UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ENGENHARIA DE ALIMENTOS

ALINE WASEM ZANOTTO

ULTRAFILTRATION OF SURFACTIN PRODUCED BY *Bacillus subtilis* LB5a USING CASSAVA WASTEWATER WITH LOW PROTEIN CONTENT

ULTRAFILTRAÇÃO DA SURFACTINA PRODUZIDA POR *Bacillus subtilis* LB5a USANDO MANIPUEIRA COM BAIXO TEOR DE PROTEÍNA

CAMPINAS
2018

ALINE WASEM ZANOTTO

ULTRAFILTRATION OF SURFACTIN PRODUCED BY *Bacillus subtilis* LB5a USING CASSAVA WASTEWATER WITH LOW PROTEIN CONTENT

ULTRAFILTRAÇÃO DA SURFACTINA PRODUZIDA POR *Bacillus subtilis* LB5a USANDO MANIPUEIRA COM BAIXO TEOR DE PROTEÍNA

Dissertação apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Mestra em Ciência de Alimentos.

Dissertation presented to the Faculty of Food Engineering of the State University of Campinas in partial fulfillment of the requisites for the degree of Master in Food Science.

Orientadora: Profª. Drª. Glaucia Maria Pastore

Coorientador: Dr. Cristiano José de Andrade

Este exemplar corresponde a versão final da dissertação defendida pela aluna Aline Wasem Zanotto e orientada pela Prof.ª Dr.ª Glaucia Maria Pastore.

CAMPINAS

2018

Agência(s) de fomento e nº(s) de processo(s): CAPES

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Engenharia de Alimentos
Márcia Regina Garbelini Sevillano - CRB 8/3647

Z17u

Zanotto, Aline Wasem, 1994-
Ultrafiltration of surfactin produced by *Bacillus subtilis* LB5a using cassava wastewater with low protein content / Aline Wasem Zanotto. – Campinas, SP : [s.n.], 2018.

Orientador: Glaucia Maria Pastore.
Coorientador: Cristiano José de Andrade.
Dissertação (mestrado) – Universidade Estadual de Campinas, Faculdade de Engenharia de Alimentos.

1. Manipueira. 2. Surfactina. 3. Ultrafiltração. I. Pastore, Glaucia Maria. II. Andrade, Cristiano José de. III. Universidade Estadual de Campinas. Faculdade de Engenharia de Alimentos. IV. Título.

Informações para Biblioteca Digital

Título em outro idioma: Ultrafiltração da surfactina produzida por *Bacillus subtilis* LB5a usando manipueira com baixo teor de proteína
Palavras-chave em inglês:
Cassava Wastewater
Surfactin
Ultrafiltration
Área de concentração: Ciência de Alimentos
Titulação: Mestra em Ciência de Alimentos
Banca examinadora:
Glaucia Maria Pastore
Eder da Costa dos Santos
Marcus Bruno Soares Forte
Data de defesa: 06-08-2018
Programa de Pós-Graduação: Ciência de Alimentos

Banca Examinadora

_____

Prof.ª Dr.ª Glaucia Maria Pastore

(Orientador)

FEA - UNICAMP

_____

Prof. Dr. Eder da Costa dos Santos
(Titular)
UTFPR – Francisco Beltrão

_____

Prof. Dr. Marcus Bruno Soares Forte
(Titular)
FEA – UNICAMP

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

Qualquer caminho que você decida tomar existe sempre alguém para te dizer que você está errado. "Existem sempre dificuldades surgindo que tentam a acreditar que as críticas estão corretas. Mapear um caminho de ação e segui-lo até o fim requer coragem".

Ralph Waldo Emerson

A minha querida mãe e meu falecido irmão

Dedico

AGRADECIMENTOS

A toda minha família e amigos.

À Michel Yudi Shinkai Kanemaru, pelo carinho, companheirismo, incentivo e principalmente paciência durante todo o mestrado.

À Deus, por todas as conquistas de minha vida.

À minha orientadora, Prof.ª Dr.ª Glaucia Maria Pastore por ter sido sempre tão atenciosa, em especial, pela confiança e oportunidade oferecida e por acreditar no desenvolvimento deste trabalho, minha eterna gratidão e admiração.

Ao meu coorientador, Dr. Cristiano José de Andrade, por sempre me incentivar, por suas correções, por ser tão paciente e generoso, por acreditar em mim em todos os momentos.

Aos membros da banca, pela disponibilidade e auxílio nas correções.

A Plaza Indústria e Comércio Ltda por doar a manipueira usada neste trabalho.

A todos os colegas do Laboratório de Bioaromas, Compostos Bioativos e Biotecnologia de Alimentos.

A Angélica, Débora, Dora e Nadir, que são fundamentais para o funcionamento dos laboratórios.

Ao laboratório de apoio central (FEA) pelo uso de alguns equipamentos, em especial Bianca Wopereis.

A todos os técnicos e funcionários do Dempster Mass Spectrometry Lab, em especial Bruno Labate Vale da Costa e Dr.ª Lidiane Maria de Andrade pela colaboração nas análises cromatográficas.

A todos os técnicos e funcionários da FEA.

Seria impossível listar todos os nomes a que devo gratidão. Por isso, a todas as pessoas que contribuíram no desenvolvimento desse trabalho, direta ou indiretamente, o meu muito obrigado e os deixo aqui um abraço.

RESUMO

A crescente preocupação com problemas ambientais relacionada ao uso de surfactantes derivados do petróleo, não-biodegradáveis, ecotóxicos, dependentes da indústria petroquímica (fonte finita), instiga o estudo de surfactantes de origem microbiana (biossurfactantes), como a surfactina. Em relação aos custos globais para obtenção de biossurfactantes, a produção e recuperação de biossurfactante ainda são os maiores empecilhos que inviabilizam a aplicação desses compostos em larga escala. O processo de recuperação dos biossurfactantes representa aproximadamente 60% dos custos de produção, enquanto que o meio de cultura entre 30-50%. Deste modo, buscam-se alternativas para reduzir os custos de produção (meio de cultura e técnicas de purificação), a fim de tornar viável economicamente a produção de biossurfactantes em escala industrial. Neste contexto, uma das estratégias mais promissoras é o uso de resíduos agroindustriais como meio de cultura associado a sistemas filtrantes (purificação); por exemplo a manipueira (resíduo da produção de farinha de mandioca) que pode ser utilizada para a produção de surfactina e associada ao processo de ultrafiltração. Portanto, essa estratégia (manipueira + ultrafiltração) representa uma alternativa para a produção e purificação da surfactina de forma potencialmente viável e econômica. Porém, o uso da manipueira como meio de cultura dificulta o processo de ultrafiltração (incrustação e/ou polarização na membrana), principalmente devido as proteínas da própria manipueira bem como as sintetizadas inerentemente pelo micro-organismo produtor de biossurfactante. Portanto, este trabalho objetiva avaliar o efeito de diferentes tratamentos de remoção das proteínas da manipueira, bem como o efeito desses tratamentos na produção e purificação (ultrafiltração) da surfactina. Os tratamentos usados para remoção das proteínas foram: HCl pH2 e HCl pH3, $(NH_4)_2SO_4$ e $C_2HCl_3O_2$ (TCA). A manipueira tratada com $(NH_4)_2SO_4$ e $C_2HCl_3O_2$ (TCA) não possibilitou o crescimento do *Bacillus subtilis* LB5a e consequente produção de surfactina. Os tratamentos com HCl pH2 e pH3 apresentaram produção de 749,80 mg/L e 625,82 mg/L com 72 horas de fermentação, valores de produtividade maiores que o controle (manipueira sem tratamento) – 598,56 mg/L. Considerando os resultados dos experimentos anteriores apenas o tratamento HCl pH2 foi submetido a purificação por ultrafiltração. A etapa de ultrafiltração consistiu em duas etapas com membranas de 100 e 50 kDa, respectivamente. As duas etapas de ultrafiltração proporcionaram uma recuperação superior a 72% e pureza de 51,49%. Portanto, foi possível observar que o tratamento da manipueira com HCl pH2 aumentou a produção de surfactina e foi possível recuperar e purificar utilizando a ultrafiltração diretamente o meio de cultura obtendo bons rendimentos de recuperação e pureza.

Palavras-chave: Manipueira, Surfactina, Ultrafiltração.

ABSTRACT

The growing concern with environmental problems related to the use of non-biodegradable petroleum-based surfactants, which are ecotoxic and dependent on the petrochemical industry (finite source), instigates the study of surfactants of microbial origin (biosurfactants), as to surfactin. In relation to the global costs to obtain biosurfactants, the production and recovery of biosurfactant are still the major obstacles that prevent the application of these compounds on a large scale. The recovery process of the biosurfactants represents approximately 60% of the costs of production, while the medium of culture between 30-50%. In this way, alternatives to reduce production costs (culture medium and purification techniques) in order to make the production of biosurfactants economically feasible on industrial scale are pursued by researches and industry. In this context, one of the most promising strategies is the use of agro-industrial residues as a culture medium associated with filtering systems (purification); for example, cassava wastewater (CWW) a residue from cassava flour production, that can be used for the production of surfactin and associated with the ultrafiltration process. Therefore, this strategy (CWW + ultrafiltration) represents an alternative for the production and purification of surfactina in a potentially viable and economical way. However, the use of cassava wastewater as a culture medium hampers the process of ultrafiltration (fouling and/or polarization in the membrane), mainly due to the proteins of the cassava wastewater itself as well as those inherently synthesized by the microorganism producing biosurfactant. Therefore, this work aims to evaluate the effect of different treatments in cassava wastewater for proteins removal, as well as the effect of these treatments on the production and purification (ultrafiltration) of surfactin. The treatments used to remove the proteins were: HCl pH2 and HCl pH3, (NH4) $_2SO_4$ and $C_2HCl_3O_2$ (TCA). The CWW treated with $(NH_4)_2SO_4$ and $C_2HCl_3O_2$ (TCA) did not allow the growth of *Bacillus subtilis* LB5a and consequent production of surfactin. On the other hand, treatments with HCl pH2 and pH3 presented a 749.80 mg/L and 625.82 mg/L production with 72 hours of fermentation, higher productivity values than the control (cassava wastewater without treatment) - 598.56 mg/L. Considering the results of the previously experiments only the HCl pH2 treatment was subjected to ultrafiltration purification. The ultrafiltration step consisted of two steps with membranes of 100 and 50 kDa, respectively. The two-ultrafiltration steps provided a recovery of greater than 72% and purity of 51.49%. Therefore, it was possible to observe that the handling of the cassava wastewater with HCl pH2 increased the surfactin production and enabled the ultrafiltration of the surfactin directly from the culture medium presenting with good yields of recovery and purity.

Keywords: Cassava Wastewater, Surfactin, Ultrafiltration.

Lista de Ilustrações

INTRODUÇÃO GERAL

CHAPTER I

CHAPTER II

Lista de Tabelas

CHAPTER I

CHAPTER II

Lista de Abreviaturas e Siglas

(NH4)2SO4	Sulfato de amônio
Ala	Alanina
Asp	Asparagina
C/N	Carbono/Nitrogênio
CAN	Acetonitrila
Cf	Concentração de surfactina
Cpp	Concentração de surfactina no permeado
Cpf	Concentração de surfactina no retido
CFU	Unidades formadoras de colônias
Cp	Concentração de proteína total
Cs	Concentração de surfactina total
CWW	Manipueira
CWW-2	Manipueira tratada com pH2
CWW-3	Manipueira tratada com pH3
EtOH	Etanol
FEA	Faculdade de Engenharia de Alimentos
Glu	Glutamina
HCl	Ácido clorídrico
HFLC	Cromatografia líquida de ultra-alta performace
HPAEC-PAD	Cromatografia de troca aniônica de alto desempenho acoplada a um sistema de detecção amperométrica pulsado
Ile	Isoleusina
lb	Libra
Leu	Leucina
MF	Microfiltração
Ms	Massa de surfactina
Msi	Massa de surfactina no retentado
Msii	Massa de surfactina na alimentação
MWCO	Corte de peso molecular
NaOH	Hidróxido de sódio
NRPS	Peptídeo sintetases não ribossômicas

o/w	Óleo em água
Pp	Pureza da surfactina
Rp	Coeficiente de rejeição para proteína
Rs	Coeficiente de rejeição para surfactina
TCA	Ácido tricloroacético
TFA	Ácido tricloroacético
TRS	Recuperação total de surfactina
TRSi	Recuperação total de surfactina na UF-1
TRSii	Recuperação total de surfactina na UF-2
UF	Ultrafiltração
UF-1	Primeira etapa de ultrafiltração
UF-2	Segunda etapa de ultrafiltração
US$	Dólar
Val	Valina
w/o	Água em óleo
α	Alfa
β	Beta

SUMÁRIO

1. INTRODUÇÃO GERAL

A maioria dos surfactantes empregados nos diversos setores industriais: indústria de alimentos, formulação de pesticidas e produtos de limpeza ainda é derivada da indústria petroquímica (KARSA, 1999; MAKKAR; CAMEOTRA; BANAT, 2011). O principal impacto causado pelo uso desses compostos são os efeitos negativos gerados para o meio ambiente, pois os surfactantes derivados da indústria petroquímica não são biodegradáveis, apresentam ecotoxicidade e capacidade de bioacumulação. Logo, há a necessidade de pesquisas/aplicações por surfactantes mais sustentáveis, como os surfactantes de origem microbiana, assim denominados biossurfactante (SOBERÓN-CHÁVEZ, 2010).

Dentre os biossurfactantes mais conhecidos (produção e purificação), a surfactina destaca-se sendo produzida por *Bacillus* sp. A surfactina apresenta estrutura heptapeptídica cíclica ligada à um ácido graxo *β*-hidróxi carbônica (12 a 16 carbonos) (KAKINUMA; TAMURA; ARIMA, 1968). A surfactina apresenta excepcional capacidade de reduzir a tensão superficial e interfacial de sistemas óleo em água (o/w em inglês) e água em óleo (w/o) de 72 para 27 mN/m (ARIMA; KAKINUMA; TAMURA, 1968; SOBERÓN-CHÁVEZ, 2010).

Além das características químicas (estrutura excepcional) e biodegradabilidade, a produção de biossurfactantes de origem microbiana é interessante, pois não é sazonal, tem-se controle das condições de produção e quantidade produzida, os biossurfactantes possuem resistência a variações de temperatura, pH e salinidade, podendo ser realizada utilizando resíduos agroindustriais como substrato, como a manipueira derivada do processamento da mandioca (ANDRADE et al., 2016; BARROS; PONEZI; PASTORE, 2008; NITSCHKE; FERRAZ; PASTORE, 2004). Deste modo a produção de biossurfactantes usando resíduos agroindustriais como meio de cultura pode contribuir de maneira indireta para a gestão de resíduos (MARÓSTICA; PASTORE, 2007).

A produção de biossurfactante em escala industrial ainda apresenta elevados custos envolvidos no processo de recuperação desses compostos, que representam aproximadamente 60% do custo de produção (ANDRADE et

al., 2016b; BARROS et al., 2007). Neste contexto, as abordagens mais utilizadas para recuperação e purificação desses compostos envolvem fracionamento da espuma e precipitação ácida (ácido clorídrico) (NITSCHKE; FERRAZ; PASTORE, 2004), extração sólido-liquido, extração líquido-líquido (hexano e acetato de etila) (CHEN; JUANG, 2008), cristalização, cromatografia por adsorção, precipitação e diafiltração (DESAI; BANAT, 1997), extração assistida por ultrasom (YUAN et al., 2012) e UF (ANDRADE et al., 2016). O uso da maioria dessas técnicas acaba gerando grandes quantidades de resíduos ou empregam solventes orgânicos, com exceção a UF que não gera resíduo (COUTTE et al., 2017).

Assim, a ultrafiltração (UF) seria uma alternativa viável e de fácil aplicação para os processos de recuperação de biossurfactantes. Porém o uso de manipueira como substrato aumenta o efeito de incrustação da membrana (*foulling*), ou seja, reduz drasticamente o fluxo e consequentemente a produtividade (ANDRADE et al., 2016). Portanto, uma alternativa seria a remoção de proteínas da manipueira previamente a fermentação.

1.1 SURFACTINA

A surfactina é produzida por *Bacillus* sp*.,* foi descoberta por Arima, Kakinuma e Tamura (1968). A surfactina possui estrutura cíclica (polar) - heptapeptídeo ($_{L}$-Glu-$_{L}$-Leu-$_{D}$-Leu-$_{L}$-Asp-$_{L}$-Asp-$_{D}$-Leu-$_{L}$–Leu) é ligado a uma cadeia ácido graxo *β*-hidróxi de cadeia carbônica variável (12 a 16 C), algumas modificações na sequência quiral dos resíduos de aminoácidos na cadeia peptídica é observada, a qual pode apresentar aminoácidos do grupo alifático como, Val, Leu e Ile nas posições 2, 4 e 7 (KAKINUMA; TAMURA; ARIMA, 1968; SOBERÓN-CHÁVEZ, 2010), surfactina com Ala na posição 4 também foi relatada (SOBERÓN-CHÁVEZ, 2010). A principal isoforma da surfactina está apresentada na Figura 1.

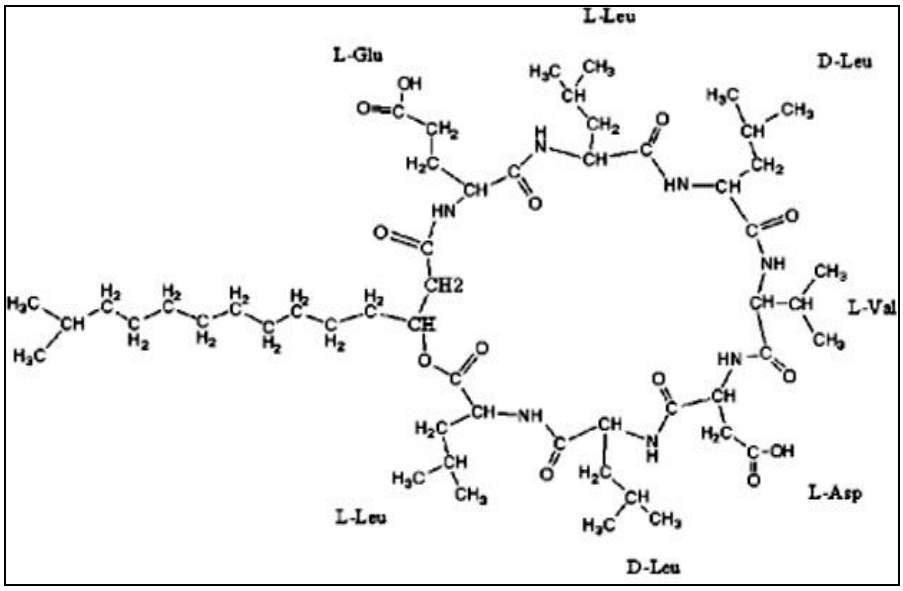


Figura 1. Principal isoforma da surfactina.

O mecanismo responsável pela síntese da surfactina é realizado por proteínas multi-enzimáticas chamadas de peptídeo sintetases não ribossômicas (NRPS), capazes de utilizar substratos não proteicos. A família da surfactina compreende aproximadamente 20 lipopetídeos distintos, essas variações podem estar associadas às condições da cultura como exemplo, as fontes de nutrientes (SOBERÓN-CHÁVEZ, 2010). Em geral, compostos biológicos de base proteica possuem resíduos de aminoácidos estereoisômeros L, enquanto que os resíduos de aminoácidos estereoisômero D não são comumente produzidos, sendo encontrados em apenas alguns peptídeos (NELSON; COX, 2014). A presença de D-aminoácidos na estrutura da surfactina pode influenciar as diferentes propriedades biológicas que ela possuiu.

Conhecida pela sua excepcional capacidade de formar emulsão e espuma, a surfactina é capaz de reduzir a tensão superficial da água de 72 para 27 $mN.m^{-1}$ em uma concentração de aproximadamente 10 mg/L (ARIMA; KAKINUMA; TAMURA, 1968; SOBERÓN-CHÁVEZ, 2010). Além de alterar as propriedades físicas/químicas de interfaces, a surfactina possui algumas atividades bioativas, como: atividade hemolítica (DUFOUR et al., 2005), antibacteriana, antiviral (KRACHT et al., 1999), antifúngica, anticancerígena, anti-inflamatória (ZHAO et al., 2017) e antimicoplasma (VOLLENBROICH; PAULI; MUHSIN, 1997).

