recovery were evaluated, separately, using artificially contaminated sand 10% ($g.g^{-1}$) of light oil, medium oil, heavy oil, benzene, toluene and xylene mixture, separately. Samples of 3 g of sand were vortexed with 0.3 g of crude oil in 20 mL Falcon tubes. All flasks were homogenized by shaking them at 100 rpm 24 h at 40 ^{o}C. Afterwards, 3 mL of biosurfactant solutions at 100 $mg.L^{-1}$ were added to each flask. The flasks were incubated at 100 rpm and 40 ^{o}C for 24 h. Finally, the supernatants were collected and measured (volume) [8]. Control assays were performed using Milli-Q water at the same conditions [9].

2.4.2. Oil displacement test

Thirty mL of Milli-Q water was placed in 15 cm diameter Petri dish. Then 200 µL crude oil was dropped onto the surface of water. Finally, 10 µL of biosurfactant solutions at

100 mg.L^{-1} was placed onto the surface of oil. The diameter the clear zone was measured using the software ImageJ (1.48v - version). Each experiment was repeated twice.

3. RESULTS AND DISCUSSION

3.1. SURFACE ACTIVITY MAINTENANCE AT EXTREME CONDITIONS

3.1.1. Study of maintenance of surface activity – extreme condition one at a time

We decided to carried out first one at time experiments, even with central composite rotational design experiments due to (i) enough biosurfactant to do both experiments and (ii) to compare data on literature, since one at time is a tradiotional methology.

The Figures 1, 2 and 3 show a comparative study of surface activity at extreme conditions of standard surfactin, produced surfactin, standard MEL and produced MEL solutions. The extreme conditions tested cover the conditions found in oil wells, in which are milder.

Ionic strength

It has been reported that surfactants, in particular anionic, are affected by eletrolytes, due to lower solubilization or even precipitation of surfactants [10]. Thus, understanding the behavior of surfactant when in solution with eletrolytes is fundamental to industrial scale applications.

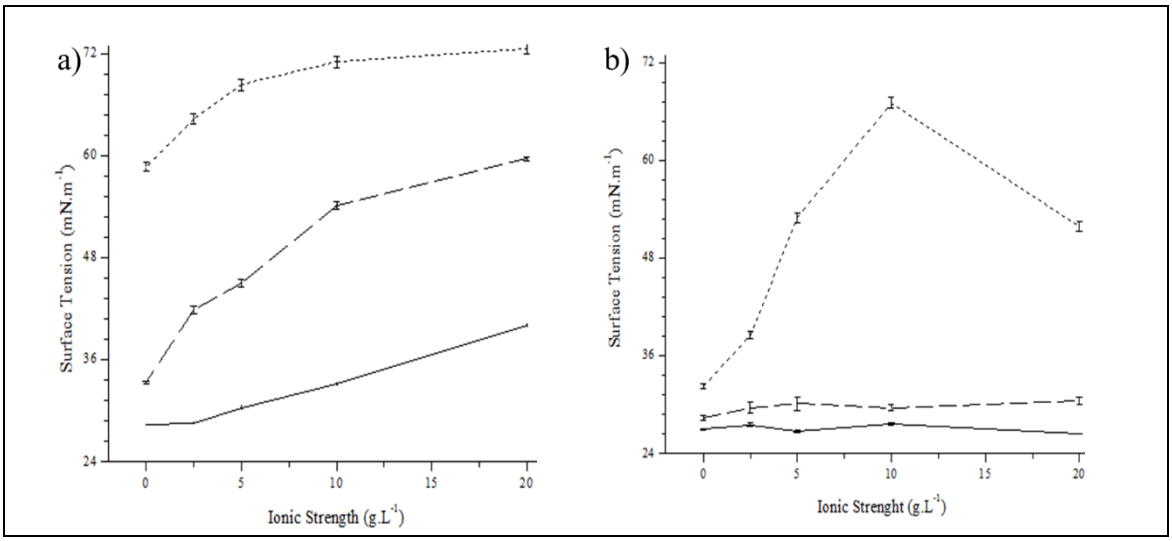


Figure 1. Surface activity of biosurfactants - surfactin (a) and MEL (b) - at range of ionic strength: ST (▬▬); CMD-1 (▬ ▬); CMD-2 (▪ ▪ ▪ ▪).

The ionic strength affect the surfactin, in which the higher ionic strength, the higher was the surface tension measurements ST, CMD-1 and CMD-2, that is low surface activity. It is worth point out the relative low ST even at high ionic strength 10 and 20 $g.L^{-1}$. 33.19 and 40.02 $mN.m^{-1}$, respectively (Figure 1).

Thimon et al. [11] evaluate the surface tension measurements of uncomplexed surfactin solution at pH 9.5 – tris buffer - and complexed with divalent ions Ca^{+2} and Ba^{+2} or monovalent cations Li^{+}, Rb^{+}, Na^{+}, K^{+}. All cations resulted in lower surface tension measurements. Vass et al. [12] studied the conformation of surfactin by Fourier transform infrared and Circular Dichroism, with or without Ca^{2+} ions and concluded that conformation of surfactin (β or γ-turn) is depending of the presence of ions. They mainly related the differences on COOH groups of Glu^{1} and Asp^{5}, as the stabilizer-key of backbone conformation of the peptide ring of surfactin.

Cations, in particular divalents, may act as a bridge between one or more molecules of surfactin. The positions for these bonds are the amino acids, Glu^{1} and Asp^{5} (anions), in the peptide loop moiety of surfactin. Therefore, due to the presence of many ions in the synthesized brine, Na^{+}, Cl^{-}, Ca^{+2}, K^{+}, etc., it is impossible to identify any effect of ions (individually) on the surface tension activity of surfactin by the use of brine. However, as a prospection of MEOR, in which usually there is mixture of ions, it can be concluded that the negative, but still feasible, effect of the presence of synthesized brine on the surface activity of surfactin (Figure 1).

As expected, the surface tension activity of MEL was less affected by ionic strength than surfactin (Figure 1). These results are aligned to previous report by Kim et al. [13], in which the named MEL-SY16 retained the surface tension activity up to 1000 mM NaCl and 10 mM $CaCl_2$.

Thus, even with the negative effects of the ionic strength on the surface tension of biosurfactants, the application of surfactin and mainly of the MEL is a feasible method for reducing the surface tension in high ionic strength system as oil wells (MEOR).

Temperature

One of the most advantages of application of biosurfactant rather than synthetic surfactants is the stability of the forms at extreme temperatures [5, 13]. Depending on the type of biosurfactant, the temperature may affect the self-aggregation or break the structure. Thus, significant changes are related to surface tension measurements.

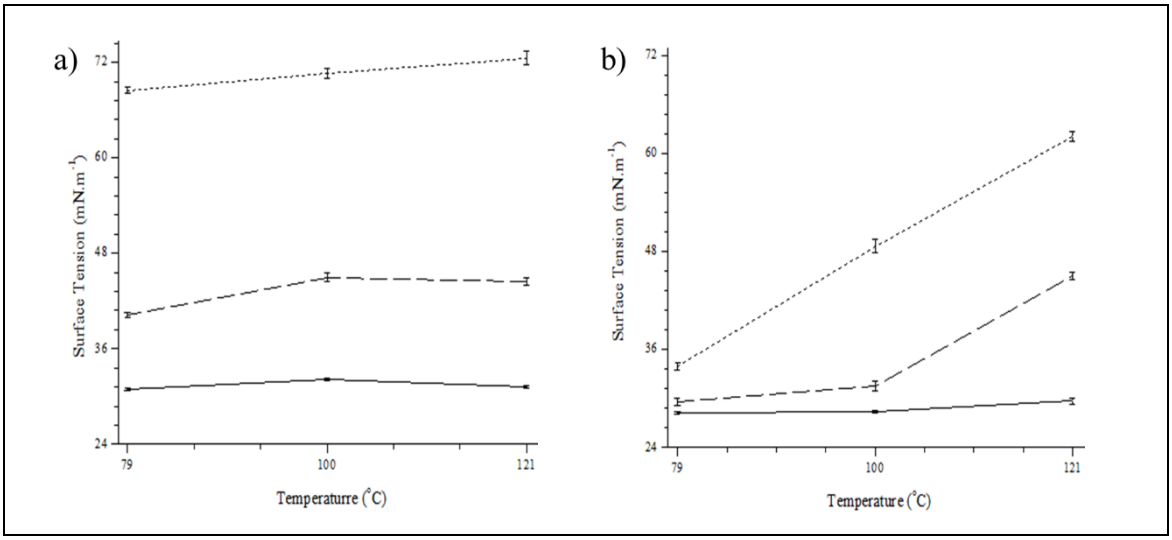


Figure 2. Surface activity of biosurfactants - surfactin (a) and MEL (b) - after being under extreme condition of temperature; ST (▬▬); CMD-1 (▬ ▬); CMD-2 (▪ ▪ ▪ ▪).

Regarding to the surfactin, no significant differences were observed among the ST and CMD values of thermal treatment at 79, 100 and 121 ^{o}C (Figure 2). Therefore, temperature was the most insignificant parameter on the surface activity of surfactin, whereas the thermal treatments 100 and 121 ^{o}C significantly affect the surface activity of MEL, may due to the carbohydrates mannose and erythritol that are components of MEL. The results of thermal threatment of MEL at 79 ^{o}C are aligned to Kim et al. [13] that reported the maintenance of surface tension activity of MEL-SY16 after 1 h of thermal treatments (20 to 90 ^{o}C).

pH

The solubility of ionic compounds is very affected by pH changes (isoelectric point), for instance the anionic biosurfactants surfactin precipitates at pH 2 [2]. On the other hand, the solubility of non-ionic surfactants, such as the glycolipid MEL, is not significantly affected by pH changes [14]. The solubility of biosurfactants is related to the surface tension activity. The higher solubility, the higher is the surface activity. Thus, the surface activity of surfactin and its CMD should be significant affect by pH changes, differently from MEL, which none or subtle changes on surface tension activity should be observed.

It is fundamental understand that the pH experiments (Figure 3) do not evaluate the stability of biosurfactants, since factors associated to decrease of solubility and changes on the self-aggregation forms are directly related to surface activity measurements. However, the

hypothesis of chemical breaking of biosurfactants at extreme conditions of pH should be not discarded.

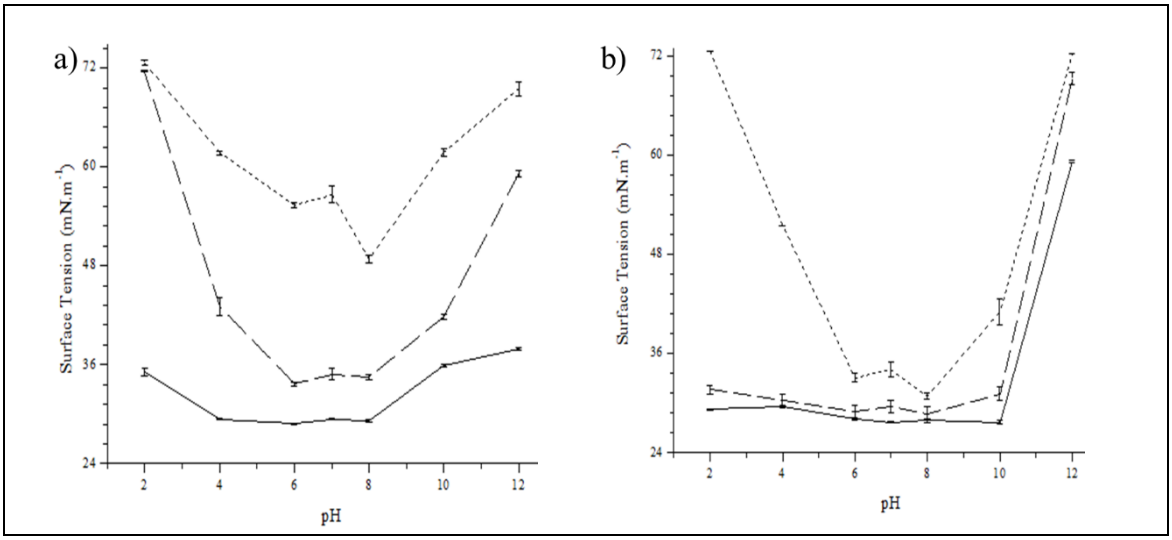


Figure 3. Surface activity of biosurfactants - surfactin (a) and MEL (b) - at range of pH; ST (▬▬); CMD-1 (▬ ▬); CMD-2 (▪ ▪ ▪ ▪).

At extreme conditions of pH (2-4, 10-12) the produced surfactin showed the highest ST and CMD values, that is, lowest surface tension activity. On one hand pH 2 precipitates surfactin reducing the surface activity [5]. On the other hand, the extreme alkaline conditions (pH 10-12) may act in the surfactin micelles or breaks the surfactin structure. The intermediaries pH ≈ 4 to 8 showed the best results, that is, lowest ST and CMD values (Figure 3). Thus, contrary to the extreme conditions of pH, intermediaries pH do not affect the surface tension of surfactin.

The analyses of ST and CMDs measurements of MEL (Figure 3) indicates an abrupt increase of values from pH 10 to 12, which could indicate the chemical breaking of MEL. On the other hand, a slight decreasing of CMD-1 values and a significant decreasing of CMD-2 values were observed from pH 2 to 6. These results follow the same trend described by Kim et al. [13], which detailed that surface activity of MEL-SY16 was relatively stable over a pH range of 4 to 10.

Therefore, the surface activity of surfactin and MEL were affected at extreme pH. However, very likely they were affected in different ways. The solubility of surfactin (ionic compound) and consequently the surface tension activity should be significantly affected by extreme pH, whereas the solubility of MEL (non-ionic) should not be significantly affected by

extreme pH. Thus, the chemical structure of MEL may be was broken at extreme pH, resulting in low surface tension activity.

In conclusion, the maintenance of surface activity properties of both surfactin and MEL were affected in different ways by the conditions tested. Surfactin was more sensitive to ionic strength and pH, whereas MEL was more sensitive to thermal treatment. However, none conditions tested preclude their application.

3.1.2. Study of maintenance of surface activity - interactions

Studies of surface activity of surfactin and MEL at extreme condition were already reported [5, 13]. However, these studies tested the surface tension activity at extreme conditions, one a time. To the best of our knowledge, this is the first study that comprehended experiments of surface tension activity in more than one extreme condition at the same time.

The understanding of surface tension activity of biosurfactants when at extreme conditions, at the same time (interactions), is fundamental for the application of biosurfactants, for instance the conditions of oil wells are high temperature, high ionic strength and extreme pH. Thus, the application of biosurfactants in oil wells implies that biosurfactants are able to reduce the surface tension of oil in the well under extreme conditions, at the same time (temperature, high ionic strength and extreme pH).

In this sense, Le et al. [4] described that in the Daqing oilfield, the temperature ranged from 45 to 89 ^{o}C and the ionic strength $\approx$ 15 g.L^{-1}, that is, the interaction of these parameters has to be considered.

Regarding CCRD of surfactin, the analysis of ANOVA of ST and CMD-1 indicated that the parameters temperature, pH and ionic strength were statistically differents (95% of confidence) [(ST - ($F_{calregression}$ 23.02; F_{tab} 3.74)); (CMD-1 - ($F_{cal\ lack\ of\ fit}$ 5.01; F_{tab} 19.41))]. The analysis of ANOVA also indicated higher coefficient of determination of CMD-1 (r^2 of 0.76) than ST. Thus, the sequence of analysis of CCRD was based only on CMD-1 and generated the following equation:

Equation (1): $Y = 67.28 + 8.5x_3 - 3.02x_3^2$

Y is CMD-1 of surfactin, x_3 ionic strength and x_3^2 ionic strength squared.

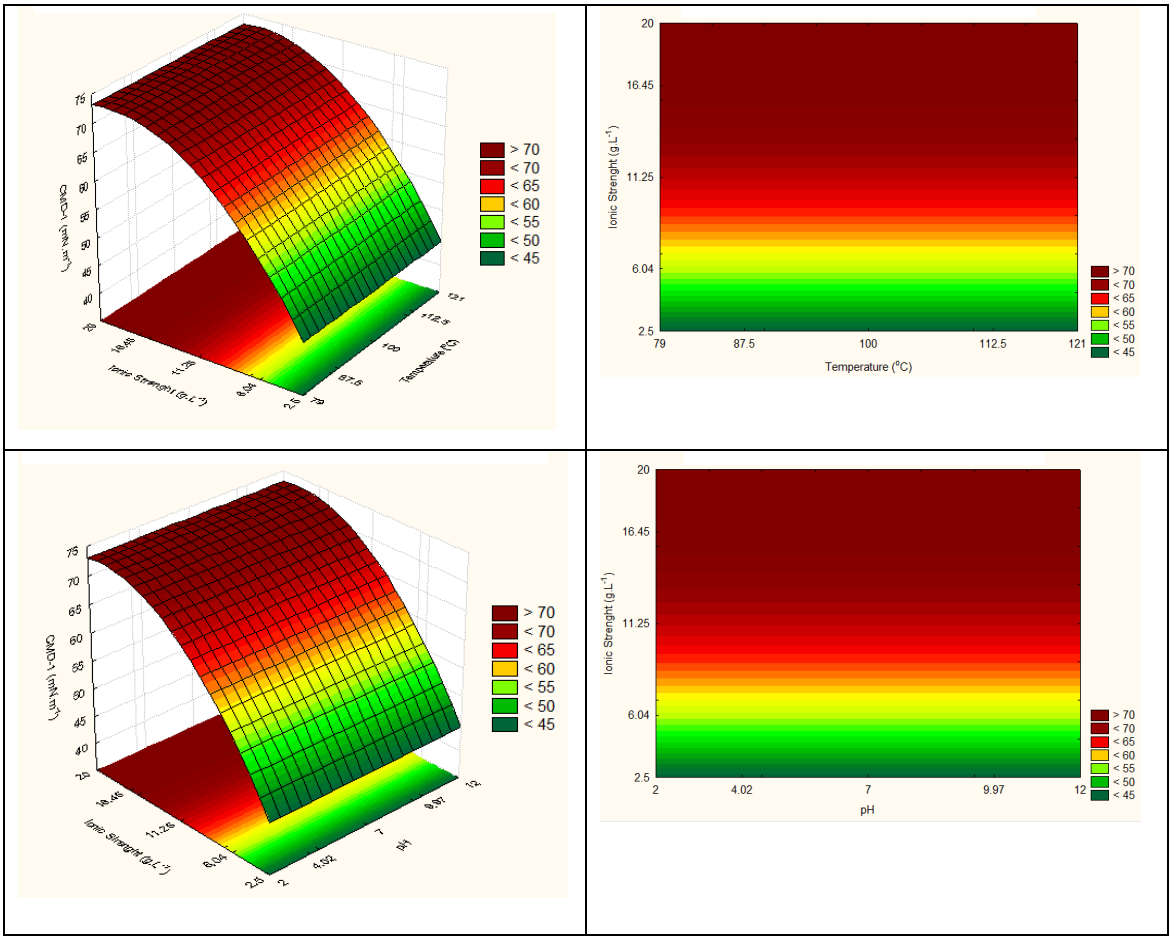


Figure 4. Response surface - CMD-1 of surfactin experiments.

It is possible to observe by the response surface (Figure 4) that ionic strength is the most significant parameters on the surface activity of surfactin. As show in Figure 4, pH and temperature minimally influenced the surface activity of surfactin. The derivate of equation 1 with Y´=0 (maximum– red area) indicates that 18.58 g.L^{-1} of brine is the threshold, when associated to extreme pH and temperature, in order to keep the surface activity.

Regarding CCRD of MEL, the analysis of ANOVA of ST, CMD-1 and CMD-2 indicated that the parameters temperature, pH and ionic strength were statistically different (95% of confidence). The result of MEL indicated the highest coefficient of determination of CMD-2 (r^2 of 0.84) than ST and CMD-1. Thus, the sequence of analysis of CCRD was based only on CMD-2 rather than ST and CMD-1 and generated the following equation.

Equation (2): $Y = 47.76 + 5.15x_1 + 9.16x_2^2 + 5.34x_3^2$

Y is CMD-2 of MEL, x_1 temperature, x_2^2 pH squared and x_3^2 ionic strength squared.

