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HYDROPHOBIC INDUCERS TO ENHANCED SURFACTIN PRODUCTION USING CASSAVA WASTEWATER AS LOW-COST CULTURE MEDIUM: A PROSPECTION ON NEW HOMOLOGUES

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Orientador: Prof. Cristiano José Andrade, Dr.
Co-orientadoras: Profa. Débora de Oliveira, Dra.
Karina Cesca, Dra.

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

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Profe. Cristiano José de Andrade, Dr.
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Florianópolis
2022

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"Durante toda a sua vida, estudara o universo, mas desprezara sua mais clara mensagem: para criaturas pequenas como nós, a vastidão só é suportável através do amor." (Carl Sagan em "Contato", 1985).

RESUMO

Biossurfactantes são moléculas anfipáticas que são sintetizadas por células vivas com inúmeras aplicações potenciais nas áreas de saúde e meio ambiente, entre outras. No entanto, o alto custo de produção limita as aplicações desses compostos em larga escala. Neste sentido, reduzir seu custo de produção é a substituição do meio de cultura sintético por resíduos agroindustriais associados a indutores hidrofóbicos. Alguns desses estudos e uma revisão recente sobre o tema, informaram que bioquimicamente, a produtividade do biossurfactante pode ser facilmente aumentada pela simples adição de indutores ao meio de cultura, que estimula crescimento microbiano e também desencadeia o metabolismo de produção de biossurfactante - novos homólogos. Ou seja, os indutores estão relacionados ao tamanho de cadeias hidrofóbicas (biossurfactantes) e consequentemente maior estabilidade e eficiência dos mesmos. Os indutores de biossurfactantes são principalmente moléculas (compostos) hidrofóbicas (e.g. óleo de soja - conjunto de moléculas saturadas e insaturadas ácidos graxos, proteínas e vitaminas). Porém há poucas informações sobre o efeito dessas moléculas de forma individual(e.g. ácido oleico) na produção e estrutura química de biossurfactantes. Neste estudo iinvestigou-se a utilização de diferentes concentrações de indutores hidrofóbicos (1, 2, 5 e 10%) na produção de surfactina por *Bacillus subtilis* ATCC 6633 utilizando o resíduo líquido gerado na produção de farinha de mandioca (manipueira) como meio de cultura alternativo (fermentação submersa, 30 °C e 150 rpm por 72 h). A análise dos resultados de HPLC para todas as fermentações com indutores óleo de soja, ácido palmítico e ácido oleico, e em todas as concentrações, indicou a possível formação de diferentes homólogos de surfactina aumentando seu rendimento em até ≈70, 17e 98%, respectivamente. A surfactina produzida utilizando indutores reduziu a tensão superficial (≈26,5 $mN.m^{-1}$) e atingiu um rendimento máximo de ≈1,3 ± 0.08 $g.L^{-1}$. Indicando um aumento na produtividade (≈2 vezes). Este estudo confirma que a utilização de indutores hidrofóbicos pode refletir em estruturas químicas diferentes, fornecendo uma base forte para explorar novas estruturas e bioatividades desconhecidas.

Palavras-chave: Lipopeptídeos, ácido palmítico, ácido oleico, indutores de biossurfactantes.

RESUMO EXPANDIDO

Introdução

A surfactina é um biossurfactante comumente produzido por *Bacillus* ssp. (e.g. *B. pumilus*, *B. licheniformis*, *B. amyloliquefacien*, e principalmente pelo *B. subtilis*). A surfactina apresenta inúmeras bioatividades (e.g. anti-inflamatória, antifúngica, antiviral e bioestimulante) e propriedades surfactantes excepcionais que mostram seu uso potencial em diversas áreas como ambiental, agrícola, petróleo e farmacêutica, entre outras. No entanto, o alto custo de produção e purificação da surfactina limita o aumento de escala e sua consequente aplicação industrial, principalmente devido à sua matéria-prima constituida por glicose sacarose e sais minerais - que representa mais de 50% do custo total de produção do biossurfactante. Assim, o emprego de técnicas com alto rendimento e baixo custo em pesquisas relacionadas a biosurfactantes tornou-se imperativo.

Uma alternativa promissora para a redução do custo de produção da surfactina é o aproveitamento de resíduos agroindustriais, como por exemplo, resíduo líquido gerado na produção de farinha de mandioca. A indústria de processamento de mandioca gera por kg de mandioca fresca processada aproximadamente ≈0,65 kg de resíduos sólidos e ≈25,3 L de efluentes. Segundo Acchar & da Silva (2021) considerando a concentração de carga orgânica e do efeito tóxico desse resíduo agroindustrial, é possível inferir a alta fonte de poluição que representa. É valido destacar que a toxicidade dos resíduos da mandioca relacionada ao cianeto, pode gerar distúrbios com sintomas agudos ou crônicos em humanos e animais. Nesse sentido, o desenvolvimento de métodos ecologicamente corretos, simples e de baixo custo para o tratamento adequado ou reaproveitamento da cassava wastewater é um desafio. De outra perspectiva, Nitschke and Pastore (2006) relataram a água residuária da mandioca se destaca como resíduo agroindustrial. Em sua composição, são identificados altos níveis de carbono e nutrientes essenciais que beneficiam a produção de surfactina. Em seu estudo Nitschke and Pastore (2004) relataram o uso das águas residuais de mandioca para a produção de surfactina, utilizando duas cepas diferentes de *B. subtilis*. Como resultado, foi produzido ≈3.0 g de surfactina bruta/L. Similarmente, Andrade et al., (2016) reportaram a produção de surfactina por *B. subtilis* LB5a utilizando água residual de mandioca como substrato alternativo. O estudo alcançou a produção de ≈1.01 $g.L^{-1}$ de surfactina pura.

Apesar da complexidade no uso de resíduos agroindustriais como a padronização do substrato devido à variação natural da composição, custos de armazenamento, transporte e purificação e/ou tratamento residual prévio, entre outros, é possível estabelecer processos controlados para produção de surfactina com características químicas pré-estabelecidas, e potencializar a sua produção com a utilização de indutores hidrofóbicos. Bioquimicamente a produtividade dos biossurfactantes pode ser facilmente aumentada pela simples adição de indutores ao meio de cultura, que estimula o crescimento microbiano e também desencadeia o metabolismo de produção deste biossurfactante. Ou seja, os indutores estão relacionados ao tamanho de cadeias hidrofóbicas dos biossurfactantes e consequentemente maior

estabilidade e eficiência. Os indutores de biossurfactantes são principalmente moléculas (compostos) hidrofóbicas (e.g. oléo de soja e azeite de oliva - compostos por um *pool* de moléculas (e.g. ácidos graxos saturados e insaturados, proteínas e vitaminas). Porém existem poucas informações sobre o efeito dessas moléculas específicas (e.g. ácido oleico e o ácido palmítico) na produção e estrutura química da surfactina. Neste trabalho, o óleo de soja e os ácidos graxos palmítico e oleico foram avaliados como indutores hidrofóbicos na produção de surfactina por *Bacillus subtilis* ATCC 6633. Seu efeito indutivo foi avaliado pela suplementação destes ácidos graxos em diferentes concentrações em meio de cultura contendo apenas manipueira. Trata-se do primeiro estudo que detalha a produção de surfactina utilizando a manipueira associada a indutores hidrofóbicos.

Objetivo

Avaliar a produção de biossurfactantes utilizando a manipueira como substrato alternativo, propondo novas estratégias para aperfeiçoar a produção de surfactina.

Objetivos especificos

- Obtenção da surfactina a partir da cepa *Bacillus subtilis* ATCC 6633 utilizando a manipueira como substrato alternativo associada a indutores hidrofóbicos;
- Avaliar a influência dos ácidos palmitico e oleico como indutores hidrofóbicos em diferentes concentrações na produção de surfactina;
- Realizar um estudo do potencial destes indutores em alterações das propriedades de superfície, rendimento e estrutura química da surfactina.

Metodologia

Aproximadamente 50 L de efluentes do processamento de farinha de mandioca foram coletados em uma indústria de farinha (Rocha & Filho Ltda - SC - Brasil) e transportados sob refrigeração. A manipueira foi fervida a 100 °C por 3 min para facilitar a remoção de material sólido insolúvel e em seguida centrifugada. O pH natural do meio foi de 5,8 e não foi ajustado. O substrato foi caracterizado pela análise de fração mineral por espectrometria de emissão ótica com plasma indutivamente acoplado e a quantificação de açúcares majoritários foi realizada por cromatografia líquida de alta eficiência. O mesmo lote de águas residuais de mandioca foi utilizado para todos os experimentos.

Os ensaios fermentativos foram realizados em frascos de Erlenmeyer de 250 mL contendo 150 mL de meio de cultura composto por águas residuais de mandioca suplementadas com diferentes concentrações (1, 2, 5, e 10% ($m.v^{-1}$)) de indutores (ácido palmítico, ácido oleico e óleo de soja, separadamente) e previamente esterilizados a 120 ºC, 1 atm por 20 min. O perfil cinético de fermentação foi avaliado por

72 h. O consumo de açúcares, variação de pH, biomassa e a redução da tensão superficial do meio foram monitorados durante o período de fermentação.

Ao final do cultivo, as amostras foram centrifugadas para separação de biomassa. O pH do sobrenadante foi ajustado para 2 utilizando solução HCl (3 e 1 M) e mantido sob decantação (5 ºC por 24 h). Após esse período, o precipitado foi centrifugado e neutralizado (pH 7) com uma solução NaOH (3 e 1 M) e liofilizado. O sólido obtido foi denominado surfactina bruta e seu rendimento final foi analisado. Para quantificar a produção de surfactina, 20 mg de amostra foram diluídas em 1 mL metanol, filtradas, diluídas a uma proporção de 1:5 e submetidos à análise HPLC.

Resultados

Foi possível concluir que o meio de cultura composto por manipueira e indutores hidrofóbicos aumentou a produção de surfactina por *Bacillus subtilis* ATCC 6633. Pela primeira vez, os ácidos graxos foram utilizados em sua forma pura para a produção de surfactina. A surfactina produzida pelos indutores reduziu a tensão superficial (≈26,5 $mN.m^{-1}$) e atingiu um rendimento máximo de ≈1,3 ± 0,08 $g.L^{-1}$ (5% PA). A análise dos resultados de todas as fermentações utilizando os indutores óleo de soja, ácido palmítico e ácido oleico, e em todas as concentrações, indicou a possível formação de diferentes homólogos de surfactina, aumentando seus rendimentos em até ≈70, 17 e 98%, respectivamente. Foi observado um aumento (≈2 vezes) na produção de biossurfactante. Não foram observadas grandes alterações no pH. A tensão superficial diminuiu 40% em 12 h, indicando a produção de surfactina. O consumo de açúcares solúveis foi quase total para todas as condições do estudo. E o ácido oleico apresentou maior diversidade de homólogos. Esses resultados referem-se à valorização de resíduos agroindustriais, como a mandioca, por meio de novas estratégias de produção. Outras sugestões para melhorar a produção de surfactina é o uso de indutores hidrofílicos associados a indutores hidrofóbicos. No entanto, mais investigações devem elucidar sua correlação com as vias metabólicas do biossurfactante.