A produção da surfactina ainda apresenta elevado custo de produção, dificultando sua aplicação em larga escala. Os custos para produção de um surfactante derivado da indústria petroquímica foi estimado em aproximadamente R$ 3,30 por kg (ANDRADE; PASTORE, 2017; MAKKAR; CAMEOTRA; BANAT, 2011). Atualmente o preço da surfactina comercializada pela Sigma Chemical Company é de aproximadamente R$ 184,40 por mg, ou seja, composto de alto valor agregado. A seleção de um meio de cultura para otimizar sua produção com baixos custos continua sendo um desafio (ISA; FRAZIER; JAUREGI, 2008). Uma alternativa para minimizar esses custos seria o emprego de resíduos agroindustriais que são uma rica fonte de nutrientes (BARROS et al., 2007). Além da redução dos custos de produção esses resíduos seriam aproveitados, podendo contribuir para a redução do impacto ambiental e a melhora da gestão de resíduos. A aplicação de processos que reduzam ou eliminem o uso de substâncias nocivas favorece o desenvolvimento de uma produção mais sustentável (*green chemistry concept*).

A surfactina produzida por *B. subtilis* LB5a utilizando manipueira como meio de cultivo foi relatada inicialmente por Nitschke et al., (2004). A produção foi realizada em pequena escala (erlenmeyer 250 mL) com 150 mL de volume de trabalho, biossurfactante foi recuperado por precipitação ácida com HCl 6 N (pH 2) *overnight,* onde foi observada uma produção de 2000 mg/L de biossurfactante bruto (NITSCHKE; PASTORE, 2004). Posteriormente uma produção em escala piloto (biorreator 80 L) com 40 L volume de trabalho foi descrita por Barros et al., (2008), o biossurfactante foi recuperado pela espuma (*foam overflow*) seguido de precipitação ácida e extração com solventes a concentração de biossurfactante obtida foi 680 mg/L. Além disso, foi reportado por Simiqueli (2014) a produção de surfactina usando manipueira como meio de cultura em modo contínuo. O bioprocesso contínuo foi realizado em biorreator de 7,5 L (volume de trabalho 4 L, 120 horas de fermentação), onde obteve-se uma produção de 1300 mg/L, após precipitação ácida e extração com solvente. Todas as determinações da concentração de surfactina foram realizadas indiretamente pela medida da tensão superficial. Recentemente, outra produção em biorreator 7,5 L (volume de trabalho de 3 L) reportou que a surfactina foi coletada pela espuma (*foam overflow*), posteriormente semi-purificada por precipitação ácida. O biossurfactante semi-purificado passou por

duas estratégias de UF e obteve-se um rendimento de 360 mg/L com 72 horas de fermentação (ANDRADE et al., 2016b).

Os estudos a serem desenvolvidos ainda visam o aumento de produtividade e melhorias nos processos de recuperação e purificação para que se possa tornar viável o emprego de manipueira para produção de biossurfactantes em escala industrial.

1.2 MANIPUEIRA

A mandioca (*Manihot esculenta* Crantz), pertence à família *Euphorbiaceae,* originária na América do Sul, popularmente conhecida como aipim ou macaxeira na região norte e nordeste e mandioca na região sul do Brasil (EMPRAPA, 2017). A família da *Manihot esculenta* Crantz é composta por cerca de 300 gêneros e 8.000 espécies, a raiz apresenta característica tuberosa, é rica em amido e pode produzir glicosídeos cianogênicos (LEBOT, 2009).

A mandioca é cultivada em mais de 100 países, incluindo algumas ilhas do pacífico, sua adaptabilidade em solos de baixa fertilidade, ácidos, com variações nos regimes pluviométricos e seu ciclo de crescimento flexível (capaz de ser produzida o ano todo) fez com que o cultivo difundisse por diversos países (LEBOT, 2009). A demanda por produtos de baixo custo e ricos em nutrientes que compõem principalmente a cesta básica da população de classe baixa também favoreceu a expansão da produção da mandioca, especialmente da farinha de mandioca (SEAB, 2015).

No panorama mundial a produção estimada de mandioca nos anos de 2015/16 foi de 281,1 e 288,4 milhões de toneladas respectivamente, apresentando um aumento na produção de 2,6% (FAO, 2016). Os três países que lideraram o ranking desta produção foram Nigéria, Tailândia e Brasil representando aproximadamente 38,1% do total produzido mundialmente (SEAB, 2015).

A mandioca é cultivada em todos estados brasileiros e se destina principalmente ao consumo na forma *in natura,* farinhas e féculas (ABAM, 2014). Os estados que possuem a maior produção são: Paraná (4.000 mil toneladas), Mato Grosso do Sul (968 mil toneladas) e São Paulo (1.326 mil

toneladas), sendo que a produção do Paraná além de favorecer a ascenção do parque industrial do estado contribui com cerca de 70% do total produzido de fécula no país (SEAB, 2015).

A expansão da produção da *Manihot esculenta* Crantz e seus derivados tecnológicos favorece a balança comercial bem como a composição da cesta básica da população de muitos países em desenvolvimento (ABAM, 2014; FAO, 2016; SEAB, 2015). No entanto, a indústria de processamento da mandioca acaba gerando grandes quantidades de resíduos tais como: resíduos sólidos (casca) e águas residuárias (manipueira) (TUMWESIGYE; OLIVEIRA; SOUSA-GALLAGHER, 2016). Manipueira é o resíduo obtido a partir da prensagem da massa triturada da raiz de mandioca já lavada e sem casca (CHISTÉ; COHEN, 2006). A produção da manipueira é estimada em cerca de 30% do peso da matéria prima (WOSIACKI; CEREDA, 2002). Assim, 730 mil toneladas foram destinadas para a produção de fécula em 2015, gerando aproximadamente 219 mil litros de manipueira (ABAM, 2014; SEAB, 2015).

Essa quantidade expressiva de resíduo agroindustrial é rica em macro-nutrientes (amido, açúcares, proteínas) e micro-nutrientes (fosforo, potássio, cálcio, magnésio, enxofre, ferro, zinco, manganês, cobre, boro, nitrato) (MARÓSTICA; PASTORE, 2007). É um potencial poluidor ambiental devido às altas concentrações de matéria orgânica (composição) bem como a presença de glicosídeos cianogênios como a linamarina (93%) e lataustralina (7%) (KUYUCAK; AKCIL, 2013; TUMWESIGYE; OLIVEIRA; SOUSA-GALLAGHER, 2016).

O cianeto tem capacidade de formar complexos com metais, por este motivo é tóxico aos seres humanos e animais. Ao complexar-se com o ferro da hemoglobina ele impede o transporte de oxigênio para as células causando sufocamento do indivíduo (KUYUCAK; AKCIL, 2013). A liberação do cianeto, a partir dos glicosídeos cianogênicos, é iniciada no momento em que o tecido vegetal da mandioca é rompido, favorecendo a ação de enzimas *β*-glicosidases (linamarase), a clivagem tem como produto glicose e *α*-hidroxinitrilas. As *α*-hidroxinitrilas quando hidrolisadas por enzimas podem ser transformadas em ânion cianeto ou ácido cianídrico (HCN) (KUYUCAK; AKCIL, 2013). Logo, devido ao potencial tóxico da manipueira é necessário o

tratamento desse resíduo agroindustrial (KUYUCAK; AKCIL, 2013; PATIL; PAKNIKAR, 2000).

No entanto, ele ainda contém grande quantidade de matéria orgânica que quando descartado em ambientes aquáticos afetam as formas de vida ali presentes (PATIL; PAKNIKAR, 2000). Neste contexto, as técnicas de gerenciamento de resíduos normalmente são de alto custo devido à necessidade de infraestrutura adequada para os tratamentos (físico-químicos). Esse cenário dificulta o tratamento de resíduos por indústrias de pequeno porte (GHOSHEH; BSOUL; ABDULLAH, 2005). Deste modo um grande desafio é o desenvolvimento de métodos de baixo custo e simples para realizar o tratamento adequado e/ou aproveitamento sem afetar negativamente o meio ambiente. Assim, uma alternativa para o aproveitamento da manipueira é seu emprego em processos biotecnológicos como fonte de carbono (ANDRADE et al., 2016b; NITSCHKE; PASTORE, 2006).

2. OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar o efeito da presença das proteínas da manipueira na produção de surfactina por *Bacillus subtilis* LB5a, e consequente purificação da surfactina por UF.

2.2 OBJETIVOS ESPECÍFICOS

Capítulo I:

- Elaborar uma revisão bibliográfica sobre a utilização de resíduos agroindustriais e técnicas de recuperação usados durante os 50 anos de estudos da surfactina para sua produção.

Capítulo II:

- Remover parcialmente as proteínas da manipueira antes da fermentação;
- Produzir surfactina utilizando manipueira como meio de cultivo;
- Estudar a cinética de crescimento na manipueira com baixo teor de proteínas;
- Verificar o efeito da remoção de proteínas sobre a produção de surfactina;
- Avaliar o efeito da remoção das proteínas no processo de UF - purificar a surfactina por UF.

REFERÊNCIAS

ABAM. **Estatísticas Produção**. [s.l: s.n.]. Disponível em: <http://www.abam.com.br/estatisticas-producao.php>. Acessado em: 20 Janeiro de 2018.

ANDRADE, C. J. DE et al. Ultrafiltration based purification strategies for surfactin produced by *Bacillus subtilis* LB5A using cassava wastewater as substrate. **Journal of Chemical Technology and Biotechnology**, v. 91, n. 12, p. 3018–3027, 2016a.

ANDRADE, C. J. DE et al. Optimizing alternative substrate for simultaneous production of surfactin and 2 , 3-butanediol by *Bacillus subtilis* LB5a. **Biocatalysis and Agricultural Biotechnology**, v. 6, p. 209–218, 2016b.

ANDRADE, C. J. DE; PASTORE, G. M. Ultrafiltration of mannosylerytritol lipids: a parallel with surfactin. In: **Ars Separatoria**. [s.l: s.n.]. v. 11p. 171–200, 2017.

ARIMA, K.; KAKINUMA, A; TAMURA, G. Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. **Biochemical and biophysical research communications**, v. 31, n. 3, p. 488–494, 1968.

BARROS, F. F. C. et al. Surfactina: Propriedades químicas, tecnológicas e funcionais para aplicações em alimentos. **Química Nova**, v. 30, n. 2, p. 409–414, 2007.

BARROS, F. F. C.; PONEZI, A. N.; PASTORE, G. M. Production of biosurfactant by *Bacillus subtilis* LB5a on a pilot scale using cassava wastewater as substrate. **Journal Indian Microbiol Biotechnol**, v. 35, n. 9, p. 1071–1078, 2008.

CHEN, H. L.; JUANG, R. S. Recovery and separation of surfactin from pretreated fermentation broths by physical and chemical extraction. **Biochemical Engineering Journal**, v. 38, n. 1, p. 39–46, 2008.

CHISTÉ, R. C.; COHEN, K. DE O. **Efeito do Processo de Fabricação da Farinha de MandiocaEmbrapa Amazônia Oriental**. Belém, Pará: [s.n.].

COUTTE, F. et al. Microbial lipopeptide production and purification bioprocesses, current progress and future challenges. **Biotechnology Journal**, v. 12, n. 7, p. 1–10, 2017.

DESAI, J. D.; BANAT, I. M. Microbial production of surfactants and their commercial potential. **Microbiology and molecular biology reviews: MMBR**, v. 61, n. 1, p. 47–64, 1997.

DUFOUR, S. et al. Hemolytic activity of new linear surfactin analogs in relation to their physico-chemical properties. **Biochimica et Biophysica Acta - General Subjects**, v. 1726, n. 1, p. 87–95, 2005.

EMPRAPA. **Mandioca**. Disponível em: <https://www.embrapa.br/mandioca-e-fruticultura/cultivos/mandioca>. Acessado em 20 de fervereiro de 2018.

FAO. **Food Outlook: Biannual report on global foof markets**. [s.l: s.n.]. Disponível em: <http://www.fao.org/3/a-i6198e.pdf>. Acessaro em 20 de fervereiro de 2018.

GHOSHEH, H. Z.; BSOUL, E. Y.; ABDULLAH, A. Y. Agro-Processing Waste Assessment and Management in Peri-Urban Hanoi, Vietnam. **Journal of Sustainable Agriculture**, v. 25, n. 1, p. 69–95, 2005.

ISA, M. H. M.; FRAZIER, R. A.; JAUREGI, P. A further study of the recovery and purification of surfactin from fermentation broth by membrane filtration. **Separation and Purification Technology**, v. 64, n. 2, p. 176–182, 2008.

KAKINUMA, A.; TAMURA, G.; ARIMA, K. Wetting of fibrin plate and apparent promotion of fibrinolysis by surfactin, a new bacterial peptidelipid surfactant. **Experientia**, v. 24, n. 11, p. 1120–1121, 1968.

KARSA, D. R. **Industrial applications of surfactants IV**. Cambridge: The Royal Society of Chemistry, 1999.

KRACHT, M. et al. Antiviral and hemolytic activities of surfactin isoforms and their methyl ester derivatives. **The Journal of Antibiotics**, v. 52, n. 7, p. 613–619, 1999.

KUYUCAK, N.; AKCIL, A. Cyanide and removal options from effluents in gold mining and metallurgical processes. **Minerals Engineering**, v. 50–51, p. 13–29, 2013.

LEBOT, V. **Tropical Root and Tuber Crops: Cassava, Sweet Potato, Yams and Aroids**. 17. ed. London, UK: CABI, 2009.

MAKKAR, R. S.; CAMEOTRA, S. S.; BANAT, I. M. Advances in utilization of renewable substrates for biosurfactant production. **AMB Express**, v. 1, n. 1, p. 1–5, 2011.

MARÓSTICA, M. R. J.; PASTORE, G. M. Production of R-(+)-α-terpineol by the biotransformation of limonene from orange essential oil, using cassava waste water as medium. **Food Chemistry**, v. 101, n. 1, p. 345–350, 2007.

NELSON, D. L.; COX, M. M. **Príncipios de bioquímica de Lehninger**. 6 edição ed. Porto Alegre: Artmed, 2014.

NITSCHKE, M.; FERRAZ, C.; PASTORE, G. M. Selection of microorganismos for biosurfactant production using agroindustrial wastes. **Brazilian Journal of Microbiology**, v. 35, n. 1–2, p. 81–85, 2004.

NITSCHKE, M.; PASTORE, G. M. Biosurfactant production by Bacillus subtilis using cassava-processing effluent. **Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology**, v. 112, n. 3, p. 163–172, mar. 2004.

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

PATIL, Y. B.; PAKNIKAR, K. M. Development of a process for biodetoxification of metal cyanides from waste waters. **Process Biochemistry**, v. 35, n. 10, p. 1139–1151, 2000.

SEAB. **Análise Da Conjuntura Agropecuária Mandioca - Safra-2015/16Departamento de Economia Rural**. http://www.agricultura.pr.gov.br/arquivos/File/deral/Prognosticos/2016/mandioca_2015_16.pdf: [s.n.]. Acessado em 21 de fevereiro de 2018.

SIMIQUELI, A. P. R. **Produção contínua de surfactina utilizando manipueira como substrato e estudo de sua aplicação em conjunto com óleos essenciais**. [s.l.] Universidade Estadual de Campinas, 2014.

SOBERÓN-CHÁVEZ, G. **Biosurfactants: from genes to applications**. Berlin, Germany: Springer-Verlag, 2010. v. 20.

TUMWESIGYE, K. S.; OLIVEIRA, J. C.; SOUSA-GALLAGHER, M. J. Integrated sustainable process design framework for cassava biobased packaging materials: Critical review of current challenges, emerging trends and prospects. **Trends in Food Science and Technology**, v. 56, p. 103–114, 2016.

VOLLENBROICH, D.; PAULI, G.; MUHSIN, O. Antimycoplasma properties and application in cell culture of surfactin , a lipopeptide antibiotic from *Bacillus subtilis*. **Applied and Environmental Microbiology**, v. 63, n. 1, p. 44–49, 1997.

WOSIACKI, G.; CEREDA, M. P. Valorização de Resíduos do Processamento de Mandioca. **Ciências Exatas e da Terra, Ciências Agrárias e Engenharias,** v. 8, n. 1, p. 27–43, 2002.

YUAN, J. et al. The ultrasound-assisted extraction and identification of antifungal substances from *B. amyloliquefaciens* strain NJN-6 suppressing Fusarium oxysporum. **Journal of Basic Microbiology**, v. 52, n. 6, p. 721–730, 2012.

ZHAO, H. et al. Biological activity of lipopeptides from *Bacillus*. **Applied Microbiology and Biotechnology**, v. 101, n. 15, p. 5951–5960, 2017.

CHAPTER I

SURFACTIN FIFTY YEARS, HOW TO PRODUCE IT AT LOW COST

Aline Wasem Zanotto, Cristiano José de Andrade, Glaucia Maria Pastore

Manuscrito a ser submetido à – Trends in Biotechnology

ABSTRACT

In 1968, Arima et al. discovered a heptapeptide known as surfactin, which belongs to a family of lipopeptides. Its non-ribosomal synthesis mechanism was later discovered (1991). Known for its ability to reduce surface tension, it also has biological activities such as, antimicrobial and antiviral. Lipopeptides represent an important class of surfactants, which can be applied in many industrial sectors such as food, pharmaceutical, fungicides, detergents and cleaning products. Currently, 75% of the surfactants used in the various industrial sectors are from the petrochemical industry. Nevertheless, there is a global current demands (green chemistry concept) for replace the petrochemical products by environmentally friendly products, such as surfactants by biosurfactants. In this sense, the biosurfactant production is costly. Thus, an alternative to reduce the production costs is the use of agro-industrial waste as a culture medium associated with an efficient and scalable purification process. This review put a light on the most promising agro-industrial residues that can be used to produce surfactin, and the techniques used for its recovery.

Keywords: Surfactin production, agro-industrial residue and surfactin purification, ultrafiltration.

1. INTRODUCTION

Surfactants are amphiphilic molecules composed of a hydrophilic and a hydrophobic moiety (KOSARIC; VARDAR-SUKAN, 2015). The simultaneous presence of polar and nonpolar groups in the structure of the surfactants allows these substances to diffuse over different polarities interfaces, reducing surface and interfacial tensions (VARJANI; UPASANI, 2017).

Biosurfactants can be produced by plants and microorganisms (KOSARIC; VARDAR-SUKAN, 2015). Microbial production of biosurfactants is mainly by bacteria, for example, *Bacillus* sp. known for the production of lipopeptides and *Pseudomonas* sp. for the production of rhamnolipids; although some fungi like *Candida* sp. and *Pseudozyma* sp. are well-known to produce biosurfactants such as sophorolipids and mannosylerithritol lipids, respectively (SOBERÓN-CHÁVEZ, 2010). Thus, microorganisms can be used to produce biosurfactants (environmentally friendly products), however about 75% surfactants applied in the most diverse industrial sectors are synthetic substances derived from petroleum (KARSA, 1999; MAKKAR; CAMEOTRA; BANAT, 2011).

Accord to their chemical structure, the biosurfactants can be classified into 5 groups (Figure 1): (I) lipopetides (II) glycolipids (III) phospholipids (IV) polymeric surfactants and (V) particulate surfactants (ANDRADE & PASTORE, 2017; NITSCHKE & PASTORE, 2002). The different chemical structures of the surfactants lead to specific micellar concentration critical (CMC) values and to different self-aggregation, for example, micellar, hexagonal, cubic and lamellar (Figure 1) (SOBERÓN-CHÁVEZ, 2010). The self-aggregation structure of biosurfactants depends on the intermolecular electrostatic interactions, polarity, ionic strength and temperature of the solvent (HOLMBERG et al., 2002; MAI; EISENBERG, 2012; YU et al., 2014).

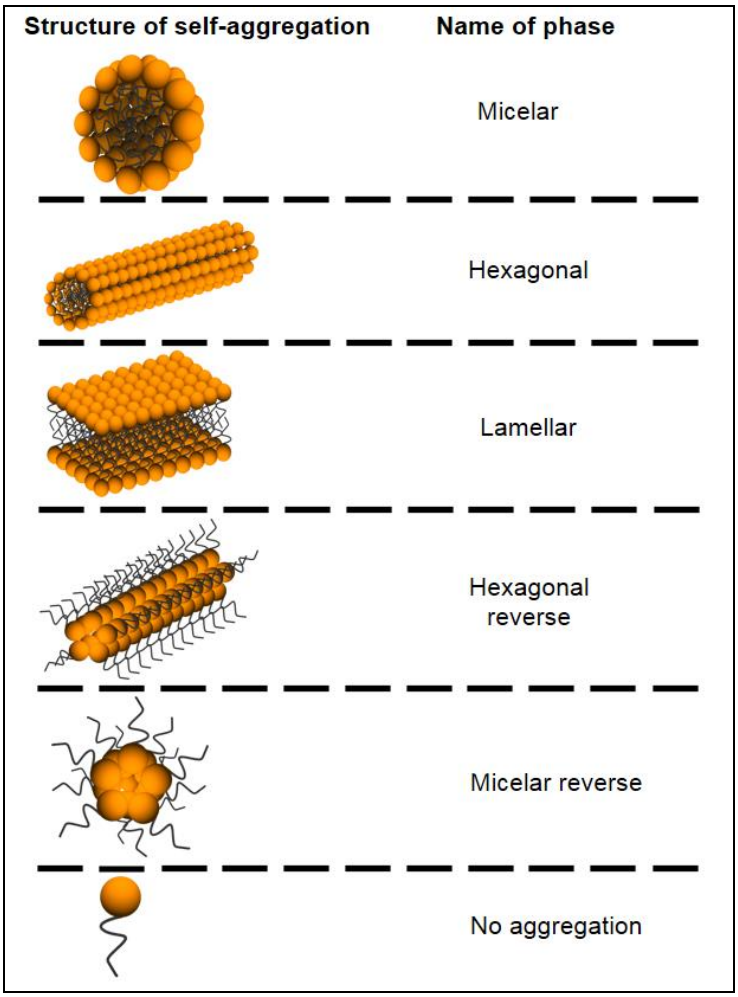


Figure 1. Forms of self-aggregation of biosurfactants and their respective denomination of phase. Adapted from (HOLMBERG et al., 2002).