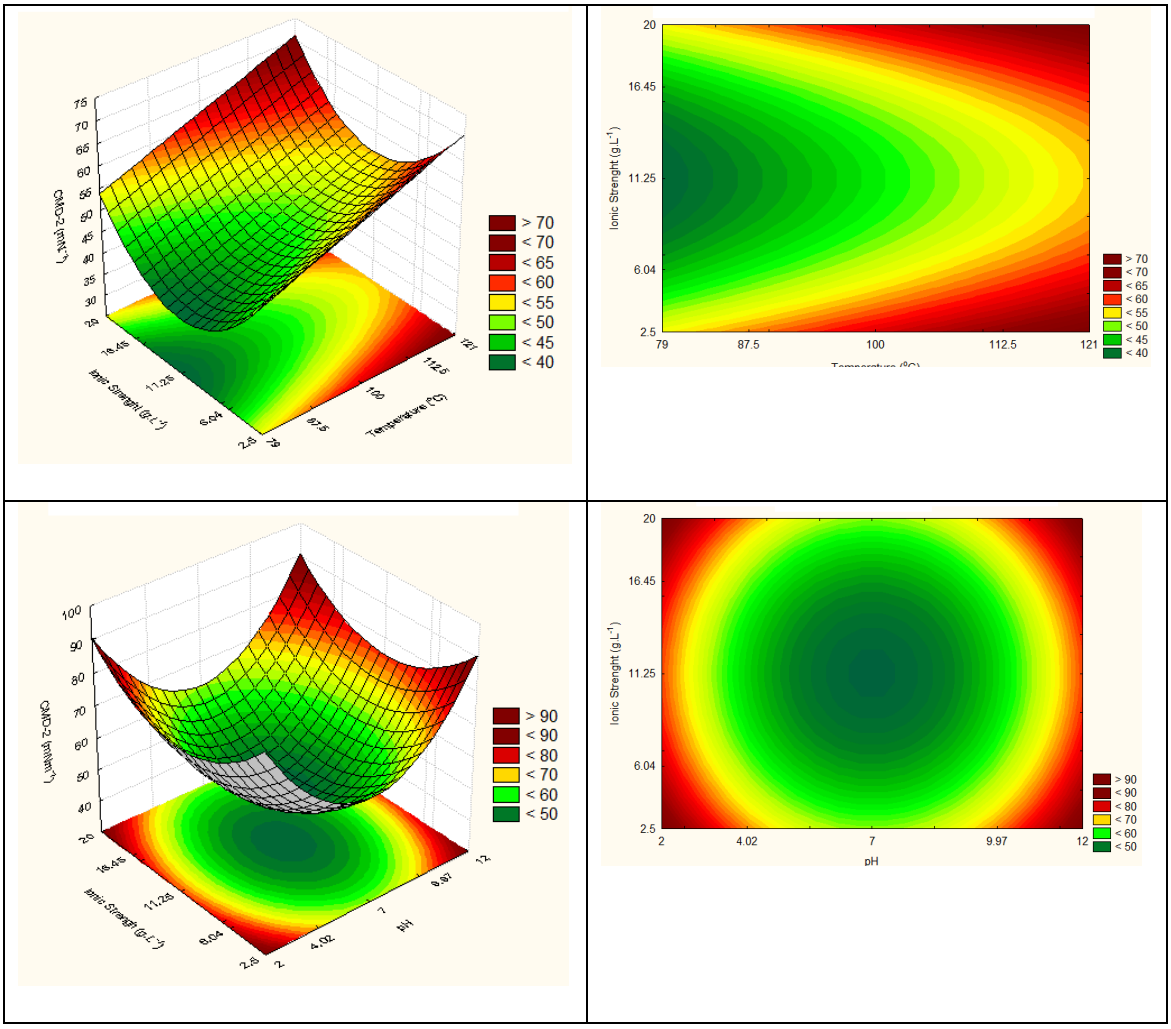


Figure 5. Response surface - CMD-2 of MEL experiments.

Differently of surfactin, the response surface analysis of MEL experiments reveled that ionic strength, pH and temperature have significant effect on the surface activity. Ionic strength and pH were squared terms, whereas, temperature linear term, that is, changes on ionic strength and pH are more significant parameters. The derivate of equation 2 with Y´=0 (minimum – green area) indicates that the central point was the lowest measurement of CMD. These results follow the same trend that the study of maintenance of surface activity – one at a time.

Table 2. Predicted and experimental data of central composite design experiments – surfactin and MEL.

Assay	Predicted ($\hat{Y}$) – CMD-1	Experimental (Y) – CMD-1	$\lvert Y - \hat{Y} \rvert$	$(Y - \hat{Y} * 100.Y^{-1})$	Predicted ($\hat{Y}$) – CMD-2	Experimental (Y) – CMD-2	$\lvert Y - \hat{Y} \rvert$	$(Y - \hat{Y} * 100).Y^{-1})$
	Surfactin				MEL			
1	55.76	48.91	6.9	14.01	57.11	59.9	2.79	4.66
2	55.76	62.44	6.7	10.70	67.41	72.09	4.68	6.49
3	55.76	48.29	7.5	15.47	57.11	57.1	0.01	0.02
4	55.76	51.23	4.5	8.84	67.41	69.07	1.66	2.40
5	72.76	72.34	0.4	0.58	57.11	61.28	4.17	6.80
6	72.76	72.54	0.2	0.30	67.41	72.46	5.05	6.97
7	72.76	72.54	0.2	0.30	57.11	50.69	6.42	12.67
8	72.76	72.71	0.1	0.07	67.41	67.59	0.18	0.27
9	67.28	72.5	5.2	7.20	39.11	49.44	10.33	20.90
10	67.28	60.95	6.3	10.39	56.41	70.93	14.52	20.47
11	67.28	72.74	5.5	7.51	73.61	72.8	0.81	1.12
12	67.28	63.6	3.7	5.79	73.61	72.31	1.30	1.80
13	44.48	49.87	5.4	10.82	62.83	37.01	25.82	69.77
14	73.04	72.21	0.8	1.14	62.83	47.77	15.06	31.53
15	67.28	71.99	4.7	6.54	47.76	52.35	4.59	8.77
16	67.28	70.28	3.0	4.27	47.76	43.83	3.93	8.97
17	67.28	67.36	0.1	0.12	47.76	48.02	0.26	0.54

As shows in the Table 2, the predicted CMD-1 values obtained of surfactin were very well aligned to experimental data. The difference among central points was minimal, also, the highest relative difference between predicted values and experimental values was 15.47%. That proves the adjustment of model in the range studied. The most predicted CMD-2 values of MEL were similar to experimental. However 4 assays have very diverged 9, 10, 13 and 14. These differences may be were related to the extreme condition tested –α and α (Table 1), in which is expected the highest errors, also, this trend is aligned to data obtained from the study of maintenance of surface activity – one a time.

3.1.2.1. Validation of rotational central composite experimental design

Regarding to surfactin, the validation test was carried out with ionic strength that 1.41 coded data, that is, 18.58 $g.L^{-1}$. Even not correlated to equation 1, temperature and pH were also included at their central points, pH 7 and 100 ^{o}C. The validation test resulted in 72.17 $mN.m^{-1}$, which is well aligned to the predicted value is 73.26 $mN.m^{-1}$. Whereas regarding to MEL, the validation should be carried out using the central point, that is, 11.25 $g.L^{-1}$, pH 7 and 100 ^{o}C, which was already done during the CCRD. The Table 2 shows the experimental values 52.35, 43.83, 48.02$mN.m^{-1}$, that were similar to predicted value 47.76 $mN.m^{-1}$.

3.2. OIL DISPLACEMENT TEST

It was already reported that the clear zone of oil displacement is directly proportional to the concentration of biosurfactants – from 50 to 2000 $mg.L^{-1}$ – with crude oil and surfactin, r^2 of 0.997 [15, 16, 17]. Morikawa et al. [16] reported a 72 cm^2 clear zone using crude oil and surfactin solution at 1036.3 $mg.L^{-1}$.

Youssef et al. [17], carried out three methods to measure surface tension activity: oil spreading, drop collapse and blood agar lysis. They reported that drop collapse method followed by the oil displacement is a reliable, simple and easy strategy to identify biosurfactants producer. It could be used to detect biosurfactant produced by a wide range of microorganisms.

Bharali et al. [15] carried out the oil displacement test for screening of biosurfactant producers. The authors described that areas obtained from oils displacement test were between 0.308-0.375 cm^2 using a 10 µL biosurfactant solution at 20.000 $mg.L^{-1}$ and 20 µL of crude oil.

Therefore, the oil displacement test is reliable for measure the concentration of unknown solution of biosurfactant or for the initial identification of biosurfactant producers [15, 16, 17]. However, we used the oil displacement test for the comparison between two biosurfactants (surfactin and MEL) using light, medium and heavy oils (Table 3). Thus, it was possible establish the best relation (highest clear zone) between biosurfactant and oil. These results could be extrapolated for a prospection on MEOR.

Table 3. Clear zone (cm^2) of surfactin and MEL (standard and produced) on light, medium and heavy oils

	Standard surfactin	Standard MEL	Produced surfactin	Produced MEL
Light oil	1.27	2.87	1.98	6.32
Medium oil	3.77	5.91	1.46	11.80
Heavy oil	4.49	6.78	3.97	15.78

Comparing with the results from both standard biosurfactants, there is a trend that both biosurfactants are more feasible to heavy oil at 100 $mg.L^{-1}$, as higher areas were obtained with heavy oil>medium oil>light oil. In this context, MEL showed higher area than surfactin.

The hydrophilic-lipophilic balance (HLB) relates the compound hydrophobicity to its chemical structure of surfactant. The values of HLB equal to 0 represents a completely lipophilic molecules, whereas values around 10 (e.g surfactin) represent equivalent hydrophilic-lipophilic moieties [18]. In this context, the application of compounds with high HLB (<10) such SDS and Tween 20 are better on emulsifying a hydrophobic substance into a water phase, that is, oil into water (o/w) resulting in higher emulsification index and stable emulsion [18]. On the other hand low HLB (>10) such MEL 7-9 are more suited in w/o Kim et al. [13].

Thus, considering that the w/o emulsion is a more hydrophobic system than o/w system, the relation between HLB of biosurfactant and degree of hydrophobicity of any substrate may follow the same trend than the already described HBL of biosurfactant and type of emulsion. That is, in order to obtain the best emulsion, the more hydrophobic substrate, the higher is the HLB of emulsifier.

The produced biosurfactants, presented the same trend that standards. MEL obtained higher clear zone and was more suitable for heavy oil rather than medium and light oils. However, it worth noting that they are product obtained by purification steps, foam overflow and ultrafiltration. Thus, surfactin and MEL were at different concentrations. The concentrations of MEL and surfactin solutions were at 870 $mg.L^{-1}$ and 73.74 $mg.L^{-1}$, respectively. These results indicate the potential of MEL to be applied as MEOR, in particular on heavy oil.

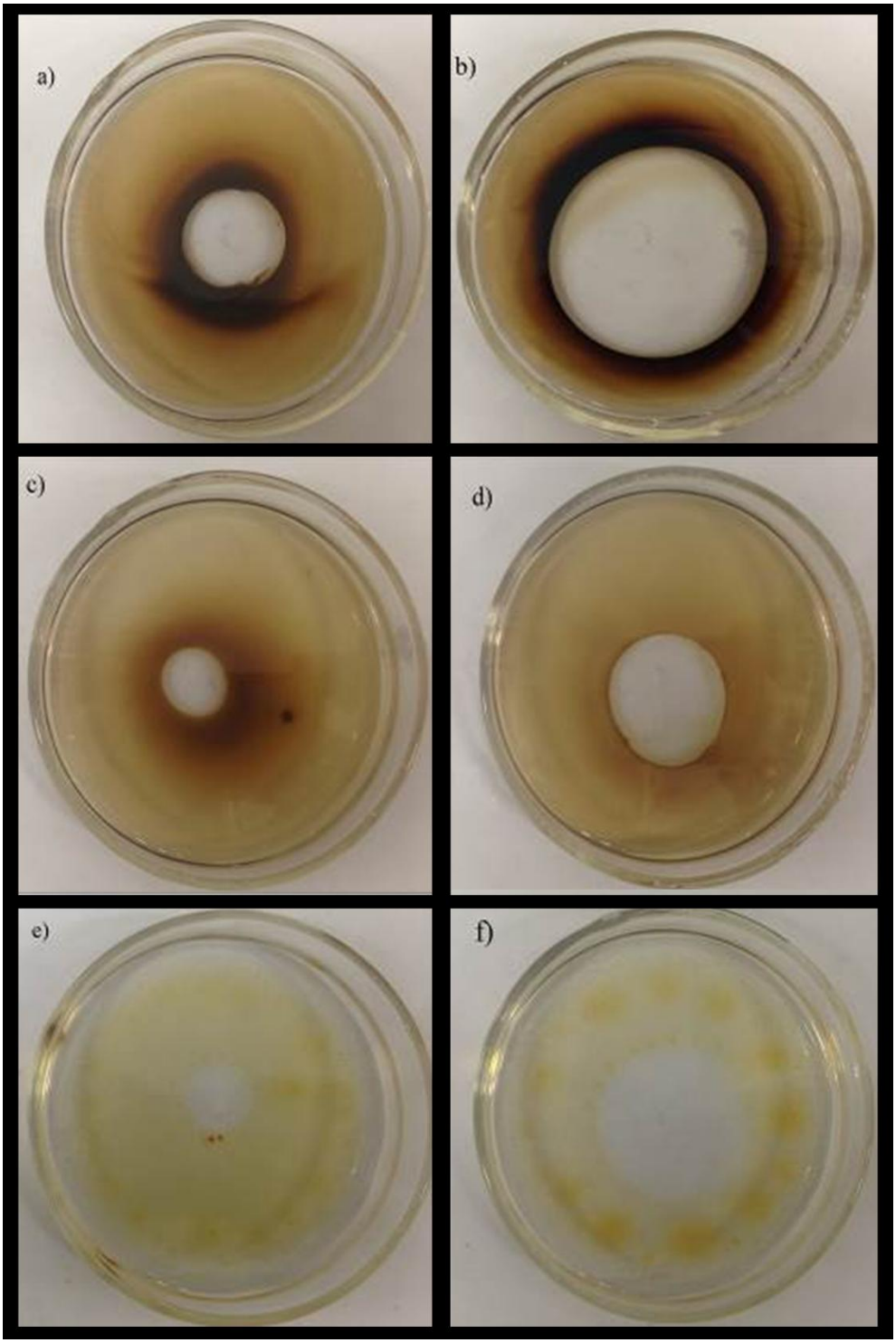


Figure 6. a) – Produced surfactin – heavy oil; b) produced MEL –heavy oil; c) Produced surfactin – medium oil; d) Produced MEL – medium oil; e) Produced surfactin – light oil; f) Produced MEL – light oil.

3.3. APPLICATION OF SYNTHETIC SURFACTANTS AND BIOSURFACTANTS IN REMOVAL OF CRUDE OIL FROM SAND

In the early 1980 was developed the first field test of MEOR in the Daqing oilfield-China, an *in situ* experiment that consist in injecting microbes biosurfactant producers and nutrient solutions into wells. The *in situ* process produces many compounds such as acids organic, enzymes, etc., and also, biosurfactant. Thus, the MEOR yields are mainly related to the presence of biosurfactant and the other compounds are impurities that may improve or decrease the MEOR yield. It was detailed that 6.3×10^4 tons of oil were recovered from 518 wells, in which the average viscosity of crude oil and the hydrocarbon components C_{23}-C_{42} decreased by 30.6% and 60.6%, respectively. On the other hand, the tubing pressure and C_{11}-C_{23}, increased 0.4 MPa and 48.31%, respectively [4].

It is worth nothing that the *in situ* MEOR has low reproducibility due to the inherent variations of any fermentation process, which in this case, is associated to variations of temperature, pH, etc., hydrocarbon composition (unique for each wells), there is no agitation and all microorganisms have to be anaerobic (lower growth rate comparing with aerobic), etc.

Pereira et al. [9] concluded that the biosurfactants are more effective in oil recovery when compared with the chemical surfactants (Enordet and Petrostep). Liu et al. [8], follows that same trend and indicated that more than 95% of petroleum ether could be remove from oil sand using surfactin or SDS solution at 300 $mg.L^{-1}$. Whereas at lower concentration 30 $mg.L^{-1}$, surfactin recovered 88% and SDS 42%.

Khajepour et al. [6] described an interesting micromodel study by comparison of images. They compared two techniques of MEOR, (i) microbial solution treatment and (ii) biosurfactant solution, that is, *in situ* and *ex situ* MEOR, in which both techniques increased the oil recovery, although better results were observed for *in situ* technique. However, as already mentioned, *in situ* MEOR presents low reproducibility, that is, even with higher production cost, *ex situ* MEOR seem a better strategy.

The higher volume recovered the better is the prospection on MEOR between the hydrophobic substrates and biosurfactants (Table 4). The results of control (Milli-Q water) showed as expected the lowest recovered volume. The results of MEL and surfactin had the same trend that oil displacement test, that is, more suitable for heavy oils, also, better results than the standards MEL and surfactin. Comparing with the results of standards MEL and surfactin, standard MEL subtly recovered higher volume.

The main contaminants of petroleum are benzene, ethyl benzene, toluene, xylenes [19]. Regarding to the experiments of removal of crude oil from sand, the differences between standards MEL and surfactin was minimal. Thus, it could be assume that both are feasible and equivalent as agent for these toxic compounds, that is, in this case, the use of MEL and surfactin in the MEOR would recovery oils and also the toxic compounds. These data are also a good prospection on the bioremediation of toxic compounds.

Table 4. Removal of crude oil from sand.

	Milli-Q Water	**Standard surfactin**	**Produced surfactin**	**Standard MEL**	**Produced MEL**
Light oil	0.71	0.75	0.80	0.80	0.80
Medium oil	0.79	0.82	0.81	0.83	0.81
Heavy oil	0.79	0.78	0.81	0.79	0.87
Benzene	0.74	0.68	0.73	0.75	0.71
Toluene	0.70	0.77	0.80	0.77	0.73
Xylene	0.77	0.78	0.80	0.78	0.75

*Recovered volume/Initial volume

Therefore, it seems that surfactin and MEL are more feasible to heavy oil>medium oil> light oil, also, both, surfactin and MEL, showed good prospection on bioremediation.

3.4. EMULSIFICATION INDEX

The relation between contaminants of petroleum (oils) and biosurfactants are one of the more important applications on bioremediation field. Thus, in order to obtain the best results, it is fundamental the understanding of parameters such as concentration of all compounds, chemical structure of biosurfactants and toxic compound, effect of impurities, etc. Broadly, all toxic compounds (benzene, toluene and xylene mixture) presented a stable emulsion up to 120 h. In this context, surfactin (standard and produced) showed emulsification indexes similar to SDS ≈ 50%, whereas MEL (standard and produced)

obtained the lowest emulsification indexes. Thus, probably the use of surfactin for bioremediation (petroleum) is more suitable than MEL.

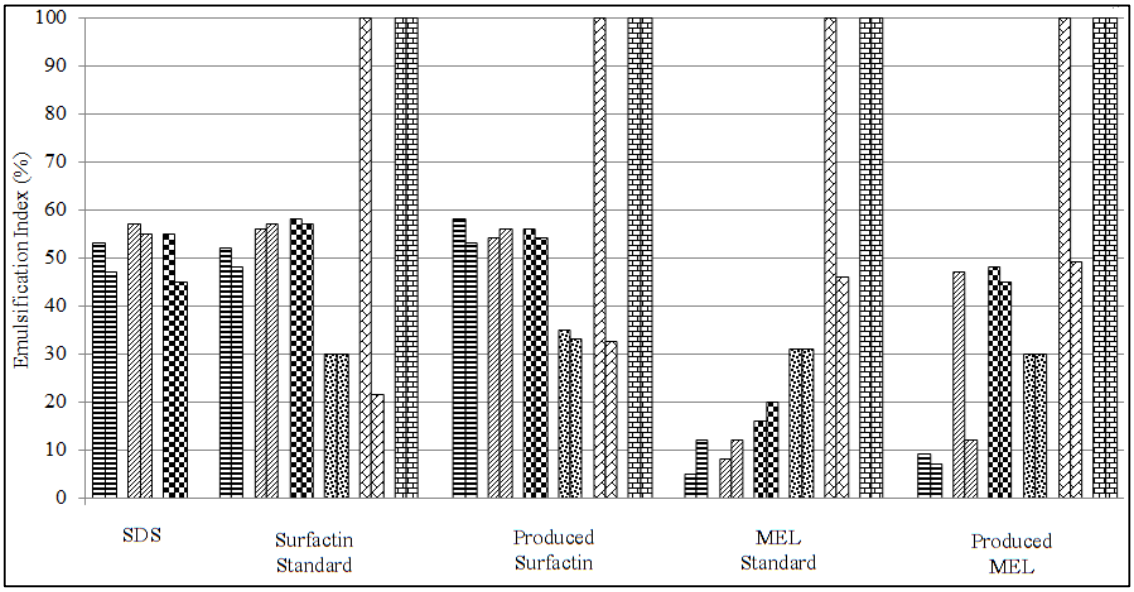


Figure 7. Emulsification Index of crude oils and its toxic compounds (%) – E_{24h} (left) and E_{120h} (right) - Benzene; Toluene; Xylene Mixture; Light Oil; Medium Oil; Heavy Oil.

The light oil showed the lowest emulsification indexes. However, they resulted in stable emulsions up to 120 h. The medium oil presented emulsification indexes of 100% for surfactin and MEL, but they were unstable at 120 h. On the other hand, heavy oil showed the highest emulsification indexes, which were stable up to 120 h. Thus, surfactin and MEL are more feasible for emulsion with heavy oil rather than light and medium oils.