Considerações finais

Os resultados obtidos fornecem uma base teórica para o desenvolvimento de novos conhecimentos sobre a produção de surfactina utilizando indutores hidrofóbicos. Influenciando positivamente a valorização de resíduos agroindustriais como a manipueira por meio de novas estratégias de produção. Estes avanços serão de grande interesse para a uma economia circular nas indústrias de beneficiamento de mandioca, tendo em vista a ampla aplicabilidade deste biossurfactante.

Palavras-chave: Lipopeptídeos, ácido palmítico, ácido oleico, indutores de biossurfante

ABSTRACT

Biosurfactants are amphipathic molecules that are synthesized by living cells with numerous potential applications in the areas of health and the environment, among others. However, the high cost of production limits the applications of these compounds on a large scale. In this sense, an interesting approach to reducing its production cost is replacing the synthetic culture medium by agro-industrial residues associated with hydrophobic inducers. Some of these studies and a recent review reported that biochemically, biosurfactant productivity can be easily increased by adding inducers to the culture medium, which stimulates microbial growth and triggers the metabolism of biosurfactant production - new homologues. The inducers are related to the size of hydrophobic chains (biosurfactants) and, consequently, their greater stability and efficiency. Biosurfactant inducers are mainly hydrophobic molecules (compounds) (e.g. soybean oil - set of saturated and unsaturated fatty acids, proteins and vitamins). However, there is little information about the effect of these molecules individual (e.g. oleic acid) on biosurfactants' production and chemical structure. In this study, we investigated the use of different concentrations of hydrophobic inducers (1, 2, 5, and 10%) in the production of surfactin by *Bacillus subtilis* ATCC 6633 using cassava as an alternative culture medium (submerged fermentation, 30 °C and 150 rpm for 72 h). The analysis of the HPLC results for all fermentations with inducers soybean oil, palmitic acid, and oleic acid, and in all concentrations, indicated the possible formation of different surfactin homologues, increasing their yield up to ≈70, 17, and 98%, respectively. Surfactin produced using inducers reduced the surface tension (≈26.5 $mN.m^{-1}$) and reached a maximum yield of ≈1.3 ± 0.08 $g.L^{-1}$. Indicating a increase in productivity (≈2 times). This study confirms that using hydrophobic inducers can reflect on different chemical structures, providing a strong foundation to explore new structures and unknown bioactivities.

Keywords: Lipopeptides, palmitic acid, oleic acid, biosurfactant inducers.

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CHAPTER 1

This chapter presents a brief introduction to the research developed and its general and specific objectives.

1 INTRODUCTION

Surfactin is a natural biosurfactant, commonly produced by bacteria of the genus *Bacillus* (e.g. *B. pumilus, B. licheniformis, B. amyloliquefacien*, and mainly by *B. subtilis*) (CHEN; JUANG; WEI, 2015; DING et al., 2021). It has numerous bioactivities (e.g. anti-inflammatory, antifungal, antiviral, and biostimulant) and exceptional surfactant properties that show its potential use in several areas such as environmental, agricultural, petroleum and pharmaceutical, among others (DING et al., 2021).

However, the high cost of production and purification of surfactin limits the scale-up and its consequent industrial application, mainly due to its raw material - which represents more than 50% of the total cost of production of the biosurfactant (GAUR et al., 2022; VICENTE et al., 2021). Thus, using techniques with high yield and low cost in research related to biosurfactants has become imperative.

A promising alternative for reducing the cost of surfactin production is the use of agro-industrial residues, such as liquid residue generated in the production of cassava flour. The cassava processing industry generates approximately ≈0.65 kg of solid waste and ≈25.3 L of effluents per kg of fresh cassava processed. According to Acchar and Da Silva, (2021), considering the concentration of organic load and the toxic effect of this agro-industrial residue, it is possible to infer the high source of pollution that it represents. It is worth noting that the toxicity of cassava residues related to cyanide can generate disorders with acute or chronic symptoms in humans and animals.

In this sense, developing ecologically correct, simple, and low-cost methods for the proper treatment or reuse of cassava is a challenge. From another perspective, Nitschke and Pastore (2006) reported that cassava wastewater stands out as an agro-industrial waste. In its composition, high levels of carbon and essential nutrients have been identified that benefit surfactin production. In their study Nitschke and Pastore, (2004) reported the use of cassava wastewater for the production of surfactin, using two different strains of *B. subtilis*. Resulted, ≈3.0 g of crude surfactin/L was produced. Similarly Andrade et al., (2016) optimized surfactin production by *B. subtilis* LB5a using cassava wastewater as an alternative substrate. The study achieved production of ≈1.01 $g.L^{-1}$ of pure surfactin.

Despite the complexity in the use of agro-industrial residues such as the standardization of the substrate due to the natural variation of the composition, costs of storage, transport, and purification, and/or previous residual treatment, among others, it is possible to establish controlled processes for the production of surfactin with pre-existing chemical characteristics - established, and enhance their products with the use of hydrophobic inducers (DE OLIVEIRA SCHMIDT et al., 2021a). Biochemically, the productivity of biosurfactants can be easily increased by adding inducers to the culture medium, which stimulate microbial growth and also trigger the metabolism

to produce this biosurfactant (PATHANIA; JANA, 2020a). The inducers are related to the size of hydrophobic chains of biosurfactants and, consequently, greater stability and efficiency. Biosurfactant inducers are mainly composed of hydrophobic molecules (e.g. soybean oil and olive oil - a pool of saturated and unsaturated fatty acids, proteins, and vitamins) (DE OLIVEIRA SCHMIDT et al., 2021a).

However, there is little information on the effect of these specific molecules (e.g. oleic acid and palmitic acid) on surfactin's production and chemical structure. In this work, soybean oil (SO) and palmitic (PA) and oleic (OA) fatty acids were evaluated as hydrophobic inducers in the production of surfactin by *Bacillus subtilis* ATCC 6633. Their inductive effect was evaluated by supplementation these fatty acids in different concentrations in a culture medium containing only cassava wastewater. To the best of our knowledge, this report is the first study that details surfactin production using cassava wastewater associated with hydrophobic inducers.

1.1 OBJECTIVES

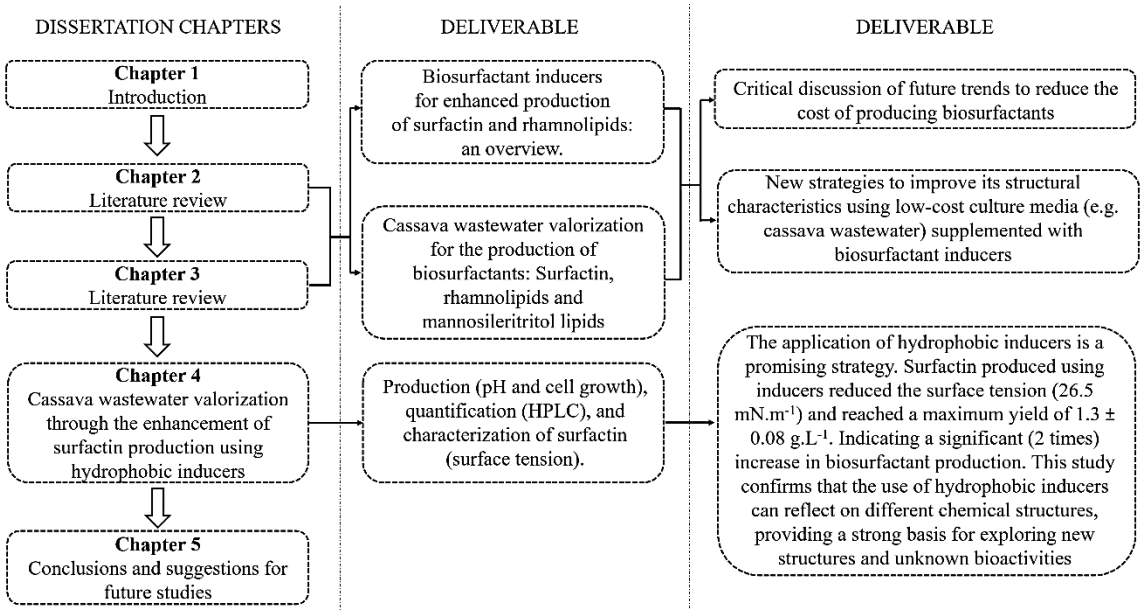
1.1.1 General Objective

Evaluate the biosurfactants production using cassava wastewater as an alternative substrate, proposing new strategies for enhanced surfactin production.

1.1.2 Specific Objectives

- Produce surfactin from *Bacillus subtilis* ATCC 6633 strain using cassava as an alternative substrate associated with hydrophobic inducers;
- Evaluate the influence of palmitic and oleic acids as hydrophobic inducers at different concentrations in the production of surfactin;
- Carry out a study of the potential of these inducers in altering the surface properties, yield and chemical structure of surfactin.

1.1.3 Conceptual diagram



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CHAPTER 2

In this section, the review article “Biosurfactant inducers for enhanced production of surfactin and rhamnolipids: an overview” is presented. This review aims to critically discuss the current state of the art and future trends on biosurfactant production, particularly biosurfactant inducers, including yield, chemical structure, production scale, and modes of operation. This review article is linked to the journal “World Journal of Microbiology and Biotechnology" published in January 2021 (doi: 10.1007/s11274-020-02970-8).

2 BIOSURFACTANT INDUCERS FOR ENHANCED PRODUCTION OF SURFACTIN AND RHAMNOLIPIDS: AN OVERVIEW

Abstract

Biosurfactants can be widely used in industries as pharmaceutical agents, for microbial enhanced oil recovery, crop biostimulation, among others. Surfactin and rhamnolipids are well-known biosurfactants. These compounds have several advantages over chemical surfactants, however they are not economically competitive, since their production cost is up to 12 times higher than chemical surfactants. In this sense, an interesting approach is to replace synthetic culture medium, which represents ≈30% of the production cost by agro-industrial wastes. In addition, biosurfactant productivity can be easily enhanced by inductor supplementation into culture medium that triggers biosurfactant metabolism. Biosurfactant inducers are mainly a pool of hydrophobic molecules (e.g. olive oil - saturated and unsaturated fatty acids, proteins and vitamins). Nevertheless, there is little information on inducer effects of specific molecules (e.g. oleic acid). In general, hydrophobic inducers lead to higher fatty acid chain lengths (biosurfactant chemical structure). Therefore, the aim of this review was to critically discuss the current state of the art and future trends on biosurfactant production, in particular biosurfactant inducers. Taking into account the last 10 years, there is a clear lack of information on correlation between "inducers" or "hydrophobic inducers" AND "biosurfactants", since only 13 documents were found (Scopus database). Thus, it is essential to deeply investigate all inducer effects on biosurfactant production, mainly yield and chemical structure.

Keywords: biopesticides; biosurfactant inducers; glycolipids; lipopeptides; microbial enhanced oil recovery.

2.1 INTRODUCTION

Synthetic surfactants, derived from petroleum sources - a finite and non-renewable resource - can impact the environment in negative ways (OLKOWSKA; RUMAN; POLKOWSKA, 2014). Therefore, it is fundamental to develop alternatives - environmentally and economically feasible - such as biosurfactants. Biosurfactants are amphipathic compounds produced by fungi, yeasts, plants and mostly by bacteria. These molecules have a wide range of applications as emulsifier, antimicrobial, surface-active agents, among others. Biosurfactants have advantages over synthetic surfactants such as higher biodegradability, similar surface tension reduction, lower toxicity, greater thermal and pH stability (ANDRADE et al., 2016b; CAVALCANTE FAI et al., 2015; EHRHARDT; SECATO; TAMBOURGI, 2015; ZANOTTO et al., 2019).