In terms of the application of biosurfactants, household detergents and cosmetic are the largest markets. Other promissing market (for biosurfactant application) is the food industry, mainly due to biosurfactants with biocidal properties such as, can used as preservative on fruits, vegetables and meat products (SHUKLA, 2017). In this sense, biosurfactants produced by *Bacillus* sp. can be used as emulsifiers in organophosphorus pesticide formulations (PATEL; GOPINATHAN, 1986). Another potential application of biosurfactants is bioremediation of petroleum contamination, which in laboratory scale showed good results in the removal of petroleum (EBADI et al., 2017).

Products derived from non-renewable resources may have their production limited due to use of these compounds in various industrial sectors. In addition, there is growing concern about environmental problems with the use of petroleum-derived surfactants. Therefore, it is necessary to advance on

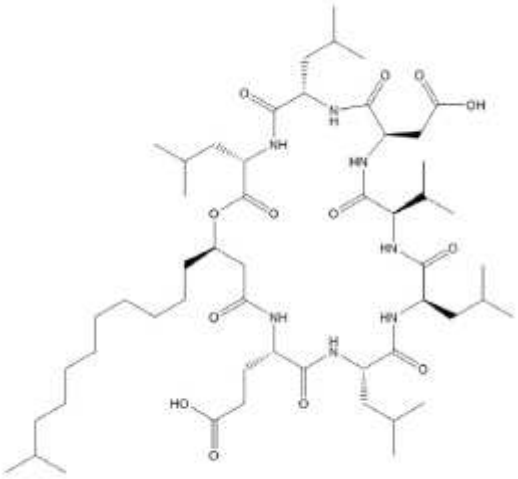
surfactants of microbial origin production and purification (KARSA, 1999; SOBERÓN-CHÁVEZ, 2010). The interest for biosurfactants is due to their characteristics of compatibility with the environment, low toxicity, biodegradable and capable of being produced with agro-industrial residues and/or alternative nutrient source (NITSCHKE; PASTORE, 2002; WHANG et al., 2008).

1.1 MAIN EVENTS AFTER THE DISCOVERY OF SURFACTIN

One of the most promising biosurfactant microorganisms producer is *Bacillus* sp. isolated around decade 1949 and 1960, known for the production of the lipopetides family. This family has more 30 peptides that gather tree different families such as, iturin, fengycin and surfactin (ZHAO et al., 2017). These families have structural differences in the chain of fatty acids and peptide sequence (SOBERÓN-CHÁVEZ, 2010). The families of iturin and fengycin were discovered in 1949 and 1986, respectively (NISHIKIORI et al., 1986; WALTON; WOODRUFF, 1949).

Surfactin discovered after iturin, is a cyclic (polar) heptapeptide attached to a β-OH (lactone) fatty acid chain (12 to 16 C). Discovered in 1968 by Arima et al., produced by several *Bacillus* species (Figure 2). Different modifications in the chiral sequence of amino acids in the peptide chain can occur, amino acids of the aliphatic group, Val, Leu and Ile in positions 2^{nd}, 4^{th} and 7^{th} was already observed (KAKINUMA; TAMURA; ARIMA, 1968). Surfactin with Ala at position 4^{th} has also been reported (SOBERÓN-CHÁVEZ, 2010).

In 1991, Nakano et al., found out that the synthesis pathway of surfactin is by multi-enzymatic proteins called non-ribosomal peptide synthetases (NRPS), capable of using non-protein substrates. The surfactin family comprises approximately 20 different lipopetides these variations may be associated with the culture conditions as an example, the sources of nutrients (SOBERÓN-CHÁVEZ, 2010). Protein based biological compounds have amino acid residues L stereoisomers, while D stereoisomer amino acid residues are not commonly found, being in only a few peptides. D-amino acids in surfactin structure may influence the different biological properties that it possesses (NELSON; COX, 2014).



1st	2nd	3rd	4th	5th	6th	7th
Glu	Leu	Leu	Ile	Asp	Leu	Ile
Glu	Ile	Leu	Ile	Asp	Leu	Ile
Glu	Leu	Leu	Ala	Asp	Leu	Leu
Glu	Leu	Leu	Val	Asp	Leu	Leu
Glu	Leu	Leu	Val	Asp	Leu	Val

Figure 2. Chemical structure of surfactin.

Known for its exceptional emulsion and foam ability, surfactin can reduce water surface tension from 72 to 27 mN/m at a concentration of ≈10 mg/L (ARIMA; KAKINUMA; TAMURA, 1968). Besides altering the physical/chemical properties of the interfaces, surfactin has some bioactive activities, such as: hemolytic activity (DUFOUR et al., 2005), antibacterial, antiviral, antifungal, anticancer, anti-inflammatory (ZHAO et al., 2017) and anti-mycoplasma (VOLLENBROICH; PAULI; MUHSIN, 1997).

Due to the high cost of surfactin production, is difficult to apply it at large scale. The costs for producing a surfactant derived from the petrochemical industry was estimated at US$ 1/lb (MAKKAR; CAMEOTRA; BANAT, 2011). Surfactin standard price marketed by Sigma Chemical Company is approximately US$ 62.75/mg (SIGMA-ALDRICH, 2018). From the economic point of view, when compared to chemical surfactants, surfactin is still not competitive.

The use of agro-industrial residues to produce surfactin has proven an economical alternative (ANDRADE et al., 2016a). However, culture medium is not the only cost in surfactin production, it represents only 30-50% of the total cost of production. In this review, we put a light on the newest approaches on the use of agro-industrial residues for the surfactin production and subsequent surfactin recovery and purification.

2. PRODUCTION OF SURFACTIN USING AGRO-INDUSTRIAL RESIDUES AS CULTURE MEDIUM

When incorrectly disposal, agro-industrial residue can causes environmental problems and for the welfare of humanity. Thus, an interesting strategy to reduce these environmental impacts is to use the agro-industrial residues as culture medium in biotechnological processes (ANDRADE et al., 2016a).

The advantage of the use of agro-industrial residues in biotechnological processes is due to its rich amount of organic matter, which contains all the macronutrients (proteins and carbohydrates) and micronutrients (minerals) that are essential for a microorganism grow and consequent production of high value-added compounds (ANDRADE et al., 2016b). Table 1, shows the residues used for the production of surfactin and the recovery techniques used.

Table 1. Agro-industrial residues applied on surfactin production with different strains of *Bacillus*, yields and recovery and purification techniques.

Agro-industrial waste	Microorganism	Yield	Recovery and purification	Reference
Cassava wastewater	*B. subtilis LB5a*	0.3 – 3.0 g/L	Acid precipitation; extraction with solvents; ultrafiltration	(ANDRADE et al., 2016; NITSCHKE & PASTORE, 2004)
Corn steep liquor	*B. subtilis #573*	1.3 – 12.3 g/kg of CSL	Acid precipitation; extraction liquid-liquid	(GUDIÑA et al., 2015; VECINO et al., 2015)
Potato waste	*B. subtilis* ATCC 21332	NQ.	Acid precipitation	(FOX & BALA, 2000)
Whey powder	*B. subtilis* ATCC 6633	0.18 – 0.24 g/L	Acid precipitation and extraction with solvents	(CAGRI-MEHMETOGLU, KUSAKLI, & VENTERT, 2012)
Okara	*B. pumilus* UFPEDA 448	126 – 359 mg/L	Acid precipitation and extraction with solvents	(SLIVINSKI et al., 2012)
Glycerol	*B. subtilis* LAMI009, LBMI005, ATCC 6633	286.69, 441.06, 146 and 71 mg/L	Acid precipitation and extraction with solvents; adsorption	(ANDRADE et al., 2016; CRUZ et al., 2018; SOUSA et al., 2012)
Cashew apple juice	*B. subtilis* LBMI005	35 – 175 mg/L	Acid precipitation and extraction with solvents	(OLIVEIRA et al., 2013)
Rice straw	*B. amyloliquefaciens* XZ-173	ND.*	Extraction with solvents and filtration 0.22 µm	(ZHU et al., 2013)
Rapeseed meal		2.68 mg/gds**		
Soybean flour		4.39 mg/gds**		
Wheat bran		2.74 mg/gds**		
Bean cake		2.18 mg/gds**		
Corn meal		1.56 mg/gds**		
Rice straw + Rapeseed meal		3.26 mg/gds**		
Rice straw + Soybean flour		6.25 mg/gds**		
Rice straw + Wheat bran		3.47 mg/gds**		
Rice straw + Bean cake		2.77 mg/gds**		
Rice straw + Corn meal		2.61 mg/gds**		
Rice straw + Soybean flour + glycerol		15.17 mg/gds**		

Rice mil polishing	*B. subtilis* MTCC 2423	4.17 g/kg of RMPR	Foam fractionation and acid precipitation	(GURJAR & SENGUPTA, 2015)
Olive oil mill	*B. subtilis* N1 and *B. subtilis* DSM 3256	3.12 mg/L – 248.5 mg/L	Extraction liquid-liquid; acid precipitation and dialise	(MAASS et al., 2015; MOYA-RAMÍREZ et al., 2015)
Olive oil mil hydrolized	*B. subtilis* N1	5.1 – 13.7 g/L	extraction liquid-liquid	(RAMÍREZ et al., 2016)
Sunflower oil	*B. amyloliquefaciens* SARCC 697	3.3 mg/L	Acid precipitation and extraction with solvents;	(NDLOVU, RAUTENBACH, KHAN, & KHAN, 2017)
Distillers' grains	*B. amyloliquefaciens* MT45	1.04 g/L	Acid precipitation and extraction with solvents;	(ZHI, WU, & XU, 2017)
Brewery wastewaters	*B. subtilis* KP7	6.6 mg/L	Acid precipitation; extraction with solvents;	(PARASZKIEWICZ et al., 2018)
Beet molasses		77.9 mg/L		
Apple peels extract		95.2 mg/L		
Carrot peels extract		140.6 mg/L		
Catla catla fish fat	*B. stratosphericus* 41KF2a	NQ.	Acid precipitation; extraction with solvents;	(SANA, MAZUMDER, DATTA, & BISWAS, 2017)

*NQ. not quantified; **gds - grams of dry substrates.

2.1 STARCH RICH RESIDUES

Cassava (*Manihot esculenta* Crantz) belongs to the family Euphorbiaceae, originating in South America, nowadays is cultivated in more than 100 countries around the Word (LEBOT, 2009). One concern in cassava processing is the amount of waste generated, is estimated that about 30% of the weight of the raw material is liquid waste (cassava wastewater). The cassava wastewater is generated from the pressing of the crushed mass of the cassava root (WOSIACKI; CEREDA, 2002).

Cassava wastewater possess large amounts of organic matter and many nutrients essential for the production of surfactin, making this residue an interesting alternative for surfactin production (NITSCHKE; PASTORE, 2004). Nitschke & Pastore (2004) reported surfactin production by *B. subtilis* LB5a using cassava wastewater as a culture medium in small-scale production (erlenmeyer 250 mL) with 150 mL working volume, followed by surfactin purification by acid precipitation, in which yield of 2,000 mg/L crude biosurfactant was reached. A pilot-scale production (80 L bioreactor) with 40 L work volume reported by Barros et al., (2008), in which surfactin was recovered by foam overflow followed by acid precipitation and solvent extraction, the yield of 680 mg/L was observed. In addition, Simiqueli (2014) reported the production of surfactin in continuous fermentation process using cassava wastewater. The continuous bioprocess realized in a 7.5 L bioreactor (4 L working volume). The continuous bioprocess mode reached 1,300 mg/L (120 hour fermentation) after acid precipitation and solvent extraction with. Another surfactin production in 7.5 L bioreactor (3 L work volume) reported the recuperation by foam overflow, then semi-purified biosurfactant (acid precipitation followed by solvent extraction) yielded 360 mg/L (ANDRADE et al., 2016).

Corn steep liquor, is a principal residue of corn starch production. Using corn steep liquor (10%) Gudiña et al., (2015) reported the production of surfactin by *Bacillus subtilis* #573 obtaining a yield of 1.3 g/L, when corn steep liquor was supplemented with metals such as iron, manganese and magnesium, which are cofactors of enzymes in surfactin synthesis, they obtained higher yields. The metals supplementation in corn steep liquor raised the yield to 4.823 g/L of surfactin (GUDIÑA et al., 2015). In another study using

corn steep liquor Vecino et al., (2015) obtained yielding 12.3 g of surfactin per kg of corn steep liquor.

Potato processing residue is another starch rich residue, Fox & Bala (2000) reported the potential of its use as culture medium with capability to reduce surface tension below 30 mN/m (quantification was not performed) after the fermentation process.

Among these residues, the more promising is cassava wastewater because some authores they got until 2,000 mg/L. The cassava wastewater residue is generated throughout the year because the production of cassava can be carried out on low fertility soils. Thus, the production of surfactin does not depend on the seasonality of the cultivar (*Manihot esculenta* Crantz). Already corn steep liquor and potato processing are dependent on planting and harvest periods. The use cassava wastewater associated with an easy scale-up recovery process with high purity would be extremely promising.

2.2 VEGETABLE OILS RESIDUES

Olive oil mill waste is a residue generated in olive oil extraction, frequently used extraction method for olive oil is the two-stage-process, which yields olive oil and the solid waste (alperujo). Alperujo is a paste with residual oil and high content of lignocellulosic material together with salts and residual oil (MOYA-RAMÍREZ et al., 2015). Moya-ramírez et al., (2015) using this residue produced 3.12 mg/L of surfactin with *B. subtilis* N1. Another study by Maass et al., (2015) with *B. subtilis* DSM 3256 had a yield of 248.6 mg/L. In order to improve surfactin productivity Ramírez et al., (2016), subjected olive oil mill to acid hydrolysis with high temperature (AH) and enzymatic hydrolyzate (EH) the yields raised from 3.12 mg/L without treatment to 5.1 and 13.7 g/L, respectively. The authors point out that acid hydrolysis it has formed toxic compounds resulting in a lower yield.

Using sunflower oil (3%) as a supplement on mineral medium Ndlovu et al., (2017) obtained 3.3 mg/L of surfactin using *B. amyloliquefaciens* SARCC 697.

In one study, the use of olive oil mill subjected the enzymatic hydrolyzate Alperujo provided higher yield (13.7 g/L of surfactin). However, the

production of olive oil depends on specific climatic conditions, and its restricted availability makes it difficult to surfactin production.

2.3 RESIDUES OF WHEY AND PROTEIN SUBSTRATES

Cheese production generate a large amount of residue (9L/Cheese kg), this residue known as whey is rich in protein and lactose. Cagri-Mehmetoglu, Kusakli, & Ventert (2012) using whey powder rehydrated at concentrations of 10, 15 and 20% rehydrated protein, obtained 0.23, 0.18 and 0.24 g/L of surfactin produced by *B. subtilis* ATCC 6633 for 72 h, respectively.

Another protein-rich (25%) residue okara solid residue is obtained during the production of soybean milk (SLIVINSKI et al., 2012). Slivinskie et al., (2012) using hydrolyzed and non-hydrolyzed okara supplemented with glycerol and sugarcane bagasse as a bulking agent for obtained 359 mg/L of surfactin for hydrolyzate and 126 mg/L for non-hydrolyzed, with *B. pumilus* UFPEDA 448.

Thus, between the residue of cheese production (whey) e soybean milk (okara), the okara was shown to be more promising for surfactin production, higher yields were obtained.

2.4 OTHER RESIDUES

Other residues already studied for surfactin production include glycerol from biodiesel production, cashew apple juice, rice straw, rapeseed meal, soybean meal, wheat bran, bean cake, corn meal, being brewery wastewaters (BW), beet molasses (M), apple peels extract (APE), carrot peels extract and catla catla fish fat.

Sousa et al., (2012) using a culture medium composed with 2% glycerol and micronutrients, produced 286.69 mg/L and 441.06 mg/L of surfactin using two different *Bacillus* strains (LAMI005 and LAMI009 respectively) (SOUSA et al., 2012). In other study Cruz et al., (2018) obtained 146 mg/L of surfactin in glycerol (5%) medium supplemented with 0.01 to 0.05 mM manganese, using the strain *Bacillus subtilis* ATCC 6633. The production with glycerol 10 g/L plus peptone 10 g/L, provided a yield of 71 mg/L (ANDRADE et al., 2016).

Cashew apple juice supplemented with different nutrients was tested by Oliveira et al., (2013), obtaining the highest surfactin production of 140 mg/L in media A (reducing sugars 46.24 g/L and $(NH_4)_2SO_4$ 1.0 g/L and 1% solution of trace elements). Another apple residue studied, apple peels extract (APE) supplemented with yeast extract was tested by Paraszkiewicz et al., (2018) obtaining 95.2 mg/L of surfactin they also evaluated brewery wastewaters (BW), beet molasses (M) and carrot peels extract supplemented with yeast extract, obtained yields of 6.6 mg/L, 77.9 mg/L, and 140.6 mg/L, respectively using *Bacillus subtilis* KP7.

Chinese liquor manufacture generates Chinese liquor distillers' grains (DGS) which are distillation-fermented grains residue. Zhi et al., (2017) exploited DGS for the production of surfactin by *B. amyloliquefaciens* MT45, the strain was able to produce 1.04 g/L.

Zhu et al. (2013), testing different agro-industrial residues reported that rice straw and soybean meal was better for the production of lipopeptides compared to rapeseed meal, soybean meal, wheat bran, bean cake and corn meal. Thus, rice straw and soybean meal combination supplemented with glycerol (2.565%) is the best combination between the residues yielding 15.17 mg of surfactin/gds (grams of dry substrates) (ZHU et al., 2013). Another residue from rice production, rice mill polishing residue (RMPR) was also studied for surfactin production using foam recovery method Gurjar & Sengupta (2015) recupered 4.17 g/kg RMPR, using *B. subtilis* MTCC 2423 producer microorganism (GURJAR; SENGUPTA, 2015).

Catla catla fat a residue from fish processing was tested by Sana et al., (2017) to produce surfactin, however the authors did not quantify the yield, they only reported the CMC value of 46.8 mg/L (SANA et al., 2017).

Among these residues, the most promising for surfactin production is rice mil polishing residue with a yield of 4.17 g/kg rice mill polishing residue.

Some residues present do not have all nutrients for the microbial growth, thus, there is a need for the supplementation with sources of carbon and nitrogen (peptone and glucose) is necessary, as reported in this review in okara) (SLIVINSKI et al., 2012) and glycerol (CRUZ et al., 2018). Thus, with this review it was possible to observe that residues rich in starch, has more potential to produce surfactin than enriched supplemented medium.

However, all these residues are derived from food processing and their generation depends on the favorable conditions of cultivation of these foods.

3. RECOVERY AND PURIFICATION OF LIPOPEPTIDE: SURFACTIN

The purification process of biosurfactants is the most significant stage in economic aspect, accounting for approximately 60% of the production costs (SIVAPATHASEKARAN; RAMKRISHNA, 2017). The five most used approaches to recovery and purify surfactin are: (1) foam fractionation and acid precipitation (NITSCHKE; FERRAZ; PASTORE, 2004), (2) liquid-liquid extraction (hexane and ethyl acetate) (VECINO et al., 2015) (3) adsorption (CHEN; JUANG, 2008a) (4) ultrasonic assisted extraction (YUAN et al., 2012) and (5) ultrafiltration (ANDRADE et al., 2016). Most of these techniques generates large quantities of residues or uses organic solvents, affecting directly the purification costs. The only exception is ultrafiltration that does not generate residues and is organic solvent free (COUTTE et al., 2017).

3.1 ACID PRECIPITATION

The easiest and oldest technique for recovering lipopeptides is based on the acid precipitation of lipopeptides. In this technique, acid is added in the cell-free fermented broth, usually hydrochloric or sulfuric acid (6 or 10 N) until it reaches pH 2. To precipitate the lipopeptides, the broth stand at 4 °C overnight. In sequence the supernatant is removed by centrifugation and the lipopeptides in the precipitate are extracted with organic solvents such as methanol, chloroform, acetone, acetonitrile, hexane (CAGRI-MEHMETOGLU; KUSAKLI; VENTERT, 2012). The most used solvents are methanol and chloroform used to obtain high recovery rates. However, the recovered crude surfactant has low purity (50-55%), which make necessary additional purification techniques (CHEN; CHEN; JUANG, 2007).