Therefore, surfactin and MEL are indicated for the emulsion with crude oils, in particular heavy oils, which is the one that, probably, will has a significant impact on the petroleum industry. This is strongh evidence that surfactin and MEL can be applied for MEOR.

SDS, surfactin and MEL showed good emulsification index (E_{24h}) for all vegetable oils, with emulsification index >30%.

As already mentioned SDS, MEL and surfactin have different HLB values. Since, the emulsification index is a o/w test, surfactants with high HLB should result in high emulsification index and more stable emulsion, that is, SDS>surfactin>MEL. Although, all emulsifier presented higher emulsification index at E_{24h}. On the other hand at E_{120h} (except for

MEL standard) at least one significant decrease in the emulsification index was observed, which indicate low stability of emulsion. A plausible explanation for this is the lower concentration that was tested 100 $mg.L^{-1}$, usually is at 1000 $mg.L^{-1}$.

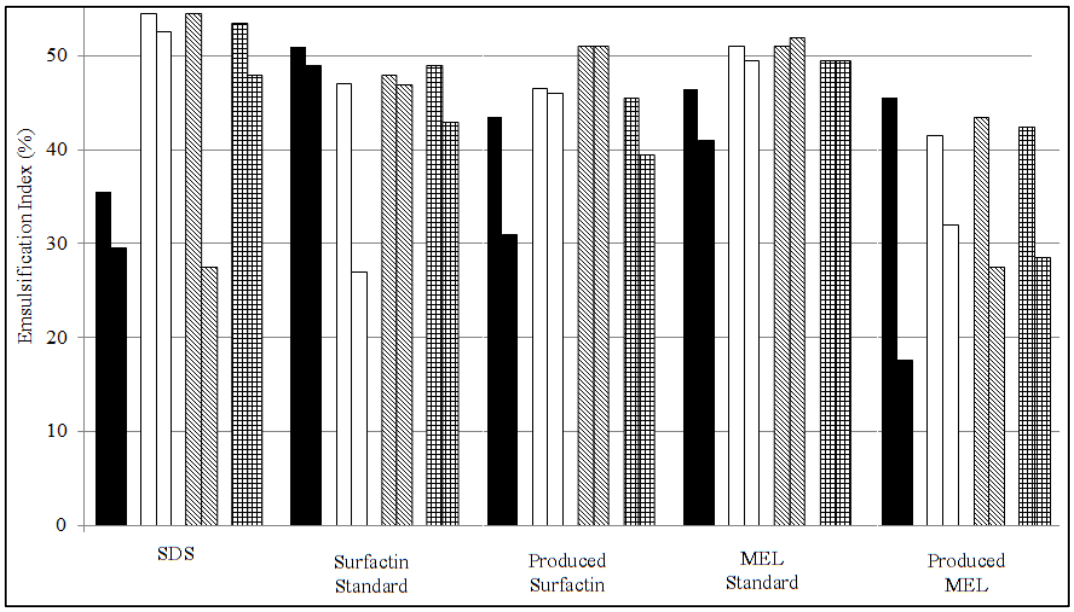


Figure 8. Emulsification Index of vegetable oils (%) – E_{24h} (left) and E_{120h} (right) ■ Sunflower Oil; □ Corn Oil; ▨ Soybean Oil; ▦ Canola Oil.

Kim et al. [13] reported that MEL exhibited similar emulsification activity of dodecylbenzene sulfonate and SDS (all emulsifier were at 20 $mg.L^{-1}$) on soybean oil. However, they used a modified turbidometric method and 1 h, 30° C, 160 rpm as emulsification process.

Santos et al. [20] evaluated the emulsification index of MEL (represented by cell-free culture-broth) using corn, soybean and sunflower oils (1 mL:1µL) and obtained the emulsification index ≈ 47% for all of oils.

Broadly, canola was the best substrate for all biosurfactants and SDS. Also, the surfactin and MEL standards showed good emulsion stability, except for surfactin with corn oil. Also, when comparing the results of biosurfactants produced with its standard, the surfactin formed more stable emulsion than MEL.

Fai et al. [21] described the emulsification index of MEL using the same vegetable oils and conditions, except the concentration of MEL, which was not mentioned. They related the hydrophobicity of oil based on the main fatty acids C16 and C18, that is, usually C16 and C18 together represent more than 85%. Thus, when comparing the

hydrophobicity of oils, the higher percentage of C18, the higher hydrophobic is the oil. Therefore the hydrophobicity order is, sunflower>corn>canola>soybean, which was relatively aligned to the data of E_{24h} proved by Fai et al. [21] sunflower (58%), corn (57%), canola (52%), soybean (51%). However, this work did not found this trend even using standard MEL.

4. CONCLUSION

Probably, MEOR will be an effective methodology in the late period of oilfield exploration. Based on the data of parameters one a time, the surface activity of surfactin was more sensitive to ionic strength and pH, whereas MEL to thermal treatment. The CCRD experimental indicated the response surface with good adjustment of model in the range studied. The oil displacement, removal of crude oil from sand and emulsification index tests followed the same trend, in which surfactin and MEL are more feasible with heavy oil than medium and light oils.

5. REFERENCES

[1] C. Zou, M. Wang, Y. Xing, G. Lan, T. Ge, X. Yan and T. Gu, “Characterization and optimization of biosurfactants produced by *Acinetobacter baylyi* ZJ2 isolated from crude oil-contaminated soil sample toward microbial enhanced oil recovery applications,” *Biochemical Engineering Journal*, vol. 90, pp. 49–58, 2014.

[2] B. Shibulal, S. N. Al-Bahry, Y. M. Al-Wahaibi, A. E. Elshafie, A. S. Al-Bemani and S. J. Joshi, “Microbial enhanced heavy oil recovery by the aid of inhabitant spore-forming bacteria: An insight review,” *The Scientific World Journal*, vol. 2014, pp. 1-12, 2014.

[3] EPA - U.S. Environmental Protection Agency. 2011. Screening-level hazard characterization. Petroleum Coke Category SPONSORED CHEMICALS Petroleum coke. green CASRN 64741-79-3 Petroleum coke. calcined CASRN 64743-05-1. Hazard Characterization Document.
http://www.epa.gov/chemrtk/hpvis/hazchar/Category_Petroleum%20Coke_June_2011.pdf

[4] J-J. Le, X-L. Wu, R. Wang, J-Y. Zhang, L-L. Bai and Z-W. Hou, "Progress in pilot testing of microbial-enhanced oil recovery in the Daqing oilfield of north China," *International Biodeterioration & Biodegradation*, vol. 97, pp. 188-194, 2015.

[5] F. F. C. Barros, A. N. Ponezi and G. M. Pastore, "Production of biosurfactant by *Bacillus subtilis* LB5a on a pilot scale using cassava wastewater as substrate," *Journal of Industrial Microbiology & Biotechnology*, vol. 35, no 9, pp. 1071-1078, 2008.

[6] H. Khajepour, M. Mahmoodi, D. Biria and S. Ayatollah, "Investigation of wettability alteration through relative permeability measurement during MEOR process: A micromodel study," *Journal of Petroleum Science and Engineering*, vol. 120, pp. 10-17, 2014.

[7] D. G. Cooper and B. G. Goldenberg, "Surface-active agents from two *Bacillus* species,"*Applied and Environmental Microbiology*, vol. 53, no 2, pp. 224-229, 1987.

[8] Q. Liu, J. Lin, W. Wang, H. Huang and S Li, "Production of surfactin isoforms by *Bacillus subtilis* BS-37 and its applicability to enhanced oil recovery under laboratory conditions,"*Biochemical Engineering Journal*, vol. 93, pp. 31-37, 2015.

[9] J. F. B. Pereira, E. J. Gudiña, R. Costa, R. Vitorino, J. A. Teixeira, J. A. P. Coutinho and L. R. Rodrigues, "Optimization and characterization of biosurfactant production by *Bacillus subtilis* isolates towards microbial enhanced oil recovery applications,"*Fuel*, vol. 111, 259-268, 2013.

[10] G. Bai, M. L. Brusseau and R. M. Miller, "Influence of cation type, ionic strength, and pH on solubilization and mobilization of residual hydrocarbon by a biosurfactants," *Journal of Contaminant Hydrology*, vol. 30, no 3-4, pp. 265-279, 1998.

[11] L. Thimon, F. Peypoux and G. Michel, "Interactions of surfactin, a biosurfactants from *Bacillus subtilis*, with inorganic cations," *Biotechnology Letters*, vol. 14, no 8, pp. 713-718, 1992.

[12] E. Vass, F. Besson, Z. Majer, L. Volpon and M. Hollósi, "Ca^{2+} -Induced changes of surfactin conformation: A FTIR and circular dichroism study," *Biochemical and Biophysical Research Communications*, vol. 282, no 1, pp. 361–367, 2001.

[13] H-S. Kim, J-W. Jeon, S-B. Kim, H-M. Oh, T-J. Kwon, B-D. Yoon, "Surface and physico-chemical properties of a glycolipid biosurfactant mannosylerythritol lipid, from *Candida antarctica*," *Biotechnology Letters*, vol. 24, no 19, pp. 1637–1641, 2002.

[14] F. Sineriz, R. K. Hommel, H. P. Kleber, "Production of biosurfactants," In: I. E. Cock. (Ed.). Encyclopedia of life support systems. Unesco-Eolls Publishers. Oxford. 2001.

[15] P. Bharali, S. Das, B. K. Konwar and A. J. Thakur, "Crude biosurfactant from thermophilic *Alcaligenes faecalis*: Feasibility in petro-spill bioremediation," *International Biodeterioration & Biodegradation*, vol. 65, no 5, pp. 68-690, 2001.

[16] M. Morikawa, Y. Hirata and T. Imanaka, "A study on the structure function relationship of lipopeptide biosurfactants," Biochimica et Biophysica Acta (BBA) - *Molecular and Cell Biology of Lipids*, vol. 1488, no 3, pp. 211-218, 2000.

[17] N. H. Youssef, K. E. Duncan, D. P. Nagle, K.N. Savage and R. M. Knapp and M. J. McInerney, "Comparison of methods to detect biosurfactant production by diverse microorganisms," *Journal of Microbiological Methods*, vol. 56, no 3, pp. 339-347, 2004.

[18] A. Y. Burch, B. K. Shimada, P. J. Browne and S. E. Lindow, "Novel high-throughput detection method to assess bacterial surfactant production," *Applied and Environmental Microbiology*, vol. 76, no 16, pp. 5363-5372, 2010.

[19] J. A. Andrade, F. Augusto, I. C. Sales and F. Jardim, "Biorremediação de solos contaminados por petróleo e seus derivados," *Eclética Química*, vol. 35, no 3, pp .17-43, 2010.

[20] D. K. F. Santos, R. D. Rufino, J. M. Luna, V. A. Santos, A. A. Salgueiro and L. A. Sarubbo, "Synthesis and evaluation of biosurfactant produced by *Candida* lipolytica using

animal fat and corn steep liquor," *Journal of Petroleum Science and Engineering*, vol. 105, pp. 43-50, 2013.

[21] A. E. C.Fai, A. P. Simiqueli, C. J. Andrade, G. Ghiselli and G. M. Pastore, "Optimized production of biosurfactant from *Pseudozyma tsukubaensis* using cassava wastewater and consecutive production of galactooligosaccharides: An integrated process," *Biocatalysis and Agricultural Biotechnology*, 2015.

DISCUSSÃO GERAL

Capítulo II - ULTRAFILTRATION IN TWO STEPS OF SURFACTIN PRODUCED BY *Bacillus subtilis* LB5A USING CASSAVA WASTEWATER AS SUBSTRATE AND ETHANOL AS MICELLE-DESTABILIZING

A produção de surfactina por *Bacillus subtilis* LB5a usando manipueira como meio de cultura já foi reportada em Erlenmeyer e escala de planta piloto de 80 L (Barros et al. 2008). No entanto, nenhum processo de purificação dessa surfactina foi avaliado (até esta tese). Além disso, também de forma inédita, foi realizada a contagem de células viáveis na espuma produzida no interior do fermentador, que permitiu estabelecer que um significante número de células foi removido durante o bioprocesso, como por exemplo em 36 h ≈ $4x10^4$ células viáveis por mL de espuma. Como o volume de espuma produzido entre 24 e 36 h foi de 330 mL, ≈ 10^6 células foram removidas do bioreator pela espuma.

A coleta de surfactina pela espuma produzida no interior do fermentador resulta em um viés, já que altas taxas de aeração são necessárias para gerar à espuma e recuperá-la, por outro lado condições de microaeração (≈ 30% de oxigênio dissolvido) favorecem a síntese de surfactin por *Bacillus subtilis*. Durante a fermentação utilizando a manipueira, os valores de oxigênio dissolvido permanecerram em 0% na maior parte do tempo, além disso foi calculado o seguinte coeficiente volumétrico de transferência de oxigênio - *Kla* 102.02 h^{-1}. Neste contexto Fahim et al. (2012) descreveu que a *Kla* ótimo para a produção de surfactina é igual a 216 h^{-1}. Portanto, os processos fermentativos foram conduzidos em boas condições de aeração, pois os valores de oxigênio dissolvido permaneceram próximos a 0% (favorecem a síntese de surfactin por *Bacillus subtilis*) e grandes volumes de espuma foram coletados (≈ 1000 mL).

O maior volume coletado de espuma foi obtido entre 24 e 36 h de fermentação, que por sua vez é alinhado com o perfil de células viáveis (células metabolicamente ativas resultam em maior produção de surfactina e consequente maior formação de espuma). Esses dados mostram que a produção de surfactina é associada ao cresimento microbiano.

A análise de cromatografia líquida de alta eficiência indicou que o biossurfactante bruto continha ≈ 36% de surfactina. Assim ≈ 1 grama de surfactina foi produzido por batelada (3 litros de meio de cultura), ou seja 336 mg de surfactina por litro de meio de cultura. Esse rendimento é menor que o indicado por Isa et al. (2007), que reportou 583 mg de surfactina por litro de meio de cultura, no qual os autores recuperaram a surfactina diretamente do meio de cultura (e nãopela formação de espuma no interior do fermentador). Vale a pena mencionar que neste estudo a produção de surfactina não foi otimizada (maiores rendimentos podem ser

obtidos), e também que o rendimento de surfactina foi subestimado pois foi considerado que toda surfactina foi recuperada pela espuma (uma pequena porcentagem de surfactina permaneceu, no meio de cultura, na paredes do bioreator, mangueiras, etc).

Comparando-se as três estratégias de ultrafiltração, a pureza do produto em termos de proteína (P_p) da estratégia iii apresentou os maiores valores P_{pi} 67% e P_{pii} 80%, primeira e segunda etapa de ultrafiltração, respectivamente. A estratégia ii também apresentou bons resultados (P_{pi} 43% e P_{pii} 59%) porém a estratégia ii apresentou problemas de incrustação e polarização por concentração.

Jauregi et al. (2013) descreveu a ultrafiltração da surfactina, depois de produzi-la usando meio de cultura sintético. Os autores reportaram que P_{pi} foi ≈ 92% usando uma membrana de polietersulfônica com 100 kDa de peso molecular de corte e P_{pii} ≈ 94%. Isa et al. (2008) obteve P_{pi} ≈ 88% e P_{pii} ≈ 96% usando uma membrana de polietersulfônica 10 kDa. Portanto, comparados a produção de surfactina com manipueira como meio de cultura (Capítulo I desta tese), melhores resultados de P_p foram obtidos quando a surfactina foi produzida usando meio sintético como meio de cultura. Provavelmente devido a menor concentração de proteína na alimentação (ultrafiltração).

A análise de ionização por dessorção a laser assistida por matriz seguida pela detecção em um analisador do tipo tempo de vôo revelou a produção dos seguintes homólogos de surfactina (1045-1080 m/z): (i) 1043.53; (ii) 1049.57; (iv) 1065.57; (v) 1066.58; (vi) 1068.58; (vii) 1079.60; (viii) 1082.57; (ix) 1093.55; (x) 1096.62 e (xi) 1109.60 (m/z). Os homólogos de surfactina foram claramente separados em 3 groups (≈ 1066, 1079 e 1093 m/z). Esses grupos são provavelmente relacionados com o comprimento do ácido graxo. Portanto, foram produzidos potencialmente 11 homólogos de surfactina por *Bacillus subtilis* LB5a usando manipueira como meio de cultura. A análise de espectroscopia no infravermelho da surfactina produzida com manipueira como meio de cultura foi similar com a reportada por Faria et al. (2011), ou seja, forte absorção da banda em 1639 cm^{-1}, que corresponde ao peptídeo. A análise de espectroscopia de ressônancia nuclear magnética indicou a presença de duas sequências de aminoácidos S e S´- Glu1-Leu2-Leu3-Val4-Asp5-Leu6-Leu7 e Glu1´-Leu2´-Leu3´-Val4´-Asp5´-Leu6´-Val7.

Capítulo III - Production, purification and identification of mannosylerylthritol lipids produced by *Pseudozyma tsukubaensis* using cassava wastewater as substrate - ultrafiltration and scale-up

Em relação aos experimentos no bioreator de bancada, a análise da contagem celular indicou que a maior taxa de desenvolvimento da fase exponencial ocorreu entre 24 e 36 h. Essa relação já era esperada, pois em 24 h a aeração e agitação foram aumentadas de 0,04 vvm e 100 rpm para 0,08 vvm e 150 rpm. A fase estacionária ocorreu em 36 h, porém nos experimentos em Erlenmeyers a fase estacionária ocorreu em 48 h. Essa diferença (12 h) é associada com as melhores condições fornecidas pelo bioreator de bancada (transferência de oxigênio, controle de temperatura, agitação, etc.). Os dados de contagem celular no bioreator de bancada foram sutilmente menores que nos experimentos em Erlenmeyers, provavelmente devido a recuperação dos manosileritritol lipídeos pela produção de espuma no interior do fermentador, que por sua vez pode carrear células do sistema.

Os valores obtidos de tensão superficial da espuma coletada durante a fermentação foram similares aos dados previamente reportados (Arutchelvi et al. 2008, Sajna et al. 2013, Yu et al. 2015). Esses dados indicam que houve a produção de manosileritritol lipídeos por *Pseudozyma tsukubaensis* utilizando manipueira como meio de cultura. É válido notar que espuma coletada foi composta majoritariamente por manosileritritol lipídeos e proteínas. Proteínas também possuem propriedades tensoativas, que podem influenciar as medidas de tensão superfical (comparando-se com os dados previamente reportados na literatura).

Portanto, muito provavelmente a manipueira é um bom meio de cultura para a produção de biossurfactantes por *P. tsukubaensis* devido ao grande volume de espuma coletada ao final da fermentação. Existem também fortes evidências que a espuma foi composta por manosileritritol lipídeos (valores obtidos de tensão superficial).

Soforolipídios e manosileritritol lipídeos são biossurfactantes largamente produzidos por micro-organismos (> 100 $g.L^{-1}$ para manosileritritol lipídeos e 300 $g.L^{-1}$ para soforolipídios) (Hubert et al. 2012, Sajna et al. 2013). Por exemplo, Konishi et al. (2011) reportou a produção de 49,2 g de manosileritritol lipídeos. por litro de meio de cultura em fermentação do tipo batelada e um consórcio de fontes de carbono (10 $g.L^{-1}$ extrato de levedura, 100 $g.L^{-1}$ glicose, e 100 $g.L^{-1}$ azeite). Em um estudo subsequente, os autores conduziram a fermentação em batelada alimentada e alcançaram 129 g de manosileritritol lipídeos por litro de meio de cultura. Sajna et al. (2013), obteve 34 g de manosileritritol

lipídeos por litro de meio de cultura usando óleo de soja (8% $w.v^{-1}$), extrato de levedura e minerais.

A ultrafiltração em equipamento de bancada levou 45 minutos e reduziu o volume inicial de 250 mL para 25 mL usando um sistema de recirculação (alimentação/retido). Durante os primeiros 25 minutos, o fluxo reduziu significativamente de 90 para 55 $L.m^{-2}.h^{-1}$. A concentração de manosileritritol lipídeos (alimentação/retido) aumentou de 294.7 $mg.L^{-1}$ para 859.52 $mg.L^{-1}$. Esses dados provam que a membrana de polietersulfônica com 100 kDa de peso molecular de corte reteve as nanopartículas de manosileritritol lipídeos, além disso a concentração de proteínas (alimentação/retido) foi significantemente reduzida (proteínas foram permeadas).

A ultrafiltração das nanopartículas de manosileritritol lipídeos é um processo tecnicamente possível e interessante. Os experimentos de ultrafiltração foram realizados com 272 mg de espuma liofilizada, que foram dissolvidos em 250 mL de tampão Tris (1091.59 $mg.L^{-1}$). Após o processo de ultrafiltração, o produto purificado (25 mL de alimentação/retido) estava em um concentração ≈ 860 mg de manosileritritol lipídeos por litro, ou seja, 21,5 mg de manosileritritol lipídeos (25 mL x 860 mg.1000 mL^{-1}). Portanto, 272 mg de espuma liofilizada resultaram em 21,5 mg de manosileritritol lipídeos. Cada processo fermentativo produziu ≈ 14,01 g de espuma liofilizada. Teoricamente ≈ 1,1 g de manosileritritol lipídeos purificado poderia ser produzido pela integração entre o processo fermentativo (uma batelada) e a ultrafiltração.