Nevertheless, biosurfactants are not widely commercially viable (ANDRADE et al., 2016). Rhamnolipid production cost is ≈US$ 20.kg^{-1} at 20 m^3, whereas at higher scale 100 m^3 the production cost is ≈US$ 5.kg^{-1} (SANTOS et al., 2016). In 2018, according to Global Market Insights, Inc., 476 thousand tons of biosurfactants were produced ≈US$ 2.21 billion dollars. In addition, in 2023 it is expected that 524 thousand tons of biosurfactants will be produced ≈US$ 2.7 billion dollars. In this sense, an interesting strategy to reduce the production cost of these important compounds is by using low-cost culture media (e.g. agro-industrial waste) supplemented with biosurfactant inducers.

Biosurfactant inducers can be classified into two main groups: hydrophilic and hydrophobic. Hydrophilic inducers (e.g. metals and glycerol) act as cell growth cofactors; whereas, hydrophobic inducers (e.g. vegetable oils) act as secondary carbon source (beta-oxidation) and also trigger the production of biosurfactant (enhanced solubilization of hydrophobic carbon sources). According to Gudiña et al. (2016) alternative culture media supplemented with hydrophobic inducers reach higher biosurfactant production, for instance 42% (flask) and 129% (reactor) - Table 1. It is worth noting that the inducers can also affect the chemical structure of biosurfactants, and thereafter changing their properties (e.g. decreasing the Critical Micelle Concentration - CMC) (BONMATIN et al., 1995; BUENO, 2014; EHRHARDT; SECATO; TAMBOURGI, 2015; HSIEH et al., 2004; INÈS; DHOUHA, 2015).

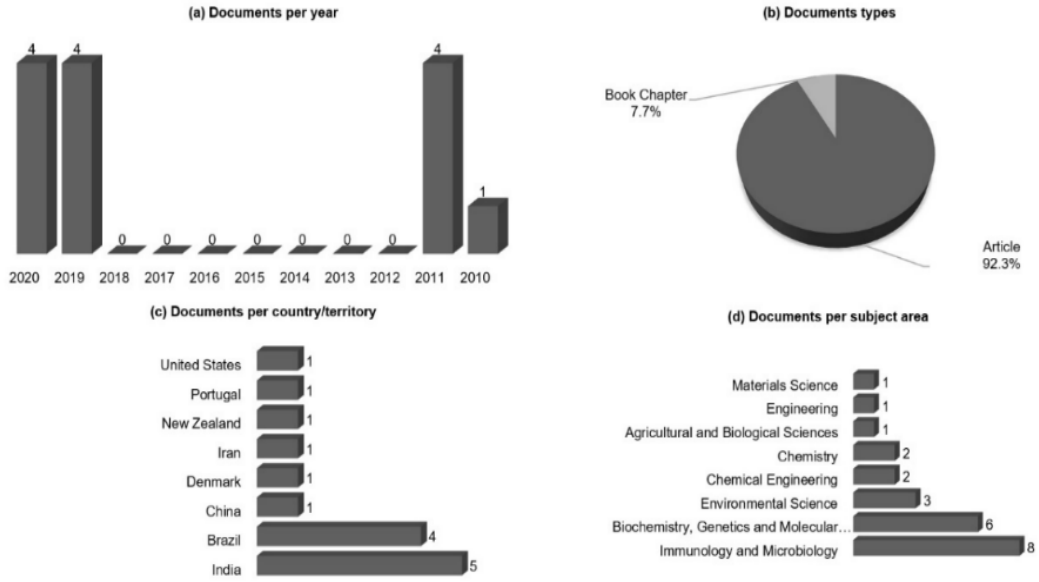
Based on these aspects, the aim of this review is to critically discuss the current state of the art and future trends on biosurfactant production, in particular biosurfactant inducers including yield, chemical structure, production scale, modes of operation, among others. In spite of the relevance on this subject, based on Scopus database from 2010 to 2020, the scientific trends on correlation between “inducers” or “hydrophobic inducers” AND “biosurfactants” indicated a clear lack of information, since only 13 documents were found - 12 (92.3%) are research articles and 1 (7.7%) book chapter (Fig. 1).

Table 1 - Surface tension (ST), emulsification index (EI), biosurfactant production (BP) and critical micelle concentrations (CMC), obtained from *Pseudomonas aeruginosa*, adapted from Gudiña et al. (2016)

*Medium	Experimental scale	Fermentation time (h)	ST ($mN.m^{-1}$)	EI (%)	BP ($g.L^{-1}$)	CMC ($mg.L^{-1}$)
No inducer	Flask	144	31.4 ± 0.1	59.0 ± 1.5	3.1 ± 0.2	50
	Reactor	96	29.0 ± 0.2	54.2 ± 2.0	2.2 ± 0.1	30
Supplemented with inducer	Flask	168	31.0 ± 0.1	64 ± 0.5	4.5 ± 0.1	14
	Reactor	168	29.2 ± 0.2	58.4 ± 1.0	5.1 ± 0.1	13

*culture medium composition: corn steep liquor (10% $v.v^{-1}$), sugarcane molasses (10% $w.v^{-1}$), and oil mil wasterwater (25% $v.v^{-1}$) as inducer.

Figure 1 - Documents per year (a), documents types (b), documents per country/territory (c) and documents per subject area (d)



Source: from the author.

2.2 BIOSURFACTANTS

Biosurfactants are remarkable surface-active agents, that is, surface and interfacial tension reducers (OLIVEIRA et al., 2015). Biosurfactants are complex chemical molecules that can be classified according to their microbial origin and/or their chemical structure. Usually, these compounds can be divided into five groups: (I) lipopeptides and lipoproteins, (II) glycolipids,

(III) fatty acids, neutral lipids and phospholipids, (IV) polymeric surfactant and (V) particles (ANDRADE et al., 2016b; ARAÚJO et al., 2012; SARWAR et al., 2017).

In general, lower molecular weight biosurfactants are effective surface and interfacial tension reducers, whereas higher molecular weight biosurfactants are powerful emulsifiers (oil in water systems) (DRAKONTIS; AMIN, 2020). Currently, two classes of low molecular weight biosurfactants are produced at industrial scale: glycolipids and lipopeptides (GEYS; SOETAERT; VAN BOGAERT, 2014). However, the high production cost restricts the massive biosurfactant applications (CHEN; JUANG; WEI, 2015). Thus, it is fundamental to enhance the biosurfactant productivity, in particular by using biosurfactant inducers.

Regarding culture media for biosurfactant production (Table 2), synthetic culture media are most commonly used. In this sense, sometimes the composition of synthetic culture media includes complex nutrients (e.g. yeast extract and peptone). These complex substances act metabolically as organic nitrogen sources, and also as carbon and mineral sources (COOPER et al., 1981; GRANT; PRAMER, 1962). Thus, the composition of culture medium must be carefully evaluated.

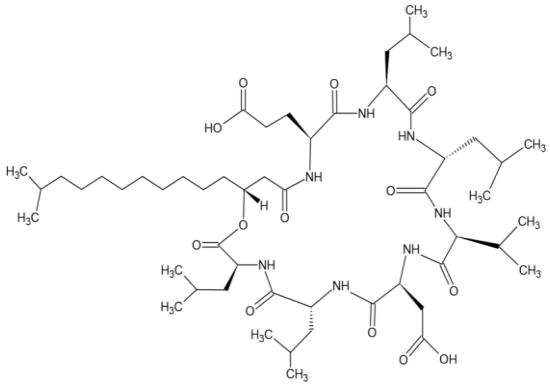
2.2.1 Lipopeptides

Lipopeptides are chemically composed of lipophilic fatty acid(s) bonded to a peptide ring. Lipopeptides are essentially produced by *Bacillus* sp. They can be sub-classified into three main classes: surfactin, iturin and fengycin, according to the number of amino acids or amino acid sequence. Surfactin is the most well-known sub-class (YOUSSEF; DUNCAN; MCINERNEY, 2005). However, the amino acid sequence can change due to the composition of culture medium composition, production scale, modes of operation, biosurfactant producing strain, among others - as briefly described below (ANDRADE et al., 2016b; HSIEH et al., 2004; INÈS; DHOUHA, 2015).

2.2.1.1 Surfactin

Surfactin (Fig. 2) is composed of a heptapeptide ring bonded to a β-hydroxy fatty acid chain (C_{12}-C_{16}). The amino acid sequence varies, in which the most common is Glu – Leu – Leu – Val – Asp – Leu – Leu. Surfactin can be also classified into A, B or C according to the 7^{th} amino acid Leu, Val or Ile, respectively (ANDRADE et al., 2017; MULLIGAN, 2005).

Figure 2 - Generic structure of surfactin.



Source: adapted from Drakontis and Amin (2020).

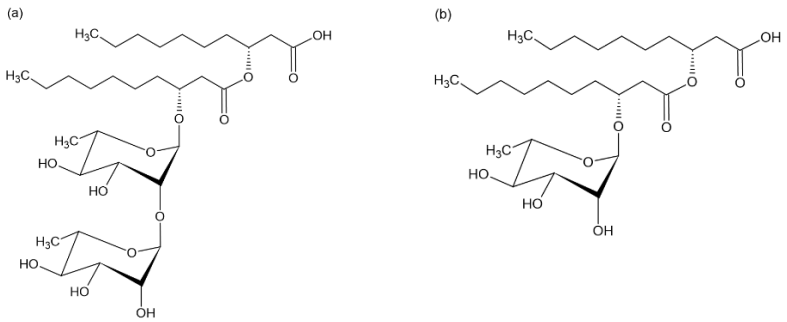
2.2.2 Glycolipids

Glycolipids are the most prominent biosurfactants. They are composed of carbohydrate(s) bounded to aliphatic or hydroxy-aliphatic acids. The most well-known sub-classes of glycolipids are rhamnolipids and sophorolipids (DE VASCONCELOS et al., 2021; DRAKONTIS; AMIN, 2020).

2.2.2.1 Rhamnolipids

Rhamnolipids (Fig. 3) are composed of β-hydroxy fatty acids bounded to rhamnose residues. Mono-rhamnose has one only rhamnose residue and di-rhamnose has two residues. They are mainly produced by *Pseudomonas aeruginosa* (DE VASCONCELOS et al., 2021; DRAKONTIS; AMIN, 2020).

Figure 3 - Structures of a di-rhamnolipid (a) and mono-rhamnolipid (b).



Source: adapted from Drakontis and Amin (2020).