3.2 FOAM FRACTIONATION

Foam fractionation is normally applied when foam formation occurs in the fermentation process, where the agitation generated using impeller and airflow favors foam formation and that can be collected by columns or traps (ALONSO; MARTIN, 2016). This technique works by the principle that soluble surface-active compounds can adsorb in gas-liquid interface layer, this allow foam formation by reducing the surface tension in gas-liquid interface (SILVA et al., 2015). Using foam column fractionation technique Gurjar & Sengupta, (2015) reported surfactin recovery rate of 69.2% produced in rice mill polishing residue (GURJAR; SENGUPTA, 2015).

3.3 ADSORPTION

Adsorption recovery method was only reported for surfactin produced in mineral medium and glycerol. This technique takes advantage of the properties of the biosurfactant, such as emulsion shape capacity and surface activity (CHEN et al., 2008). Chen & Juang (2008a) studied the adsorption on microporous polyvinylidene fluoride (PVDF) and desorption using n-hexane where they obtained a purity of 78%.The high initial concentration of surfactin in the broth affected adversely its recuperation, a concentrated solution provided a recovery of only 20% and using a 300 mg/L the recovery was 60%. Coupling with other techniques is necessary. For example, the cell-free broth was subjected to a two-stage ultrafiltration process whereby a purity of 76% was obtained, so an ion exchange adsorption process with Amberlite XAD-7 neutral resin (slightly polar) with 98% recovery and 88% purity (CHEN et al., 2008).

Recently, macroporous adsorption resin column chromatography was used for separation of iturin, fengycin and surfactin directly from the cell free broth. This strategy was accomplished by simultaneously varying the solvent composition and pH of the mobile phase, purities up to 68.3% iturin, 77.6% fengycin and 91.6% surfactin (DHANARAJAN; RANGARAJAN; SEN, 2015).

3.4 EXTRACTION LIQUID-LIQUID

The extraction liquid-liquid is a process using immiscible liquids, in most cases the cell-free broth (water phase) and organic solvent, and the interest compound has more affinity with solvent phase. Using liquid-liquid extraction Chen & Juang (2008c) extracted surfactin with ethyl acetate and *n*-hexane obtaining a 99% and 59% extraction with 84% and 63% purity, respectively (CHEN & JUANG, 2008b). In other study Vecino et al., (2015) carried the extraction of surfactin produced in corn steep liquor with chloroform in a single stage. After extraction the chloroform was removed by rotary evaporation, the biosurfactant extract was dissolved in water with the aid of sonication, yielding 12.3 g of crude biosurfactant per kg of corn steep liquor.

The main problem of liquid-liquid extraction is the use of large amounts of solvents making it unfeasible for large-scale application (CHEN & JUANG, 2008b; VECINO et al., 2014).

3.5 ULTRAFILTRATION

Ultrafiltration (UF) is a membrane separation process, with a pore size range ranging from 0.01 µm to 0.1 µm (CHERYAN, 2000). Currently UF is the most promising technique for lipopeptide recovery due low costs and low scale-up complexity (ANDRADE et al., 2016; COUTTE et al., 2017). However, one problem in UF biosurfactant recovery is the low permeate flux and progressive flux reduction due to the concentration polarization mechanism and fouling, one way to minimize the problem is using cross flow UF (CHEN; CHEN; JUANG, 2008; RANGARAJAN; DHANARAJAN; SEN, 2014).

Surfactin micelles can be recuperated in the retentate and separated from low molecular weight compounds such as amino acids, mineral salts, organic acids, alcohols, small peptides and other small sub-products derived from metabolism (ANDRADE et al., 2016; JAUREGI et al., 2013). This is possible because surfactin molecules above their CMC associate to form supramolecular structures, such as micelles with diameters ranging from 5 to 105 nm, two to three times larger than their monomer units, which allows the the use of high molecular weight cut-off (MWCO) membranes to separate from

low molecular compounds (JAUREGI et al., 2013). When present in the form of monomers can permeate and separated from high molecular weight compounds such as proteins (ANDRADE & PASTORE, 2017). This interesting feature allowed the development of sequential purification strategies UF in two steps, using membranes with different MWCO (COUTTE et al., 2017; JAUREGI et al., 2013).

The two-step UF process with Amicon-Ultra 15 (regenerated cellulose) membranes with a MWCO of 30 and 10 kDa for UF-1 and UF-2 (50% methanol for destabilization) showed a yield of 96 ± 5% with purity greater than 90% (ISA et al., 2007). Isa et al., (2007) further measured the zeta potential for a better understanding of the effects on the interactions between surfactin and UF membrane structures. The authors concluded that surfactin in methanolic solution has a negative surface charge promoting the electrostatic repulsion between the surfactin molecules and the membrane that also has a negative charge.

Andrade et al. (2016) obtained 78.25% recovery of surfactin with 80% purity produced by *Bacillus subtilis* LB5a in cassava wastewater (bioreactor - 3 L work volume) using two stages UF purification process with Vivaspin 20 membrane (PES-100 and 50 kDa), combined with the acid precipitation. Surfactin micelle were separated in the retentate (100 kDa), then these aggregate were destabilized with 75% ethanol to separate them from proteins. After micelles destabilization, the broth of surfactin monomers and proteins, were passed to the second UF-stage (50 kDa) surfactin is present in the permeate.

Surfactin micelles may exhibit variations in their mean diameter depending on the concentration in the medium. Micelles with high concentrations of surfactin in the medium (500 mg/L) has a diameter of 10 nm, at lower concentrations (10 to 100 mg/L) the diameters range from 100 to 200 nm. The micelles formation occurs due to the intercellular hydrogen interactions that lead to the surfactin aggregates. In higher concentrations, the repulsive interactions are more intense that can impair the aggregates formation, that fact may justify the formation of different micellar sizes as a function of different concentrations (JAUREGI et al., 2013). Thus, the formation of micelles with larger diameters may favor their retention in the first stage of UF.

Among the techniques used for surfactin recovery and purification, ultrafiltration is possible the most attractive technique. Since, it is not necessary to use organic solvents, easier to scale-up to a large scale, recovery and purity are satisfactory (ANDRADE et al., 2016). In addition, the reuse of ultrafiltration membranes can help reduce production costs (ISA, FRAZIER, & JAUREGI, 2008).

4. SURFACTIN PRODUCTION AT INDUSTRIAL SCALE – AN STRATEGY

The potential use of these residues complex associated with an easy scale-up recovery process, throws the real challenges and offers tremendous opportunities to make industrial biosurfactant production a success story. The starch rich residues is the most promising for surfactin production. In addition, to presenting good yields, it is not necessary to supplement it.

The combination for example of cassava wastewater as a culture medium and UF in several stages as a recovery technique for the production of surfactin is an excellent strategy to reduce production costs (ANDRADE et al., 2016; ISA et al., 2007). Furthermore, it is also easy to apply on a large scale.

Various biprocesses for producing lipopeptides was developed, each with its advantages and disadvantages. Integrated processes using acid precipitation followed by UF in different stages, this technique provides purities higher than 80% (JAUREGI et al., 2013). Another strategy commonly employed is fractionation of foam combined with UF, the difficulty encountered in this process is controlling the kinetics of foam production (COUTTE et al., 2018).

5. CONCLUSION

After 50 years of the discovery of surfactin, its production is still not competitive with surfactants derived from the petrochemical industry. With the technologies currently available, it might be possible to market surfactin for US$ 100 to US$ 1000/ kg. Thus, its application would be restricted to specific products where it needs a low critical micellar concentration or a great reduction in the interfacial surface tension with for example, dispersion of pigment and emulsifier for preparation of new drugs. The use of agro-industrial residues is

one of the best strategies to reduce the total costs of surfactin production. Among the existing techniques the ultrafiltration is applied in other large-scale industries as in the recovery of whey, being possible its application in the recovery of surfactin. The recovery and purification techniques already studied the ultrafiltration demonstrates a greater potential to improve the recovery processes of surfactin. However, a strain that produces only surfactin would raise the efficiency of ultrafiltration process, since ultrafiltration is not able to separate lipopeptides selective.

Acknowledgements

The authors are grateful to the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001 for their financial support.

Conflict of interest

We have no conflict of interest to declare.

REFERENCES

ALONSO, S.; MARTIN, P. J. Impact of foaming on surfactin production by *Bacillus subtilis*: Implications on the development of integrated in situ foam fractionation removal systems. **Biochemical Engineering Journal**, v. 110, p. 125–133, 2016.

ANDRADE, C. J. DE et al. Optimizing alternative substrate for simultaneous production of surfactin and 2,3-butanediol by *Bacillus subtilis* LB5a. **Biocatalysis and Agricultural Biotechnology**, v. 6, p. 209–218, 2016a.

ANDRADE, C. J. DE et al. Ultrafiltration based purification strategies for surfactin produced by *Bacillus subtilis* LB5A using cassava wastewater as substrate. **Journal of Chemical Technology and Biotechnology**, v. 91, n. 12, p. 3018–3027, 2016b.

ANDRADE, C. J. DE et al. Comparative study: bench-scale surfactin production from *Bacillus subtilis* using analytical grade and concentrated glycerol from the biodiesel industry. **International Journal of Scientific World**, v. 5, n. 1, p. 28–37, 2016d.

ANDRADE, C. J. DE; PASTORE, G. M. Ultrafiltration of mannosylerytritol lipids: a parallel with surfactin. In: **Ars Separatoria**. [s.l: s.n.]. v. 11p. 171–200, 2017.

ARIMA, K.; KAKINUMA, A; TAMURA, G. Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. **Biochemical and biophysical research communications**, v. 31, n. 3, p. 488–494, 1968.

BARROS, F. F. C.; PONEZI, A. N.; PASTORE, G. M. Production of biosurfactant by *Bacillus subtilis* LB5a on a pilot scale using cassava wastewater as substrate. **Journal Indian Microbiol Biotechnol**, v. 35, n. 9, p. 1071–1078, 2008.

CAGRI-MEHMETOGLU, A.; KUSAKLI, S.; VENTERT, M. VAN DE. Production of polysaccharide and surfactin by *Bacillus subtilis* ATCC 6633 using rehydrated whey powder as the fermentation medium. **American Dairy Science Association**, v. 95, n. 7, p. 3643–3649, 2012.

CHEN, H. L. et al. Purification of surfactin in pretreated fermentation broths by adsorptive removal of impurities. **Biochemical Engineering Journal**, v. 40, n. 3, p. 452–459, 2008.

CHEN, H. L.; CHEN, Y. S.; JUANG, R. S. Separation of surfactin from fermentation broths by acid precipitation and two-stage dead-end ultrafiltration processes. **Journal of Membrane Science**, v. 299, n. 1–2, p. 114–121, 2007.

CHEN, H. L.; CHEN, Y. S.; JUANG, R. S. Flux decline and membrane cleaning in cross-flow ultrafiltration of treated fermentation broths for surfactin recovery. **Separation and Purification Technology**, v. 62, n. 1, p. 47–55, 2008.

CHEN, H. L.; JUANG, R. S. Recovery and separation of surfactin from pretreated fermentation broths by physical and chemical extraction. **Biochemical Engineering Journal**, v. 38, n. 1, p. 39–46, 2008a.

CHEN, H. L.; JUANG, R. S. Extraction of surfactin from fermentation broth with n-hexane in microporous PVDF hollow fibers: Significance of membrane adsorption. **Journal of Membrane Science**, v. 325, n. 2, p. 599–604, 2008b.

CHERYAN, M. **Ultrafiltration and microfiltration**. Second Edi ed. Boca Raton, Flórida: CRC Press, 2000.

COUTTE, F. et al. Microbial lipopeptide production and purification bioprocesses, current progress and future challenges. **Biotechnology Journal**, v. 12, n. 7, p. 1–10, 2017.

CRUZ, J. M. et al. Agricultural feedstock supplemented with manganese for biosurfactant production by *Bacillus subtilis*. **Waste Biomass Valor**, v. 9, p. 613–618, 2018.

DHANARAJAN, G.; RANGARAJAN, V.; SEN, R. 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**, v. 143, p. 72–79, 2015.

DUFOUR, S. et al. Hemolytic activity of new linear surfactin analogs in relation to their physico-chemical properties. **Biochimica et Biophysica Acta - General Subjects**, v. 1726, n. 1, p. 87–95, 2005.

EBADI, A. et al. Effective bioremediation of a petroleum-polluted saline soil by a surfactant-producing *Pseudomonas aeruginosa* consortium. **Journal of Advanced Research**, v. 8, n. 6, p. 627–633, 2017.

FOX, S. L.; BALA, G. A. Production of surfactant from *Bacillus subtilis* ATCC 21332 using potato substrates. **Bioresource Technology**, v. 75, p. 235–240, 2000.

GUDIÑA, E. J. et al. Biosurfactant production by *Bacillus subtilis* using corn steep liquor as culture medium. **Frontiers in Microbiology**, v. 6, p. 1–7, 2015.

GURJAR, J.; SENGUPTA, B. Production of surfactin from rice mill polishing residue by submerged fermentation using *Bacillus subtilis* MTCC 2423. **Bioresource Technology**, v. 189, p. 243–249, 2015.

HOLMBERG, K. et al. **Surfactants and polymers in Aqueous Solution**. 2 edition ed. England: John Wiley & Sons, LTD, 2002.

ISA, M. H. M. et al. Recovery and purification of surfactin from fermentation broth by a two-step ultrafiltration process. **Journal of Membrane Science**, v. 296, n. 1–2, p. 51–57, 2007.

ISA, M. H. M.; FRAZIER, R. A.; JAUREGI, P. A further study of the recovery and purification of surfactin from fermentation broth by membrane filtration. **Separation and Purification Technology**, v. 64, n. 2, p. 176–182, 2008.

JAUREGI, P. et al. Micelle size characterization of lipopeptides produced by *B. subtilis* and their recovery by the two-step ultrafiltration process. **Separation and Purification Technology**, v. 104, p. 175–182, 2013.

KAKINUMA, A.; TAMURA, G.; ARIMA, K. Wetting of fibrin plate and apparent promotion of fibrinolysis by surfactin, a new bacterial peptidelipid surfactant. **Experientia**, v. 24, n. 11, p. 1120–1121, 1968.

KARSA, D. R. **Industrial applications of surfactants IV**. Cambridge: The Royal Society of Chemistry, 1999.

KOSARIC, N.; VARDAR-SUKAN, F. **Biosurfactants: Production and Utilization—Processes, Technologies, and Economics**. Boca Raton, Flórida: CRC Press, 2015.

LEBOT, V. **Tropical Root and Tuber Crops: Cassava, Sweet Potato, Yams and Aroids**. 17. ed. London, UK: CABI, 2009.

MAASS, D. et al. Two-phase olive mill waste (alpeorujo) as carbon source for biosurfactant production. **Journal of Chemical Technology & Biotechnology**, v. 91, p. 1990–1997, 2015.

MAI, Y.; EISENBERG, A. Self-assembly of block copolymers. **Chemical Society Reviews**, v. 41, n. 18, p. 5969–5985, 2012.

MAKKAR, R. S.; CAMEOTRA, S. S.; BANAT, I. M. Advances in utilization of renewable substrates for biosurfactant production. **AMB Express**, v. 1, n. 1, p. 1–5, 2011.

MOYA-RAMÍREZ, I. et al. Rhamnolipid and surfactin production from olive oil mill waste as sole carbon source. **Bioresource Technology**, v. 198, p. 231–236, 2015.

NDLOVU, T. et al. Variants of lipopeptides and glycolipids produced by Bacillus amyloliquefaciens and *Pseudomonas aeruginosa* cultured in different carbon substrates. **AMB Express**, v. 109, n. 7, p. 2–13, 2017.

NELSON, D. L.; COX, M. M. **Príncipios de bioquímica de Lehninger**. 6 edição ed. Porto Alegre: Artmed, 2014.

NISHIKIORI, T. et al. Plipastatins: new inhibitors of phospholipase A2, produced by *Bacillus cereus* BMG302-fF67. III. Structural elucidation of plipastatins. **The Journal of antibiotics**, v. 39, n. 6, p. 755–761, 1986.

NITSCHKE, M.; FERRAZ, C.; PASTORE, G. M. Selection of microorganismos for biosurfactant production using agroindustrial wastes. **Brazilian Journal of Microbiology**, v. 35, n. 1–2, p. 81–85, 2004.

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

NITSCHKE, M.; PASTORE, G. M. Biosurfactant production by *Bacillus subtilis* using cassava-processing effluent. **Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology**, v. 112, n. 3, p. 163–172, mar. 2004.

OLIVEIRA, D. W. F. DE et al. Kinetic study of biosurfactant production by *Bacillus subtilis* LAMI005 grown in clarified cashew apple juice. **Colloids and Surfaces B: Biointerfaces**, v. 101, p. 34–43, 2013.

PARASZKIEWICZ, K. et al. Structural identification of lipopeptide biosurfactants produced by *Bacillus subtilis* strains grown on the media obtained from renewable natural resources. **journal of Environmental Management**, v. 209, p. 65–70, 2018.

PATEL, M. N.; GOPINATHAN, K. P. Lysozyme-sensitive bioemulsifier for immiscible organophosphorus pesticides. **Applied and Environmental Microbiology**, v. 52, n. 5, p. 1224–1226, 1986.

RAMÍREZ, I. M. et al. Hydrolysis of olive mill waste to enhance rhamnolipids and surfactin production. **Bioresource Technology**, v. 205, p. 1–6, 2016.

SANA, S. et al. Towards the development of an effective in vivo wound healing agent from *Bacillus* sp. derived biosurfactant using Catla catla fish fat. **Royal Society of Chemistry**, v. 7, p. 13668–13677, 2017.

SHUKLA, S. **Surfactants market by type (cationic, anionic, non-ionic, amphoteric) and application (household detergents, personal care, industrial & intitutional cleaners, emulsion polymerization, food processing, oilfield chemicals)- Global opportunity analysis and** . [s.l: s.n.]. Disponível em: <https://www.alliedmarketresearch.com/surfactant-market>. Accessed in 30 of may of 2018.

SIGMA-ALDRICH. Disponível em: **https://www.sigmaaldrich.com/brazil.html**. Accessed in 15 of july of 2018.

SILVA, M. T. S. et al. Integral production and concentration of surfactin from *Bacillus* sp. ITP-001 by semi-batch foam fractionation. **Biochemical Engineering Journal**, v. 104, 2015.

SIMIQUELI, A. P. R. **Produção contínua de surfactina utilizando manipueira como substrato e estudo de sua aplicação em conjunto com óleos essenciais**. [s.l.] Universidade Estadual de Campinas, 2014.

SIVAPATHASEKARAN, C.; RAMKRISHNA, S. Origin , Properties , Production and Purification of Microbial Surfactants as Molecules with Immense Commercial Potential. **Tenside, Surfactants, Detergents**, v. 54, n. 2, p. 92–107, 2017.

SLIVINSKI, C. T. et al. Production of surfactin by *Bacillus pumilus* UFPEDA 448 in solid-state fermentation using a medium based on okara with sugarcane bagasse as a bulking agent. **Process Biochemistry**, v. 47, p. 1848–1855, 2012.

SOBERÓN-CHÁVEZ, G. **Biosurfactants: from genes to applications**. Berlin, Germany: Springer-Verlag, 2010. v. 20.

SOUSA, M. et al. Screening of biosurfactant-producing *Bacillus* strains using glycerol from the biodiesel synthesis as main carbon source. **Bioprocess and Biosystems Engineering**, v. 35, p. 897–906, 2012.

VARJANI, S. J.; UPASANI, V. N. Critical Review on Biosurfactant Analysis, Purification and Characterization Using Rhamnolipid as a model Biosurfactant. **Bioresource Technology**, v. 232, p. 389–397, 2017.

VECINO, X. et al. Study of the surfactant properties of aqueous stream from the corn milling industry. **Journal of Agricultural and Food Chemistry**, v. 62, n. 24, p. 5451–5457, 2014.

VECINO, X. et al. Optimization of liquid–liquid extraction of biosurfactants from corn steep liquor. **Bioprocess and Biosystems Engineering**, v. 38, p. 1629–1637, 2015.

VOLLENBROICH, D.; PAULI, G.; MUHSIN, O. Antimycoplasma properties and application in cell culture of surfactin, a lipopeptide antibiotic from *Bacillus subtilis*. **Applied and Environmental Microbiology**, v. 63, n. 1, p. 44–49, 1997.

WALTON, R. B.; WOODRUFF, B. A crystalline antifungal agent, mycosubtilin, isolated from subtilin broth. **The Journal of clinical investigation**, v. 28, n. 5, p. 924–926, 1949.

WHANG, L. M. et al. Application of biosurfactants, rhamnolipid, and surfactin, for enhanced biodegradation of diesel-contaminated water and soil. **Journal of Hazardous Materials**, v. 151, n. 1, p. 155–163, 2008.