A análise de cromatografia gasosa acoplada à espectrometria de massa indicou a presença dos seguintes ácidos graxos C8:0; C10:0; C12:1; C12:0; C14:1 e C18:1, no qual C8:0, C12:1 e C14:1 foram os ácidos graxos majoritários. Esses resultados são relativamente similares aos descritos por Sajna et al. (2013), C14:1, C16:0, C16:1 e também por Fukuoka et al. (2008) C12 e C14 molecules.

Pseudozyma tsukubaensis produziu vários homólogos de manosileritritol lipídeos, no qual os maiores picos foram 683,41 e 657,42 m/z. Essa diversidade pode ser atribuida ao comprimento dos ácidos graxos ligados aos C-2´ e C-3´ da manose (Figuras 6 e 7 do Capítulo III), como demostrado pela análise de ácidos graxos (cromatografia gasosa acoplada à espectrometria de massa).

Em teoria e desconsiderando os ácidos graxos e o eritritol, a razão massa/carga (m/z) dos manosileritritol lipídeos-B e manosileritritol lipídeos-C é idêntica. Sajna et al. (2013) analizou a produção de manosileritritol lipídeos-C por análise de ionização por dessorção a laser assistida por matriz seguida pela detecção em um analisador do tipo tempo

de vôo e observou que os principais picos foram 607,42, 634,57 e 660,57 m/z. Assim, a análise dos dados de ionização por dessorção a laser assistida por matriz seguida pela detecção em um analisador do tipo tempo de vôo deste estudo mostrou alta similaridade com dados previamente reportados, que por sua vez é uma forte enviência da produção de manosileritritol lipídeos-B ou manosileritritol lipídeos-C. A análise de espectroscopia no infravermelho indicou alta absorção em 3400 (O-H), 1730 (C=O), 1240 (C-O) e 1075 (-O-), que por sua vez são resultados semelhantes aos obtidos por Kitamoto et al. (1990).

A análise dos dados de espectroscopia de ressônancia nuclear magnética indicou que a amostra de manosileritritol lipídeos purificada é do tipo manosileritritol lipídeos-B, no qual R_1 (C-2) e R_2 (C-3) são do grupo acil, R_3 é uma hidroxila e R_4 é um grupo acetil (Figura 7 do Capítulo 3). Foi também identificado a presença de um segundo estereoisômero (8-10%).

Capítulo IV - Comparative study on microbial enhanced oil recovery using mannosylerithritol lipids or surfactin and their emulsification properties.

Em relação a avaliação dos fatores (separadamente), a força iônica afetou as propriedades tensoativas da surfactina, na qual os ensaios com maior força iônica apresentaram as maiores medidas de tensão superficial e também de diluição micelar crítica. No entanto vale a pena mencionar o baixo valor de tensão superficial (alta atividade tensoativa) mesmo nos ensaios com alta força iônica 10 e 20 $g.L^{-1}$, 33,19 e 40,02 $mN.m^{-1}$, respectivamente.

Thimon et al. 1992, avaliaram as medidas de tensão superficial da surfactina em diferentes condições, complexadas e não-complexidas (Ca^{+2}, Ba^{+2}, Li^{+}, Rb^{+}, Na^{+} e K^{+}) e concluiram que todos os cátions resultaram em menores valores de tensão superficial (maior tensoatividade). Vass et al. 2001 estudaram a conformação da surfactina por espectroscopia de infravermelho com transformada de Fourier e dicroísmo circular, na presença e ausência de íons de Ca^{+2} e concluiram que a conformação da surfactina (β ou γ-turn) é dependente da presença de íons.

Em relação a surfactina, nenhuma diferença foi observada entre as medidas de tensão superficial ou diluição micelar crítica para os tratamentos térmicos (79, 100 e 121 ^{o}C). Por outro lado, o mesmo tratamento térmico afetou significativamente as medidas de tensão superficial e diluição micelar crítica dos manosileritritol lipídeos tratados térmicamente. O resultado referente aos manosileritritol lipídeos é semelhante ao reportado por Kim et al.

(2002) que descreveram a manutenção da atividade tensoativa dos manosileritritol lipídeos-SY16 após 1 h de tratamento térmico (20 to 90 ^{o}C).

A solubilidade de compostos iônicos é altamente afetada por mudanças no pH (ponto isoelétrico), por exemplo a surfactina (biossurfactantes aniônico) precipita em pH 2 (Shibulal et al. 2014). Por outro lado, a solubilidade de compostos não-iônicos tais como os glicolipídeos manosileritritol lipídeos, não é significativamente afetada por alterações no pH (Sineriz et al. 2001). A solubilidade dos biossurfactantes é relacionada com os valores de tensão superficial, no qual a maior solubilidade resulta em maior atividade superficial (menor valor de tensão superficial). Assim, a atividade superficial da surfactina e diluição micelar crítica devem ser significativamente afetadas por mudanças no pH, diferentemente dos manosileritritol lipídeos, no qual nenhuma ou sutis mudanças na atividade superficial devem ser observadas.

É fundamental compreender que os experimentos relacionados as alterações do pH não avaliaram a estabilidade dos biossurfactantes, visto que fatores associados com a redução da solubilidade dos biossurfactantes e mudanças na conformação estrutural dos biossurfactantes são diretamentes relacionadas com as medidas de atividade superficial. Entretanto, a hipóteses de ruptura estrutural química dos biossurfactantes, quando expostos a pH extremos não pode ser discartada.

Baseado nos experimentos (interação entre os parâmetros - pH, temperatura e força iônica), a força iônica foi o parâmetro mais significante na atividade superficial da surfactina. Por outro lado, o pH e a temperatura influênciaram minimamente a atividade tensoativa da surfactina. Diferentemente da surfactina, os experimentos com manosileritritol lipídeos indicaram que pH, força iônica e temperatura apresentaram efeitos significativos na atividade superficial.

Em relação aos experimentos de dispersão de óleo, os biossurfactantes produzidos com manipueira como meio de cultura e depois ultrafiltrados (manosileritritol lipídeos e surfactina) apresentaram as mesmas tendências, ou seja, os manosileritritol lipídeos apresentaram maior área de dispersão (em relação a surfactina) e melhores resultados com petróleo pesado (petróleo leve e intermediário).

Os experimentos com o petróleo leve obtiveram os menores índices de emulsão, porém apresentaram emulsões estáveis até 120 h. Por outro lado, os experimentos com petróleo intermediário apresentaram máximos índices de emulsão para a surfactina e manosileritritol lipídeos. No entanto, em ambos os casos as emulsões não foram estáveis até 120 h. Os experimentos com petróleo pesado apresentaram altos índices de emulsão com

estabilidade até 120 h. Portanto, a aplicação de surfactina e manosileritritol lipídeos são mais indicadas para petróleo pesado do que para os petróleos intermediário e leve.

REFERÊNCIAS

J. I. Arutchelvi, S. Bhaduri, P. V. Uppara and M. Doble, "Mannosylerythritol lipids: a review," *J Ind Microbiol Biotechnol* **35**:1559-1570, (2008).

F. F. C. Barros, A. N. Ponezi and G. M. Pastore, "Production of biosurfactant by *Bacillus subtilis* LB5a on a pilot scale using cassava wastewater as substrate," *J Ind Microbiol Biotechnol* **35**:1071-1078, (2008).

S. Fahim, K. Dimitrov, F. Gancel, P. Vauchel, P. Jacques and I. Nikov, "Impact of energy supply and oxygen transfer on selective lipopeptide production by *Bacillus subtilis* BBG21," *Bioresour Technol* **126**:1-6, (2012).

A. F. Faria, D. S. Teodoro-Martinez, G. N. O. Barbosa, B. G. Vaz, I. S. Silva, J. S. Garcia, M. R. Tótola, M. N. Eberlin, M. Grossman, O. L. Alves L. R and Durrant, "Production and structural characterization of surfactin (C14 /Leu7) produced by *Bacillus subtilis* isolate LSFM-05 grown on raw glycerol from the biodiesel industry," *Process Biochem* **46**:1951-1957. (2011).

F. Fukuoka, T. Morita, M. Konishi, T. Imura and D. Kitamoto, A "*basidiomycetous* yeast, *Pseudozyma tsukubaensis*, efficiently produces a novel glycolipid biosurfactant. The identification of a new diastereomer of mannosylerythritol lipid-B," *Carbohy Res*, **343**:3555-560, (2008).

J. Hubert, K. Ple´, M. Hamzaoui, G. Nuissier, I. Hadef, R. Reynaud, A. Guilleret and J-H. Renault, "New perspectives for microbial glycolipid fractionation and purification processes," *Comptes Rendus Chimie* **15**:18-28, (2012).

M. H. M. Isa, D. E. Coraglia, R. A. Frazier and P. Jauregi, "Recovery and purification of surfactin from fermentation broth by a two steps ultrafiltration process," *J Membrane Sci* **296**: 51-57, (2007).

P. Jauregi, F. Coutte, L. Catiau, D. Lecouturier and P. Jacques, "Micelle size characterizationof lipopeptides produced by *B. subtilis* and their recovery by the two-step ultrafiltrationprocess," *Sep Purif Technol*, **104**:175-182, (2013).

M. Konishi, T. Nagahama, T. Fukuoka, T. Morita, T. Imura, D. Kitamoto and Y. Hatada, "Yeast extract stimulates production of glycolipid biosurfactants, mannosylerythritol lipids, by *Pseudozyma hubeiensis* SY62," J Biosci Bioeng **111**:702-705, (2011).

D. Kitamoto, S. Akiba, C. Hioki and T. Tabuchi, "Extracellular accumulation of mannosylerythritol lipids by a strain of *Candida Antarctica*," *Agric Biol Chem*, **54**:31-36, (1990).

K. V. Sajna, R. K. Sukumaran, H. Jayamurthy, K. K. Reddy, S. Kanjilal, R. B. N. Prasad, A. Pandeya, "Studies on biosurfactants from *Pseudozyma* sp. NII 08165 and their potential application as laundry detergent additives," *Biochem. Eng. J.* **78**:85-92, (2013).

B. Shibulal, S. N. Al-Bahry, Y. M. Al-Wahaibi, A. E. Elshafie, A. S. Al-Bemani and S. J. Joshi, "Microbial enhanced heavy oil recovery by the aid of inhabitant spore-forming bacteria: An insight review," *Sci W J*, **2014**:1-12, (2014).

F. Sineriz, R. K. Hommel and H. P. Kleber, "Production of biosurfactants," In: I. E. Cock. (Ed.). Encyclopedia of life support systems. Unesco-Eolls Publishers. Oxford, (2001).

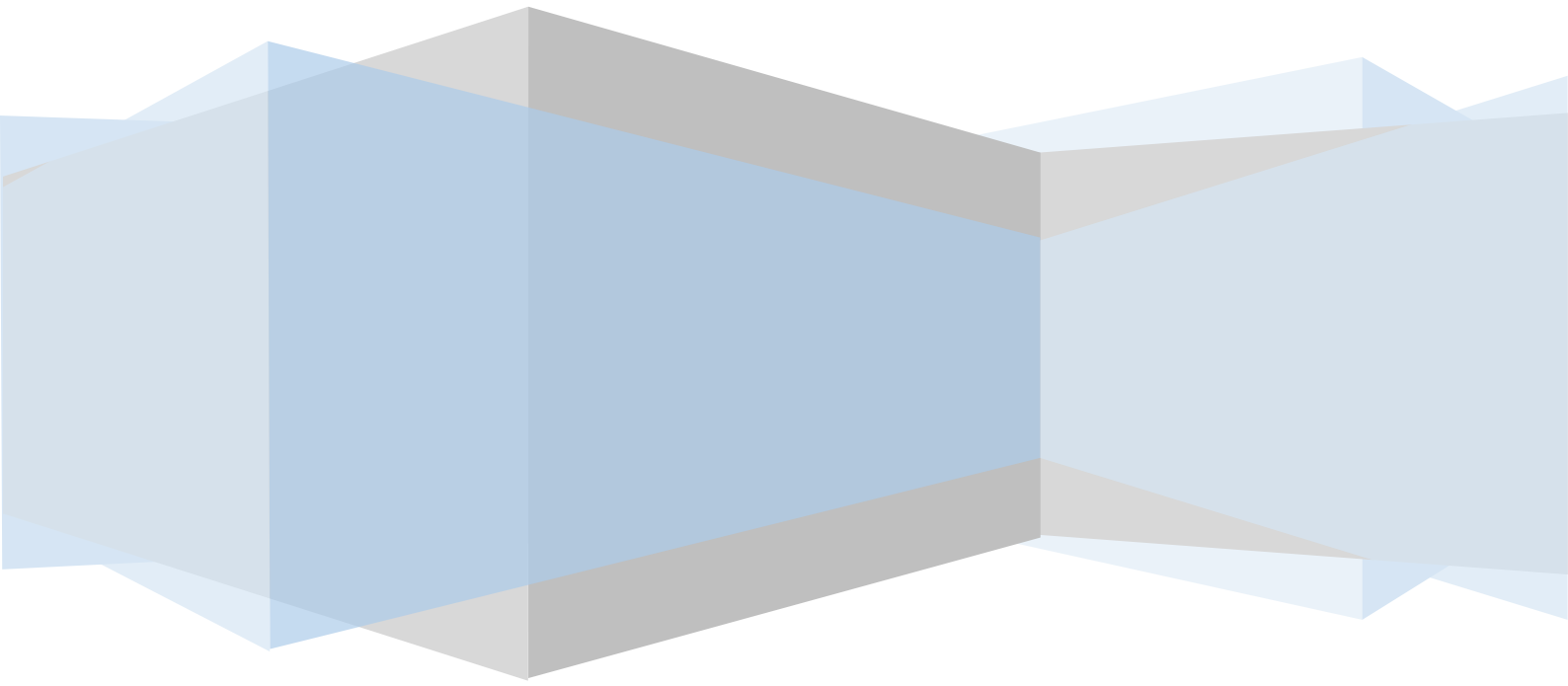
E. Vass, F. Besson, Z. Majer, L. Volpon and M. Hollósi, "Ca^{2+} -Induced changes of surfactin conformation: A FTIR and circular dichroism study," *Biochemical Biophy R Commun*, **282**:361–367, (2001).

L. Thimon, F. Peypoux and G. Michel, "Interactions of surfactin, a biosurfactants from *Bacillus subtilis*, with inorganic cations," *Biotechnol Letters*, **14**:713-718, (1992).

H-S. Kim, J-W. Jeon, S-B. Kim, H-M. Oh, T-J. Kwon and B-D. Yoon, "Surface and physico-chemical properties of a glycolipid biosurfactant mannosylerythritol lipid, from *Candida antarctica*," *Biotechnol Letters*, **24**:1637–1641, (2002).

M. Yu, Z. Liu, G. Zeng, H. Zhong, Y. Liu, Y. Jiang, M. Li, X. He and Y. He, “Characteristics of mannosylerythritol lipids and their environmental potential,” *Carbohy Res* **407**:63-72, (2015).

CONCLUSÃO GERAL



CONCLUSÃO GERAL

A surfactina foi produzida por *Bacillus subtilis* LB5a em bioreator (3 litros de volume de trabalho) usando manipueira como meio de cultura. A espuma (alto teor de surfactina) foi coletada pelo topo do bioreator e utilizada para os cálculos de rendimento do processo e avaliação da purificação por ultrafiltração. Foram produzidos ≈ 336,66 mg de surfactin por litro de meio de cultura. Em relação a surfactina, a ultrafiltração foi realizada em duas etapas (i) na qual as micelas de surfactinas foram retidas e, (ii) na qual a adição de solvente orgânico (EtOH) desestabilizou as micelas de surfactina, permitindo que moléculas de surfactina livres (não agregadas) fossem recuperadas no permeado. O processo de ultrafiltração utilizou membranas de polietersulfônica com dois pontos de corte molar, 100 kDa e 50 kDa. Sendo a melhor estratégia a utilização da membrana de 100 kDa na primeira etapa de ultrafiltração e 50 kDa na segunda etapa de ultrafiltração. A ultrafiltração do biossurfactante bruto foi associada com incrustração e/ou polarização por concentração. A ultrafiltração do biossurfactante semipurificado foi adequada, resultando em alta recuperação da surfactina (78,25%) com elevada separação das proteínas e problemas mínimos de incrustração e polarização por concentração. Assim, por um lado o uso de manipueira para a produção de surfactina reduz o custo de produção, por outro lado, dificulta o processo de purificação. Já que a produção, purificação e aplicação devem ser avaliadas sequencialmente, o uso da manipueira como meio de cultura deve ser integrado a processos de purificação alternativos a ultrafiltração, ou as proteínas da manipueira devem ser retiradas anteriormente ao processo fermentativo. A determinação estrutural química da surfactina foi realizada por duas análises, (i) ionização por dessorção a laser assistida por matriz seguida pela detecção em um analisador do tipo tempo de vôo (MALDI-TOFMS) e, (ii) espectroscopia de ressônancia nuclear magnética (RNM). Foram identificadas 11 isoformas, que por sua vez são compostas por diferentes β-ácidos graxos e duas sequencias de aminoácidos S e S´- Glu1-Leu2-Leu3-Val4-Asp5-Leu6-Leu7 e Glu1´-Leu2´-Leu3´-Val4´-Asp5´-Leu6´-Val7´.

Manosileritritol lipídeos (MEL) foram produzido por *Pseudozyma tsukubaensis* em bioreator (3 litros de volume de trabalho) usando manipueira como meio de cultura. A espuma (alto teor de MEL) foi coletada pelo topo do bioreator e utilizada para os cálculos de rendimento do processo e avaliação da purificação por ultrafiltração. Foram produzidos ≈ 1,26 g de MEL por litro de meio de cultura, ou seja, manipueira é um meio de cultura adequado a produção de MEL por *P. tsukubaensis*. Os experimentos de ultrafiltração com o MEL, removeram ≈ 95% de proteínas e retiveram (vesículas) ≈ 80% dos MEL. Portanto, uma

única etapa de ultrafiltração foi necessária para a purificação dos MEL. O processo de ultrafiltração foi escalonado de 20 mL (dispositivo de centrifugação) para 500 mL (equipamento de ultrafiltração de bancada), e os resultados não mostraram disparidade.

A recuperação dos MEL pela formação de espuma integrada a ultrafiltração é uma notável estratégia, já que não utiliza solvente orgânico, ou seja, alinhado com o conceito de química verde, e também teoricamente de menor custo

A determinação estrutural química dos MEL produzido neste estudo foi realizada por três análises, (i) MALDI-TOFMS, (ii) RNM, e (iii) cromatografia gasosa acoplada a espectrometria de massa (CG-MS). A análise dos dados obtidos com a MALDI-TOFMS indicou que foram produzidas duas principais isoformas de MEL, 683,41 m/z and 657,42 m/z. A análise dos dados de RNM confirmou a produção de MEL-B e revelou a produção de um segundo esterioisômero (≈ 9%). A análise dos dados de CG-MS indicou que os principais ácidos graxos associados ao MEL foram C8:0, C12:1 e C14:1.

Como trabalhos futuros, indicamos a avaliação da produção de manosileritritol lipídeos utilizando manipueira suplementada com compostos hidrofóbicos, com o objetivo de avaliar o aumento do rendimento de produção e efeitos no processo de ultrafiltração.

O aumento da recuperação de petróleo por micro-organismos ou seus metabótitos é uma eficiente metodologia na fase final da exploração de poços de petróleo. Os experimentos de dispersão de óleo, remoção de petróleo da areia e índice de emulsificação apresentaram a mesma tendência, no qual surfactina e manosileritritol lipídeos apresentaram melhores resultados com o petróleo pesado. Os testes de emulsão apresentaram melhores resultados (índice de emulsão e estabilidade) com petróleo do que com óleos vegetais.

APÊNDICE

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Comparative study: bench-scale surfactin production from *Bacillus subtilis* using analytical grade and concentrated glycerol from the biodiesel industry

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Abstract

The market price of glycerol worldwide tends to decrease, since it is a by-product of biodiesel production. Thus its biotechnological use might lead to significant reduction in the cost of fermentations. The aim of this study was to compare the production of surfactin in peptone culture media supplemented with analytical grade glycerol (AGG) and concentrated glycerol from biodiesel production (CGBP). Differences were observed between the two processes including cell growth and dissolved oxygen consumption. The semi-purified biosurfactant produced with AGG was composed of about 21 surfactin isoforms, whereas the semi-purified biosurfactant with CGBP showed only 6 surfactin isoforms. Interestingly the lower molecular weight surfactin isoforms were not produced when CGBP was used. Surfactin yield was 325.19 mg/L with AGG and 71.13 mg/L with CGBP, which proves the impact and importance of the purity of glycerol both on the yield of surfactin as in the composition of surfactin isoforms. Therefore, as surfactin is a high value-added product, the use of glycerol with high purity is fundamental to achieve higher productivity and broad spectrum of surfactin isoforms.