Table 2 - Main classes of biosurfactants and strain producers

Microorganism	Biosurfactant	Primary carbon source	Inducer	Biosurfactant concentration ($g.L^{-1}$)	Fermentation time (h)	Biomass ($g.L^{-1}$)	Surface tension ($mN.m^{-1}$)	References
Pseudomonas aeruginosa	Rhamnolipids	Glucose (2% $w.v^{-1}$)	Soybean oil fried (2% $v.v^{-1}$)	4.20	96	1.48	30	Pathania; Jana (2020)
Agrobacterium fabrum	Lipopeptide	*YE and glucose	Crude oil	5.77	144	5.77	34.5	Sharma et al., (2019)
Pseudomonas aphidis	Mannosylerythritol lipids	*YE, malt extract, peptone, and glucose.	Waste cooking oil and soybean oil	61.50 and 51.26	240	19 and 15	32.83 and 30.63	Niu et al., (2019)
Aureobasidium thailandense LB01	not specified	*YE (2 $g.L^{-1}$) and glucose (6 $g.L^{-1}$)	Olive oil (1.5% $w.w^{-1}$)	0.14	48	not specified	33	Meneses et al., (2017)
P. aeruginosa	Rhamnolipids	Corn steep liquor (10% $v.v^{-1}$) and sugarcane molasses (10% $v.v^{-1}$)	Olive oil mill wastewater (25% $v.v^{-1}$)	4.51	168	3.93	31	Gudiña et al., (2016)
P. aeruginosa and *Bacillus pumilus*	not specified	*YE (10 $g.L^{-1}$)	Soybean oils (1% $v.v^{-1}$)	not specified	120	not specified	34 and 39	Decesaro et al., (2013)
Bacillus subtilis MTCC2423	Surfactin	*YE and peptone	Fried sunflower oil (50 $g.L^{-1}$) and fried rice bran oil residue (50 $g.L^{-1}$)	0.65 and 0.45	120	3.67 and 4.67	31.9 and 34.5	Vedaraman; Venkatesh (2011)
B. subtilis	Surfactin	Glucose (4% $w.v^{-1}$)	Crude oil (2% $v.v^{-1}$)	not specified	27	not specified	25.9	Queiroga; Nascimento; Serra (2003)
Candida bombicola	Sophorolipids	*YE (1 $g.L^{-1}$) and glucose (100 $g.L^{-1}$)	Sunflower oil (100 $g.L^{-1}$)	50	180	6	not specified	Casas; García-Ochoa (1999)

*synthetic culture medium; YE - Yeast extract

2.3 POTENTIAL APPLICATIONS

The specific chemical bonds and hydrophilic-lipophilic balance of biosurfactants are correlated to their unique properties, and thereafter their applications (DESAI; BANAT, 1997; JAGTAP et al., 2010). Thus, there is a wide range of biosurfactant applications (e.g. pharmaceutical, agricultural and environmental) (JIMOH; LIN, 2019), as shown in Table 3. The main biotechnological applications of surfactin and rhamnolipids are briefly described below.

2.3.1 Agriculture

In agriculture, biosurfactants can act as exogenous elicitors, that is, stimulating the defense response in plants and/or plant growth (sprouting and rooting) (DE VASCONCELOS et al., 2021; YAMAMOTO; SHIRAISHI; SUZUKI, 2015). In this sense, Yamamoto et al. (2015) proved that surfactin and iturin triggered the systemic resistance against *Colletotrichum gloeosporioides* (anthracnose) in strawberry (elicitor effect). When compared to the control (digested soy casein), foliar gene expression of chitinase and β-1,3-glucanase increased significantly (≥ 500%) after application of surfactin and iturin.

Similarly, Le Mire et al. (2018) applied surfactin and elicitor Bion®50WG (chemical reference) in wheat. Both surfactin and Bion®50WG triggered the systemic resistance against *Zymoseptoria tritici*, since they protected wheat by 70%. It is worth noting that *in vitro* biocidal assays revealed no antifungal activities of surfactin against the pathogen *Z. tritici*. Hence, surfactin does not act as fungicidal (*Z. tritici*), nevertheless it induced significant systemic resistance in plants, stimulating signaling pathways dependent on salicylic acid and jasmonic acid. Biosurfactants are the most promising sustainable alternative to pesticides. Thus, ideally, biosurfactants act as biopesticides and biostimulants.

2.3.2 Environmental and petroleum

Currently, the biosurfactants are strongly related to the petroleum industry in applications such as bioremediation, in cleaning tanks and microbial enhanced oil recovery (DECESARO et al., 2015; FARIA, 2010; SANTOS et al., 2016).

Lai et al. (2009) compa red the bioremediation rates of total petroleum hydrocarbon by using two biosurfactants (rhamnolipids and surfactin) and two synthetic surfactants (Tween 80 and Triton X-100). The results showed higher efficiency of the biosurfactants in the removal of total petroleum hydrocarbon. The removal of total petroleum hydrocarbon from the soil

contaminated with ca. 3,000 mg.kg^{-1} were 23, 14, 6 and 4%, using rhamnolipids, surfactin, Tween 80 and Triton X-100, respectively.

The same trend was observed for more contaminated soil ca. 9,000 mg.kg^{-1} 63, 62, 40 and 35%, respectively. Recently Zhao et al. (2020) investigated the removal of oil present in the sludge using rhamnolipids. The sludge (containing 22.91% oil) was treated with rhamnolipids solution at 200 mg.L^{-1}. The authors reached ≈34% removal rate.

Based on the potential applications, it is essential to enhance the biosurfactant production in order to develop economically and environmentally viable technology for bioremediation.

2.3.3 Pharmaceuticals and medicine

The potential application of biosurfactant as pharmaceutical agents is notable - for example, targeted drug interactions with specific cells and tissues. Regarding lipopeptides and glycolipids, these applications include immunomodulatory activities, carriers of antitumor drugs, the binding system for G and M immunoglobulins (mediators of the human humoral immune response), induction of ion channel formation in lipid bilayers, transfection studies and gene therapy (COELHO et al., 2020; SEYDLOVÁ; SVOBODOVÁ, 2008; WANG et al., 2019). Wang et al. (2019) developed a system of "mosaic type" nanoparticles using surfactin for selective release of drugs directly to the hypoxic cancer cells. Surfactin increased the therapeutic efficacy by 70%.

Similarly, Abdelli et al. (2019) demonstrated that the lipopeptide produced by *Bacillus safensis* F4 exhibited polyvalent activity (antibiofilm and anti-tumor properties).

Guo et al. (2021) showed that minimum inhibitory concentration of surfactin against antiplankton is ≈6.25 mg.mL^{-1}, whereas antibiofilm rate reached 80% against *Staphylococcus epidermidis* S61, an opportunistic pathogen responsible primarily for nosocomial infections (e.g. associated with the use of indwelling or implanted foreign bodies). In addition, the lipopeptide presented antitumor activity against T47D breast cancer cells and B16F10 mouse melanoma cells with the half-maximal inhibitory concentration (IC_{50}) of 0.66 mg.mL^{-1} and 1.17 mg.mL^{-1}, respectively. The antitumor activity of surfactin is due to the cell cycle arrest in phase G1 due to inhibition of DNA synthesis, which negatively influences the proliferation of cancer cells (DUARTE et al., 2014). Magalhães et al. (2014) demonstrated the synergistic effect between rhamnolipids and nisin against *Listeria monocytogenes* - a widespread foodborne intracellular pathogen. Therefore, biosurfactants are versatile molecules that have remarkable industrial applications, including pharmaceutical agents (Table 3). Nevertheless, the high degree of purity required is the main drawback.

Table 3 - Biosurfactant applications

Industry	Application	Biosurfactant properties	References
Pharmaceutical/ Medicine	Microbiological Pharmaceuticals and therapeutics	Carriers of antitumor drugs, anti-adhesive, antibacterial, antifungal and antiviral agents, gene therapy, immunomodulatory molecules, transfection studies and gene therapy in addition to cell stimulation and differentiation.	Abdelli et al. (2019); Coelho et al. (2020); Wang et al. (2019)
Agriculture	Biocontrol, fertilizers Exogenous elicitors of molecules	Emulsification, dispersion, suspension of pesticides and fertilizers, facilitation of microbial biocontrol mechanisms, elimination of plant pathogens and defense response in plants.	De Vasconcelos et al., (2021); Le Mire et al., (2018); Yamamoto et al. (2015)
Environment/ Petroleum	Bioremediation Oil spill cleanup operations Enhanced oil recovery de-emulsification	Emulsification, solubilization and dispersion of oils, lowering of interfacial tension, wetting of solid surfaces, spreading, detergency, foaming, corrosion inhibition in fuel oils and equipment, soil flushing, viscosity reduction	Decesaro et al. (2015); Santos et al. (2016); Zhao et al. (2020)
Food	Emulsification and de-emulsification Functional ingredients Microbial inhibition	Solubilization of flavored oils, control of consistency, emulsification, wetting agent, spreading, detergency, foaming and thickener.	Campos et al. (2013); Ribeiro et al. (2020)
Mining	Heavy metal cleanup operations Soil remediation Flotation	Wetting and foaming, collectors and frothers, removal of metal ions from aqueous solutions, soil and sediments, heavy metals sequestrants, spreading, corrosion inhibition in oils.	Campos et al. (2013); Sarubbo et al. (2015)
Textiles	Preparation of fibers Finishing of textiles	Wetting, penetration, solubilization, emulsification, detergency and dispersion, wetting and emulsification in finishing formulations, softening.	Banat et al., (2010); Helmy; Kardena (2011)
Cosmetics	Health and beauty products	Emulsification, foaming agents, solubilization, wetting agents, cleansers, antimicrobial agents, mediators of enzyme action.	Vijayakumar; Saravanan (2015)

2.4 ALTERNATIVE CULTURE MEDIA FOR BIOSURFACTANT PRODUCTION

Biosurfactants have a wide range of chemical structures, even within sub-classes (homologues), for instance, the amino acid sequences of surfactin and also its fatty-acid chain length. The production of specific homologues is correlated to culture medium used, in particular carbon source, biosurfactant strain producer and modes of operation, among others. Undoubtedly, these homologues have unique biological and physical-chemical properties (SANTOS et al., 2016; WADDINGTON et al., 2010).

De Araujo et al. (2013) emphasized the complexity of agro-industrial wastes as alternative culture media such as standardization, seasonality, storage, among others. However, they claim that it is technically feasible to control the biosurfactant production.

In this context, Fox and Bala (2000) used starch-rich wastes from potato processing industries as alternative culture medium for biosurfactant production by *Bacillus subtilis* 21332. The analysis of results showed significant surface tension reduction from 71.3 to 28.3 $mN.m^{-1}$ and critical micelle concentration ≈0.10 $mg.L^{-1}$.

Paraszkiewicz et al. (2018) reported the potential of agro-industrial residues as alternative culture medium for biosurfactant production by two *Bacillus subtilis* strains (KP7 and I'-1a): brewers' spent grain, beet molasses, apple peel extract and carrot peel extract supplemented with 0.25% yeast extract or 0.25% peptone). Luria Bertani and Cooper (synthetic media) were used as control. The biological process using carrot peel culture medium and *B. subtilis* KP7 reached 1.09 $mg.L^{-1}h^{-1}$, which was similar to Luria Bertani 1.04 $mg.L^{-1}h^{-1}$. The authors concluded that the concentration of production and rate of specific biosurfactant homologues were highly correlated to medium composition.

Thus, it is evident that agro-industrial residues provide all essential nutrients to microbial biosurfactant producers - Table 4 (ANDRADE et al., 2016b; SARUBBO et al., 2015). The production of biosurfactant can be carried out by hydrophilic carbon sources such as glucose and sucrose. Nevertheless, the productivity of biosurfactants can be significantly enhanced supplementing the culture medium with hydrophilic carbon sources - biosurfactant inducers (HOMMEL et al., 1994).