WOSIACKI, G.; CEREDA, M. P. Valorização de Resíduos do Processamento de Mandioca. **Ciências Exatas e da Terra, Ciências Agrárias e Engenharias,** v. 8, n. 1, p. 27–43, 2002.

YU, X. et al. Giant surfactants based on molecular Nanoparticles: Precise synthesis and solution self-assembly. **Journal of Polymer Science, Part B: Polymer Physics**, v. 52, n. 20, p. 1309–1325, 2014.

YUAN, J. et al. The ultrasound-assisted extraction and identification of antifungal substances from *B. amyloliquefaciens* strain NJN-6 suppressing *Fusarium oxysporum*. **Journal of Basic Microbiology**, v. 52, n. 6, p. 721–730, 2012.

ZHAO, H. et al. Biological activity of lipopeptides from *Bacillus*. **Applied Microbiology and Biotechnology**, v. 101, n. 15, p. 5951–5960, 2017.

ZHI, Y.; WU, Q.; XU, Y. Production of surfactin from waste distillers' grains by co-culture fermentation of two *Bacillus amyloliquefaciens* strains. **Bioresource Technology**, v. 235, p. 93–103, 2017.

ZHU, Z. et al. The usage of rice straw as a major substrate for the production of surfactin by *Bacillus amyloliquefaciens* XZ-173 in solid-state fermentation. **Journal of Environmental Management**, v. 127, p. 96–102, 2013.

CHAPTER II

CASSAVA WASTEWATER PRETREATMENTS: ENHANCED SURFACTIN PRODUCTION FOLLOWED BY ITS PURIFICATION BY ULTRAFILTRATION

Aline Wasem Zanotto, Cristino José de Andrade, Bruno Labate Vale da costa, Claudio Augusto Oller do Nascimento, Glaucia Maria Pastore

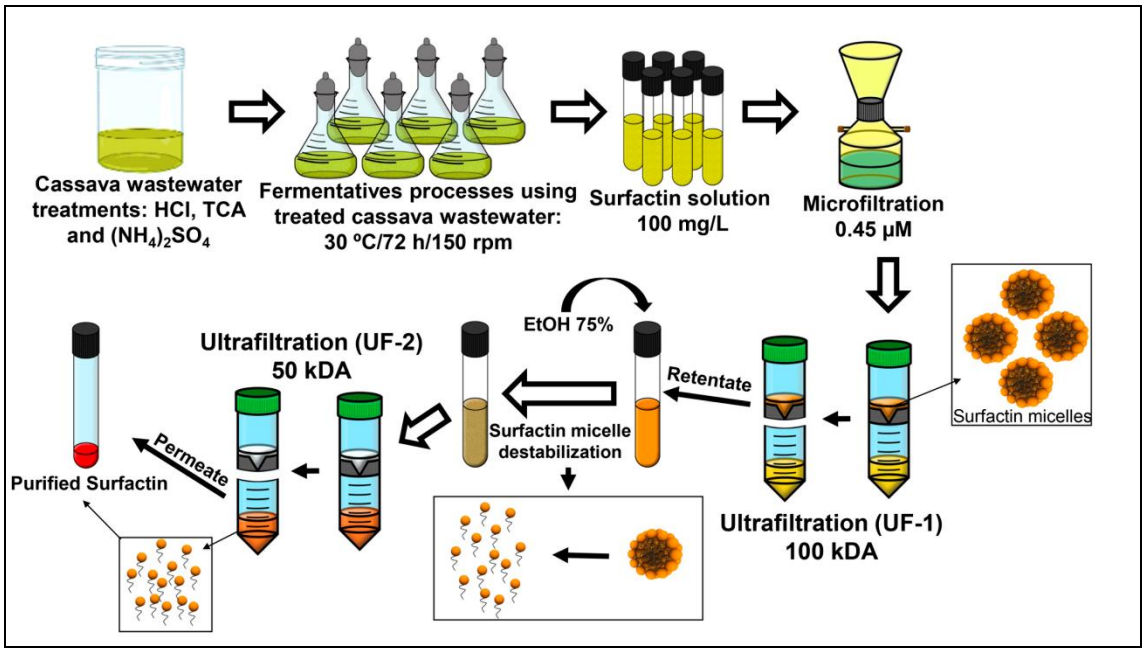
Manuscrito a ser submetido à – Applied Microbiology and Biotechnology

ABSTRACT

The surfactin purification step represents approximately 60% - total production. In this sense, the most promising technique for surfactin purification is the ultrafiltration, mainly due to the low operational complexity and easy scale-up. The ultrafiltration is negatively affected by proteins – that are often present in agro-industrial residue - which leads to membrane fouling. Thus, on the one hand, the use of agro-industrial residues as a culture medium for surfactin production does reduce de total cost production, on the other hand makes harder the surfactin purification by ultrafiltration. Therefore, the aim of this work was to partially remove or hydrolyze the cassava wastewater proteins (prior to bioprocess) and then to evaluate effects of the treatments on the surfactin production and ultrafiltration processes. The treatments used for protein removal/hydrolyze were: HCl pH 2 and pH 3, $(NH_4)_2SO_4$ and $C_2HCl_3O_2$ (TCA). The culture media treated with $(NH_4)_2SO_4$ and TCA, did not favor microbial growth and consequent surfactin production, whereas the culture medium treated with HCl pH 2 reached 749.80 mg of surfactin/L, which is higher when compared to control condition (untreated cassava wastewater, which reached 598.56 mg of surfactin/L). The enhanced surfactin production (HCl pH 2) took place, very likely, due to the chances in nitrogen metabolism (protein hydrolysis) of *Bacillus subtilis* LB5a (surfactin producer). Hence, when compared to control, the HCl pH 2 treatment improved the surfactin production by 2 days. In addition, the two-stage ultrafiltration steps yielded satisfactory results - total recovery up to 72% and purity of 51.49%. In conclusion, the HCl pH 2 treatment (very simple technique) enhanced the surfactin production and also allowed the surfactin ultrafiltration process.

Keywords: Agro-industrial residue, cassava wastewater, protein removal, surfactin production, ultrafiltration.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Surfactants are chemicals derived from petroleum that are widely used in many industrial sectors (KARSA, 1999; MAKKAR, CAMEOTRA, & BANAT, 2011). Nowadays, there is great concern about either the negative impacts of synthetic surfactants on the environment or oil depletion (ANDRADE & PASTORE, 2016). Thus, it is essential to develop environmentally friendly alternatives, e.g. surfactants synthesized by microorganisms (biosurfactants) (NITSCHKE & COSTA, 2007; SOBERÓN-CHÁVEZ, 2010). One of the most well-known biosurfactant producers is *Bacillus* spp., which synthesizes lipopetides such as iturin, fengicin and surfactin (ZHAO et al., 2017).

Surfactin, discovered by Arima et al., (1968), is known for its exceptional ability to form emulsion and foam, presenting also some bioactive activities such as antibacterial (KRACHT et al., 1999), antifungal, anti-inflammatory (ZHAO et al., 2017), among others. Surfactin has a cyclic structure (polar) with an heptapeptide (L-Glu-L-Leu-D-Leu-L-Asp-L-Asp-D-Leu-L-Leu), in which a *β*-hydroxy carbon chain (variable from 12 to 16 carbon atoms) is attached. Some modifications in the chiral sequence of the 7 amino acids were already observed (Val, Leu and Ile at 2nd, 4th and 7th positions) (ARIMA; KAKINUMA; TAMURA, 1968; SOBERÓN-CHÁVEZ, 2010).

The production of surfactin is costly, the conventional purification process represents up to 60% of total production cost whereas culture medium represents up to 30% (ANDRADE & PASTORE, 2017). However, surfactin is a high value-added compound. Currently, surfactin is commercially available by Sigma Chemical Company and Lipofabrik at US$ 62.75/mg, and US$ 19.79/mg, respectively.

Thus, an alternative to minimize the total surfactin production cost is the use of agro-industrial residue as culture medium associated to a solvent-free purification method, in particular ultrafiltration (UF) (COUTTE et al., 2017).

Regarding surfactin UF, the two-steps UF strategy is the most promising techniques for lipopeptides recovery/purification – low cost and easy scale up – Figure 1 (ANDRADE, et al., 2016; ISA, FRAZIER, & JAUREGI, 2008).

A few reports described surfactin purification by UF. Surfactin micelles can be recovered (retentate) in the first step of UF (100 kDa), in which low molecular weight compounds permeate (JAUREGI et al., 2013). Then in the second step of the UF (50 kDA), surfactin micelles are destabilized to monomers by organic solvents treatment (e.g. ethanol, methanol). The monomers of surfactin permeate, while high molecular weight compounds are retained (ANDRADE et al., 2016; COUTTE et al., 2017; JAUREGI et al., 2013).

Cassava wastewater (CWW) is nutrient-rich residue from floury industries. Our research group reported the use of CWW as culture medium for two biosurfactant productions; surfactin and mannosylerythritol lipids, at different bioprocess scales and modes, for instance Nitschke et al., (2004) reported the small-scale production (shake flask 250 mL) followed by surfactin purification by acid precipitation, in which yield of 2,000 mg/L crude biosurfactant was reached. Then, it was reported the pilot scale production (80 L bioreactor) by Barros et al., (2008), in which surfactin was recovered by foam overflow followed by acid precipitation and solvent extraction, the yield of 680 mg/L was observed. The continuous production mode was reported by Simiqueli (2014), by the use of 7.5 L bioreactor. The continuous bioprocess mode reached 1,300 mg/L (120 hour fermentation) after acid precipitation and solvent extraction. Both concentrations of the biosurfactant were determined by measurement indirectly through surface tension (BARROS; PONEZI; PASTORE, 2008; SIMIQUELI, 2014). Simultaneous production of surfactin and 2,3 butanediol (7.5 L - bioreactor) using CWW supplemented with activated charcoal and whey was also reported, which yielded 27.07 mg/L and 275 mg/L, respectively (ANDRADE et al., 2016). Recently, another production in 7.5 L bioreactor reported by Andrade et al., (2016) that surfactin was collected by foam overflow, then semi-purified biosurfactant (acid precipitation followed by solvent extraction) yielded 360 mg/L. Then, the authors applied UF to surfactin purification. They concluded that the major bottleneck on the surfactin production, using CWW as culture medium followed by the surfactin UF, is the membrane fouling caused by the proteins present inherently in the CWW (ANDRADE et al., 2016).

Therefore, the aim of this work was to evaluate different treatments for protein removal CWW prior to fermentation, and then to evaluate the effect on surfactin production and two-steps UF.

2. MATERIAL AND METHODS

2.1. CHEMICALS

The chemicals used included hydrochloric acid (Química moderna 37%), sodium hydroxide (Synth -97%), ammonium sulfate (Synth -99%), trichoroacetic acid (Merck ≥98%), acetonitrile (J.T. Baker ≥99.9%), trifluoroacetic acid (Sigma-Aldrich® ≥99.0%), ethanol (Sigma-Aldrich® -96%), surfactin (Sigma-Aldrich® ≥98%), D-(+)-glucose (Sigma-Aldrich® ≥99.5%), fructose (Sigma-Aldrich® ≥99%), sucrose (Sigma-Aldrich® ≥99.5%).

2.2. MICROORGANISM

The biosurfactant producer was *Bacillus subtilis* LB5a belonging to the collection of the Laboratory of Bioaromas (FEA-UNICAMP) (NITSCHKE et al., 2004).

2.3. CULTURE MEDIUM

The CWW culture medium was the by-product from cassava flour production (variety IAC-13), kindly provided by Plaza Indústria e Comércio Ltda. CWW was transported at room temperature to the laboratory. In order to remove cyanide (evaporation), CWW was boiled for 3 minutes at 100 °C. Then, the starch was removed by centrifugation 10.000 g at 5 °C for 20 minutes (Thermo Scientific, Sorvall RC-6 Plus), and the supernatant stored at -18 °C. Finally, 3 different CWW treatments were performed to remove and/or hydrolyze.

2.3.1. Cassava wastewater treatments

Three strategies were used to remove and/or hydrolyzed CWW proteins. The first strategy was: (I) Protein hydrolysis by hydrochloric acid (HCl) - the partial hydrolysis/precipitation of the proteins was performed by the addition of HCl solution (6 N) to CWW, the treatments were performed at pH 2 (CWW-2) and pH 3 (CWW-3). Then, the acidified CWW was incubated (shaker) at 30 °C and 200 rpm where it remained for 2 hours. (II) Protein precipitation by ammonium sulfate [$(NH_4)_2SO_4$]: the salt was added to the CWW at final concentration of 30% and 50% (w/v) in CWW (ENGLARD; SEIFTER, 1990). (III) Protein hydrolysis/precipitation by trichoroacetic acid (TCA): the CWW was treated with 10%, 20% and 30% (v/v) TCA for the precipitation/hydrolysis of proteins (RAJALINGAM et al., 2009). Both treatments II and III were incubated on shaker at 200 rpm, 20 and 25 °C for 2 hours, respectively. Subsequently the CWW was centrifuged at 2000 g, 5 °C and 20 minutes (Thermo Scientific, Sorvall RC-6 Plus) and supernatant used as culture medium. Before the fermentative processes, all treated CWW (culture media) were adjusted to pH 5.5 (natural pH of the CWW) and both treatments were sterilized by autoclaving. Preliminary experiments showed that the CWW - treatments $(NH_4)_2SO_4$ and TCA followed by thermal sterilization - do not allowed *B. subtilis* LB5a growth. Thus the filtration, 0.45 µm, was chosen as sterilization method (Figure 1).

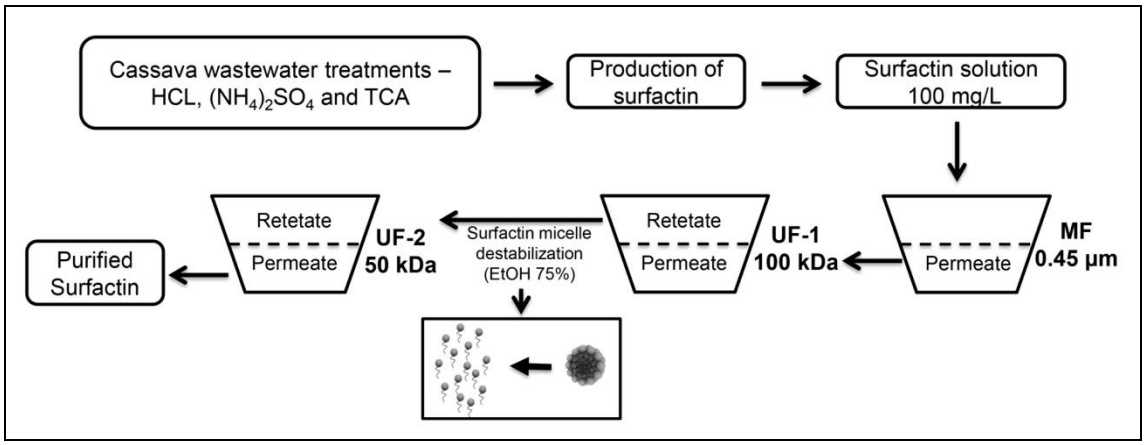


Figure 1. CWW pretreatments, surfactin production and ultrafiltration (UF); an overview.

2.3.2. The best culture medium treatment

The parameters used to choose the best treatment were: (I) protein removal, (II) microbial growth and (III) surfactin production.

2.4. DETERMINATION OF PROTEIN CONCENTRATION AND CELL GROWTH

The determination of the protein concentration of the CWW samples (before and after treatments) was performed by the Bradford method (BRADFORD, 1976).

Cell growth was determined by the spread-plate counting technique (nutrient agar), in which samples were taken at 36 and 72 hours of fermentation (ISO, 2013).

2.5 FERMENTATION KINETICS

After choosing the best CWW treatment. The microbial growth kinetics, surfactin production and consumption of fermentable sugars were analyzed on 1 hour basis in the first 12 hours of fermentation and then on 2 hours basis from 12 to 72 hours of fermentation.

2.5.1 Inoculum; surfactin production and microbial growth kinetics

B. subtilis LB5a was maintained by periodically preparing a fresh culture from the previous stock culture - agar nutrient solution and incubated at 30 °C for 24 hours. Then, a single colony was transferred to 50 mL of nutrient broth, previously sterilized and then incubated at 30 °C under shaking at 150 rpm for 12 hours. Then, the standardization of the inoculums (0.5 ≈ $2x10^7$ CFU/mL) was carried out by optical density λ 600 nm (Spectrophotometer UV-Vis, Beckman Coulter DU730). After standardization of the *inoculum* 7% (v/v) of culture medium was added in each erlenmeyer. The fermentation was carried out in 250 mL erlenmeyer flask with 50 mL of CWW (working volume). The

erlenmeyers were then incubated at 30 °C at 150 rpm for 72 h. (Shaker, New Brunswick Scientific™, INNOVA® 4335) (BARROS; PONEZI; PASTORE, 2008).

For microbial growth kinetics by absorbance measurement (OD_{600}), periodic sampling were performed (1 hour basis in the first 12 hours and then on 2 hours basis from 12 to 72 hours).

After fermentations, all samples of the culture medium were centrifuged at 10.000 g for 20 minutes. The supernatants were used for the determination of surfactin concentration (HFLC), protein concentration (Bradford) and sugars concentration (HPAEC-PAD).

2.5.2 Consumption of sugars

The consumption and quantification of sugars during the bioprocesses were performed by high performance anion exchange chromatography coupled to a pulsed amperometric detection system (HPAEC-PAD) (DIONEX ICS-5000 model; Thermo Fisher Scientific, Waltham, MA). The chromatographic column used was Carbopac PA1 column (250 mm x 4 mm, 10 µm particle size) for determination of mono- and disaccharides (glucose, fructose and sucrose). The system was equipped with a quaternary pump, with gradient elution where the mobile phase is composed of: solvent A (200 mM NaOH) and solvent B (Milli-Q water). Fermentation samples were filtered with a 0.22 µm filter, and the injection volume was 25 µL, the run duration was 35 minutes. The initial gradient was 40 to 69% (A) for the first 25 minutes, then 100% (A) in 25 to 30 min., and returned to 40% (A) in 30 to 35 minutes. The column temperature was maintained at 30 °C all the time.

Glucose, fructose and sucrose standards were used for the calibration curve (Sigma-Aldrich). The sugars in the sample were identified by comparing the retention times obtained in the samples with the standards; whereas the quantification was determined from the calibration curve with commercial standards (0.4 to 20 µg/mL). Data acquisition was performed by Chromeleon v 7.0 software (PEREIRA et al., 2017).

2.6 SURFACTIN PURIFICATION BY THE TWO-STAGE ULTRAFILTRATION PROCESS

The UF process was carried out using Amicon® Ultra membrane with regenerated cellulose - 50 and 100 kDa, containing 7.6 cm^2 of active area. Initially, all culture medium samples (composed also by surfactin) was solubilized in Milli-Q water, in order to reach 100 mg of surfactin/L, which was then filtered in a 0.45 µm pore membrane, the permeated was used in the UF process. The first UF step (UF-1) was performed using a 100 kDa membrane, where 12 mL of that solution was added to the UF device and centrifuged at 2200 g (10 minutes at 20 °C). The UF-1 retained was solubilized in 75% (v/v) ethanol (micelle destabilizing) to the same initial volume. Subsequently this solution was used to the second stage of UF (UF-2), nevertheless using the 50 kDa membrane device and then centrifuged under the same conditions as in the UF-1 (ANDRADE et al., 2016).

All retained and permeate samples (UF-1 and UF-2) were analyzed in order to determine protein concentration (Bradford) and surfactin concentration (RF-HFLC). The membrane rejection coefficient (R) was calculated according to equation (1), for surfactin (Rs) and for protein (Rp).

Equation:

$$R = [(Cf - Cpp)/ Cf)] \quad (1)$$

Where, Cf is the concentration of surfactin and Cp protein concentration, in the feed (Cpf) and permeate (Cpp).

The purity of surfactin (Pp) was determined in terms of the amount of protein in relation to the total surfactin (Cs) and protein (Cp) in UF-1 and UF-2, according to equation (2) below.

Equation:

$$Pp = [(Cs/ (Cs + Cp)] \times 100 \quad (2)$$

The determination of total recovery of surfactin (TRS) was measured according to equation (3), where Ms is the mass of surfactin.

Equation:

$$TRS = [(Msi/ Msii)] x100 \quad (3)$$

Thus, in UF-1 (TRSi) Msi is the mass of surfactin in the retained and Msii is the mass of surfactin in the initial solution which was ultrafiltered (feed). For UF-2 (TRSii) Msi is the mass of surfactin in the permeate while Msii is the mass of surfactin in the ultrafiltered initial solution (feed) which in turn is equal to the mass of the retained in (UF-1). For the determination of TRSt in UF-1 and UF-2, Msi is the mass of surfactin in the initial feed (UF-1) and Msii is the mass of surfactin in the permeate (UF-2). To obtain Ms, multiply the Cs by the volume of the solution (ANDRADE et al., 2016).