Key-words: *Bacillus subtilis*; biodiesel; fermentation; glycerol; surfactin.

[1] Analytical Grade Glycerol - AGG

[2]Concentrated glycerol from biodiesel production - CGBP

1. Introduction

Brazil ranks among the top 5 world's largest producers and consumers of biodiesel, which produced ≈ 2,696.00 m^3 and 2,741.115 m^3 in 2011 and 2012, respectively [1-2]. Glycerol is the main by-product of biodiesel production. It represents approximately 10% of the volume of a reaction [1, 3]. However, glycerol from the biodiesel industry has a low aggregate value due to the presence of impurities [3, 4]. Thus, in years to come, due to increasing biodiesel production the price of glycerol will tend to decrease.

Glycerol is a fermentable polyol (sugar alcohol) nutrient for most bacteria and yeasts. In addition, depending on the source of triglycerides used in biodiesel production, raw glycerol can contain nutritional elements such as phosphorus, sulfur, magnesium, calcium, nitrogen and sodium, which can be used by microorganisms in the fermentation process [5]. Thus, the by-product from biodiesel industry can be used as a low-cost substrate for bioproduction of high added value products such as biosurfactants [1, 3, 5]. It is known that a wide variety of microorganisms produce biosurfactants, including *Bacillus subtilis*, which synthesizes lipopeptides (e.g. surfactin) [3]. Surfactin (98% purity) is available from Sigma Chemical Company at approximately $ 15.3/mg. Makkar et al. [6] suggested that the perfect scenario would be to have biosurfactants priced at ≈ $ 0.011/mg, which would make the biosurfactants economically equivalent to surfactants.

One way of reducing bioproduction cost is by using low cost nutrients as culture medium (fermentation) such as industrial waste or by-product, for instance, glycerol from the biodiesel industry. At the same time the use of glycerol from biodiesel industry could improve the profitability of biodiesel in a broader sense for biorefineries. However, a few papers have detailed surfactin production from *Bacillus subtilis* using glycerol from biodiesel production as carbon source. In particular, there is a lack of knowledge on the effect of glycerol purity on productivity.

We speculate that the purity of glycerol from industrial biodiesel production has significant effect on the productivity of surfactin. Thus, the aim of this study was to evaluate the surfactin production from *Bacillus subtilis* LB2b, mainly, on a bench-scale bioreactor using glycerol of two different purities: (1) concentrated glycerol from biodiesel production (by-product of biodiesel industry after removal of methanol) (CGBP), (2) analytical grade glycerol (AGG).

2. Materials and Methods

2.1. Chemicals

The chemicals used: acetonitrile (Synth ≈ 99.8%), analytical grade glycerol (Sigma-Aldrich ≈ 86-89%), bicinchoninic acid kit (Sigma-Aldrich), bovine serum albumin (Sigma-Aldrich ≥ 98%), chloroform (Synth ≈ 99.8%), hydrochloric acid (Lafan ≈ 37%), methanol (Sigma-Aldrich ≥ 99.6%), periodic acid (Vetec ≥ 99%), potassium dichromate (Impex ≥ 99%), phosphoric acid (Sigma-Aldrich ≥ 85%), sodium hydroxide (Sigma-Aldrich ≥ 97%), sodium iodide (Synth – analytical grade), sodium thiosulfate (Synth–0.05 M), sulfuric acid (Merck 98%), surfactin (Lipofabrik ≥ 99%), and trifluoroacetic acid (Sigma-Aldrich ≥ 99%).

2.2. Preliminary study – Culture medium

A preliminary study with different culture media (flask fermentation) was conducted to assess the growth and surface tension of *B. subtilis* LB2b: (i) peptone plus raw glycerol from biodiesel industry, (ii) AGG plus peptone; (iii) CGBP plus peptone. Then, based on the results of flask experiments, bench-scale bioreactor experiments were carried out to investigate in more detail the effect of glycerol purity on surfactin productivity and surfactin isoforms. For this, two glycerol types were investigated separately: (1) AGG and (2) CGBP.

2.3. Microoganisms and inoculum

Bacillus subtillis LB2b pertaining to laboratory collection of Bioflavour/Fea/UNICAMP collection, previously identified as biosurfactants producer was used [7]. The inoculum was standardized according to Barros et al. [8].

2.4. Culture media

The culture media were prepared with the following compositions (g/L in distilled water): bacto-peptone 10.0 and glycerol 10.0. In view of the objectives of this study, glycerol from three different sources was used separately: analytical grade glycerol (AGG), concentrated glycerol from biodiesel production (CGBP) and raw glycerol from biodiesel industry. Raw glycerol was used only on the flask experiments. Raw glycerol was produced by the base-catalyzed transesterification (NaOH) of soybean oil with methanol, obtained at BrasBio Industry (Rio Claro-SP, Brazil).

Regarding bench-scale bioreactor experiments, a volume of 3.5 L of both culture media described above were adjusted to pH 7 with NaOH 0.05 M, placed into the bench-scale bioreactor (Bioflo® & Celligen® 310-New Brunswick Scientific-7.5 L) and sterilized (121 °C for 20 minutes).

2.4.1. Concentration of raw glycerol

The raw glycerol was adjusted to pH 3 by phosphoric acid (0.66 M) and then it was left to rest for 24 h. Subsequently the solution was separated into three phases. According to Rivaldi et al. [5] the intermediate part has the highest concentration of glycerol; thereby, it was isolated using a separating funnel. Then, methanol was removed from the intermediate part by a rotary evaporator at 50 ^{o}C for 4 h. The material (glycerol) was collected from rotary evaporator and used as the culture medium in the bench-scale bioreactor experiments [3-5].

2.5. Fermentation procedures and sampling

2.5.1. Flask fermentation

The flask experiments were carried out as a preliminary screening to evaluate the fermentation process using three culture media, one with AGG, the second with CGBP and a third with raw glycerol which contained methanol. The Erlenmeyer flasks, containing each culture medium, were inoculated and then incubated at 150 rpm and 30 ^{o}C. Samples (≈ 12 mL) of the culture medium were collected on a 12-hour basis and centrifuged at 10,000 x *g* for 10 minutes at 5 ^{o}C. Finally, the viable cell count, surface tension (ST) of the samples and their dilutions were analyzed [7-8].

2.5.2. Bench-scale fermentation

All experiments were carried out at least 3 times. The process conditions were**:** 150 rpm, 30 ^{o}C [8] and an aeration rate (air) of 0.266 vvm. The dissolved oxygen (DO) sensor (Mettler Toledo - INPRO 6830/12/320) was set to measure every thirty seconds during the entire fermentation process. Samples (≈ 30 mL) of the culture medium were collected on a 24-hour basis, and subsequently the viable cell count, ST dilutions [7-8] and consumption of glycerol [2] were used as process parameters. Foam was collected during production from the top of the bench-scale bioreactor (foam overflow) into a Büchner flask through a hose [8]. The foam volume was measured on a 24-hour basis, centrifuged (10,000 x *g* for 10 minutes at 5 ^{o}C) and had its surface activity (ST and its dilution) measured.

2.6. Purification of surfactin

Two purification methods were applied: (1) acid precipitation method [8-9] and (2) acid precipitation followed by solvent extraction (chloroform:methanol-81:19) and solvent evaporation [8-9]. The resulting product (in powder form) from (1) was named crude biosurfactant and the product from (2) semi-purified biosurfactants. The yield in both methods was calculated by dividing each mass obtained by the total volume of culture medium (3.5 L).

2.7. Analytical methods

2.7.1. Determination of methanol in raw glycerol and CGBP

The free methanol contents of CGBP and raw glycerol were determined by HPLC-Shimadzu Prominence (Kyoto, Japan), using a LC-20AD HPLC system (Shimadzu, Columbia, USA) equipped with a RID-20A refractive index detector and HPX-87H column of dimensions 300 mm × 7.8 mm, and a particle size of 9 µm (Aminex, London, England). The analyses were performed using 5 mM H_2SO_4 as mobile phase and the flow rate was 0.6 mL/min. The total run time was 25 min. All the samples were previously filtered through a 0.45 µm teflon membrane (Millipore). The samples were injected (10 µL) at 4 °C. The column and RID temperatures were maintained at 60 and 50 °C, respectively.

The chromatograms were analyzed and integrated by the LCSolutions data acquisition software, version 5.73 (Shimadzu, Columbia, USA). An external calibration curve was constructed by analyzing standard methanol solutions at different concentration levels and the methanol content of samples were determined.

2.7.2. Fermentation process

Curves of viable cellular growth were plotted using CFU/mL data [7-8]. The data of DO were obtained from a probe submerged in the culture medium. Additionally, the glycerol concentrations were measured by titration of a centrifuged culture medium [2]. The concentration of micronutrients in the culture medium comprised of bacto-peptone and CGBP was sterilized (121 ^{o}C for 20 minutes) and analyzed by ICP-OES, the Kjeldahl' method (N), distillation (ammonia and nitrate) and the Walkley-Black' method (organic carbon).

2.7.3. Measurement of surface activity and critical micelle concentration

The ST measurements were carried out by using the plate method at room temperature in a Krüss GmbH K-12 tensiometer (K-12 model, Krüss GmbH) [7-8].

The surface activity was measured in culture media, collapsed foam and solutions (1 mg/mL) of crude and semi-purified biosurfactants. The ST, critical micelle dilution (CMD), and critical micelle concentration (CMC) were determined. The CMD corresponds to the surface tension value of a sample diluted 10 times (CMD-1) and 100 times (CMD-2). The CMC was determined by a serial dilution from 0.006 to 0.3 mg/L, where the objective was to identify the curve inflection point, that is, the CMC [10]. The CMC determination was carried-out using semi-purified biosurfactants from all experiments with the same medium.

2.7.4. Determination of surfactin concentration

Semi-purified biosurfactants (AGG-23.42 mg/50 mL and CGBP-7.95 mg/50 mL) were analyzed by reverse phase-HPLC using a Gilson 306 (Rockford, IL, USA), with a C18 column of dimensions 250 mm × 4.6 mm, and a particle size of 5 μm. The flow rate of the mobile phase was 1.1 mL/min with initial gradient starting from 50 to 80% acetonitrile in 0.1% trifluoroacetic acid in the first 15 min. The gradient increased, then, remained at 80% for 20 min before increasing (4%/min) to 100% for 5 min as a washing step before returning (6%/min) back to 50% and remained for 10 min. A 50 μL of sample was injected in each run, which lasted for 60 min, and eluent absorbance was monitored at 214 nm. The system was calibrated using standard surfactin (>99.8%) [11-12]. The surfactin concentration was determined by HPLC and the purity in terms of mass of surfactin over the total dry weight mass.

2.7.5. Protein concentration

The concentration of protein in the solutions of semi-purified biosurfactant was determined by the bicinchoninic acid method [11-12].

2.7.6. MALDI-TOFMS (Matrix Assisted Laser Desorption Ionization Time-of-Flight)

Solutions of semi-purified biosurfactant were analyzed using MALDI-TOF-MS spectrometer (Bruker Daltonics). Volumes of 1 μL of samples were used directly onto a target. After drying the samples at the room temperature was added 1 μL of matrix solution (2 mg of α-hydroxycinnaminic acid per mL in acetonitrile-methanol-water, 1:1:1) and allowed to dry at room temperature. External calibration was performed by using the [M+H]+ signals of peptide calibration standard which containing Angiotensin II, Angiotensin I, Substance P, Bombesin, ACTH clip 1-17, ACTH clip 18-39 and Somatostatin 28 (Bruker Daltonics). MALDI-TOFMS spectra were acquired in a m/z range of 600-3,500 Da by using

Smartbeam^{TM} laser irradiation with a frequency of 2,000 Hz for desorption and ionization. The mass spectrometer was operated in the refraction mode at an accelerating voltage of 22.45 kV. The delay time was 110 ns, the matrix-suppression was set to 500 Da, and the mass spectra were averaged over 1,500 laser shots. The laser intensity was set just above the threshold for ion production. Surfactin isomers were anticipated to have an m/z range of 1,000–1,050Da. The variance of the m/z of ± 0.8 Da was considered acceptable [13].

3. Results and Discussion

3.1. Flask fermentation

Surface tension measurements can be used to monitor production of biosurfactants during the fermentation. The surface tension value and its dilution are inversely proportional to the biosurfactants concentration [3, 7-8].

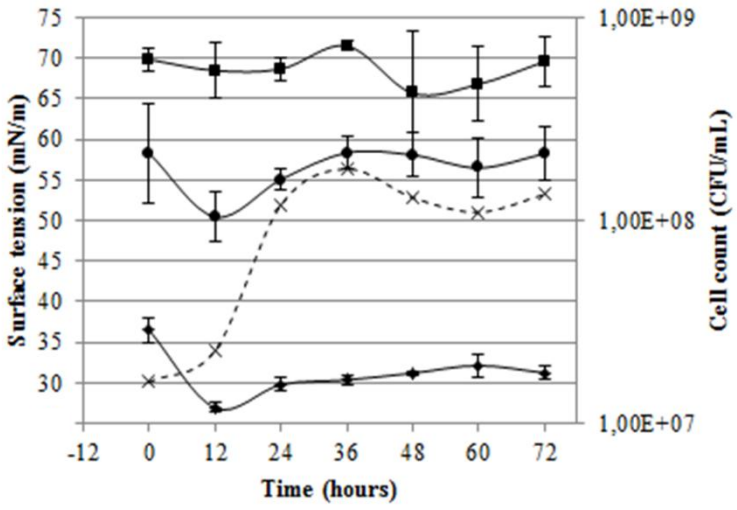

Figure 1. Growth curve and surface activity in the culture medium - AGG plus peptone - (—◆—) ST; (—●—) CMD-1; (—■—) CMD-2; (- - ✕ - -) Viable Cells.

The total cell count in Figure 1 showed a relative good microbial growth between 0-9 h, followed by a growth phase (the highest microbial growth rate) up to 36 h and a stationary phase up to 72 h. It is worth noting that the lag phase took place within the interval of 0-9 h, probably during the 1st or 2nd hour of fermentation. A strong reduction in the ST occurred in the first hours of fermentation, where the value dropped from ≈ 40 mN/m to ≈ 27 mN/m. The same behavior was observed in CMD-1 and CMD-2, the first of which showed

significant reduction from ≈ 59 mN/m to ≈ 50 mN/m. On the other hand, most cell growth was observed between 9 and 23 h, where cell count increasead from 1.71x107 CFU/mL to 1.12x108 CFU/mL. A subtle increase in all parameters (ST and CMDs) was observed after 9 h.

The CMD-2 data were similar to the surface tension of distilled water (72 mN/m). In other words, due to the high dilution (100 times), no significant content of surfactin was observed. On the other hand, CMD-1 showed values around 55 mN/m, which is lower than that of distilled water, indicating a relevant content of surfactin even when it was diluted 10 times. It is worth noting that the highest difference of CMD-1 values took place between 0 and 9 h ($\Delta \approx 10$ mN/m), which is aligned with the ST data. Thereby, when comparing samples collected subsequently, that period had the highest production of biosurfactants. After that, subtle changes occurred until the 70 hours, which indicates the maintenance of surfactin concentration. Therefore, the culture medium composed by AGG and peptone was very suitable to *B. subtilis* LB2b growth and biosurfactants production.

Then, experiments evaluated the microbial growth and biosurfactants using a culture medium composed by raw glycerol and peptone (Fig. 2).

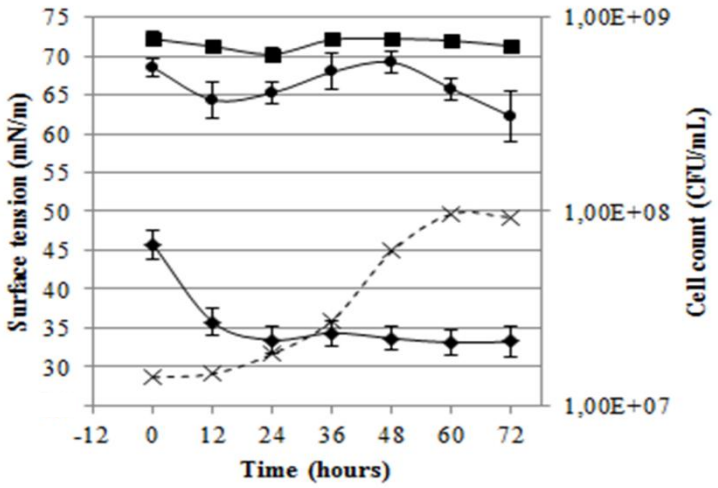

Figure 2. Growth curve and surface activity in the culture medium - raw glycerol from biodiesel industry plus peptone - (—◆—) ST; (—●—) CMD-1; (—■—) CMD-2; (- - ✕ - -) Viable Cells.

The fermentation using a culture medium composed by peptone and raw glycerol from biodiesel industry showed significant lower microbial growth rate and biosurfactant production (Fig. 2). Contrary to what was observed in the fermentation with AGG, the characteristic ST value of surfactin at concentrations equal or higher than CMC (≈ 27 mN/m) were not obtained. The CMD-1 was also higher (≈ 65 mN/m), that is, a lower biosurfactants production was achieved using raw glycerol. Salakkam and Webb [14] studied the effect of methanol on *Cupriavidus necator* DSM4058. It was found that methanol at any concentration (up to 125 g/L) had a negative influence on microbial growth. Thus, we speculate that the difference in biosurfactant production was mainly due to the high concentration of methanol in the raw glycerol.

Thus, based on the experimental data obtained with AGG and raw glycerol from biodiesel industry, further experiments were carried out using treated raw glycerol, CGBP - (Fig. 3).

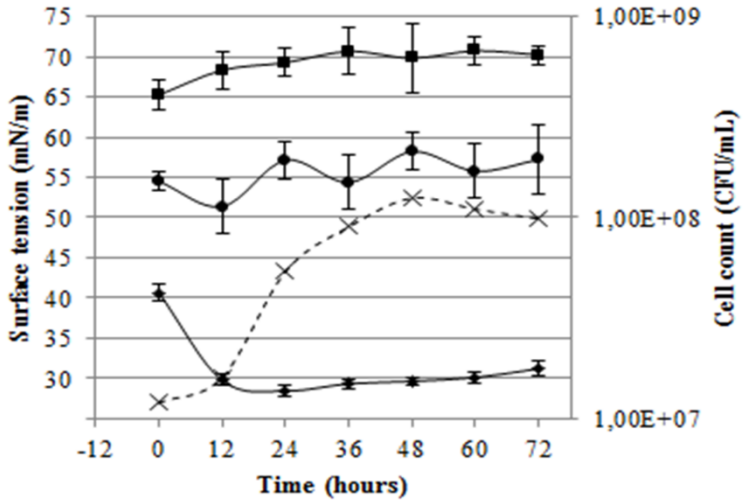

Figure 3. Growth curve and surface activity in the culture medium - CGBP plus peptone - (—◆—) ST; (—●—) CMD-1; (—■—) CMD-2; (- - ×- -) Viable Cells.

The Figure 3 shows that the *B. subtilis* Lb2b growth in the medium composed by CGBP and peptone showed similar microbial growth and biosurfactants production compared to AGG plus peptone, that is, ST ≈ 27 mN/m, CMD-1 ≈ 55 mN/m and microbial growth curve.

3.1.1. Composition of culture medium (concentrated glycerol from biodiesel industry)

Fermentation with AGG and CGBP led to good and similar production of biosurfactants. The main difference between raw and CGBP glycerol is the removal of salts, soap, but mainly methanol (32.41% in raw and 4.41% in CGBP, Table 1). On the other hand, the raw glycerol from biodiesel industry experiments showed lower production. Thus, there is strong evidence that *B. subtilis* Lb2b is very sensitive to the presence of methanol.

Most of the metal present in the culture medium with CGBP was higher than 0.01 ppm (Table 1). However Fe, Mn, Cu and Ca were below the detectable limits of the test. Also, the composition, compared with Cooper's medium, most of the minerals were at a higher concentration [9].

Table 1. Nutritional composition of the culture medium comprised of bacto-peptone and concentrated glycerol from biodiesel production.

Nutrient	[mg/L]	Nutrient	[mg/L]
P	0.3	Zn	0.8
K	0.1	NH_3	43.1
Ca	<0.01	Mg	0.02
C*	9.1	S	0.1
NO_3^-	4.3	B	8.0
N*	1.2	Mn	<0.01
Cu	<0.01	MeOH†	4.41
Fe	<0.01		

* - g/Kg

† - %

The C/N ratio ≈ 7.52 was very similar to Cooper's medium, which was one of the first papers on the content of minerals and production of surfactin by *Bacillus subtilis* [9]. Obviously, this result is due to the positive combination of glycerol and peptone, since both are carbon sources. In addition, the peptone could also be a nitrogen source.