Table 4 - Alternative culture medio for the biosurfactant production

Biosurfactant	Carbon source	Biosurfactant producer	Application	Experimental conditions	Biosurfactant concentration	Reference
Glycolipid not specified	Waste soybean oil 1.0% (w.v^{-1}) and corn steep liquor 1.0% (w.v^{-1})	*Saccharomyces cerevisiae* URM 6670	Food industry	Laboratorial scale using conical flasks (volume not informed), with 2% (v.v^{-1}) of inoculum; at 28 °C, pH 6.8; 150 rpm during 120 h.	5.84 g.L^{-1}	Ribeiro et al., (2019)
Lipopeptide not specified	Soybean waste frying (10 g.L^{-1}) oil and corn steep liquor (20 g.L^{-1})	*Streptomyces* sp. DPUA 1566	Bioremediation and antimicro bial	Laboratorial scale using conical flasks containing 70 mL of culture medium, with 1% (v.v^{-1}) of inoculum; at 28 °C, pH 8.5; 150 rpm during 144 h.	1.9 g.L^{-1}	Dos Santos et al., (2019)
Surfactin	Hemicellulosic corncob liquor	*Bacillus subtilis*	Bioremediation	Laboratorial scale using conical flasks containing 100 mL of culture medium, with 5% (v.v^{-1}) of inoculum; at 30 °C, pH 6.8; 120 rpm during 72 h.	3.95 g.L^{-1}	Prado et al., (2019)
Surfactin	Light-paraffin oil 3.8% (v.v^{-1})	*Bacillus subtilis* MG 495086	Enhanced oil recovery	Laboratorial scale using conical flasks (volume not informed), with 1% (v.v^{-1}) of inoculum; at 37 °C, pH 7.7; 180 rpm during 72 h.	6.3 g.L^{-1}	Datta et al., (2018)
Surfactin	Brewery wastewater, Beet molasses 2% (v.v^{-1}), Apple peel* and Carrot peel*	*Bacillus subtilis* KP7 *Bacillus subtilis* I'-1a	Bioremediation	Laboratorial scale using conical flasks containing 150 mL of culture medium, with 2% (v.v^{-1}) of inoculum; at 28 °C, pH 7; 120 rpm during 96 h.	0.099 g.L^{-1} 0.077 g.L^{-1} 0.095 g.L^{-1} 0.123 g.L^{-1}	Paraszkiewicz et al., (2018)
Mannosylerythritol lipids	Cassava wastewater	*Pseudozyma tsukubaensis*	Bioremediation	Bioreactor (Bioflo & Celligen 310 - 3.0 L working volume), with 7% (v.v^{-1}) of inoculum, at 30°C, 100 rpm and aeration rate of 0.4 vvm were kept in the firsts 24 h then 150 rpm and 0.8 vvm from 24 to 84 h.	1.26 g.L^{-1}	Andrade et al., (2017)
Rhamnolipids	Crude glycerol 1% (v.v^{-1})	*Pseudomonas aeruginosa*	Bioremediation	Laboratorial scale using conical flasks containing 100 mL of culture medium, with 1% (v.v^{-1}) of inoculum; at 37 °C, pH 6; 180 rpm during 120 h.	2.160 g.L^{-1}	Waala et al. (2016)
Surfactin	Crude glycerol 2% (v/v)	*Bacillus subtilis* ATCC 6633	Bioremediation and antimicrobial	Laboratorial scale using conical flasks containing 50 mL of culture medium	0.158 g.L^{-1}	De Sousa et al., (2014)

2.5 BIOSURFACTANT INDUCERS

In general, inducers are an efficient strategy to enhance yields of biotechnological processes. Usually, biosurfactant inducers are defined as supplementary carbon sources, that is, they are at low concentration (HANSON; DESAI; DESAI, 1993; NURFARAHIN; MOHAMED; PHANG, 2018). In this sense, Vasilyeva and Strijakova, (2007) reported that biphenyl was an efficient biosurfactant inducer. Thus, soils and sediments contaminated with polychlorinated biphenyls could be bioremediated by inoculating microbial biosurfactant producers. Similarly, Salam and Das (2013) detailed that biosurfactant production by *Rhodotorula* sp. VITJzN03 (yeast) was enhanced, when olive oil was added into culture medium. Bonmatin et al. (1995) used *Bacillus subtilis* and basal medium supplemented with 5 $g.L^{-1}$ leucine and 5 $g.L^{-1}$ L-isoleucine. Interestingly, L-valine in the 4^{th} position (amino acid sequence) was replaced by leucine or L-isoleucine.

The biosurfactant inducers can be hydrophobic molecules (e.g. vegetable oils and hydrocarbons derived from petroleum) and also hydrophilic ones (e.g. metals) (BUENO, 2014; EHRHARDT; SECATO; TAMBOURGI, 2015; FONTES; AMARAL; COELHO, 2008; SANTOS et al., 2016; WEI; CHU, 2002)

Recently, a study conducted by Zhao et al., (2020) compared two culture media (hydrophilic and hydrophobic) for rhamnolipid production by *Pseudomonas aeruginosa*. The authors reached 7.06 and 10.32 $g.L^{-1}$ of rhamnolipids using glucose and soy oil medium, respectively. In addition, rhamnolipids produced from glucose showed higher surface activity 26.3 $mN.m^{-1}$ and 50 $mg.L^{-1}$ CMC, whereas rhamnolipids produced from soy oil 28.1 $mN.m^{-1}$ and 60 $mg.L^{-1}$ CMC. On the other hand, rhamnolipids produced from soy oil presented higher emulsifying activity index of petroleum (76.1%), when compared to rhamnolipids produced from glucose (65.5%). These results, very likely, are correlated to homologues rates of rhamnolipids.

2.5.1 Hydrophilic inducers

The biosurfactant production is strictly regulated by interaction among microbial producer, nutrients and modes of operation. It is important to note that hydrophilic inducers are not primary carbon sources, hydrophilic inducers are co-factors molecules that assist cell growth and thereafter, biosurfactants synthesis.

Wei and Chu (2002) added metallic salts into culture medium ($CuSO_4$, $MnSO_4$, $MgSO_4$, $CoSO_4$ and $NiSO_4$). Mn^{2+} (0.01 mM) enhanced the surfactin production from 0.33 to 2.6 $g.L^{-1}$. Subsequently, the same research group used iron to induce *Bacillus subtilis* growth for the surfactin production. The biosurfactant production was carried out using mineral salt medium

containing glucose (40 $g.L^{-1}$) as the main carbon source and $FeSO_4.7H_2O$ (varying 0, 2, 5 and 10 mM). It was found that iron is a hydrophilic inducer (WEI et al., 2003).

Recently, Ortega Ramirez et al., (2020) reported the rhamnolipid production by *Burkholderia* sp. C3 using glycerol as hydrophilic inducer and dibenzothiophene as main carbon source. It was observed a significant increasing of rhamnolipid production ≈250% by using both glycerol and dibenzothiophene.

Gudiña et al., (2015) described the surfactin production by *B. subtilis* using corn steep liquor as alternative culture medium supplemented with hydrophilic inducers (Mg^{2+}, Mn^{2+} and Fe^{2+}). The supplementation with three hydrophilic inducers presented the highest biosurfactant production 4.829 ± 0.193 $g.L^{-1}$ (3.6 times), when compared to control.

Table 5 - Hydrophilic surfactin inducers

Inducers	**Concentration (mM)**	**Surface tension ($mN.m^{-1}$)**	**Biosurfactant production ($g.L^{-1}$)**	**Fermentation time (h)**
Control	-	59.8 ± 1.8	11.311 ± 0.109	48
$FeSO_4$	2.0	46.8 ± 1.0	4.170 ± 0.054	72
$MnSO_4$	0.2	46.0 ± 0.9	4.467 ± 0.158	72
$MgSO_4$	0.8	48.7 ± 1.2	3.519 ± 0.102	72
$FeSO_4/MgSO_4$	2.0/0.8	51.6 ± 2.0	2.704 ± 0.132	24
$FeSO_4/MnSO_4$	2.0/0.2	47.4 ±1.9	3.933 ± 0.205	24
$MgSO_4/MnSO_4$	0.8/0.2	46.6 ± 1.3	4.224 ± 0.157	48
$FeSO_4/MgSO_4/MnSO_4$	2.0/0.8/0.2	45.1 ± 1.9	4.829 ± 0.193	72

Wei et al., (2007) enhanced the surfactin production by *B. subtilis* ATCC 21332 using the MMS medium (40 $g.L^{-1}$ glucose, 50 mM NH_4NO_3, 30 mM KH_2PO_4, 40 mM Na_2HPO_4, 7 mM $CaCl_2$, 4 mM sodium EDTA, 800 mM $MgSO_4.7H_2O$, 0.3 mM $FeSO_4.7H_2O$ and 0.2 mM $MnSO_4.H_2O$) supplemented individually with Mg^{2+}, K^+, Mn^{2+} and Fe^{2+}. The highest surfactin production 2.23 ± 0.10 $g.L^{-1}$ was reached with Mg^{2+} (0.6 mM) ion. Interesting, when 0.6 mM Mg^{2+}, 10 mM K^+, 0.1 mM Mn^{2+}, 1.2 mM Fe^{2+} were used simultaneously, a significant reduction on biosurfactant production was observed (1.30 ± 0.10 $g.L^{-1}$).

Hydrophilic inducers can easily enhance the biosurfactant production. However, further investigation must elucidate their correlation to biosurfactant metabolic pathways.

2.5.2 Hydrophobic inducers

Hydrophobic inducers (e.g. hydrocarbons derived from petroleum and vegetable oils) act as secondary carbon source for microbial growth and also assist the synthesis fatty acid moiety of

biosurfactants (DE ARAUJO; FREIRE; NITSCHKE, 2013; NIU et al., 2019; PATHANIA; JANA, 2020a). Thus, hydrophobic inducers affect directly the production rate of biosurfactant homologues, as shown in Table 2.

Decesaro et al., (2013) described the biosurfactant production by *Pseudomonas aeruginosa* and *Bacillus pumilus* using mineral medium supplemented with diesel and soybean oils as hydrophobic inducers at 1% and 2%. For both biosurfactant producers, the lowest surface tension was obtained with soybean oil at 1%. It is worth noting that soybean oil is easier metabolized by microbial cells, when compared to diesel oil (SATPUTE et al., 2010).

Regarding specific hydrophobic inducers, the effect of complex hydrophobic sources in yeasts was described by Kitamoto et al., (2001).They observed that culture medium containing octadecane led to highest cellular growth and biosurfactant production.

Hence, it is essential to correlate the fatty acid profile of hydrophobic inducer to biosurfactant production (BONMATIN et al., 1995; NIU et al., 2019). The fatty acid profile of vegetable oils that are used as hydrophobic inducers is illustrated in Table 6, most of them are polyunsaturated fatty acids, for example, 71.6% (C18:1) in olive oil and 56.5% (C18:2) in sunflower oil (BACKES et al., 2017; DHIFI et al., 2015).