2.7 QUANTIFICATION OF SURFACTIN

The quantification of surfactin was performed by ultra-high performance liquid chromatography (RP-HFLC) (Pominence model, Shimadzu, SPD-20A). The chromatographic column used was analytical C18 (Hypersil ™ ODS) dimensions 4.6 mm x 250 mm, 5 µm particle size. The flow of the mobile phase was 1.1 mL/min with the initial gradient was 2 to 50% acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA) for the first 4 min., and then 50 to 80% between 4 and 19 min. The 80% concentration remained for 20 min. Finally the concentration increased to 100% in 5 min. and returned to 50% in 59 minutes. The injection volume was 50 µL, the run duration was 70 min., and the eluent monitored at 214 nm. The column temperature was maintained at 30 °C all the time. Data acquisition was performed by LCsolution software.

The calibration curve for quantification of surfactin produced by *B. subtilis* LB5a was performed with the surfactin standard (Sigma-Aldrich) (standard curve between 25 and 150 mg/L) (ANDRADE et al., 2016).

2.8 STATISTICAL ANALYSIS

Analysis of variance followed by the Tukey test were used to compare the means ($p \leq 0.05$) using the ActionStat 3.4 program an Excel tool (EQUIPE ESTATCAMP, 2018).

3. RESULTS AND DISCUSSION

3.1 CASSAVA WASTEWATER TREATMENTS; PROTEIN REMOVAL

Regarding CWW treatments, we describe a novel approach. Usually, the techniques applied to remove proteins aim to the isolation of proteins and further application. In this case, protein removal from the CWW is essential, since CWW proteins make the surfactin purification by UF unfeasible (ANDRADE et al., 2016).

Table 1. CWW treatments - protein removal – effects on *B. subtilis* LB5a growth and surfactin production.

Treatment	Removed protein concentration (mg/mL)	*B. subtilis* LB5a *growth*		Surfactin production (mg/L)
		36 h# (CFU/mL)	72 h# (CFU/mL)	
CWW*	0.28	3.29E+10	2.46E+11	598.56
CWW-2	0.19	3.29E+10	2.46E+11	749.80
CWW-3	0.19	6.77E+10	2.16E+11	625.82
$(NH_4)_2SO_4$ (30%) **	0.79	0	0	0
$(NH_4)_2SO_4$ (50 %) **	0.75	0	0	0
$(NH_4)_2SO_4$ (30 %) ***	0.79	0	1.08E+10	30
$(NH_4)_2SO_4$ (50 %) ***	0.75	0	7.98E+09	20
TCA (10 %) **	0.83	0	0	0
TCA (20%) **	0.35	0	0	0
TCA (30%) **	0.45	0	0	0
TCA (10%) ***	0.83	0	1.00E+10	30
TCA (20%) ***	0.35	0	5.96E+09	20
TCA (30%) ***	0.45	0	7.70E+07	20

*CWW: Cassava wastewater control. #h: hours. **sterilized by autoclaving. ***sterilized by filtration.

The HCl treatments, namely CWW-2 and CWW-3, reduced the protein concentration from 0.28 mg of the control condition (CWW) to 0.19 mg (Table 1). In addition, the HCl treatments allowed a microbial growth which was very similar to the control condition, approximately 10^{11} CFU/mL. Surprisingly, the HCl treatments allowed the surfactin production 25% (CWW-2) and 5% (CWW-3) higher than CWW condition – 749.80 mg/L and 630 mg/L, respectively. The acid treatments hydrolyzed CWW proteins, which released amino acids and/or peptides. Consequently, an enhanced surfactin production was observed, very likely due to the changes in nitrogen metabolism of *B.*

subtilis LB5a. On the other hand, the treated culture media with $(NH_4)_2SO_4$ 30 and 50% and TCA 10, 20 and 30% showed higher percentage of protein removal, when compared to HCl treatments.

Thus, very likely the HCl treatments affected the carbon/nitrogen ratio of CWW. There is an ideal C/N ratio for each microorganism that favors the production of biosurfactant. Fonseca et al. (2007) observed that C/N ratio of 3 provided greater reduction of the surface tension when compared to C/N ratio of 15.

The CWW treatment with $(NH_4)_2SO_4$ partially removed the protein, however the prevalence of inorganic NH_4^+ ions, associated with the sterilization by heat, very likely produced caramel colors product by the thermal modification of carbohydrates. Caramel colors can be produced by ammonia process, this compound can affects the production of piridoxal phosphate and nonphosphorylated, the biologically active form of vitamin B_6 (piridoxal phosphate), is an essential cofactor for numerous metabolic enzymes, probably preventing the *B. subtilis* growth and consequent biosurfactant production (BELITSKY, 2004; SPECTOR; HUNTOON, 1982). Besides, the high amount of $(NH_4)_2SO_4$ (30 and 50%) required for protein precipitation in this study was, *per se* responsible for affecting the microbial osmotic regulation (ionic force) (MÜLLER et al., 2006), and consequent *B. subtilis* growth. Nevertheless, the reduction of organic nitrogen using $(NH_4)_2SO_4$ promoted the increase of inorganic nitrogen. The C/N ratio between the sugars analyzed and addition of $(NH_4)_2SO_4$ (30 and 50%) for both concentrations was 1/6 and 1/10, in which the yield obtained was 30 and 20 mg surfactin/L, respectively.

Some studies report the use of inorganic nitrogen sources for biosurfactant production. Davis et al., (1999) reported the *B. subtilis* growth in a culture medium with ammonium nitrate (4 g/L) as inorganic nitrogen source. This strategy allowed production of 439 mg/L, whereas the culture medium with $(NH_4)_2SO_4$ (4 g/L) allowed production of only 53 mg/L of surfactin.

Therefore, the HCl treatments: CWW-2 and CWW-3 were chosen as the most promising CWW treatments for surfactin production, due to the higher production of surfactin and better growth of *B. subtilis* LB5a.

3.2 FERMENTATION KINETICS, CONSUMPTION OF SUGARS AND SURFACTIN PRODUCTION

Fermentation kinetics were performed using control CWW and treated conditions (CWW-2 and CWW-3) as culture media (Figure 2).

Regarding microbial growth, there were no significant differences in the first 8 hours of fermentation. After 10 hours of fermentation, a propensity of higher microbial growth was observed in the experiments with CWW-2 and CWW-3 culture media.

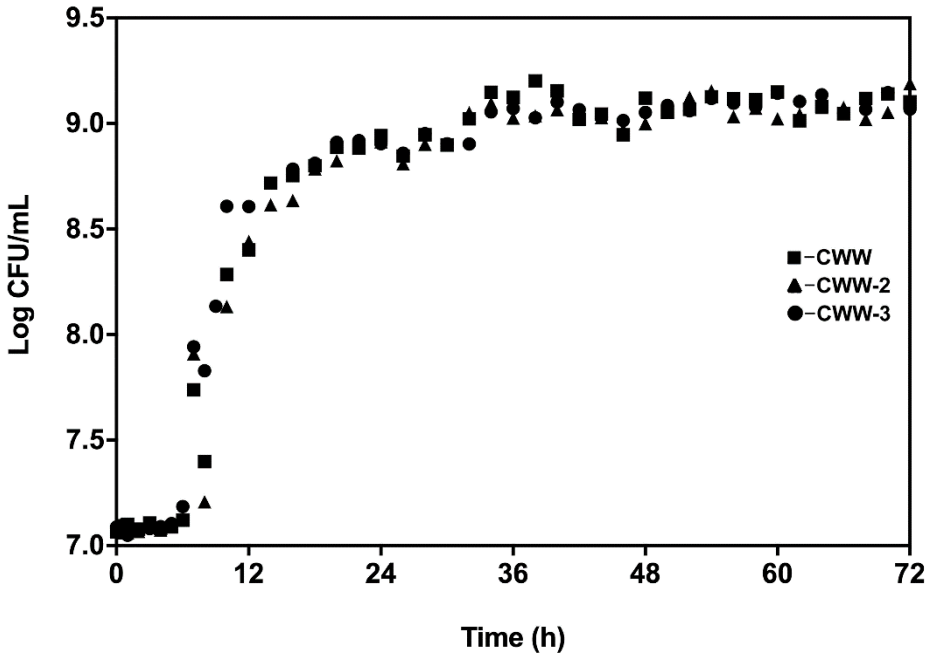


Figure 2. *Bacillus subtilis* LB5a growth kinetics in control CWW (not treated), CWW-2 and CWW-3 conditions (treated with HCl).

The quantification of sugars in the CWW, CWW-2 and CWW-3 media (Table 2) indicates a reduction in glucose concentration by 1.45% for both CWW-2 and CWW-3 treatments and in sucrose concentration by 10.52% for CWW-2 and 3.58% for CWW-3, due to the HCl treatment. On the other hand, the fructose concentration increased by 16.5% and 14.97% in CWW-2 and CWW-3, respectively. Usually the hydrolysis of sugars is performed at high temperatures (>100 °C), Thompson et al. (2000) performed pretreatment in potato process effluent using sulfuric acid and autoclaving the 121 °C. To the

best of our knowledge there are no reports on hydrolysis in mild conditions, thus further investigations are necessary. Cassava wastewater is residue complex and its sugars have never been completely defined. The acid treatment probably promoted the partial hydrolysis of sugars, such as oligosaccharides resulting in increased fructose, another possibility is the precipitation of these oligosaccharides reducing the concentration of glucose and sucrose, corroborating with the results obtained during the fermentation without treatment (CWW), where to an increase in sucrose, glucose and fructose during the initial fermentation phase occurring with less intensity in the CWW-3 treatment and almost no occurring in CWW-2.

Thus, the acid may have promoted partial hydrolysis of sugars resulting in increased fructose, another possibility and the precipitation of glycoproteins reducing the glucose and sucrose concentration.

Table 2. Quantification of sugars in control CWW and after acid treatments

Treatment	Glucose (g/L)	Fructose (g/L)	Sucrose (g/L)
CWW	4.86 ± 0.043	3.24 ± 0.63	24.05 ± 1.56
CWW-2	4.79 ± 0.038	3.88 ± 0.038	21.52 ± 1.55
CWW-3	4.79 ± 0.029	3.81 ± 0.052	23.19 ± 1.54

All determinations were carried out in triplicate and the values were reported as the mean ± standard deviation.

The analysis of glucose, fructose and sucrose of the culture medium (control CWW, CWW-2 and CWW-3) - Figure 3, indicates that there was an increase in the concentration of these sugars at the culture media after 4 hours of fermentation. In relation to the glucose concentration in CWW (Figure 3a) it is possible to observe that there was an increase of 8 to 18 hours (5.84 to 11.96 g/L), which subsequently declines with 72 hours of fermentation is practically 0 (0.19 g/L) being considered the end of the fermentation. The opposite is observed in the CWW-2 and CWW-3 experiments, where the increase in glucose concentration starts at 12 hours and the decline starts at 18 hours (CWW-2 5.60 to 9.22 g/L and CWW-3 4.94 to 16.25 g/L), reaching close to 0 at the end of the fermentation. The acid treatment in CWW probably improved the nutrient availability of the medium, slower consumption of glucose in CWW-2 and CWW-3.

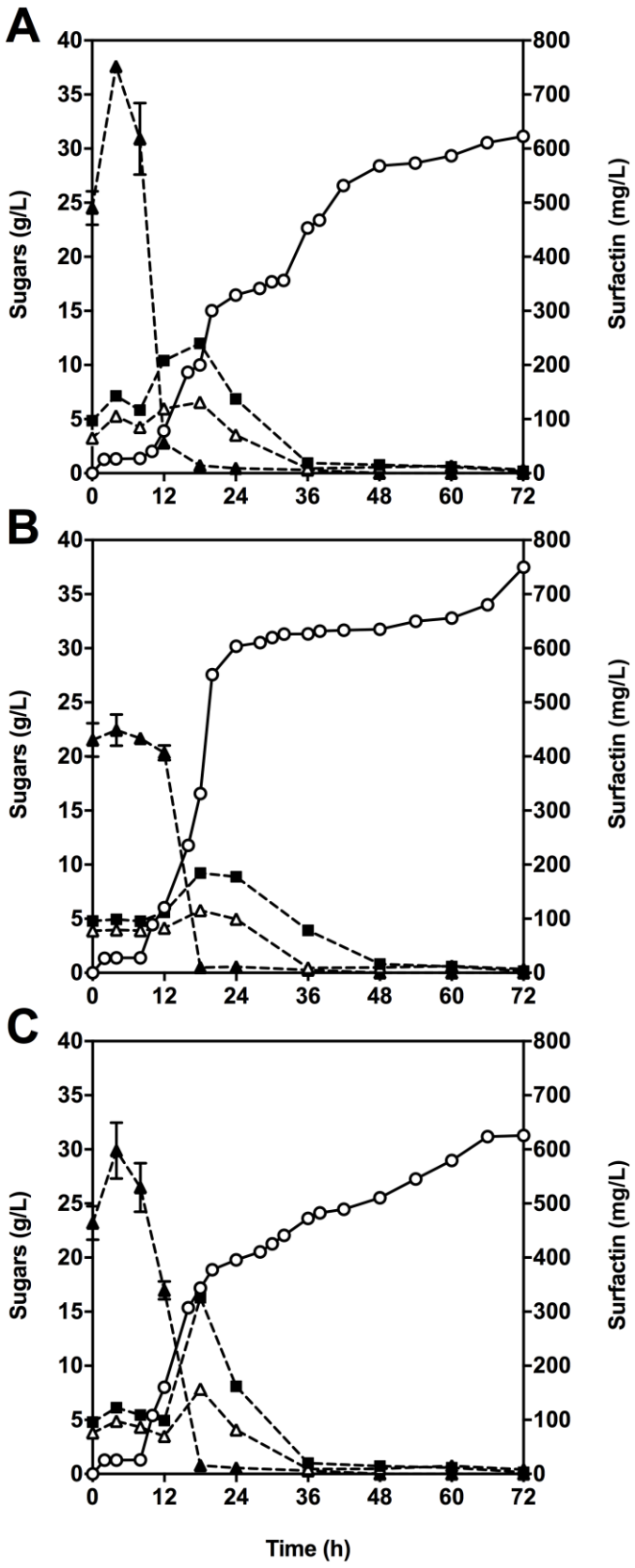


Figure 3. Kinetics of surfactin production (○), glucose (■), fructose (△) and sucrose (▲) consumption by *Bacillus subtilis* LB5a in the control CWW (A), CWW-2 (B) and CWW-3 (C).

The fructose concentration (Figure 3c) in the CWW medium increased from 8 hours (4.24 g/L) to 18 hours (6.56 g/L). In the treatments CWW-2 and CWW-3, the increase of fructose was observed from 12 to 18 hours, with values of 4.13 to 5.76 g/L and 3.48 to 7.85 g/L, respectively. At the end of the fermentation the fructose concentration in CWW-2 and CWW-3 was still at 0.3 g/L. Sucrose consumption (Figure 3) increased from the start of the fermentation up to 4 hours for both control CWW medium (24.72 to 37.59 g/L) and CWW-3 (23.19 to 29.89 g/L) and CWW-2 there was no increase. The increase of glucose and fructose at different times for culture medium indicate that the treatments with HCl affected the initial composition of the CWW favoring the increase in the production of surfactin.

It can be observed a tendency of *B. subtilis* to consume first complex sugars like oligosaccharides. The increase in the concentration of the sugars analyzed after the beginning of the fermentation occurs due to the action of enzymes to depolymerize complex nutrients produced by *B. subtilis* (BARROS; PONEZI; PASTORE, 2008; STULKE; HILLEN, 2000). Among the sugars analyzed, sucrose was the first consumed. Rapid consumption of sucrose was also observed within the first 12 hours of fermentation. The hydrolysis of sucrose consequently promoted the increase of glucose and fructose during fermentation in the control CWW, CWW-2 and CWW-3 medium, which is considered when the adaptation (lag phase) of the microorganism begins.

The opposite was observed in CWW-2 that there is no increase of sucrose at the beginning of the fermentation. Due to the non-sucrose increase, lower glucose and fructose concentration were obtained for CWW-2. The concentration of glucose and fructose in control CWW (22.91% and 12.2%, respectively) higher than those obtained at CWW-2. On the other hand, CWW-3 this increase was more evident with 43.26% and 26.63% higher than CWW-2.

There is a difference in glucose pattern consumption at CWW-2, as it ends up with only 48 hours, which indicates that the presence of glucose affects much less the amount of surfactin produced. The rapid increase in surfactin production is observed with consumption of sucrose in the culture medium for the three treatments, more pronounced in CWW-2. At the three treatments, after sucrose was depleted, it was observed an increase surfactin production until glucose and fructose were completed depleted. This might

occur due to the *B. subtilis* surfactin synthesis for nutrient assimilation (SWIFT; ROWE; KAMATH, 2008). It is also possible that CWW and CWW-3 had oligosaccharides or glycoproteins, this can explaining the increase of sucrose, glucose and fructose in the first 8 hours of fermentation in this two treatment. Possibly oligosaccharides made more difficult to *B. subtilis* produce surfactin, since he needs produce enzymes to reduce that in simple is sugar and uses in his metabolism. In the treatment CWW-2 we don`t observed this initial sucrose, glucose and fructose increase, this maybe occurred by the elimination of theses precursor (oligosaccharides or glycoproteins) by precipitation or hydrolysis. Obviously, further research on the sugar profile and consumption present in CWW and CWW treated is needed to understand complete *B. subtilis* surfactin production in complex media.

In relation to the surfactin production (Figure 3), it was observed that for the CWW-2 condition, the production in 36 h of fermentation was 626.75 ± 0.008 mg/L and 72 h of 749.80 ± 0.006 mg/L. However, in control CWW and CWW-3 the production was 440.90 ± 0.017 and 472.52 ± 0.021 mg/L at 36 h and 598.56 ± 0.033 and 625.82 ± 0.017 mg/L with 72 h, respectively. According to statistical analysis the production in control CWW and CWW-3 showed no differences in production ($p \leq 0.05$). Both yields in the treatments with CWW and CWW-3 were statistically different from CWW-2 in 36 and 72 h times.

Both CWW (control) and CWW-3 presented yields statistically different surfactin production from CWW-2 at times of 36 and 72 hours.

In addition, both the microbial enzyme action and HCl treatment have affected the partial hydrolysis of proteins and sugars, in other words, increasing the amount of free amino acids and mono- or di-saccharides. The higher concentration of free amino acids and mono- or di-saccharides very likely was te key factor that favored surfactin production.

Interestingly, when compared to CWW (control) – in terms of fermentation time (productivity) – the CWW-2 can reduce from 72 h (CWW - 598.56 mg/L) to 24 h (603.68 mg/L). The yields of surfactin production during the fermentation process are shown in Table 3.

Table 3. Surfactin production yield during the fermentation process.

CWW					
Fermentation time (h)	Growth* (g/L)	Surfactin (mg/L)	Yxs** Biomass (g/g)	Yps*** Surfactin (mg/L)	Pr# Surfactin (mg/L.h)
24	45.62	329.42	2.10	15.14	13.73
36	73.47	440.90	2.37	14.25	12.25
72	72.72	598.56	2.26	18.64	8.31
CWW-2					
Fermentation time (h)	Growth* (g/L)	Surfactin (mg/L)	Yxs** Biomass (g/g)	Yps*** Surfactin (mg/L)	Pr# Surfactin (mg/L.h)
24	51.18	603.68	3.25	38.29	25.15
36	64.76	626.75	2.53	24.85	17.41
72	100.28	749.80	3.38	25.43	10.41
CWW-3					
Fermentation time (h)	Growth* (g/L)	Surfactin (mg/L)	Yxs** Biomass (g/g)	Yps*** Surfactin (mg/L)	Pr# Surfactin (mg/L.h)
24	46.89	395.77	2.46	20.77	16.49
36	84.57	472.52	2.82	15.74	13.13
72	79.85	625.82	2.56	20.04	8.70

*Humid biomass; **Yxs - Yield of biomass per substrate consumed (x/s); ***Yps - Yield of the product (p/s); #Pr - Productivity of biomass per process time.

Meanwhile, it was observed that in CWW-2 the product yield was 153% and 84% higher than the CWW and CWW-3 treatments in the first 24 hours of fermentation. Finally, this increase in fermentation remained 36.43% and 26.89% higher than CWW and CWW-3, respectively, with 72 hours of fermentation.

Therefore the treatment CWW-2 was chosen for the additional experiments of UF.