Peptides can be absorbed into the cell and metabolized into amino acids. Then, by deamination or oxidative deamination, these amino acids are converted into intermediates of tricarboxylic acid cycle such as serine → pyruvate, aspartate → oxaloacetate, glutamate → 2-oxoglutarate [15-16]. However, the catabolic pathways of many amino acids remain unknown or only partially characterized. In this context, arginine and histidine are known to provide energy [15-16]. Yan et al. [16] evaluated the aflatoxin production from *Aspergillus flavus* using a culture medium comprised by salts and peptone as sole carbon source. They indicated that *Aspergillus flavus* preferred peptone as a sole carbon source for growth rather than traditional fermentable sugars. Thus, peptone can be used by microorganisms as carbon source.

The first reports on biosurfactant production using glycerol from biodiesel production were carried out with *Pseudomonas sp*, which synthesizes rhamnolipids. To the best of our knowledge, De Faria et al. [17] published the first relevant report on the production of lipopeptides: surfactin (C_{14}/Leu_7) from *B. subitils* using raw glycerol (5% v/v) from biodiesel production as the sole carbon source.

Sousa et al. [3] neutralized the raw glycerol and then removed the methanol by evaporation. Finally, the remaining product was added to the culture medium. As a result, 4 out of 7 strains of *Bacillus subtilis* reached ST values around 27 mN/m. Thus, there are differences in glycerol metabolism, even among the same species of a microorganism [3].

In summary, results above confirmed that both the culture media peptone plus AGG and peptone plus CGBP are better suited for *B. subtilis* LB2b growth and biosurfactant production than raw glycerol. Further experiments were carried out with culture media containing either AGG or CGBP at bench scale and a comparative study was carried out in terms of biosurfactant production.

3.2. Bench-scale fermentation

3.2.1. Fermentation parameters

In the experiments with AGG, DO dropped to 0% at ≈ 4.5 h of fermentation and started to increase at ≈ 30 h (Fig. 4). On the other hand, tests with CGBP, DO decreased to 0% at ≈ 9 h and maintained this level until 72 h (Fig. 4). In both cases, the experiments remained stable at 0% DO for the majority of the time, 25.5 and 63 h, respectively. It is worth noting that, after 54 h of fermentation, there was a great difference in DO between both bioprocesses, AGG and CGBP.

In the experiments with AGG, the number of viable cells increased from $1.6x10^8$ at 0 h to $1.3x10^{11}$ CFU/mL after 48 h; then, at 72 h, this value was ≈ $7x10^{10}$. On the other hand, when CGBP was used, the count reached only $3.5x10^{10}$ CFU/mL after 48 h, and at 72 h, it was ≈ $8.3x10^9$. In experiments with CGBP, there was a delay in overall lower cellular development, in a similar way to that reported by Salakkam and Webb [14]. This difference is consistent with the curves of the DO (Fig. 4), which has a direct relationship to cellular growth. Low DO values in cellular growth indicate high oxygen absorption (high consumption per cell or high cell content). It is worth mentioning that, after 48 h, the number of cells decreased in both cases and the bench-scale bioreactor is a semi-closed system in which the foam was collected during its production. Thus, many cells were removed from the system (bench-scale bioreactor) by foam overflow. This inference is strengthened by the fact that the DO levels rose strongly after this time in the fermentation in which AGG was used.

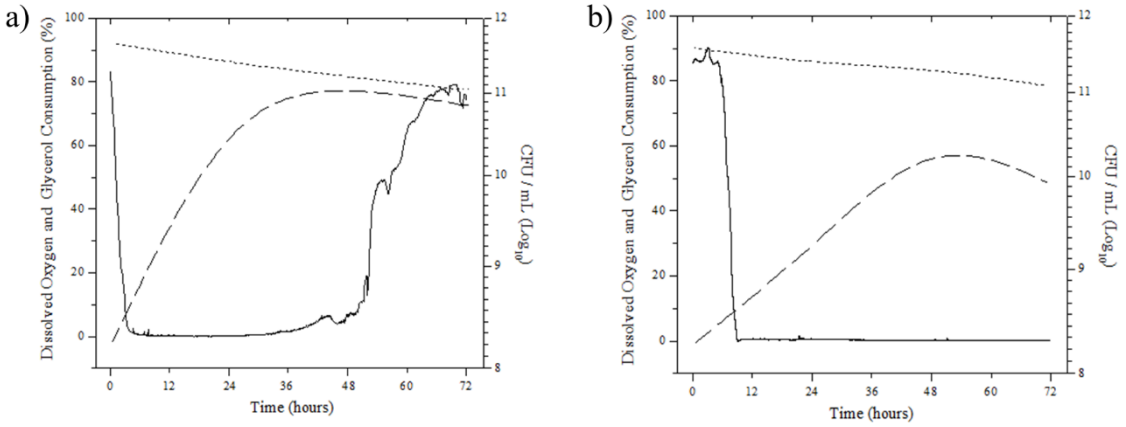

Figure 4. Fermentation parameters – AGG (a); CGBP (b) - microbial growth (- - - - -), dissolved oxygen (——) and glycerol consumption (·····).

Raw glycerol contains ≈ 5% NaCl and up to ≈ 30% methanol [14] and even though most of soap and methanol were removed from the by-product from biodiesel industry (raw glycerol→CGBP), their presence, even at low concentrations, may have had a significant effect upon the *B. subtilis* metabolism.

There are very few reports that evaluated the relation between microbial kinetics and toxicity of methanol, in particular for bacteria. One of those few reports was developed by Salakkam and Webb [14], who studied the effect of methanol upon the microbial growth rate of bacteria using glycerol as carbon source. They reported that the microbial growth rate

(inversely proportional) and lag phase (proportional) were very affected by the presence of methanol, in which the hypothesis are (i) reduction of membrane stability, (ii) denaturation of protein, including enzymes, (iii) changes in fatty acid and acid nucleic composition, (iv) similar influence of intermediate, methanol → formaldehyde → formic acid. Thus, it is strongly recommended to eliminate methanol from any culture medium [14].

Some species of *Bacillus* are classified as methylotrophic microorganisms and may use methanol as a carbon source via the ribulose monophosphate (RuMP)[18]. The experiments with CGBP contained 4.41% of methanol and probably, due to the absence of the RuMP in this strain, the methanol might have been oxidized to formaldehyde, which could have started alkylation reactions within the cytoplasm. As a result of this, cell metabolism was reduced, and consequently, substrates were consumed at a lower rate, which allowed consumption of oxygen (0% of DO) until 72 h. Alternatively, experiments with AGG did not have methanol or other impurities in the medium. Thereby, high oxygen intake (0% of DO) was readily reached after 9 h, hence, a lack of nutrients or excess of secondary metabolites may have occurred after 48 h, which is aligned with increase of DO after that time. Therefore, there is evidence that CGBP, even after the purification steps described above, contains other molecule(s) with significant deleterious effect on growth. In other words, the medium with AGG was the best for microbial growth.

Taking this into account, research on more efficient processes and techniques for glycerol purification can increase cell viability, and, therefore, biosurfactant production.

Glycerol consumption showed similar results in both experiments. Glycerol, when used as a carbon source, is mainly degraded by glycerol kinase pathways, which is better expressed in an aerobic condition [19]. Surfactin produced from *B. subtilis* is synthesized in the log phase. Thereby, considering the process as non-segregated and structured, the maximum metabolism state took place at that phase [19]. Therefore, the intake of glycerol should be similar to the oxygen consumption curve (or the opposite of DO), Fig. 4. However, glycerol consumption curves showed linearity (gradually absorbed during the fermentation). Thus, it could mean that glycerol was not used as carbon source, but the presence of glycerol may improve the fermentation, for instance by increasing the cellular membrane permeability.

It is worth nothing that both culture media (AGG and CGBP) show similar composition, however impurities from biodiesel industry remained in the CGBP. These impurities have a negative effect in the bioprocess, for instance reduced cell growth, production of biosurfactant.

3.2.1.1. Measurement of surface activity – collapsed foam and culture media

Biosurfactant concentration is inversely proportional to the ST; the lower the CMD values are, the higher the biosurfactant concentration. Also, an increase in foam production is expected with higher biosurfactant concentrations (Table 2).

The ST values for the clarified foams from experiments with AGG and CGBP did not show statistical differences at a significance level of 0.05 and were 29.42 mN/m (+/- 3.02) and 29.97 mN/m (+/- 4.27), respectively (Fig. 5). This is most likely due to the fact that in both samples the biosurfactant concentration was higher than its CMC and this resulted in a constant value for ST. This obviously indicates that in both cases good biosurfactant production and recovery was obtained.

For both culture media (AGG and CGBP), the ST remained constant ≈ 34 mN/m, after 24 h, similar to CMD-2 ≈ 72 mN/m. However, CMD-1 data for the experiments with AGG were lower, indicating a higher biosurfactant production (Fig. 5). These results converged with the results of purity and yields of surfactin (Table 2), viable cells and DO, that is, comparing with CGBP medium, AGG showed higher purity and yield of surfactin, viable cell count and absorption of oxygen.

Finally, the ST data for culture media – higher than surface tension at CMC (27 mN/m) - indicates that the recovery of surfactin by foam is a good strategy, since less than 10 mg of surfactin per liter of culture medium remained in the system during the fermentation. Also, high concentration of surfactin in the culture medium may inhibit the growth of *B. subtilis* and foam overflow could be a strategy to avoid it. Henceforth, surfactin production was calculated based only in the collapsed foam, that is, it was assumed that 100% (theoretically) of surfactin produced was recovery by foam overflow.

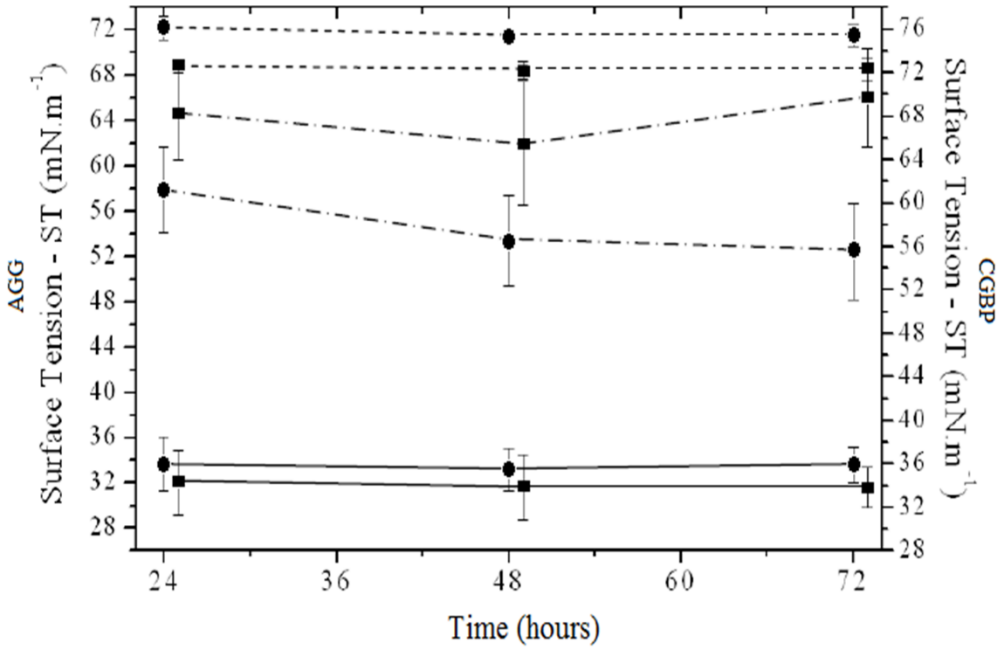

Figure 5. The ST, CMD-1 and CMD-2 values for the centrifuged culture media; bench fermentation: (■) experiments with CGBP [(—■—) ST, (•-■-•) CMD-1, (•-■-•) CMD-2], - (●) experiments with AGG: [(—●—) ST, (•-●-•) CMD-1, (••●••) CMD-2].

3.2.1.2. Volume of collapsed foam, crude and semi-purified biosurfactant yields, protein concentration in semi-purified and purity of surfactin

Table 2 illustrates all yields of collapsed foam produced, crude and semi-purified biosurfactants and the purity of semi-purified biosurfactant. Volumes of foam produced were statistically different (Tukey test 95%) and their yields (foam/culture medium) were 0.18 and 0.10 (v/v) in the experiments with AGG and CGBP, respectively. This difference is clearly related to a higher yield of surfactant.

Table 2. Yields of biosurfactant production.

	AGG medium	CGBP Medium
Collapsed foam produced - (mL)	657	360
Crude biosurfactant - (1) acid precipitation method - (g/L of foam)	7.85	4.89
Semi-purified biosurfactant - (2) acid precipitation followed by solvent extraction - (g/L of foam)	1.58	1.13
Purity of surfactin in semi-purified biosurfactant- (%w/w)*	72.02	22.03
Concentration of protein in semi-purified biosurfactant–BCA kit- (%w/w)	26.52	48.08
Micelle size-DLS-(nm)	152.3	176.3

* The surfactin concentration was determined by HPLC and the purity in terms of mass of surfactin over the total dry weight mass.

Differences of crude biosurfactant yields were observed between both culture media. This may be due to the decrease in solubility of peptone residue in the medium during the acidification step, or to peptones and/or proteins synthesized by the strain.

The concentration of protein in the solutions of semi-purified biosurfactant was: 124.23 mg/L(AGG) and 76.45 mg/L (CGBP), that is, 26.52 and 48.08%, respectively. These results follow the same trend as crude and semi-purified yields, in which the products (crude and semi-purified biosurfactant) obtained from AGG showed higher surfactin concentration, that is, lower impurities (mainly proteins) concentration (see Table 2).

Thus, probably the impurities of CGBP decrease the surfactin production and also, increased protein production. A plausible explanation for the higher concentration of protein when using CGBP is that the impurities (toxic molecules) suppressed the metabolic pathway of surfactin production and induced the strain to synthesize more enzymes to keep itself alive or the impurities diverted the metabolic pathway of surfactin.

The CMC of semi-purified biosurfactant from experiments with AGG and CGBP were determined as 11 mg/L and 19 mg/L, respectively. These results converge with the definition that a powerful biosurfactant has a CMC value between 1mg/L and 2000 mg/L

[20]. Even using a new culture medium (reported for the first time), the results are similar to those reported by Nitschke et al. [7]; Barros et al. [8], ≈ 11 mg/L, and better than the 14 mg/Lreported by Sheppard & Mulligan [10], and 25 mg/L reported by Cooper et al. [9], respectively. However, it is possible to notice that a higher value of CMC was identified for the culture grown in CGBP than in the medium with AGG. This data follows the same trend that already presented in this part of the study (ST measurement and purity).

The literature describes the production of surfactin per liter of culture medium (extracted direct from the culture medium) ≈ 500 mg/L [11-12].The fermentation with AGG medium obtained ≈ 325 mg of surfactin/liter of culture medium, whereas the CGBP medium 71 mg/L. De Faria et al. [17] used a synthetic culture medium for surfactin production, then recovered it by foam overflow and purified it by absorption column chromatography. They obtained the following surfactin yields: 230 mg/L of foam, or 89.93 mg/L of medium. The same fermentative process was used to identify the fengycin homologues (decapeptide attached to a β-hydroxy fatty acid) [21]. In this context,it should be noted that the aim of this study was not the production of surfactin but the effect of the purity of glycerol on productivity. We speculate the reasons for the relative low production as: (i) glucose is more assimilable carbon source than glycerol; (ii) no optimization experiments were performed (agitation, inoculum, temperature, proportion of glycerol and peptone, etc) and (iii) the recovery of surfactin by foam overflow (collapsed foam) did not recover 100% of surfactin (remainders: in the culture medium, foam (bioreactor), hose, etc). Further studies will be carried to optimize the production of surfactin.

3.2.1.3. Confirmation of surfactin by MALDI-TOFMS

Bacillus produces lipopeptides, which are classified in three families: surfactin, iturin and fengycin. Each family has a specific number of aminoacids, but with different residues at specific position. It also has different lengths and isomery of β-hydroxyl fatty acids, that is, lipopeptides have a remarkable heterogeneity of molecular weight [22].

Ayed et al. [23] analyzed lipopeptides from *Bacillus* by MALDI-TOFMS. They found 13 peaks and attributed them to isoforms of surfactin between 1045 to 1080 m/z. In this research, the cluster of peaks related to semi-purified biosurfactant from AGG showed 21 potential isomers of surfactin, whereas, for the semi-purified biosurfactant from CGBP only 6 isomers of surfactin were found, in which all 6 were also present in the semi-purified biosurfactant from AGG (Figure 7). The semi-purified biosurfactant from CGBP, also showed heavier molecular weight isoforms, from 1065.55 to 1081.56 m/z; whereas semi-purified

biosurfactant from AGG ranged from 1044.55 to 1083.53 m/z. Al-Ajlani et al. [13] evaluated the surfactin produced by different culture media (defined, semi-defined, and complex media) by MALDI-TOFMS analysis. They observed that the production of surfactin isomers was not determined by the culture medium.

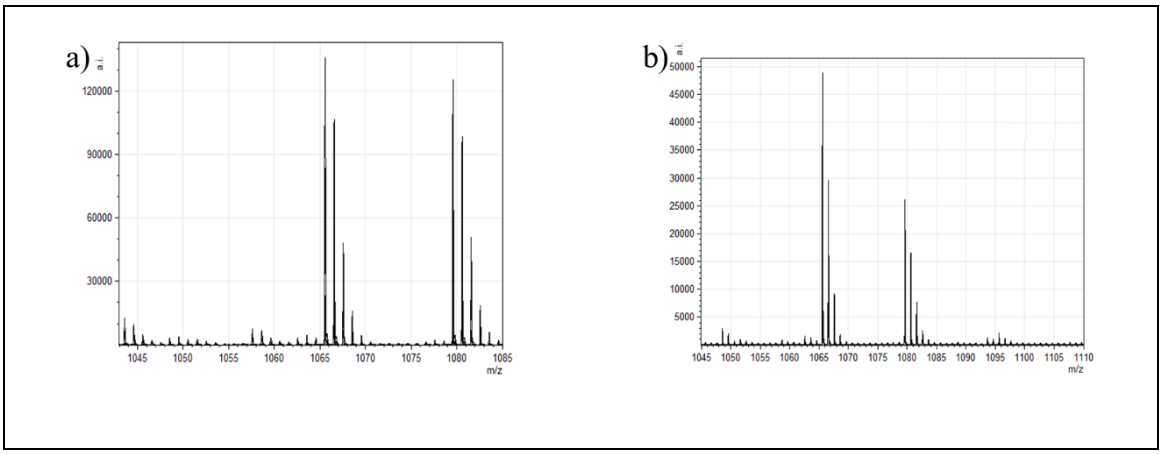

Figure 6. MALDI-TOFMS results: from experiments with AGG (a) and CGBP (b).

Our results suggest that the impurities from biodiesel production (eg: methanol) affect the productivity of surfactin, which leads to the production of heavier isoforms of surfactin.

3.3. Economic impact on surfactin production and its prospection on production and application

The surfactin production of each batch was ≈ 1.14 g (AGG) and 0.25 g (CGBP), respectively. That is, 0.89 g (1.14 – 0.25) of surfactin was not produced probably, due to the effect of impurities of biodiesel production (most probably methanol). If both productions of surfactin (1.14 g AGG and 0.25 g CGBP) were purified (> 98% purity), they would represent (based on the market price -$ 15.3/mg of surfactin >98 % purity) US$ 17,442 (AGG) and US$ 3,825 (CGBP). Thus, it is obviously unacceptable the production of surfactin by the use of CGBP.

Therefore, it is clear that the higher-purity glycerol used in the culture medium or the lower concentration of methanol, the higher is the surfactin production, that is, the use of high-purity glycerol is fundamental to achieve high surfactin production. Also, since the methanol is separated from glycerol, it could be used again in the biodiesel industry (transesterification). Additionally, it is also fundamental to consider the relation between the surfactin isoforms to their application, that is, if the heavier molecular weight isoforms of

surfactin show better outcome (e.g antimicrobial properties), the surfactin production with CGBP, even with significant lower productivity would be favored.

4. Conclusions

Flask experiment data indicated a negative influence of impurities (present on by-product from biodiesel industry) on growth of *B. subtilis* LB2b. However, good growth and biosurfactant production were obtained using a medium comprised of peptone and AGG, which was scaled up to a bench-scale bioreactor (3.5 L working volume). Higher surfactin production (4.6 times) was obtained with glycerol of highest purity (AGG) and this was related to the following differences: cell growth, volume of foam and oxygen consumption absorption. However no difference in glycerol consumption was observed. Although, significant differences were observed on the purity (protein concentration), which may be associated to the effect of impurities on metabolic pathways of protein and/or surfactin production. The semi-purified biosurfactant from AGG contained ≈ 4 times more isoforms of surfactin than semi-purified biosurfactant from CGBP. Therefore, the downstream processing of biodiesel derived glycerol should provide a product with purity level equivalent to that of AGG when used as fermentation medium for the production of surfactin in order to get improved yield.