Table 6 - Fatty acids profile (% $g.g^{-1}$) of hydrophobic biosurfactant inducers

Inducers	***C16:0**	***C16:1**	***C18:0**	***C18:1**	***C18:2**	***C18:3**	**References**
Olive oil	13.8	1.4	2.8	71.6	9.0	1.0	Dhifi et al., (2015)
Soy oil	11.1	0.09	3.25	23.28	54.58	6.66	Souza et al., (2019)
Corn oil	11.6	0.0	2.5	38.7	44.7	1.4	Dhifi et al., (2014)
Sunflower oil	5.2	0.1	3.7	33.7	56.5	0.0	Dhifi et al., (2014)
Canola oil	3.8	0.16	1.80	51.41	32.91	4.69	Backes et al., (2017)

*Cx:y, where x = carbons and y = unsaturation.

It should also take into account that hydrophobic inducers at high concentrations may lead to low oxygen transfer (DUSANE et al., 2010). Therefore, it is evident that an interesting alternative to reduce the production cost of biosurfactant is to use alternative culture medium supplemented with inducers. However, they should be carefully evaluated (type, purity, concentration, among others).

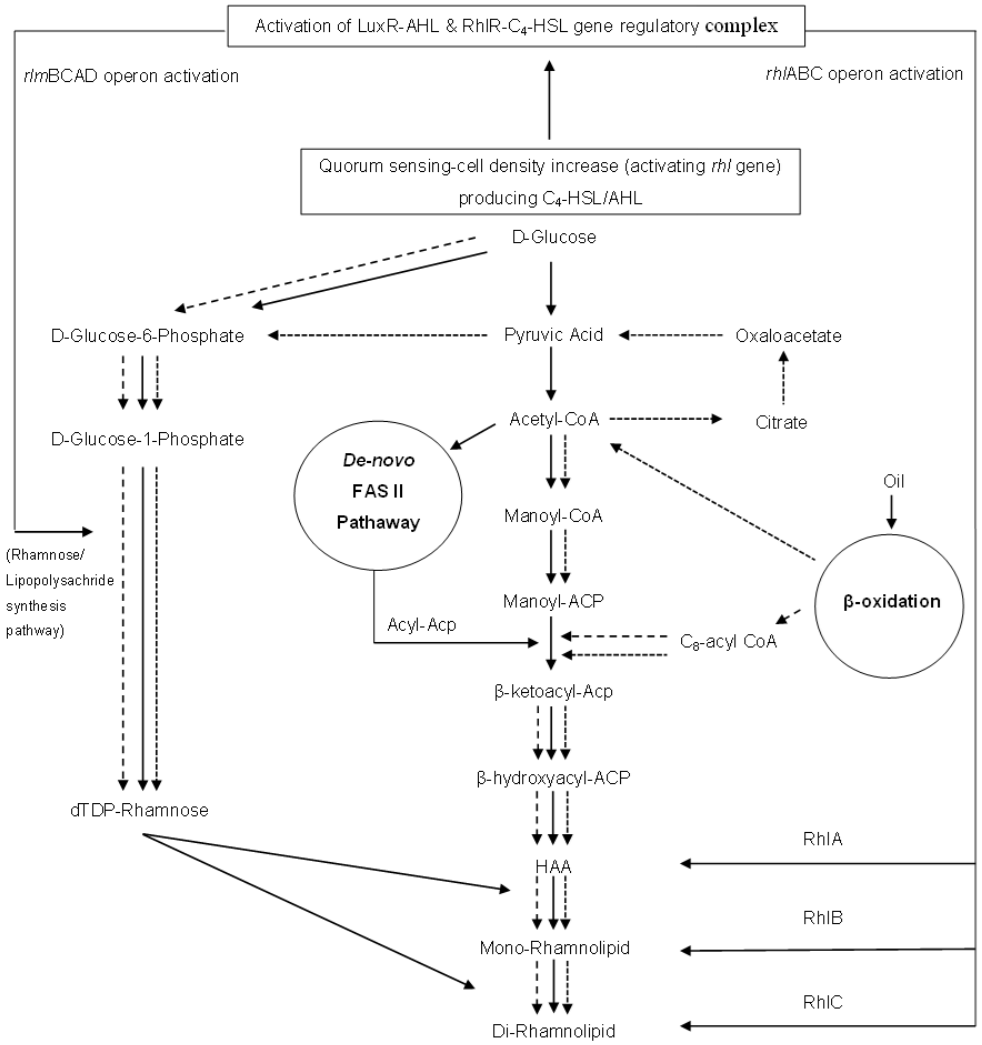
2.5.3 Biosurfactant metabolism correlated to inducers

Regarding biosurfactant metabolism, there are very complex integration among metabolic pathways. According to Zhao et al., (2020) the production of biosurfactants is triggered by quorum sensing - small signaling molecules (e.g. intra and extracellular autoinducers) - that induce or repress metabolic pathways, including gene expression correlated to biosurfactant production.

Gram-positive bacteria, such as *Bacillus subtilis*, have a subtle more complex metabolic pathways than Gram-negative bacteria, in particular due to their peptide post-translational modifications (Dusane et al. 2010). Surfactin, for example, is synthesized by non-ribosomal multienzyme peptides (DAS et al., 2008). However, Gram-negative bacteria, such as *Pseudomonas aeruginosa*, have a quorum sensing control related to the control of self-inducing molecules of acetyl-homoserine lactones (AHLs) - N-(3-oxododecanoyl)-L-homoserine lactone (C_{12}) and the N-butyl-L-homoserin lactone (C_4) - formed by the reductive condensation cycle of the chemical biosynthesis and are part of the LuxR, *las* and *rhl* type systems and the quinolones *Pseudomonas* signal (*pqs*). Fatty acids as hydrophobic inducers lead to early formation of AHLs compared to other hydrophobic substrates (e.g. mineral oil) (DUSANE et al., 2010; PATHANIA; JANA, 2020a).

In this sense, when different carbon sources are used for the glycolipid production, for example, the carbon flow is regulated to lipogenic pathways for the formation of the hydrophobic portion, and also glycolytic pathway for the hydrophilic formation of biosurfactants (ORTEGA et al., 2020; PATHANIA; JANA, 2020). Biosynthesis of rhamnolipids (Fig. 4), depends on two metabolic pathways, it requires a precursor (β-ketoacyl-ACP) lipidic 3-(3-hydroxyalkanoiloxy) alkanoic produced from the oxidation pathway (De-novo FAS II) and a precursor (glycose-6-phosphate) of dTDP-rhamnose sugar (PATHANIA; JANA, 2020b). When microorganisms use hydrophilic carbon sources (e.g. glucose), the synthesis of the rhamnose moiety occurs directly via the glycolytic pathway, whereas the lipid moiety is synthesized via the De-novo FAS II route.

Figure 4 - Biochemical and genetic regulation route for the biosynthesis of rhamnolipids. (dotted arrow) oil; (continuous line arrow) glucose and (dash arrow) path proposed for mixed substrate



Source: Pathania and Jana (2020).

On the other hand, when microorganisms use hydrophobic compounds (oil), alkanoic is synthesized directly via β-oxidation and not via De-novo FAS II and the sugar moiety is synthesized via glycolysis. Since lipid and sugar precursors are produced, RhlA, RhlB, and RhlC mediate the formation of mono- or di-rhamnolipids (PATHANIA; JANA, 2020b).

According to Pathania and Jana, (2020a), the biosurfactant production by *Pseudomonas aeruginosa* was enhanced using mixed carbon sources (glucose and soybean oil fried 2% $w.v^{-1}$). They obtained an accumulation of 3.0 $g.g^{-1}$ of rhamnolipids using mixed substrates and 2.3 and 0.63 $g.g^{-1}$ when glucose and fried oil were used separately.

Micronutrients are also essential to microbial cell growth. Micronutrients (e.g. iron, phosphorus and manganese) act as cofactors of important enzymes in microbial metabolism

(AMARAL et al., 2010). Regarding macronutrients, organic and inorganic nitrogen sources, for example, are used for biosurfactant production such as ammonium sulfate, urea, peptone, ammonium chloride and ammonium nitrate (AMARAL et al., 2010; LEATHERS et al., 2015). The C:N ratio is also quite significant on biosurfactant production, since low C:N ratio triggers the metabolism to biomass production (MARCELINO et al., 2020). In this sense, Albrecht et al. (1996) reported that the balance C:N ratio lead to lower activity of isocitrate dehydrogenase, enzyme responsible for oxidation of isocitrate to β-ketoglutarate in the Krebs cycle (MARCELINO et al., 2020).

Thus, isocitrate and citrate are accumulated in mitochondria and then transported to cytosol, where the citrate is hydrolyzed into acetyl-CoA - precursor of fatty acids, increasing the biosurfactant production (MARCELINO et al., 2020). Both macro and micronutrients should be used at optimal concentrations, which strictly depend of biosurfactant producers. Therefore, the elucidation of these mechanisms is fundamental to make biosurfactant commercially viable.

2.6 CONCLUSION AND PERSPECTIVES

Biosurfactants have a wide range of applications (pharmaceutical, agricultural and environmental), including new ones such as exogenous plant elicitors, drug carriers, among others. Nonetheless, they are not commercially viable. Biosurfactant inducers, directly, affect biosurfactant productivity and also their chemical structure. However, they have been poorly investigated, since, usually, a molecule pool is used as biosurfactant inducer, instead of a specific molecule. Thus, it is essential to deeply investigate all inducer effects on biosurfactant production. Biosurfactant inducers are versatile molecules that can: i) Hydrophilic inducers - To act as cofactors for microbial growth, and thereafter enhanced biosurfactant production; ii) Hydrophobic inducers - To synthesis, directly the hydrocarbon moiety of biosurfactants, changing their chemical structure, and thereafter their potential applications.

Nevertheless, there are still no studies on culture medium supplemented with specific fatty acids (e.g. saturated or unsaturated) for biosurfactant production. Therefore, a promising strategy is to associate agro-industrial waste (e.g. culture medium) with hydrophilic (e.g. iron) and hydrophobic (e.g. olive oil) inducers.

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CHAPTER 3

This section discusses a book chapter on the fundamental concepts and future trends on biosurfactants production using cassava wastewater as an alternative low-cost culture medium. General aspects of cassava including nutritional values, production and residues process, plant defense mechanism and toxicity inherent, cassava wastewater composition, effluent treatment, and finally the biorefinery to achieve biosurfactant production, are addressed. This review chapter is linked to the book "The Cassava Crop: Cultivation, Potential Uses, and Food security" submitted to the Nova Science Publishers in December 2021.

3 CASSAVA WASTEWATER VALORIZATION FOR THE PRODUCTION OF BIOSURFACTANTS: SURFACTIN, RHAMNOLIPIDS AND MANNOSILERITRITOL LIPIDS.