3.3. SURFACTIN PURIFICATION BY THE TWO-STAGE ULTRAFILTRATION PROCESS

Surfactin micelles exhibit variations in their average diameter, which is mainly related to surfactin concentration. Surfactin micelle, at high surfactin concentrations (e.g 500 mg/L) has a low diameter ≈10 nm, on the other hand, at lower concentrations (e.g 10 to 100 mg/L) the diameters range from 100 to 200

nm. The formation of micelles occurs due to the intermicellar hydrogen interactions that lead to the surfactin aggregates. At higher surfactin concentrations the repulsive interaction is stronger that can impair the formation of aggregates, (JAUREGI et al., 2013). Thus, the formation of micelles with larger diameters favors their retention in membranes of MWCO larger, separating surfactin and proteins from low molecular weight compounds. To separate surfactin from proteins the surfactin micelles need to be destabilized.

The surfactin obtained from the culture medium supernatant was analyzed by HFLC which had an initial concentration of 700 mg/L, this initial concentration indicates that surfactin micelles have small diameters around 10 nm as observed by Jauregi et al., (2013). To obtain micelles with larger diameters the crude broth was diluted to obtain approximately 100 mg of surfactin/L, after dilution the surfactin concentration was confirmed 112 mg/L (Table 4). The surfactin solution (112 mg/L) after microfiltration at 0.22 µm showed purity of 53.47% in relation to low molecular weight compounds.

The determination of the surfactin concentration after UF-1 shows that part of the surfactin passed through the membrane (16 mg/L) but most of the surfactin was rejected (94 mg/L), presenting a rejection coefficient (Rs) of 0.86 whereas for proteins Rp=0.42 (PC=0.067). Therefore, UF-1 was able to recovery surfactin at high yield (83.76%) and also separated the surfactin from smaller molecules, such as salts and amino acids or peptides. In relation to UF-2, the retentate of UF-1 after being solubilized in 75% ethanol was used as feed for UF-2. The feed of UF-2 is basically composed of surfactin monomers (micelles have been destabilized with ethanol) and proteins.

In the UF-2 most of the surfactin passed through the membrane, the rejection coefficient was only Rs=0.073 with a surfactin concentration in the retentate of 8 mg/L whereas for proteins Rp=0.011 (PC=0.079). The UF-2 permeate had 87 mg/L surfactin and 82 mg/L protein. Total surfactin recovery was 72.37% (TRS_t), and its purity was only 51.49%.

Table 4. Recovery of surfactin from fermentation culture.

	Ultrafiltration – first step (UF-1 100kDa)		
	Feed	R	P
SC	112	94	16
PC	114	83	67
Rs		0.86	
Rp		0.42	
TRSi		83.76	
Ppi	49.54	53.47	20.18
	Ultrafiltration – second step (UF-2 50kDa)		
	Feed	R	P
SC	94	8	87
PC	83	79	82
Rs		0.073	
Rp		0.011	
TRSii		84.18	
TRSt		72.37	
Ppii	53.47	29.95	51.49

R - retentate; P - permeate; SC - surfactin concentration (mg/L); PC - protein concentration (mg/L); Rs and Rp - rejection coefficient (by equation 1); TRS – total recovery of surfactin – (by equation 3); Purity of surfactin as mass fraction of surfactin in relation to sum of mass of surfactin and protein (%w/w) – Ppi (UF-1) and Ppii (UF-2).

The protein concentration in the UF-2 permeate was similar to the retentate concentration, and the surfactin concentration in the permeate was only 5.75% higher than the protein concentration. In a study carried out by our research group, the surfactin was produced in bioreactor (7.5 liter – 3 working volume) and recovered by foam overflow technique. The surfactin in the foam was purified by acid precipitation, solvent extraction (chloroform: methanol 65:15) and then the obtained powder was used in the two stages UF. It was reported a purity of 80% and a yield of 78.25% (ANDRADE et al., 2016).

This is the first study that purified surfactin directly from the CWW supernatant (complex culture medium) through two strategies of ultrafiltration. The recovery up to 72% and purity of 51.49% were promissing - green chemistry concept.

4. CONCLUSIONS

The strategies of CWW protein removal with $(NH_4)_2SO_4$ and TCA negatively affected the surfactin production. Whereas, the HCl treatment, partially hydrolyzed the proteins, and surprisingly it enhanced the surfactin production (750 mg/L), since the control experiment (without any treatment) reached 620 mg/L. The recovery of surfactin by the two stages ultrafiltration

proved to be satisfactory where a yield of greater than 72% was obtained with 51.49% purity. The enhanced surfactin production is a significant step further the feasible surfactin production, since it use an agro-industrial residue as culture medium; associated to a low cost purification process (ultrafiltration) – directly from culture medium.

Acknowledgements

The authors are grateful to the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) - Finance Code 001 for their financial support, Dempster Mass Spectrometry Lab, Polytechnic School of University of São Paulo for Bradford and HFLC analysis. Plaza Indústria e Comércio Ltda for provide cassava wastewater.

Conflict of interest

We have no conflict of interest to declare.

REFERENCES

ANDRADE, C. J. DE et al. Optimizing alternative substrate for simultaneous production of surfactin and 2 , 3-butanediol by *Bacillus subtilis* LB5a. **Biocatalysis and Agricultural Biotechnology**, v. 6, p. 209–218, 2016a.

ANDRADE, C. J. DE et al. Ultrafiltration based purification strategies for surfactin produced by *Bacillus subtilis* LB5A using cassava wastewater as substrate. **Journal of Chemical Technology and Biotechnology**, v. 91, n. 12, p. 3018–3027, 2016b.

ANDRADE, C. J. DE; PASTORE, G. M. Comparative study on microbial enhanced oil recovery using mannosylerithritol lipids and surfactin. **International Journal of Scientific World**, v. 4, n. 2, p. 69–77, 2016.

ANDRADE, C. J. DE; PASTORE, G. M. Ultrafiltration of mannosylerytritol lipids: a parallel with surfactin. In: **Ars Separatoria**. [s.l: s.n.]. v. 11p. 171–200, 2017.

ARIMA, K.; KAKINUMA, A; TAMURA, G. Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. **Biochemical and biophysical research communications**, v. 31, n. 3, p. 488–494, 1968.

BARROS, F. F. C.; PONEZI, A. N.; PASTORE, G. M. Production of biosurfactant by *Bacillus subtilis* LB5a on a pilot scale using cassava wastewater as substrate. **Journal Indian Microbiol Biotechnol**, v. 35, n. 9, p. 1071–1078, 2008.

BELITSKY, B. R. Physical and enzymological interaction of *Bacillus subtilis* proteins required for de novo pyridoxal 5′-phosphate biosynthesis. **Journal of Bacteriology**, v. 186, n. 4, p. 1191–1196, 2004.

BRADFORD, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical Biochemistry**, v. 72, n. 1–2, p. 248–254, 1976.

COUTTE, F. et al. Microbial lipopeptide production and purification bioprocesses, current progress and future challenges. **Biotechnology Journal**, v. 12, n. 7, p. 1–10, 2017.

DAVIS, D. A.; LYNCH, H. C.; VARLEY, J. The production of surfactin in batch culture by *Bacillus subtilis* ATCC 21332 is strongly influenced by the conditions of nitrogen metabolism. **Enzyme and Microbial Technology**, v. 25, n. 3–5, p. 322–329, 1999.

DESAI, J. D.; BANAT, I. M. Microbial production of surfactants and their commercial potential. **Microbiology and molecular biology reviews : MMBR**, v. 61, n. 1, p. 47–64, 1997.

ENGLARD, S.; SEIFTER, S. Precipitation Tecniques. In: M. P. DEUTSCHER (Ed.). **Methods in Enzymology**. [s.l.] Academic Press Inc., 1990. v. 182p. 285–300.

EQUIPE ESTATCAMP. **Software action**, 2018. Available in: <http://www.portalaction.com.br/>. Accessed in april of 2018.

FONSECA, R. R. et al. Optimizing carbon/nitrogen ratio for biosurfactant production by a *Bacillus subtilis* strain. **Applied Biochemistry and Biotechnology**, v. 137–140, n. 1–12, p. 471–486, 2007.

ISA, M. H. M.; FRAZIER, R. A.; JAUREGI, P. A further study of the recovery and purification of surfactin from fermentation broth by membrane filtration. **Separation and Purification Technology**, v. 64, n. 2, p. 176–182, 2008.

ISO, I. O. FOR S. Colony count at 30°C by the surface plating technique, ISO 4833-2. v. 44, n. 0, p. 1–19, 2013.

JAUREGI, P. et al. Micelle size characterization of lipopeptides produced by *B. subtilis* and their recovery by the two-step ultrafiltration process. **Separation and Purification Technology**, v. 104, p. 175–182, 2013.

KAKINUMA, A.; TAMURA, G.; ARIMA, K. Wetting of fibrin plate and apparent promotion of fibrinolysis by surfactin, a new bacterial peptidelipid surfactant. **Experientia**, v. 24, n. 11, p. 1120–1121, 1968.

KARSA, D. R. **Industrial applications of surfactants IV**. Cambridge: The Royal Society of Chemistry, 1999.

KOSARIC, N.; VARDAR-SUKAN, F. **Biosurfactants: Production and Utilization—Processes, Technologies, and Economics**. Boca Raton, Flórida: CRC Press, 2015.

KRACHT, M. et al. Antiviral and hemolytic activities of surfactin isoforms and their methyl ester derivatives. **The Journal of Antibiotics**, v. 52, n. 7, p. 613–619, 1999.

MAKKAR, R. S.; CAMEOTRA, S. S.; BANAT, I. M. Advances in utilization of renewable substrates for biosurfactant production. **AMB Express**, v. 1, n. 1, p. 1–5, 2011.

MÜLLER, T. et al. Ammonium toxicity in bacteria. **Current Microbiology**, v. 52, n. 5, p. 400–406, 2006.

NITSCHKE, M.; COSTA, S. G. V. A. O. Biosurfactants in food industry. **Trends in Food Science and Technology**, v. 18, n. 5, p. 252–259, 2007.

NITSCHKE, M.; FERRAZ, C.; PASTORE, G. M. Selection of microorganismos for biosurfactant production using agroindustrial wastes. **Brazilian Journal of Microbiology**, v. 35, n. 1–2, p. 81–85, 2004.

NITSCHKE, M.; PASTORE, G. M. Biosurfactant production by *Bacillus subtilis* using cassava-processing effluent. **Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology**, v. 112, n. 3, p. 163–172, mar. 2004.

PEREIRA, G. A. et al. Extraction optimization and profile analysis of oligosaccharides in banana pulp and peel. **Journal of Food Processing and Preservation**, v. 41, n. 1, p. 1–10, 2017.

RAJALINGAM, D. et al. Trichloroacetic acid-induced protein precipitation involves the reversible association of a stable partially structured intermediate. **Protein science : a publication of the Protein Society**, v. 18, n. 5, p. 980–993, 2009.

SIMIQUELI, A. P. R. **Produção contínua de surfactina utilizando manipueira como substrato e estudo de sua aplicação em conjunto com óleos essenciais**. [s.l.] Universidade Estadual de Campinas, 2014.

SIVAPATHASEKARAN, C.; RAMKRISHNA, S. Origin , Properties , Production and Purification of Microbial Surfactants as Molecules with Immense Commercial Potential. **Tenside, Surfactants, Detergents**, v. 54, n. 2, p. 92–107, 2017.

SLIVINSKI, C. T. et al. Production of surfactin by *Bacillus pumilus* UFPEDA 448 in solid-state fermentation using a medium based on okara with sugarcane bagasse as a bulking agent. **Process Biochemistry**, v. 47, p. 1848–1855, 2012.

SOBERÓN-CHÁVEZ, G. **Biosurfactants: from genes to applications**. Berlin, Germany: Springer-Verlag, 2010. v. 20.

SPECTOR, R.; HUNTOON, S. Effects of caramel color (ammonia process) on mammalian vitamin B6 metabolism. **Toxicology and Applied Pharmacology**, v. 62, n. 1, p. 172–178, 1982.

STULKE, J.; HILLEN, W. Regulation of carbon catalobism in *Bacillus* species. **Annual Review of Microbiology**, v. 54, p. 849–880, 2000.

SWIFT, S.; ROWE, M. C.; KAMATH, M. **Bacterial physiology: a molecular approach**. [s.l.] Springer-Verlag, 2008.

ZHAO, H. et al. Biological activity of lipopeptides from *Bacillus*. **Applied Microbiology and Biotechnology**, v. 101, n. 15, p. 5951–5960, 2017.

DISCUSSÃO GERAL

A produção de biossurfactante ainda apresenta elevado custo e um dos principais desafios é realizar uma produção com alto rendimento e purificação com pureza superior a 80%. Porém nem sempre é possível, e no cenário atual os processos de recuperação de surfactina empregam várias etapas o que muitas vezes dificulta sua produção em larga escala ou até mesmo inviabiliza a comercialização a preços mais baixos.

O capítulo I deste trabalho relatou o uso de resíduos agroindustriais para produção de surfactina. O uso de resíduos é uma alternativa para minimizar os custos de produção de surfactantes pela substituição de meios de cultura. Alguns resíduos possuem algumas vantagens com relação a outros, como no caso dos resíduos ricos em amido, onde não é necessário realizar a suplementação do meio com outras fontes de carbono. Por outro lado, resíduos de óleos vegetais necessitam ser suplementados para propiciar melhores condições para produção.

Apesar de que alguns resíduos proporciona rendimentos maiores que outros, isso pode estar associado não somente aos componentes do resíduo, mas sim as diferentes cepas de *Bacillus* usadas para produção de lipopetídeos.

Dentre as técnicas mais usadas para recuperação e purificação de surfactina tais como, precipitação ácida fracionamento da espuma, extração com solventes, a filtração com diferentes tipos de membrana demostra-se promissora para recuperação de lipopeptídeos, por ser de fácil escalonamento e por poder ser associada ao fracionamento da espuma, podendo ser aplicado sem o uso de solventes orgânicos.

No capítulo II foi realizada a produção de surfactina em manipueira e como técnica de recuperação aplicou-se a UF em duas etapas. Foram empregadas três estratégias para remoção de proteínas na manipueira, a fim de minimizar os efeitos causados por incrustação de membrana (UF) com o objetivo de melhorar a recuperação da surfactina. Das três estratégias utilizadas: HCl pH 2 e pH3, $(NH_4)_2SO_4$, TCA o tratamento ácido demostrou-se o mais promissor. Pois a produção de surfactina foi 749, 80 mg/L e na manipueira sem tratamento a produção foi de 598,56 mg/L. Os tratamentos

com: $(NH_4)_2SO_4$ e TCA afetaram negativamente o crescimento do *B. subtilis* e a produção de surfactina sendo esta de 30 e 20 mg/L, respectivamente.

Durante o estudo foi possível observar um aumento de glicose, frutose e sacarose no meio de cultura após 4 horas de fermentação na manipueira (CWW), que declina subsequentemente com 72 horas de fermentação sendo praticamente 0 (0,19 g / L). O oposto é observado nos tratamentos com ácido a pH2 e pH3 (CWW-2 e CWW-3), onde o aumento na concentração de glicose começa às 12 horas e a queda começa às 18 horas (CWW-2 5,60 a 9,22 g / L e CWW-3 4,94 a 16,25 g / L), chegando perto de 0 no final da fermentação. O tratamento com ácido em CWW provavelmente melhorou a disponibilidade de nutrientes do meio, e observou-se um consumo mais lento de glicose em CWW-2 e CWW-3.

A concentração de frutose em CWW aumentou de 8 horas (4,24 g/L) para 18 horas (6,56 g/L). Nos tratamentos CWW-2 e CWW-3, observou-se aumento de frutose de 12 a 18 horas, com valores de 4,13 a 5,76 g/L e 3,48 a 7,85 g/L, respectivamente. No final da fermentação, a concentração de frutose em CWW-2 e CWW-3 ainda estava em 0,3 g/L. O consumo de sacarose aumentou desde o início da fermentação até 4 horas tanto para o meio CWW controle (24,72 a 37,59 g/L) quanto para o CWW-3 (23,19 a 29,89 g/L), enquanto que em CWW-2 não houve aumento. O aumento de glicose e frutose em diferentes tempos para o meio de cultura indicam que os tratamentos com HCl afetaram a composição inicial da CWW, favorecendo o aumento da produção de surfactina.

Porém, neste trabalho não foi possível identificar o que especificamente o tratamento ácido afetou que ocasionou aumento da produção.

CONCLUSÃO GERAL

A utilização de diferentes estratégias para remoção das proteínas presente na manipueira, demostrou que os tratamentos com $(NH_4)_2SO_4$ e TCA afetaram negativamente a produção de surfactina e crescimento do *Bacillus subtilis* LB5a (0 mg/L), provavelmente pela reação de caramelização. Devido a formação de uma coloração escura (após autoclavagem) os tratamentos foram filtrados (meio de esterilização) e a produção de surfactina foi de 30 mg/L. O tratamento com HCl pH2 surpreendentemente aumentou a produção de surfactina (749,80 mg/L), uma vez que o experimento controle (sem qualquer tratamento) atingiu uma produção de 598,56 mg/L.

Como a maior produção foi obtida com o tratamento HCl pH2, apenas este passou pelo processos de recuperação e purificação. A recuperação da surfactina pelas duas etapas de ultrafiltração mostrou-se satisfatória onde obteve-se um rendimento superior a 72% com pureza de 51,49%.

A recuperação superior a 72% e o aumento da produção de surfactina é um passo importante na viabilização da produção, uma vez que utiliza como meio de cultura um resíduo agroindustrial associado a um processo de purificação de baixo custo (ultrafiltração) diretamente do meio de cultura.

PERSPECTIVAS FUTURAS

Este trabalho foi o primeiro a desenvolver um método para remover as proteínas da manipueira. E ainda há uma série de estudos que devem ser realizados a fim de melhorar mais o processo.

- Avaliar se o tratamento ácido afetou a forma química da surfactina;
- Investigar o perfil de outros açúcares presentes na manipueira;
- Analisar o perfil proteômico durante a produção de surfactina na manipueira tratada, possibilitando melhor entendimento do metabolismo do *Bacillus subtilis*;
- Otimizar as condições de produção da surfactina;
- Aumentar a escala do processo fermentativo;
- Avaliar se a recuperação da espuma em biorreator possibilita maior pureza quando associada as estratégias de ultrafiltração.

REFERÊNCIAS GERAIS

ABAM. **Estatísticas Produção**. [s.l: s.n.]. Disponível em: <http://www.abam.com.br/estatisticas-producao.php>. Acessado em: 20 Janeiro de 2018.

ALONSO, S.; MARTIN, P. J. Impact of foaming on surfactin production by *Bacillus subtilis*: Implications on the development of integrated in situ foam fractionation removal systems. **Biochemical Engineering Journal**, v. 110, p. 125–133, 2016.

ANDRADE, C. J. DE et al. Comparative study: bench-scale surfactin production from *Bacillus subtilis* using analytical grade and concentrated glycerol from the biodiesel industry. **International Journal of Scientific World**, v. 5, n. 1, p. 28–37, 2016.

ANDRADE, C. J. DE et al. Optimizing alternative substrate for simultaneous production of surfactin and 2,3-butanediol by *Bacillus subtilis* LB5a. **Biocatalysis and Agricultural Biotechnology**, v. 6, p. 209–218, 2016.

ANDRADE, C. J. DE et al. Ultrafiltration based purification strategies for surfactin produced by *Bacillus subtilis* LB5A using cassava wastewater as substrate. **Journal of Chemical Technology and Biotechnology**, v. 91, n. 12, p. 3018–3027, 2016.

ANDRADE, C. J. DE; PASTORE, G. M. Ultrafiltration of mannosylerytritol lipids: a parallel with surfactin. In: **Ars Separatoria**. [s.l: s.n.]. v. 11p. 171–200, 2017.

ARIMA, K.; KAKINUMA, A; TAMURA, G. Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. **Biochemical and biophysical research communications**, v. 31, n. 3, p. 488–494, 1968.

BARROS, F. F. C. et al. Surfactina: Propriedades químicas, tecnológicas e funcionais para aplicações em alimentos. **Química Nova**, v. 30, n. 2, p. 409–414, 2007.

BARROS, F. F. C.; PONEZI, A. N.; PASTORE, G. M. Production of biosurfactant by *Bacillus subtilis* LB5a on a pilot scale using cassava wastewater as substrate. **Journal Indian Microbiol Biotechnol**, v. 35, n. 9, p. 1071–1078, 2008.

BELITSKY, B. R. Physical and enzymological interaction of *Bacillus subtilis* proteins required for de novo pyridoxal 5′-phosphate biosynthesis. **Journal of Bacteriology**, v. 186, n. 4, p. 1191–1196, 2004.

BRADFORD, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical Biochemistry**, v. 72, n. 1–2, p. 248–254, 1976.

CAGRI-MEHMETOGLU, A.; KUSAKLI, S.; VENTERT, M. VAN DE. Production of polysaccharide and surfactin by *Bacillus subtilis* ATCC 6633 using rehydrated whey powder as the fermentation medium. **American Dairy Science Association**, v. 95, n. 7, p. 3643–3649, 2012.