Conflict of interest

The authors declare no conflict of interest

Acknowledgements

This project was sponsored by the São Paulo State Research Foundation-(Fapesp)

5. References

[1] Leoneti AB, Aragão-Leoneti V, De Oliveira SVWB. Glycerol as a by-product of biodiesel production in Brazil: Alternatives for the use on unrefined glycerol. Renew Energ 2012; 45: 138-145.

[2] ANP - Brazilian National Agency for Petroleum, Natural Gas and Biofuel. Resolution Nº 42, 24.11.2004.

http://nxt.anp.gov.br/nxt/gateway.dll/leg/resolucoes_anp/2004/novembro/ranp%2042%20-%202004.xml. Acessed September, 19, 2010.

[3] Sousa M, Melo VMM, Rodrigues S, Sant'ana H B, Gonçalves LRB. Screening of biosurfactant-producing *Bacillus* strains using glycerol from the biodiesel synthesis as main carbon source. Bioprocess Biosystems Eng 2012; 35: 897-906.

[4] Sousa JR, Correia JAC, Almeida JGL, Rodrigues S, Pessoa ODL, Melo VMM, Gonçalves LRB. Evaluation of a co-product of biodiesel production as carbon source in the production of biosurfactant by *P. aeruginosa* MSIC02. Process Biochem 2011; 46: 1831-1839.

[5] Rivaldi JD, Sarrouh BF, Fiorilo R. Glicerol de biodiesel - Estratégias biotecnológicas para o aproveitamento do glicerol gerado da produção de biodiesel. Biotecnologia Cienc Desenvol 2008; 37: 44-51.

[6] Makkar RS, Cameotra SS, Banat IM. Advances in utilization of renewable substrates for biosurfactant production. AMB Express 2011; 1: 1-19.

[7] Nitschke M, Ferraz C, Pastore GM. Selection of microorganisms for biosurfactant production using agroindustrial wastes. Brazilian Journal of Microbiology. 2004; 35:81-85.

[8] Barros FFC, Ponezi AN, Pastore GM. Production of biosurfactant by *Bacillus subtilis* LB5a on a pilot scale using cassava wastewater as substrate. J Ind Microbiol Biot 2008; 35: 1071-1078.

[9] Cooper DG, Macdonald CR, Duff SJB, Kosaric N. Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions. Appl Environ Microbiol 1981; 42: 408-412.

[10] Sheppard J.D, Mulligan CN. The production of surfactin by *Bacillus subtilis* grown on peat hydrolysate. Appl Microbiol Biotechnol 1987; 27: 110-116.

[11] Isa MHM, Coraglia DE, Frazier RA, Jauregi P. Recovery and purification of surfactin from fermentation broth by a two steps ultrafiltration process. J Membrane Sci 2007; 296: 51-57.

[12] Jauregi P, Coutte F, Catiau L, Lecouturier D, Jacques P. Micelle size characterization of lipopeptides produced by *B. subtilis* and their recovery by the two-step ultrafiltration process. Sep Purif Technol 2013;104: 175-182.

[13] Al-Ajlani MM, Sheikh MA, Ahmad Z, Hasnain S. Production of surfactin from *Bacillus subtilis* MZ-7 grown on pharmamedia commercial medium. *Microbial Cell Fact* 2007; *6*: 17-24.

[14]Salakkam A and Webb C. The inhibition effect of methanol, as a component of crude glycerol, on the growth rate of *Cupriavidus necator* and other micro-organisms. Biochemical Engineering Journal. 2015; 98: 84-90.

[15] Christesen JE, Dudley GE, Pederson JA, Steele JL. Peptidase and amino acid catabolism in lactic acid bacteria. Antonie van Leeuwenhock, 1999; 76: 217-246.

[16] Yan S, Liang Y, Zhang J, Liu C-M. *Aspergillus flavus* grown in peptone as the carbon source exhibits spore density- and peptone concentration-dependent aflatoxin biosynthesis. BMC Microbiology. 2012; 12:106-119.

[17] De Faria AF, Teodoro-Martinez DS, Barbosa GNO, Vaz BG, Silva IS, Garcia JS, Tótola MR, Eberlin MN, Grossman M, Alves OL, Durrant LR. Production and structural characterization of surfactin (C 14 /Leu 7) produced by *Bacillus subtilis* isolate LSFM-05 grown on raw glycerol from the biodiesel industry. Process Biochem 2011a; 46: 1951-1957.

[18] Orita I, Sato T, Yurimoto H, Kato N, Atomi H, Imanaka T, Sakai Y. The ribulose monophosphate pathway substitutes for the missing pentose phosphate pathway in the *archaeon Thermococcus kodakaraensis*. J Bacteriol 2006; 188: 4698-4704.

[19] Pasteris SE, Strasser de Saad AM. Aerobic glycerol catabolism by *Pediococcus pentosaceus* isolated from wine. Food Microbiol 2005; 22: 399-407.

[20] Mulligan CN, Gibbs BF. Factors influencing the economics of biosurfactants. In: N Kosaric editor. Biosurfactants: production, properties, applications. (pp. 392-37). New York: E-Publishing Inc; 1993. p. 392-437.

[21] De Faria AF, Stéfani D, Vaz BG, Silva IS, Garcia JS, Eberlin MN, Grossman M J, Alves OL, Durrant LR. Purification and structural characterization of fengycin homologues produced by *Bacillus subtilis* LSFM-05 grown on raw glycerol. J Ind Microbiol Biotechnol 2011b; 38: 863-871.

[22] Raaijmakers JM, Bruijn I, Nybroe O, Ongena, M. Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. FEMS Microbiol Ver 2010; 34: 1037–1062.

[23] Ayed HB, Hmidet N, Béchet M, Chollet M, Chataigné G, Leclère V, Jacques P. Identification and biochemical characteristics of lipopeptides from *Bacillus mojavensis* A21. Process Biochem2014; 49: 1699-1707.

ANEXO

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PROCESSO DE OBTENÇÃO DE MANOSILERITRITOL LIPÍDIOS (MEL), COMPOSIÇÕES E USOS DAS MESMAS

Campo da invenção:

[001] A presente invenção refere-se a um processo de obtenção de manosileritritol lipídios (MEL) usando manipueira como meio de cultura, composições com alto teor de MEL obtido através do processo descrito e uso.

[002] A invenção se aplica no campo de gerenciamento de resíduos, de forma mais específica na área de aproveitamento de resíduos, preferencialmente manipueira (resíduo da indústria farinheira), que não utiliza solvente orgânico. A composição obtida pelo processo da presente invenção pode ser aplicada em cosméticos, como agente tensoativo, como emulsificante na recuperação de petróleo ou para pulverização em cultivares.

Fundamentos da invenção:

[003] Biossurfactantes são compostos que consistem em subprodutos metabólicos de bactérias, fungos e leveduras e que exibem propriedades surfactantes, isto é, diminuem a tensão superficial e possuem alta capacidade emulsificante.

[004] Em comparação com seus homólogos químicos, os biossurfactantes apresentam menor toxicidade, biodegradabilidade e estabilidade em condições extremas. Um exemplo de biossurfactante é o manosileritritol lipídio (MEL) dos tipos -A, -B, ou -C ou, o qual consiste em uma mistura de derivados parcialmente acilados de 4-O-β-D-manopiranosil-D-eritritol, contendo ácidos graxos diferentes na sua porção hidrofóbica. Este biossurfactante é produzido por leveduras do gênero *Pseudozyma*, sendo reportado na literatura que a espécie *Pseudozyma tsukubaensis* produz

apenas o MEL-B.

[005] Atualmente, em escala industrial, a produção do manosileritritol lipídio (MEL) é realizada com o uso de meio de cultura sintético (solução de sais minerais). Além disso, o processo de purificação é dispendioso, pois engloba as etapas de: extração líquido-líquido (acetato de etila) do manosileritritol lipídio diretamente do meio de cultura, seguido de secagem do mesmo, sua dissolução em clorofórmio e cromatografia em coluna de sílica.

[006] Os passos de purificação podem representar cerca de 60% do custo de produção. Dessa maneira, devido ao alto custo associado a métodos ineficientes de recuperação do produto e ao uso de substratos caros, o uso comercial de biossurfactantes é restritivo. Contudo, uma alternativa é a produção de MEL por meio de cultura através de resíduos agroindustriais.

[007] A manipueira é um líquido de cor amarelada que sai da mandioca depois dela prensada, durante a fabricação da farinha. Se a mesma for despejada na natureza, provoca a poluição do solo e das águas (rios, riachos e açudes), causando grandes prejuízos ao meio ambiente e ao homem.

[008] Portanto, visto que a manipueira é um resíduo da indústria de farinha, rica em minerais e carboidratos, a presente invenção propõe um processo de obtenção de MEL, preferencialmente o MEL-B, usando manipueira como meio de cultura, através da recuperação do biossurfactante pela formação de espuma no biorreator e purificação desta por membranas em uma única etapa de ultrafiltração, o qual não é utilizado solvente orgânico.

[009] Com isso, as principais vantagens obtidas com a presente invenção podem ser divididas em duas grandes seções: (i) impacto ambiental e (ii) redução do custo de produção.

[010] A primeira (i) é relacionada à utilização de um resíduo agroindustrial como meio de cultura, que atualmente, é um problema para a indústria que gera tal resíduo e que deve realizar o seu descarte de maneira ambientalmente correta. Além disso, a invenção não utiliza solventes orgânicos, que são potencialmente prejudiciais ao meio ambiente.

[011] A segunda (ii) é relacionada ao fator econômico, pois o meio de cultura é obtido com custo nulo (uma vez que é um resíduo) e a indústria que gera o resíduo economiza as despesas relacionadas ao tratamento e ao correto descarte deste.

[012] Ainda, a recuperação do MEL através da coleta da espuma produzida durante a fermentação não implica em altos investimentos de equipamentos. E finalmente, a etapa de purificação com a ultrafiltração é simples, rápida, de baixo custo (uma vez que as membranas são reutilizáveis) e resulta em um elevado nível de pureza do biossurfactante.

[013] Assim, de forma geral, o novo processo aqui proposto reduz o impacto ambiental e econômico da obtenção e purificação do biossurfactante MEL.

Estado da técnica:

[014] Alguns documentos do estado da técnica descrevem um processo no qual a ultrafiltração é uma etapa comum para a purificação dos produtos relativos a cada documento ou descrevem um processo de obtenção de

biossurfactante.

[015] Em WO200420647, é descrito um processo de obtenção de manosileritritol lipídio com um meio de cultura composto por fontes hidrofóbicas de carbono (óleos vegetais) e de nitrogênio (nitrato) por *Pseudozyma aphidis*. Além disso, este documento reporta a recuperação e a purificação do manosileritritol lipídio (MEL) da seguinte forma: aquecendo e resfriando o meio de cultura após a fermentação (para obter duas fases) e utilizando soluções de álcool para purificar o mesmo.

[016] Diferentemente, a presente invenção utiliza meio de cultura não hidrofóbico. Em uma modalidade preferida, utiliza a espécie *Pseudozyma tsukubaensis* e recupera o MEL do tipo B através da formação de espuma no biorreator, a qual é purificada com ultrafiltração.

[017] O documento US20060194294 se refere à purificação de estaurosporina por etapas especificas de ultrafiltração, diafiltração (utilizando solventes orgânicos solúveis em água) e precipitação.

[018] A invenção proposta promove a recuperação do MEL através da formação de espuma formada durante a fermentação. Já o documento US20060194294 extrai o composto de interesse do meio de cultura. Além disso, a presente invenção utiliza apenas um processo de purificação por membranas e utiliza apenas água como solvente, enquanto o documento de anterioridade adiciona solvente orgânico solúvel em água anteriormente ao processo de ultrafiltração e diafiltração, seguido de precipitação. Resumindo, o método da invenção proposta é muito mais simples, pois não inclui uso de solvente orgânico, precipitação e diafiltração.

[019] Os documentos WO9915464 e US6004466 se referem a etapas de tratamento de resíduo, com a utilização de surfactantes não iônicos, em que o surfactante é adicionado ao resíduo. Em seguida, a solução é ultrafiltrada e os surfactantes e água são recuperados no permeado, enquanto os contaminantes são retidos.

[020] A invenção proposta difere destes documentos principalmente pelo fato de que clama pela produção seguida da purificação do MEL, preferencialmente o MEL-B. A ultrafiltração retém o biossurfactante, enquanto as impurezas são permeadas. Ou seja, é o oposto ao descrito nos referidos documentos.

[021] O documento US5071751 se refere ao aumento do rendimento da produção de ácido hialurônico (HA) pela adição de soro do sangue (diversos animais) ao meio de cultura, seguido de purificação do HA por centrifugação ou filtração, diafiltração ou ultrafiltração, adição de álcool ao permeado, coleta do precipitado e finalmente, purificação do mesmo por coluna de troca iônica ou permeação em gel.

[022] A invenção proposta difere do documento US5071751 principalmente pelo fato de que aborda a produção de MEL (compostos muito diferentes). Além disso, há a etapa de recuperação pela formação de espuma. A etapa de purificação da invenção proposta é muito mais simples, com apenas uma etapa de ultrafiltração, em que o produto de interesse é retido. Ou seja, não utiliza solvente, colunas de troca iônica ou permeação em gel.

[023] O documento US20100137579 se refere à produção e purificação do ácido hialurônico (HA) por *Streptococcus zooepidemicus*, em meio sintético, seguida da

diafiltração do meio de cultura. A invenção proposta difere do referido documento principalmente pelo fato de que clama pela produção seguida da purificação do MEL, preferencialmente o MEL-B. Ou seja, trata-se de produções e purificações de compostos totalmente diferentes, no qual o documento US20100137579 utiliza o ajuste de pH e diafiltração para purificar o HA, enquanto a invenção proposta utiliza a recuperação do biossurfactante pela formação de espuma seguida da ultrafiltração.

[024] A tecnologia descrita nos documentos PT 106959A e WO2014185805A1 reporta a obtenção de MEL utilizando substratos alternativos. Porém, a tecnologia descrita nesses documentos engloba apenas substratos lignocelulósicos, ou seja, trata-se de uma fermentação em estado sólido. A invenção proposta também utiliza resíduos, entretanto, são resíduos não lignocelulósicos, tal como a manipueira, e aplica-se um processo de fermentação submersa. Além disso, não é mencionado nenhum processo de purificação, enquanto a invenção proposta purifica o MEL com membranas.

[025] Portanto, nenhum dos documentos do estado da técnica descreve um processo de obtenção de MEL, preferencialmente o MEL-B por *Pseudozyma tsukubaensis*, usando manipueira como meio de cultura, em que este processo promove a recuperação do MEL pela formação de espuma no biorreator e purificação desta por membranas em uma única etapa de ultrafiltração, em que não se utiliza solvente orgânico, tal como o descrito na presente invenção.

[026] Consequentemente, também não há documentos que fazem referência à composição com alto teor de MEL, preferencialmente o MEL-B, obtidos através do processo

descrito, bem como seus usos para aplicações em cosméticos, como agente tensoativo, emulsificante na recuperação de petróleo e na pulverização em cultivares.

Breve descrição da invenção:

[027] A presente invenção refere-se a um processo de obtenção de manosileritritol lipídios (MEL) usando manipueira (resíduo da indústria farinheira) como meio de cultura.

[028] No processo, o MEL é recuperado pela formação de espuma no biorreator e purificado por membranas em uma única etapa de ultrafiltração, que não utiliza solvente orgânico.

[029] Em uma modalidade preferida da invenção, propõe-se um processo de obtenção do biossurfactante manosileritritol lipídio do tipo B (MEL-B) por *Pseudozyma tsukubaensis*.

[030] Adicionalmente, a invenção faz referência a composições com alto teor de MEL obtido através do processo descrito. Em uma modalidade preferida, a presente invenção se refere à composição com alto teor de MEL-B obtida, assim como seus usos para aplicações em cosméticos, como agente tensoativo, emulsificante na recuperação de petróleo e na pulverização em cultivares.

Breve descrição das figuras:

[031] Para obter um total e completa visualização do objeto desta invenção, são apresentadas as figuras as quais se faz referências, conforme se segue.

[032] A Figura 1 representa graficamente o perfil dos parâmetros fermentativos, sendo estes a tensão superficial do meio de cultura (círculo preto), a diluição

micelar crítica - 10x (triângulo), a diluição micelar crítica - 100x (losango) e a contagem de células (círculo branco).

[033] A Figura 2 representa um diagrama da produção e purificação do manosileritritol lipídio tipo B.

[034] A Figura 3 representa graficamente o processo de ultrafiltração baseado no fluxo do sistema.

[035] A Figura 4 representa graficamente o perfil do processo de ultrafiltração baseado no volume de alimentação através das concentrações do manosileritritol lipídio tipo B retido (círculo), proteína no retido (triângulo) e proteína no permeado (quadrado).

[036] A Figura 5 representa graficamente a análise de infravermelho da amostra de manosileritritol lipídio purificada por ultrafiltração.

[037] A Figura 6 representa graficamente a análise de MALDI-TOF-MS da amostra de manosileritritol lipídio purificada por ultrafiltração.

[038] A Figura 7 representa graficamente a análise de CG-MS (perfil dos ácidos graxos) da amostra de manosileritritol lipídio purificada por ultrafiltração, após extração e esterificação dos ácidos graxos.

Descrição detalhada da invenção:

[039] A presente invenção se refere a um processo de obtenção de manosileritritol lipídios (MEL) usando manipueira (resíduo da indústria farinheira) como meio de cultura.

[040] O processo de obtenção de MEL desta invenção compreende as etapas de:

a) Tratar a manipueira;

b) Produzir o MEL por fermentação;

b.1) Fermentação nas primeiras 24 horas

b.2) Fermentação de 24 a 84 horas

c) Recuperar e centrifugar a espuma produzida;

d) Secar e reidratar a solução obtida na etapa "c";

e) Filtrar a solução obtida em "d" em um equipamento de ultrafiltração com agitação magnética;

f) Obter a solução com concentração de MEL que varia de 800 a 900 mg/L.

[041] Os MELs que podem ser obtidos através do processo descrito são selecionados dos grupos que consistem em manosileritritol lipídios dos tipos -A, -B, -C e -D, preferencialmente manosileritritol lipídio do tipo B (MEL-B).

[042] A manipueira, que é um líquido de cor amarelada que sai da mandioca depois que ela é prensada, durante a processo de fabricação da farinha, é utilizada no presente processo como meio de cultura devido ao seu alto teor de nutrientes, conforme exemplo de composição apresentado na Tabela 1.

[043] No entanto, alternativamente, a manipueira pode ser substituída por quaisquer outros resíduos, desde que seja um resíduos agroindustriais com elevada solubilidade em água, ou meio de cultura, desde que os mesmos apresentem componentes e possuam condições que supram as necessidades do micro-organismo usado para a produção do MEL de interesse, preferencialmente os que contêm açúcar; e meios de cultura sintéticos e naturais.

Tabela 1 - Composição da manipueira

Alumínio (mg/L)	138-158	**Zinco (mg/L)**	1,4-7,38

Fósforo total (mg/L)	83,3-369	**Nitrogênio total (g/L)**	1,72-2,67
Potássio (mg/L)	895-3641	**Nitrato (mg/L)**	15,2-17,2
Cálcio (mg/L)	184-293	**Amônia (mg/L)**	129-133
Magnésio (mg/L)	173-519,09	**DQO (g O_2/L)**	45,36-60
Enxofre (mg/L)	38-154	**pH**	5,3-6,0
Ferro (mg/L)	2,72-8,0	**Açúcares Totais (g/L)**	33,78-58,18
Manganês (mg/L)	1,5-3,46	**Açúcares Redutores (g/L)**	12,23-38
Cobre (mg/L)	0,3-1,11	**Açúcares não redutores (g/L)**	20,1-23,3
Boro (mg/L)	3,0-5,0	**Sólidos totais (g/L)**	60-62

[044] Sendo assim, na etapa "a", o tratamento da manipueira ocorre através de aquecimento até seu ponto de ebulição durante um intervalo de tempo que varia de 1 a 5 minutos, preferencialmente 3 minutos. Em seguida, a mesma é centrifugada a uma força centrífuga que varia de 10^3 a $5x10^4$g, preferencialmente 10^4g. Assim, o sobrenadante obtido é utilizado como substrato no processo fermentativo.

[045] Na etapa "b", a produção do MEL é realizada com a manipueria tratada na etapa "a", a qual deve ser previamente esterilizada em uma temperatura que varia de 110 a 130°C, preferencialmente 121°C, durante um intervalo de tempo que varia de 10 a 30 minutos, preferencialmente 20 minutos, em bioreator industrial de 7,5 litros com volume de trabalho de 3,0 litros.

[046] Deste modo, a sub-etapa "b.1" compreende as primeiras 24 horas do processo fermentativo e ocorre nas

condições em que a taxa de agitação varia de 50 a 150 rpm, preferencialmente 100 rpm, e a taxa de aeração varia de 0,1 a 1,0 vvm, preferencialmente 0,4 vvm (volume de ar por volume de meio, por minuto). A sub-etapa "b.2" compreende entre 24 a 84 horas, a taxa de agitação varia de 100 a 200 rpm, preferencialmente 150 rpm, e a taxa de aeração varia de 0,4 a 1,6 vvm, preferencialmente 0,8 vvm (Figura 1).