Abstract

The global production of cassava was estimated at ≈303 million tons. In 2019, Nigeria, Democratic Republic of the Congo, Thailand, Ghana, and Brazil were the major producers, since they represented ≈56% out of total production. Due to this high production, the cassava processing industry (cassava flour and starch) generates approximately ≈0.65 kg of solid residue and ≈25.3 L of wastewaters per kg of fresh processed cassava root. The composition of the liquid effluent varies according to its origin; for example, the effluent from cassava flour production, when compared to the wastewater from the starch processing, presents a higher organic load (≈12 times) and total cyanide (≈29 times). It is worthy to highlight the toxicity of cassava residues regarding the cyanide presence, which could generate disorders with acute or chronic symptoms in humans (e.g. Tropical ataxic neuropathy, Konzo, and Goiter) and animals (the ingestion of cassava plant can cause intense muscle tremors). The cyanide liberation, through the cyanogenic glycoside (linamarin (92-98%) and lotaustralin (2-8%)) starts with the disruption of the cassava vegetable tissue. In this sense, the development of simple and low-cost eco-friendly methods for the proper treatment or reuse of cassava wastewater is a challenge but promising way. Cassava wastewater is rich in macro-nutrients (proteins, starch, sugars) and micro-nutrients (iron, magnesium, manganese, nitrate, phosphorus, zinc), enabling its use as a low-cost culture medium for biotechnological processes, such as the production of biosurfactants. There is a research trend regarding the evaluation of the effects of iron, manganese and magnesium as hydrophilic inducers, i.e. co-factors that enhance the microbial growth and, consequently the biosurfactants production yield. These compounds are amphipathic molecules that are synthesized by living cells and can be widely used in industries as pharmaceutical agents, for microbial enhanced oil recovery, crop biostimulation, among others. Currently, two classes of low molecular weight biosurfactants are produced at an industrial scale: glycolipids and lipopeptides. Amongst these biosurfactants, surfactin, rhamnolipids, and mannosileritritol lipids show remarkable properties such as antimicrobial, biodegradability, demulsifying and emulsifying capacity, low toxicity, and resilience to pH. However, the high production cost restricts the massive biosurfactant applications. Therefore, this chapter aims to present the state of the art and challenges on the production of biosurfactants using cassava wastewater as an alternative culture medium.

Keywords: Cassava wastewater; Cyanogenic glycoside; Biosurfactants; Hydrophilic inducers; Green production.

3.1 INTRODUCTION

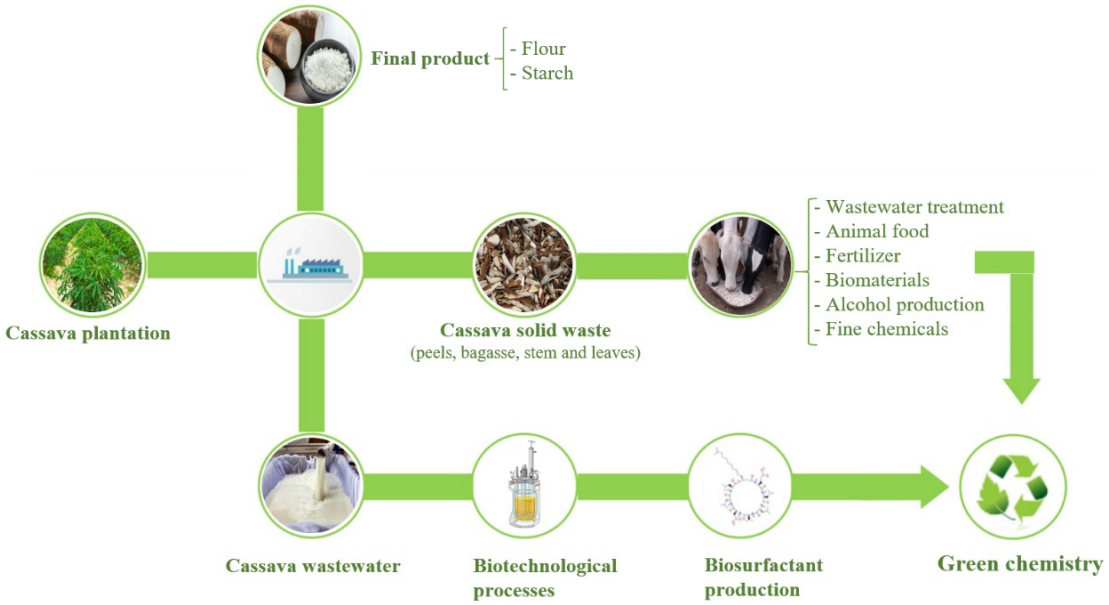
Indigenous to South America, Cassava (*Manihot esculenta* Crantz) is a staple food in developing countries due to its rich nutritional composition (LEBOT, 2009). It is among the top five crops in the world in terms of produced tons (FAOstat 2019). Cassava stands out for its use in the food, textile, alcohol, pharmaceutical, and paper industries, in addition to being widely used for human and animal consumption (ACCHAR; DA SILVA, 2021; DE SOUZAYES FERNANDES et al., 2019).

However, the cassava processing industry generates large amounts of waste (Figure 5) such as solid residue (peels, bagasse, stem, and leaves) and cassava wastewater (TUMWESIGYE; OLIVEIRA; SOUSA-GALLAGHER, 2016). Cassava wastewater is the residue obtained from pressing the crushed mass of the cassava root that has already been washed and peeled (CHISTÉ; COHEN, 2006). Cassava wastewater can be considered an ambiental problem, since it represents about 30% of the weight of the raw material (WOSIACKI; CEREDA, 2002).

The significant amount of agro-industrial residue is mostly composed of macro-nutrients (e.g. starch, sugars, and proteins) and micro-nutrients (e.g. phosphorus, potassium, calcium, magnesium, sulfur, iron, zinc, manganese, copper, and nitrate) (MARÓSTICA; PASTORE, 2007). It has a potential environmental pollution factor due to the high concentrations of organic matter (composition) as well as the presence of cyanide-generating cyanide glycosides such linamarin (92-98%) and lotaustralin (2-8%) (KUYUCAK; AKCIL, 2013; TUMWESIGYE; OLIVEIRA; SOUSA-GALLAGHER, 2016). Cyanide is capable of complexing with metals (for example, the iron of hemoglobin, preventing the transport of oxygen to the cells and promoting the individual suffocation). Thus, it is toxic to humans and animals. Therefore, due to the toxic potential of cassava wastewater, treatments are demanded (KUYUCAK; AKCIL, 2013).

Due to the large amounts of organic load in the cassava wastewater, its discharge in aqueous environments can cause negative impacts (PATIL; PAKNIKAR, 2000). In this perspective, the techniques of residue management have a high cost involved due to the requirement of appropriate infrastructure for physical-chemical treatments. This scenario hampers the correct treatment of cassava residues at small scale industries (PETERS; NGAI, 2005). Therefore, an alternative to the cassava wastewater destination is its promising and challenging use in biotechnological processes, contributing to the concept of biorefinery. The main advantages are the low-cost, ecofriendly (green chemistry), simple methods, and potential carbon source (DE ANDRADE et al., 2016; NITSCHKE; PASTORE, 2006).

Figure 5 - General flowchart of cassava processing and ecofriendly potential of its residues in the concept of biorefinery.



Source: authors (2021).

In this context, this chapter aims to explore the current state of the art and future trends on biosurfactant production using cassava wastewater as an alternative low-cost culture medium. General aspects of cassava including nutritional values, production and residues process, plant defense mechanism and toxicity inherent, cassava wastewater composition, effluent treatment, and finally the biorefinery to achieve biosurfactant production are addressed.

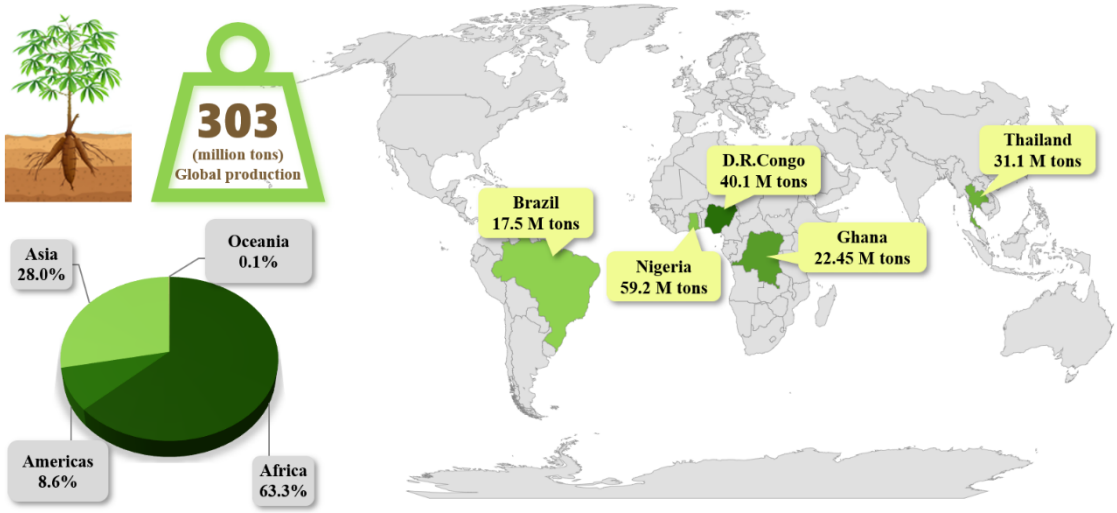
3.2 CASSAVA

Cassava or manioc is a tropical root crop from a group of approximately 100 species of the genus *Manihot* of the Euphorbiaceae (Dicotyledons) family (HOWELER; LUTALADIO; THOMAS, 2013). It is a perennial plant with starchy roots (lateral subterranean organs), and a growth rate that lasts for years. Its use as an agricultural crop was found to have its first occurrence in the Amazonian rain forest (ALLEM, 2009; HOWELER; LUTALADIO; THOMAS, 2013; SHIGAKI, 2016), being an important nutritional source for the Amerindians. According to Carter et al. (1997), the first evidence of cassava globalization was registered between the years of 1558-1600 through ship supplies traveling between Africa, Europe, and Brazil.

During the 19th and 20th centuries, cassava production was stimulated as an ‘anti-famine’ crop in Eastern and Central Africa, due to its ability to survive extended periods, adaptability to environments with bush vegetation, resistance in drought areas, and stem cutting propagation

process (LEBOT, 2009). Currently, cassava is produced in ≈110 countries with a total output of 303 million tons, on approximately 27 million hectares worldwide. The cassava production ranking (Fig. 6) is led by Nigeria, Democratic Republic of the Congo, Thailand, Ghana, and Brazil, which production corresponds to ≈56% out of the global production crop in 2019 (FAOstat 2019).

Figure 6 - Data on cassava global production per continent and country in 2019



Source: adapted from FAOstat (2019).

Cassava is the 4th most important staple crop globally after maize corn, rice, and wheat concerning production and caloric intake (FAOstat 2019). More than 60% out of the global production is in Africa (≈191 million tons). Cassava is recognized as a primary foodstuff for people mostly from sub-tropical regions and the least industrialized tropical of the world due to its adaptability to acid soils (low fertility, with variations in pluviometric regime), its flexible growth cycle (capable of being cultivated and harvested year-round), and resistance to pest and diseases. The expansion of cassava crops and the production of its technological products favors the commercial balance as well as the nutritional content of the basic diet of developing countries' populations (Howeler et al., 2013; Lebot, 2009; Rees et al. 2012).

The main use of cassava is for livestock feed, starch production, and further industrial applications. According to Nweke et al. (2002), cassava transformation has four stages, depending on the use of the crop: (i) famine reserve, (ii) rural staple, (iii) cash crop for urban consumption, and (iv) as livestock feed and industrial raw material. In Kenya, Malawi, Tanzania, and Zambia the role of cassava prevails as a famine reserve crop, due to drought. Countries such as Congo, Côte d'Ivoire, and Uganda have the crop mainly as a rural staple, whereas Nigeria and Ghana

present cassava crops for urban consumption. Some producing countries in Asia and Latin America are at the last stage of cassava transformation. From the total cassava production in Africa, approximately 50% is used as a staple human food, and only 2% is applied in industrial processes. Notwithstanding in Asia, Europe, and Oceania, the application for non-food purposes, such as livestock feed and starch production,is highlighted.