CHEN, H. L. et al. Purification of surfactin in pretreated fermentation broths by adsorptive removal of impurities. **Biochemical Engineering Journal**, v. 40, n. 3, p. 452–459, 2008.

CHEN, H. L.; CHEN, Y. S.; JUANG, R. S. Flux decline and membrane cleaning in cross-flow ultrafiltration of treated fermentation broths for surfactin recovery. **Separation and Purification Technology**, v. 62, n. 1, p. 47–55, 2008.

CHEN, H. L.; CHEN, Y. S.; JUANG, R. S. Separation of surfactin from fermentation broths by acid precipitation and two-stage dead-end

ultrafiltration processes. **Journal of Membrane Science**, v. 299, n. 1–2, p. 114–121, 2007.

CHEN, H. L.; JUANG, R. S. Extraction of surfactin from fermentation broth with n-hexane in microporous PVDF hollow fibers: Significance of membrane adsorption. **Journal of Membrane Science**, v. 325, n. 2, p. 599–604, 2008.

CHEN, H. L.; JUANG, R. S. Recovery and separation of surfactin from pretreated fermentation broths by physical and chemical extraction. **Biochemical Engineering Journal**, v. 38, n. 1, p. 39–46, 2008.

CHERYAN, M. **Ultrafiltration and microfiltration**. Second Edi ed. Boca Raton, Flórida: CRC Press, 2000.

CHISTÉ, R. C.; COHEN, K. DE O. **Efeito do Processo de Fabricação da Farinha de MandiocaEmbrapa Amazônia Oriental**. Belém, Pará: [s.n.].

COUTTE, F. et al. Microbial lipopeptide production and purification bioprocesses, current progress and future challenges. **Biotechnology Journal**, v. 12, n. 7, p. 1–10, 2017.

CRUZ, J. M. et al. Agricultural feedstock supplemented with manganese for biosurfactant production by *Bacillus subtilis*. **Waste Biomass Valor**, v. 9, p. 613–618, 2018.

DAVIS, D. A.; LYNCH, H. C.; VARLEY, J. The production of surfactin in batch culture by *Bacillus subtilis* ATCC 21332 is strongly influenced by the conditions of nitrogen metabolism. **Enzyme and Microbial Technology**, v. 25, n. 3–5, p. 322–329, 1999.

DESAI, J. D.; BANAT, I. M. Microbial production of surfactants and their commercial potential. **Microbiology and molecular biology reviews : MMBR**, v. 61, n. 1, p. 47–64, 1997.

DHANARAJAN, G.; RANGARAJAN, V.; SEN, R. 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**, v. 143, p. 72–79, 2015.

DUFOUR, S. et al. Hemolytic activity of new linear surfactin analogs in relation to their physico-chemical properties. **Biochimica et Biophysica Acta - General Subjects**, v. 1726, n. 1, p. 87–95, 2005.

EBADI, A. et al. Effective bioremediation of a petroleum-polluted saline soil by a surfactant-producing *Pseudomonas aeruginosa* consortium. **Journal of Advanced Research**, v. 8, n. 6, p. 627–633, 2017.

EMPRAPA. **Mandioca**. Disponível em: <https://www.embrapa.br/mandioca-e-fruticultura/cultivos/mandioca>. Acessado em 20 de fervereiro de 2018.

ENGLARD, S.; SEIFTER, S. Precipitation Tecniques. In: M. P. DEUTSCHER (Ed.). **Methods in Enzymology**. [s.l.] Academic Press Inc., 1990. v. 182p. 285–300.

EQUIPE ESTATCAMP. **Software action**, 2018. Available in: <http://www.portalaction.com.br/>. Accessed in april of 2018.

FAO. **Food Outlook: Biannual report on global foof markets**. [s.l: s.n.]. Disponível em: <http://www.fao.org/3/a-i6198e.pdf>. Acessaro em 20 de fervereiro de 2018.

FONSECA, R. R. et al. Optimizing carbon/nitrogen ratio for biosurfactant production by a *Bacillus subtilis* strain. **Applied Biochemistry and Biotechnology**, v. 137–140, n. 1–12, p. 471–486, 2007.

FOX, S. L.; BALA, G. A. Production of surfactant from *Bacillus subtilis* ATCC 21332 using potato substrates. **Bioresource Technology**, v. 75, p. 235–240, 2000.

GHOSHEH, H. Z.; BSOUL, E. Y.; ABDULLAH, A. Y. Agro-Processing Waste Assessment and Management in Peri-Urban Hanoi, Vietnam. **Journal of Sustainable Agriculture**, v. 25, n. 1, p. 69–95, 2005.

GUDIÑA, E. J. et al. Biosurfactant production by *Bacillus subtilis* using corn steep liquor as culture medium. **Frontiers in Microbiology**, v. 6, p. 1–7, 2015.

GURJAR, J.; SENGUPTA, B. Production of surfactin from rice mill polishing residue by submerged fermentation using *Bacillus subtilis* MTCC 2423. **Bioresource Technology**, v. 189, p. 243–249, 2015.

HOLMBERG, K. et al. **Surfactants and polymers in Aqueous Solution**. 2 edition ed. England: John Wiley & Sons, LTD, 2002.

ISA, M. H. M. et al. Recovery and purification of surfactin from fermentation broth by a two-step ultrafiltration process. **Journal of Membrane Science**, v. 296, n. 1–2, p. 51–57, 2007.

ISA, M. H. M.; FRAZIER, R. A.; JAUREGI, P. A further study of the recovery and purification of surfactin from fermentation broth by membrane filtration. **Separation and Purification Technology**, v. 64, n. 2, p. 176–182, 2008.

ISO, I. O. FOR S. Colony count at 30°C by the surface plating technique, ISO 4833-2. v. 44, n. 0, p. 1–19, 2013.

JAUREGI, P. et al. Micelle size characterization of lipopeptides produced by *B. subtilis* and their recovery by the two-step ultrafiltration process. **Separation and Purification Technology**, v. 104, p. 175–182, 2013.

KAKINUMA, A.; TAMURA, G.; ARIMA, K. Wetting of fibrin plate and apparent promotion of fibrinolysis by surfactin, a new bacterial peptidelipid surfactant. **Experientia**, v. 24, n. 11, p. 1120–1121, 1968.

KARSA, D. R. **Industrial applications of surfactants IV**. Cambridge: The Royal Society of Chemistry, 1999.

KOSARIC, N.; VARDAR-SUKAN, F. **Biosurfactants: Production and Utilization—Processes, Technologies, and Economics**. Boca Raton, Flórida: CRC Press, 2015.

KRACHT, M. et al. Antiviral and hemolytic activities of surfactin isoforms and their methyl ester derivatives. **The Journal of Antibiotics**, v. 52, n. 7, p. 613–619, 1999.

KUYUCAK, N.; AKCIL, A. Cyanide and removal options from effluents in gold mining and metallurgical processes. **Minerals Engineering**, v. 50–51, p. 13–29, 2013.

LEBOT, V. **Tropical Root and Tuber Crops: Cassava, Sweet Potato, Yams and Aroids**. 17. ed. London, UK: CABI, 2009.

MAASS, D. et al. Two-phase olive mill waste (alpeorujo) as carbon source for biosurfactant production. **Journal of Chemical Technology & Biotechnology**, v. 91, p. 1990–1997, 2015.

MAI, Y.; EISENBERG, A. Self-assembly of block copolymers. **Chemical Society Reviews**, v. 41, n. 18, p. 5969–5985, 2012.

MAKKAR, R. S.; CAMEOTRA, S. S.; BANAT, I. M. Advances in utilization of renewable substrates for biosurfactant production. **AMB Express**, v. 1, n. 1, p. 1–5, 2011.

MARÓSTICA, M. R. J.; PASTORE, G. M. Production of R-(+)-α-terpineol by the biotransformation of limonene from orange essential oil, using cassava waste water as medium. **Food Chemistry**, v. 101, n. 1, p. 345–350, 2007.

MOYA-RAMÍREZ, I. et al. Rhamnolipid and surfactin production from olive oil mill waste as sole carbon source. **Bioresource Technology**, v. 198, p. 231–236, 2015.

MÜLLER, T. et al. Ammonium toxicity in bacteria. **Current Microbiology**, v. 52, n. 5, p. 400–406, 2006.

NDLOVU, T. et al. Variants of lipopeptides and glycolipids produced by Bacillus amyloliquefaciens and *Pseudomonas aeruginosa* cultured in different carbon substrates. **AMB Express**, v. 109, n. 7, p. 2–13, 2017.

NELSON, D. L.; COX, M. M. **Príncipios de bioquímica de Lehninger**. 6 edição ed. Porto Alegre: Artmed, 2014.

NISHIKIORI, T. et al. Plipastatins: new inhibitors of phospholipase A2, produced by *Bacillus cereus* BMG302-fF67. III. Structural elucidation of plipastatins. **The Journal of antibiotics**, v. 39, n. 6, p. 755–761, 1986.

NITSCHKE, M.; COSTA, S. G. V. A. O. Biosurfactants in food industry. **Trends in Food Science and Technology**, v. 18, n. 5, p. 252–259, 2007.

NITSCHKE, M.; FERRAZ, C.; PASTORE, G. M. Selection of microorganismos for biosurfactant production using agroindustrial wastes. **Brazilian Journal of Microbiology**, v. 35, n. 1–2, p. 81–85, 2004.

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

NITSCHKE, M.; PASTORE, G. M. Biosurfactant production by Bacillus subtilis using cassava-processing effluent. **Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology**, v. 112, n. 3, p. 163–172, mar. 2004.

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

OLIVEIRA, D. W. F. DE et al. Kinetic study of biosurfactant production by *Bacillus subtilis* LAMI005 grown in clarified cashew apple juice. **Colloids and Surfaces B: Biointerfaces**, v. 101, p. 34–43, 2013.

PARASZKIEWICZ, K. et al. Structural identification of lipopeptide biosurfactants produced by *Bacillus subtilis* strains grown on the media obtained from renewable natural resources. **journal of Environmental Management**, v. 209, p. 65–70, 2018.

PATEL, M. N.; GOPINATHAN, K. P. Lysozyme-sensitive bioemulsifier for immiscible organophosphorus pesticides. **Applied and Environmental Microbiology**, v. 52, n. 5, p. 1224–1226, 1986.

PATIL, Y. B.; PAKNIKAR, K. M. Development of a process for biodetoxification of metal cyanides from waste waters. **Process Biochemistry**, v. 35, n. 10, p. 1139–1151, 2000.

PEREIRA, G. A. et al. Extraction optimization and profile analysis of oligosaccharides in banana pulp and peel. **Journal of Food Processing and Preservation**, v. 41, n. 1, p. 1–10, 2017.

RAJALINGAM, D. et al. Trichloroacetic acid-induced protein precipitation involves the reversible association of a stable partially structured intermediate. **Protein science : a publication of the Protein Society**, v. 18, n. 5, p. 980–993, 2009.

RAMÍREZ, I. M. et al. Hydrolysis of olive mill waste to enhance rhamnolipids and surfactin production. **Bioresource Technology**, v. 205, p. 1–6, 2016.

SANA, S. et al. Towards the development of an effective in vivo wound healing agent from *Bacillus* sp. derived biosurfactant using Catla catla fish fat. **Royal Society of Chemistry**, v. 7, p. 13668–13677, 2017.

SEAB. **Análise Da Conjuntura Agropecuária Mandioca - Safra-2015/16Departamento de Economia Rural**. http://www.agricultura.pr.gov.br/arquivos/File/deral/Prognosticos/2016/mandioca_2015_16.pdf: [s.n.]. Acessado em 21 de fevereiro de 2018.

SHUKLA, S. **Surfactants market by type (cationic, anionic, non-ionic, amphoteric) and application (household detergents, personal care, industrial & intitutional cleaners, emulsion polymerization, food processing, oilfield chemicals)- Global opportunity analysis and** . [s.l: s.n.]. Disponível em: <https://www.alliedmarketresearch.com/surfactant-market>. Accessed in 30 of may of 2018.

SIGMA-ALDRICH. Disponível em: **https://www.sigmaaldrich.com/brazil.html**. Accessed in 15 of july of 2018.

SILVA, M. T. S. et al. Integral production and concentration of surfactin from *Bacillus* sp. ITP-001 by semi-batch foam fractionation. **Biochemical Engineering Journal**, v. 104, 2015.

SIMIQUELI, A. P. R. **Produção contínua de surfactina utilizando manipueira como substrato e estudo de sua aplicação em conjunto com óleos essenciais**. [s.l.] Universidade Estadual de Campinas, 2014.

SIVAPATHASEKARAN, C.; RAMKRISHNA, S. Origin , Properties , Production and Purification of Microbial Surfactants as Molecules with Immense Commercial Potential. **Tenside, Surfactants, Detergents**, v. 54, n. 2, p. 92–107, 2017.

SLIVINSKI, C. T. et al. Production of surfactin by *Bacillus pumilus* UFPEDA 448 in solid-state fermentation using a medium based on okara with sugarcane bagasse as a bulking agent. **Process Biochemistry**, v. 47, p. 1848–1855, 2012.

SOBERÓN-CHÁVEZ, G. **Biosurfactants: from genes to applications**. Berlin, Germany: Springer-Verlag, 2010. v. 20.

SOUSA, M. et al. Screening of biosurfactant-producing *Bacillus* strains using glycerol from the biodiesel synthesis as main carbon source. **Bioprocess and Biosystems Engineering**, v. 35, p. 897–906, 2012.

SPECTOR, R.; HUNTOON, S. Effects of caramel color (ammonia process) on mammalian vitamin B6 metabolism. **Toxicology and Applied Pharmacology**, v. 62, n. 1, p. 172–178, 1982.

STULKE, J.; HILLEN, W. Regulation of carbon catalobism in *Bacillus* species. **Annual Review of Microbiology**, v. 54, p. 849–880, 2000.

SWIFT, S.; ROWE, M. C.; KAMATH, M. **Bacterial physiology: a molecular approach**. [s.l.] Springer-Verlag, 2008.

TUMWESIGYE, K. S.; OLIVEIRA, J. C.; SOUSA-GALLAGHER, M. J. Integrated sustainable process design framework for cassava biobased packaging materials: Critical review of current challenges, emerging trends and

prospects. **Trends in Food Science and Technology**, v. 56, p. 103–114, 2016.

VARJANI, S. J.; UPASANI, V. N. Critical Review on Biosurfactant Analysis, Purification and Characterization Using Rhamnolipid as a model Biosurfactant. **Bioresource Technology**, v. 232, p. 389–397, 2017.

VECINO, X. et al. Optimization of liquid–liquid extraction of biosurfactants from corn steep liquor. **Bioprocess and Biosystems Engineering**, v. 38, p. 1629–1637, 2015.

VECINO, X. et al. Study of the surfactant properties of aqueous stream from the corn milling industry. **Journal of Agricultural and Food Chemistry**, v. 62, n. 24, p. 5451–5457, 2014.

VOLLENBROICH, D.; PAULI, G.; MUHSIN, O. Antimycoplasma properties and application in cell culture of surfactin , a lipopeptide antibiotic from *Bacillus subtilis*. **Applied and Environmental Microbiology**, v. 63, n. 1, p. 44–49, 1997.

WALTON, R. B.; WOODRUFF, B. A crystalline antifungal agent, mycosubtilin, isolated from subtilin broth. **The Journal of clinical investigation**, v. 28, n. 5, p. 924–926, 1949.

WHANG, L. M. et al. Application of biosurfactants, rhamnolipid, and surfactin, for enhanced biodegradation of diesel-contaminated water and soil. **Journal of Hazardous Materials**, v. 151, n. 1, p. 155–163, 2008.

WOSIACKI, G.; CEREDA, M. P. Valorização de Resíduos do Processamento de Mandioca. **Ciências Exatas e da Terra, Ciências Agrárias e Engenharias,** v. 8, n. 1, p. 27–43, 2002.

YU, X. et al. Giant surfactants based on molecular Nanoparticles: Precise synthesis and solution self-assembly. **Journal of Polymer Science, Part B: Polymer Physics**, v. 52, n. 20, p. 1309–1325, 2014.

YUAN, J. et al. The ultrasound-assisted extraction and identification of antifungal substances from *B. amyloliquefaciens* strain NJN-6 suppressing Fusarium oxysporum. **Journal of Basic Microbiology**, v. 52, n. 6, p. 721–730, 2012.

ZHAO, H. et al. Biological activity of lipopeptides from *Bacillus*. **Applied Microbiology and Biotechnology**, v. 101, n. 15, p. 5951–5960, 2017.

ZHI, Y.; WU, Q.; XU, Y. Production of surfactin from waste distillers' grains by co-culture fermentation of two *Bacillus amyloliquefaciens* strains. **Bioresource Technology**, v. 235, p. 93–103, 2017.

ZHU, Z. et al. The usage of rice straw as a major substrate for the production of surfactin by *Bacillus amyloliquefaciens* XZ-173 in solid-state fermentation. **Journal of Environmental Management**, v. 127, p. 96–102, 2013.

ANEXOS

CADASTRO DE ACESSO DE ATIVIDADE DO PATRIMÔNIO GENÉTICO

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Comprovante de Cadastro de Acesso

Cadastro nº AA11715

A atividade de acesso ao Patrimônio Genético, nos termos abaixo resumida, foi cadastrada no SisGen, em atendimento ao previsto na Lei nº 13.123/2015 e seus regulamentos.

Número do cadastro:	**AA11715**
Usuário:	**UNICAMP**
CPF/CNPJ:	**46.068.425/0001-33**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa e Desenvolvimento Tecnológico**

Espécie

Bacillus subtilis

Manihot esculenta

Título da Atividade:	**ULTRAFILTRAÇÃO DA SURFACTINA PRODUZIDA POR Bacillus subtilis LB5A USANDO MANIPUEIRA COM BAIXO TEOR DE PROTEÍNA**

Equipe

Aline Wasem Zanotto	**UNICAMP**
Glaucia Maria Pastore	**Unicamp**
Cristiano José de Andrade	**Unicamp**

Resultados Obtidos

Divulgação de resultados em meios científicos ou de comunicação

Identificação do meio onde foi divulgado:	**Publicação de resumo no Simpósio Latino Ame**

Data do Cadastro:	**26/06/2018 15:19:47**
Situação do Cadastro:	**Concluído**

Conselho de Gestão do Patrimônio Genético
Situação cadastral conforme consulta ao SisGen em **23:13** de **26/06/2018**.

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO - SISGEN

TERMO DE SIGILO REFERENTE ÀS INFORMAÇÕES CONSTANTES EM DEFESA DE TESES E DISSERTAÇÕES

Título da Tese/Dissertação: ULTRAFILTRAÇÃO DA SURFACTINA PRODUZIDA POR *Bacillus subtilis* LB5A USANDO MANIPUEIRA COM BAIXO TEOR DE PROTEÍNA
Aluno: *Aline Wasem Zanotto* ***Data:*** *08/2018*
Orientador: Glaucia Maria Pastore

1. São tidas como **"Informações Confidenciais"** todas as informações relacionadas à Dissertação de Mestrado do **Sra**. **Aline Wasem Zanotto,** cujo o título é *"* ULTRAFILTRAÇÃO DA SURFACTINA PRODUZIDA POR *Bacillus subtilis* LB5a USANDO MANIPUEIRA COM BAIXO TEOR DE PROTEÍNA*"* no dia 06/08/2018 às 10:00 horas expressas por qualquer meio seja oral, escrito ou eletrônico, constantes em documentos, planilhas, sistemas, fotografias, relatórios, disquetes, disco laser, desenhos, modelos, dados, especificações, relatórios, compilações, programas de computador, pedidos de patentes e patentes, produtos e processos e outros, a que venham a ser apresentadas ou de alguma forma disponibilizadas aos examinadores e demais presentes.

2. Os membros da banca examinadora e os presentes manterão sigilo, em relação às **"Informações Confidenciais"** mesmo que estas não contenham nem sejam acompanhadas de qualquer tipo de advertência de sigilo, devendo tal condição ser sempre presumida.

3. Os membros da banca examinadora e os presentes têm ciência e anuência que as informações confidenciais serão protegidas pelos meios legais e asseguram desde já que estas **"Informações Confidenciais"** a que tiverem acesso, não serão mecanicamente copiadas ou de qualquer outra forma reproduzidas, bem como não serão divulgadas, publicadas nem serão circuladas, sem a autorização do aluno, do orientador e da Agência de Inovação da Unicamp – INOVA.

4. O descumprimento dos itens ou condições previstas neste Termo sujeitará o signatário que incorrer na infração ao presente, à responsabilidade criminal e ou civil e conseqüente indenização.

5. Os membros da banca examinadora, abaixo assinados, manifestam sua total ciência e anuência com as condições estabelecidas neste termo.

Profª.
Instituição:
RG

Profª.
Instituição:
RG

Profª.
Instituição:
RG

Profª.
Instituição:
RG

Profª.
Instituição:
RG

Os demais presentes assinam no verso deste documento (nome completo, RG e assinatura).

Nome Completo	RG	Assinatura