[047] Por conseguinte, o processo fermentativo é iniciado com a inoculação de meio de cultura YEPD que varia de 100 a 300 ml, preferencialmente 210 mL, na concentração que varia de 0,02 a 0,231 g, preferencialmente 0,02155 gramas de células de levedura por 100 mL, em que estas leveduras são selecionadas do grupo que compreende Schizonella melanogramma, *Kurtzmanomyces sp*, *Ustilago* sp e *Pseudozyma tsukubaensis*.

[048] Em uma modalidade preferida da presente invenção, as células de levedura utilizadas são do tipo *Pseudozyma tsukubaensis* para a obtenção de MEL-B (Figura 2).

[049] Na etapa "c", a recuperação primária de MEL é realizada durante o processo fermentativo, devido à agitação e aeração descrita na etapa anterior, em que de 0,5 a 1,5L, preferencialmente 1L, de espuma é recuperada por orifício no topo do fermentador.

[050] Este processo separa os compostos que são capazes de formar espuma, neste caso, separar o MEL de interesse do meio de cultura (que contém uma concentração alta de impurezas), ou seja, é uma metodologia que aproveita as características do processo fermentativo (formação de espuma) para recuperar os referidos biossurfactantes e realizar uma etapa de purificação ao mesmo tempo, sem a

adição de nenhum material ou aparatos de alta dificuldade.

[051] Em seguida, a espuma é centrifugada à força centrífuga que varia de 10^3 a $5x10^4$ g, preferencialmente 10^4, durante um intervalo de tempo que varia de 10 a 30 minutos, preferencialmente 20 minutos. Assim, o sobrenadante resultante é utilizado para a sequência dos procedimentos.

[052] Na etapa "d", a secagem é realizada por liofilizador de bandeja e o pó remanescente resultante de uma massa que varia entre 10 e 18 g, preferencialmente a 14 g, é armazenado a uma temperatura que varia de -20 a -1°C, preferencialmente -18°C. A etapa "d" é opcional.

[053] Posteriormente, é realizada a reidratação (adição de água) do pó obtido no processo de secagem em concentração que varia de 700 a 1300mg, preferencialmente 1000 mg por litro.

[054] Na etapa "e", essa solução, que varia de 200 a 300 mL, preferencialmente 250mL, é adicionada a um equipamento de ultrafiltração, tal como *TFF system*, com agitação magnética que varia de 40 a 60 rpm e purificação por membrana tal como membrana de polietersulfona (PES), celulose regenerada, polipropileno, ácido poliático, entre outras, preferencialmente membrana de polietersulfona (PES), em que seu peso molecular de corte é de 100 kDa e área efetiva de 50 cm^2. A pressão nas válvulas de alimentação é mantida entre 10 a 30 psi, preferencialmente 20 psi, pressão nas válvulas do retido (retroalimentação) deve ser mantido entre 9 e 11 psi, preferencialmente 10 psi. Além disso, não há a adição de solvente orgânico.

[055] Sendo assim, a purificação por membranas é possível devido à separação do MEL de diversas impurezas

(recuperação da espuma), uma vez que soluções com alto teor de impureza resultam em *fouling* - entupimento dos poros - ou seja, não há filtração.

[056] Deste modo, o fluxo da ultrafiltração é de 90 a 45L/h.m^2(+/- 5) e deve ser calculado baseado na Equação 1 abaixo:

Equação 1 - *(LMH or L/m2 h) = [flow rate (mL/min)/membrane aerea (cm^2)] x 600.*

[057] Cabe ressaltar que o sistema deve ser condicionado (limpeza) antes e após a ultrafiltração e a membrana deve ser armazenada de acordo com o protocolo do fabricante.

[058] Assim, após um intervalo de tempo que varia de 40 a 50 minutos, preferencialmente 45 minutos de filtração, o volume inicial da alimentação é reduzido a 1/10 do valor inicial. Por exemplo, se o volume inicial da alimentação é de 250 mL, este é reduzido a 25 mL (Figura 3).

[059] Vale ressaltar que, devido às etapas de coleta da espuma, liofilização e ultrafiltração, há uma perda natural de material. Entretanto o diferencial da presente invenção é o fato da ultrafiltração e purificação por membrana do substrato ser realizada em uma única etapa (etapa "e"), melhorando, assim, a produtividade e o rendimento do volume final.

[060] Portanto, na etapa "f", o volume final da solução, obtido após filtração, apresenta alta concentração de MEL que varia de 800 a 900 mg/L.

[061] Em uma modalidade alternativa da presente invenção, o volume final obtido na etapa "f" é submetido à secagem da solução por liofilização, visando à obtenção de

pó com alta concentração de MEL, o qual varia de 200 a 300 mg.

[062] Cabe ressaltar que o rendimento do processo e a necessidade de etapas adicionais ou modificadas no processo de purificação podem ser dependentes de cada MEL, tais como do tipo -A, -B, -C, -D ou mistura dos mesmos.

[063] Em uma modalidade preferida, a presente invenção se refere ao processo de obtenção do biossurfactante MEL-B por *Pseudozyma tsukubaensis*.

[064] Assim sendo, na etapa "f", o volume final da solução obtido após filtração, apresenta alta concentração de MEL-B, o qual varia de 800 a 900 mg/L, preferencialmente 859,52 mg/L (Figura 4).

[065] Ainda, o volume final obtido na etapa "f" de MEL-B pode ser submetido à secagem da solução por liofilização, para obtenção de pó com alta concentração de MEL-B, o qual varia de 200 a 300 mg.

[066] Desta forma, adicionalmente, a presente invenção faz referência a composição com alto teor de MEL que varia de 800 a 900 mg/L e de 200 a 350 mg, respectivamente, na forma de solução e de pó obtida conforme processo aqui descrito.

[067] Em uma modalidade preferida da presente invenção a composição obtida compreende alto teor de MEL-B, que varia de 800 a 900 mg/L e de 200 a 300 mg, respectivamente, na forma de solução e de pó.

[068] Adicionalmente, a invenção proposta se refere ao uso da composição com alto teor de MEL-B na forma de solução para a aplicação como agente tensoativo, emulsificante na recuperação de petróleo e na pulverização

em cultivares (atividade antimicrobiana); e, na forma de pó, para a aplicação em cosméticos, preferencialmente os dermocosméticos.

- Testes Realizados:

- Proporção de MEL-B obtido por cada fermentação utilizando o processo aqui descrito:

[069] Em um teste de obtenção de MEL-B utilizando o processo da presente invenção, a espuma produzida durante cada fermentação resultou em cerca de 1060 mL. Após a etapa de liofilização, cerca de 14,01 g de pó foram recolhidos. O pó foi, então, solubilizado em uma concentração de 1091,59 mg/L e essa solução foi utilizada para o processo de ultrafiltração.

[070] O processo de ultrafiltração resultou em 25 mL de solução com concentração de manosileritritol lipídio igual a 859,52 mg/L, ou seja, 21,49 mg de manosileritritol lipídio. Logo, extrapolando a solubilização de 1091,59 mg/L para os 14,01 g produzidos em cada fermentação, é possível obter aproximadamente 275 mg de manosileritritol lipídio purificado.

- Identificação química do MEL-B:

[071] A identificação química do manosileritritol lipídio pode ser realizada com análises de infravermelho do manosileritritol lipídio, CG-MS e MALDI-TOF-MS.

[072] A análise de infravermelho do manosileritritol lipídio deve indicar alta absorção nos seguintes números de ondas: 3400 (O-H), 1730 (C=O), 1240 (C-O), 1075 (-O-). Na Figura 5, os manosileritritol lipídios são identificados por infravermelho.

[073] A análise de MALDI-TOF-MS deve indicar a

produção de uma série de homólogos, porém com os principais picos com razão carga massa (m/z) de 683,41 e 657,42. Na Figura 6, os manosileritritol lipídios são identificados por MALDI-TOF-MS (espectro).

[074] A análise dos ácidos graxos por CG-MS deve identificar a presença de C8; C10; C12:1; C12:0; C14:1 e C18:1, no qual C12 e C14:1 devem ser majoritários. Na Figura 7, é possível observar o perfil dos ácidos graxos do manosileritritol lipídio analisados via CG-MS.

[075] Os versados na arte valorizarão os conhecimentos aqui apresentados e poderão reproduzir a invenção nas modalidades apresentadas e em outras variantes, abrangidas no escopo das reivindicações anexas.

REIVINDICAÇÕES

1. Processo de obtenção de manosileritritol lipidio (MEL) **caracterizado** por utilizar manipueira como meio de cultura e compreender as etapas de:

a) Tratar a manipueira;

b) Produzir o MEL:

b.1) Fermentação nas primeiras 24 horas;

b.2) Fermentação de 24 a 84 horas;

c) Recuperar e centrifugar a espuma produzida;

d) Secar e reidratar a solução obtida na etapa "c";

e) Filtrar a solução obtida em "d" em um equipamento de ultrafiltração com agitação magnética;

f) Obter a solução com MEL.

2. Processo, de acordo com a reivindicação 1, **caracterizado** pelo fato de os MELs obtidos serem selecionados do grupo que consiste em manosileritritol lipídios dos tipos -A, -B, -C e -D, preferencialmente manosileritritol lipidio do tipo B (MEL-B).

3. Processo, de acordo com a reivindicação 1, **caracterizado** pela manipueira ser alternativamente substituída por resíduos agroindustriais com elevada solubilidade em água ou meios de cultura sintéticos e naturais, preferencialmente os que contêm açúcar.

4. Processo, de acordo com a reivindicação 1, **caracterizado** por, na etapa "a", a manipueira ser previamente esterilizada em uma temperatura que varia de 110 a 130°C, preferencialmente 121°C, durante um intervalo de tempo que varia de 10 a 30 minutos, preferencialmente 20 minutos; o tratamento da manipueira ser através de aquecimento até seu ponto de ebulição durante um intervalo de tempo que varia de

1 a 5 minutos, preferencialmente 3 minutos, seguido de centrifugação à força centrífuga que varia 10^3 a $5x10^4$g, preferencialmente 10^4g.

5. Processo, de acordo com a reivindicação 1, **caracterizado** por, na etapa "b", o processo fermentativo ser iniciado com a inoculação de meio de cultura YEPD em um volume que varia de 100 a 300 ml, preferencialmente 210 mL, na concentração que varia de 0,02 a 0,231 g, preferencialmente 0,02155 gramas de células de levedura por 100 mL.

6. Processo, de acordo com a reivindicação 1, **caracterizado** por a fermentação da sub-etapa "b.1" ocorrer a uma taxa de agitação que varia de 90 a 110 rpm, preferencialmente 100 rpm, e uma taxa de aeração que varia de 0,35 a 0,45 vvm, preferencialmente 0,4 vvm, nas primeiras 24 horas apresentar.

7. Processo, de acordo com a reivindicação 1, **caracterizado** por a fermentação da sub-etapa "b.2" ocorer a uma taxa de agitação que varia de 100 a 200 rpm, preferencialmente 150 rpm, e uma taxa de aeração que de 0,4 a 1,6 vvm, preferencialmente 0,8 vvm.

8. Processo, de acordo com a reivindicação 5, **caracterizado** pelo fato de as células de levedura serem selecionadas do grupo que compreende Schizonella melanogramma, *Kurtzmanomyces sp*, *Ustilago* sp e *Pseudozyma tsukubaensis*, preferencialmente *Pseudozyma tsukubaensis*.

9. Processo, de acordo com a reivindicação 1, **caracterizado** por, na etapa "c", a recuperação primária de MEL ser realizada durante o processo fermentativo, em que de 0,5 a 15,0L, preferencialmente 1L, de espuma é recuperada no

topo do fermentador; e posteriormente centrifugada com uma força variável entre 10^3 e $5x10^4$ g, preferencialmente 10^4, durante um intervalo de tempo que varia de 10 a 30 minutos, preferencialmente 20 minutos.

10. Processo, de acordo com a reivindicação 1, **caracterizado** pela etapa "d" ser opcional e a secagem ser realizada por liofilizador de bandeja e o pó remanescente ser armazenado a uma temperatura que varia de -20 a -1°C, preferencialmente -18°C; e, posteriormente, a reidratação do pó obtido no processo de secagem ser de forma a se obter concentração que varia de 700 a 1300mg, preferencialmente 1000 mg por litro.

11. Processo, de acordo com a reivindicação 1, **caracterizado** por, na etapa "e", a solução que varia de 200 a 300 mL, preferencialmente 250mL, ser adicionada a um equipamento de ultrafiltração, preferencialmente *TFF system*, com agitação magnética que varia de 40 a 60 rpm e purificação por membrana, em que a pressão nas válvulas de alimentação seja mantida entre 10 a 30 psi, preferencialmente 20 psi e pressão na válvula do retido ser mantida entre 9 e 11 psi, preferencialmente a 10 psi; o fluxo da ultrafiltração ser de 90 a 45L/h.m^2 (+/- 5) e ser calculado baseado na Equação 1; e o intervalo de tempo de filtração variar de 40 a 50 minutos, preferencialmente 45 minutos.

12. Processo, de acordo com a reivindicação 11, **caracterizado** por, na etapa "e", a membrana utilizada ser selecionada do grupo que compreender membrana de polietersulfona (PES), celulose regenerada, polipropileno, ácido poliático, entre outras, preferencialmente membrana de polietersulfona (PES), em que seu peso molecular de corte é

de 100 kDa.

13. Processo, de acordo com a reivindicação 1, **caracterizado** pela solução obtida em "f" poder sofrer processo de secagem, preferencialmente liofilização, e obter um produto final, preferencialmente MEL-B, em quantidade que varia entre 200 a 350 mg na forma de pó.

14. Composição **caracterizada** por ser obtida conforme processo definido nas reivindicações de 1 a 13 e compreender pelo menos 800 mg/L, na forma de solução, ou pelo menos 200 mg, na forma de pó, de teor de MEL, preferencialmente MEL-B.

15. Uso da composição conforme definida na reivindicação 14 **caracterizado** por ser na aplicação como agente tensoativo, emulsificante na recuperação de petróleo e na pulverização em cultivares, quando na forma de solução; e, quando na forma de pó, ser na aplicação de cosméticos, preferencialmente dermocosméticos.

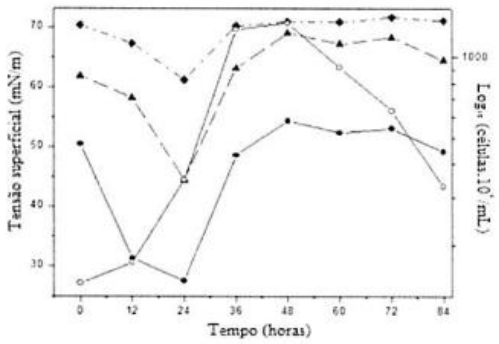

FIGURA 1

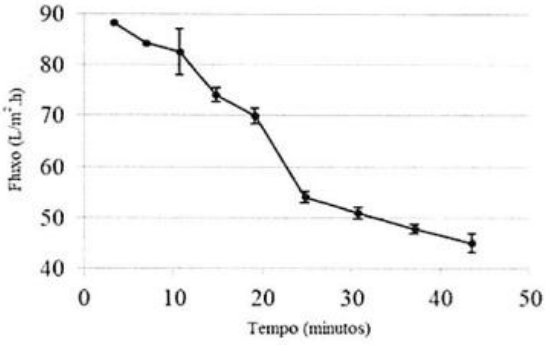

FIGURA 2

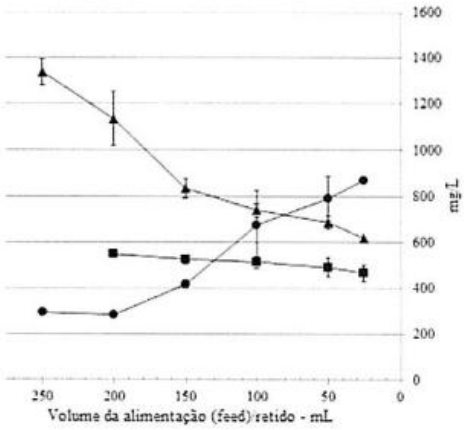

FIGURA 3

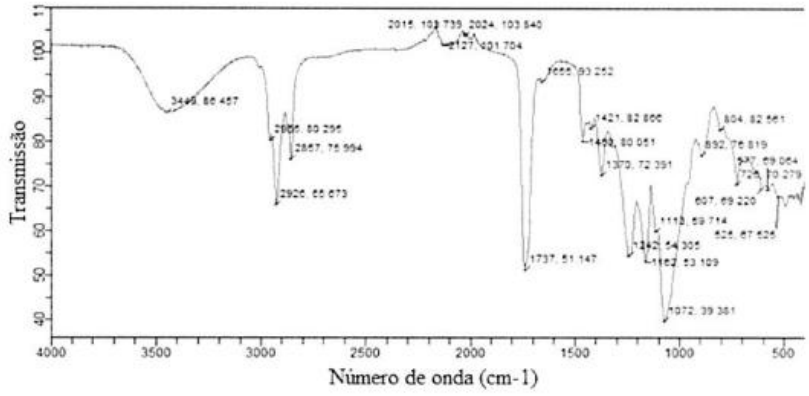

FIGURA 4

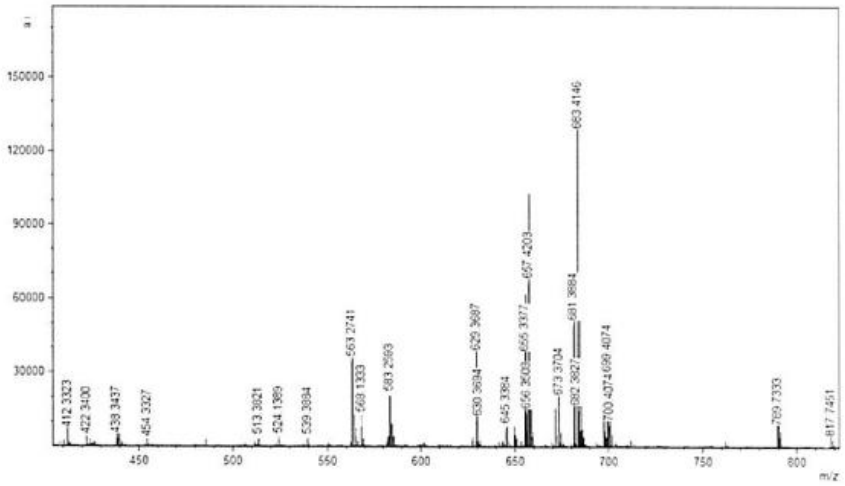

FIGURA 5

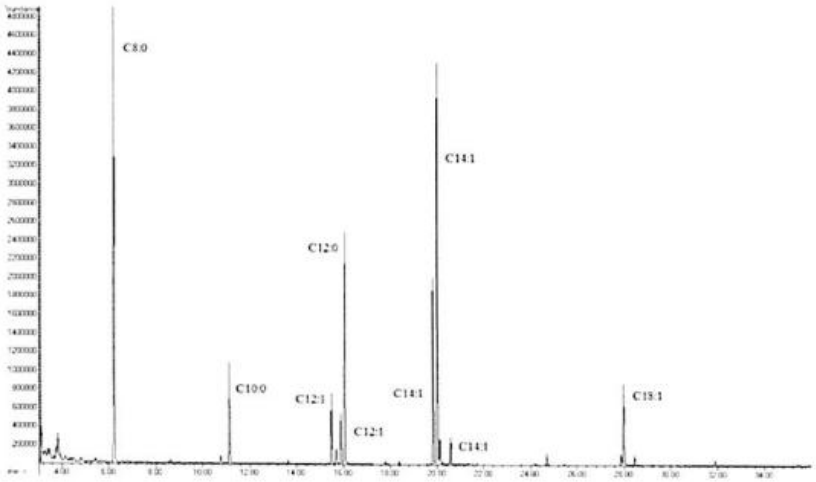

FIGURA 6

Resumo

PROCESSO DE OBTENÇÃO DE MANOSILERITRITOL LIPÍDIO (MEL), COMPOSIÇÕES E USOS DAS MESMAS

A presente invenção faz referência a um processo de obtenção de manosileritritol lipídio (MEL), preferencialmente do tipo B (MEL-B), por meio da fermentação, preferencialmente pela levedura *Pseudozyma tsukubaensis*, de manipueira (resíduo da indústria farinheira) como meio de cultura. Neste processo, o MEL é recuperado pela formação de espuma no biorreator e purificação desta por membranas em uma única etapa de ultrafiltração, que não utiliza solvente orgânico. Adicionalmente, a invenção faz referência a composições compreendendo alto teor de MEL, preferencialmente MEL-B, obtidos através do processo descrito, assim como seu uso para aplicações em cosméticos, como agente tensoativo, emulsificante na recuperação de petróleo e na pulverização em cultivares.