Apart from its relevance as an important staple food, cassava can be used as industrial raw material and livestock feed. The economic profitability of cassava is increasing with enhanced application in several industrial segments such as the production of animal feed, beverages, biofuel, glue, paper, plywood, and textiles (KLEIH; PHILLIPS; WORDEY, 2013; UCHECHUKWU-AGUA; CALEB; OPARA, 2015). The major cassava and its products exporters are Cambodia, Thailand, and Vietnam while the greatest importers are China, Japan, South Korea, United Kingdom, and the United States (PARMAR; STURM; HENSEL, 2017). A deeper understanding of nutritional composition, production process and residues, defense mechanism and toxicity inherent of cassava is essential to associate its well-payed contribution to various biotechnological applications.

3.2.1 Nutritional value

Cassava, a traditional and subsistence crop, is now being recognized for its commercial potential as a raw material in the production of energy (bioethanol) (PRADYAWONG et al., 2018), livestock feed (BUI et al., 2018), starch (Yangyang Jin et al., 2018), starch-based products (Tianyu Jiang et al., 2020), and as a forage (fresh or dehydrated meal) due to the beta-carotenoids and proteins from cassava leaves (PARMAR; STURM; HENSEL, 2017).

The cassava dry matter consists primarily of starch and sugars (about 90%) and corresponds to approximately 40% of cassava roots (LEBOT, 2009), a high level compared to other root crops such as potato (20%), sweet potato (30%), taro (30%), and yams (26%). Vitamin C and calcium are found at great levels in white flesh cassava roots. However, taking into account the daily lipid and protein requirements for human consumption, the cassava nutritional content is insufficient (Table 7), being necessary the diet supplementation in terms of protein sources such as animal protein, leafy vegetables, and legumes. Other vitamins and minerals such as folate, iron, magnesium, potassium, and thiaminare also at low content considering the daily human requirement. Therefore, genetic engineering is being explored and applied to maximize the nutrient content of cassava roots (SHIGAKI, 2016).

Cassava leaf is also used in the culinary of some Africa countries, it has a nutritional composition that varies according to age, cultivar, weather conditions, and soil. Nevertheless, it is considered a good source of calcium, magnesium, phosphorous, potassium, riboflavin, protein, and vitamins, with a protein concentration enough to offer most of the amino acids

(LANCASTER; BROOKS, 1983; LATIF; MÜLLER, 2015). As an example, during the Nigerian Civil War, leaves of cassava were consumed in the eastern regions, which were rarely eaten in normal conditions (LANCASTER; BROOKS, 1983). Flour from cassava leaves combined with cereals (maize and wheat) was incorporated in the national food supplement program to child nutrition for Northeastern Brazil low-income households (CÂMARA; MADRUGA, 2001). The cyanogenic glycosides (GCs) amount present on the leaves is similar when compared to roots. The cyanogenic toxicity of cassava originates from the formation of hydrocyanic acid (HCN), from the enzymatic hydrolysis of GCs, which degradation is achieved by cooking or processing (Sections 2.4 and 2.5 will focuse on the main features related to the mechanisms of cyanogenic glycosides and linamarin hydrolysis).

African household cooking process of cassava leaf was investigated concerning the composition. No change was detected in the levels of ash, calcium, carotene, copper, fiber, lipids, manganese, magnesium, phosphorus, potassium, protein, sodium, total carbohydrate, and zinc. However, reduction in ascorbic acid (77.7%), cyanogenic potential (>99%), free sugars (23.2%), tannin (55.2%), and thiamine (37.1%) were promoted by the cooking process (ACHIDI et al., 2008).

Production of cassava pellets and silage (leaves and roots) for animal feed is leading the production chain in countries such as Thailand, and Vietnam, great cassava exporters. Cassava roots are sun-dried, mechanical peeled and chopped; and then processed into pellets. Protein content enhancement is demanded to supply balanced diets for livestock, in this context the addition of fish or soybean meal is made.

The inherent presence of lactic acid bacteria and yeasts provides excellent digestibility characteristics to cassava meal (PARMAR; STURM; HENSEL, 2017). Howeler et al. (2013) reported that microdoses of HCN improved the natural antibacterial efficiency of enzymes like lactoperoxidase secreted from mammary, mucosal, and salivary glands.

Despite the limitations in its nutritional composition, cassava has a wide applicability potential. Cassava flour can serve as a functional food and is appropriate to be used for gluten-free food products. Starch compounds about 80% of cassava, it has great gelatinization ability, and greater water binding capacity and viscosity compared to different starch sources. These technological properties allow its use in adhesives (gums with and without additives), bakery, bioethanol, biodegradable plastic, citric and lactic acids, dextrin, liquid glucose, fructose, maltose, syrups and sweets production (BALAGOPALAN, 2009; RAY; SWAIN, 2011; SHIGAKI, 2016).

Table 7 - Nutritional composition of raw cassava roots and leaves.

Nutrient	Unit	Raw cassava[a] Per 100 g	Fresh leaves[b] Per 100 g dry matter	Daily adult requirements[c]
Water	g	59.7	71.7 – 90.0	-
Energy	Kcal	160	-	-
Protein	g	1.36	19.7 – 38.1	0.75
Total lipid (fat)	g	0.28	3.5 – 7.3	-
Carbohydrate, by difference	g	38.1	31.9 – 64.7	-
Fiber, total dietary	G	1.8	8.3 – 19.5	-
Sugars, total	G	1.7	-	-
Minerals				
Calcium, Ca	mg	16	430 – 1140	1000 – 1300
Potassium, K	mg	0.27	15 – 27	-
Phosphorus, P	mg	21	260 – 370	-
Magnesium, Mg	mg	27	180 – 420	220 – 260
Sodium, Na	mg	271	1380 – 2260	-
Zinc, Zn	mg	14	38 – 120	3 – 14
Iron, Fe	mg	0.34	12 – 21	7.5 – 58.8
Vitamins				
Vitamin C, total ascorbic acid	mg	20.6	28.3 – 431.6	25
Thiamin	mg	0.087	0.3 – 10.3	1.1 – 1.2
Riboflavin	mg	0.048	0.6 – 2.1	1.1 – 1.3
Niacin	mg	0.854	2.4 – 9.5	14 – 16
Vitamin B-6	mg	0.088	-	1.3 – 1.7
Beta-carotene	mg	-	3.0 – 43.6	-
Folate, DFE	µg	27	-	400
Vitamin B-12	µg	-	-	2.4
Vitamin A, ERA	µg	1	-	-
Vitamin A, IU	IU	13	-	-
Vitamin E (alpha-tocopherol)	mg	0.19	-	-
Vitamin D (D2 + D3)	mg	-	-	-
Vitamin D	IU	-	-	5 – 10
Vitamin K (phylloquinone)	µg	1.9	-	55 – 65
Lipids				
Fatty acids, total saturated	g	0.074	-	-
Fatty acids, total monounsaturated	g	0.075	-	-
Fatty acids, total polyunsaturated	g	0.048	-	-

[a] USDA (2019) National Nutrient Database for Standard Reference, United States Department of Agriculture.

[b] Latif and Müller (2015) (The values are on a dry matter basis).

[c] World Health Organization and Food and Agriculture Organization of the United Nations (1998).

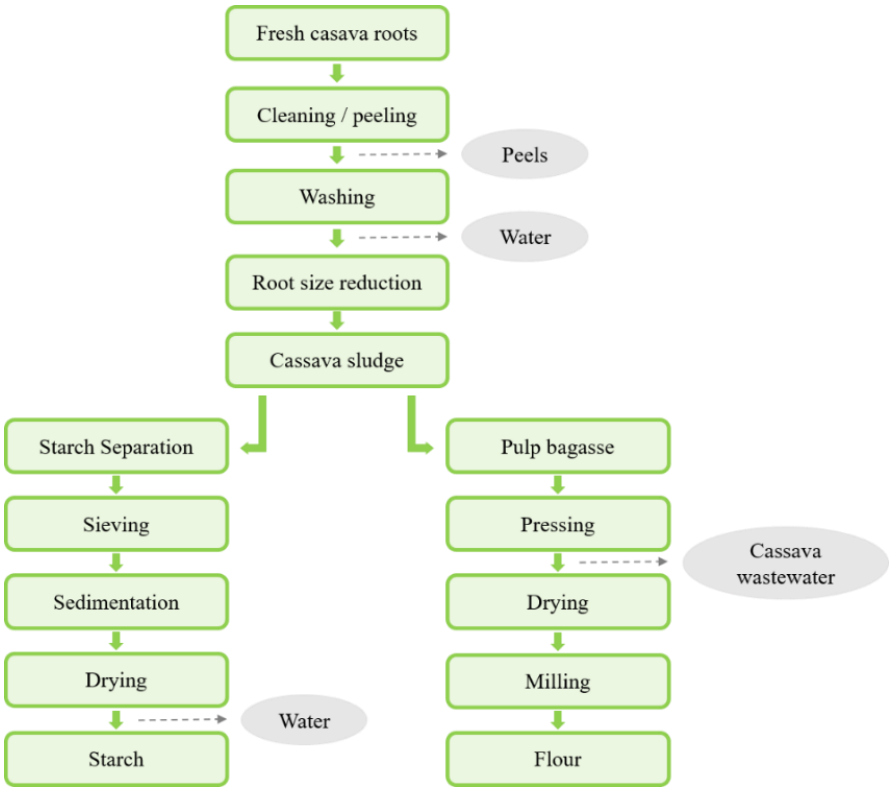
3.2.2 Production process

The cassava processing chain starts with its cultivation. Vegetative propagation using stem cuttings is the most used method at a household (subsistence) level as well as for commercial production (NDUWUMUREMYI et al., 2016; WADDINGTON et al., 2010). Nevertheless, the reproduction rates are low compared to others cultures. For example, one maize seed can produce 300 seeds in three months, while one cassava stalk produces 10 stalks in a year (HOWELER; LUTALADIO; THOMAS, 2013). An estimative of 10,000 plants per hectare is an optimal plant density for root production. Cassava root harvest is mainly manual, supported by simple soil loosening tools, tractor-based, and self-propelled cassava harvesters (PARMAR; STURM; HENSEL, 2017). Harvesting cassava roots is recommended after 12 months when the starch content is maximum (SRIROTH et al., 1999).

Cassava flour is the dry, and fibrous product from cassava roots. To produce cassava flour (Fig. 7), the fresh roots are firstly washed and peeled. The peeling removal can be carried out by an abrasive or cutting mechanism. Cutting methods demand less water for washing. Peeled roots are then washed and crushed (≈ 5 x 0.5 x 0.2 cm particles) such as chipping, grating (or rasping), and mincing to improve subsequent unit operations as drying, fermentation, pressing, and starch extraction. Fermentation is applied to produce eatable products from raw cassava roots. Cassava root fermentation can be used for solid-state fermentation and submerged fermentation, both by lactic acid bacteria activity. The fermented or grated cassava mash is pressed to reduce the moisture ≤30%. Drying or dehydration (sun-dried for two or three days, or dried in a hot air oven at 55 °C) is often one of the last stages of cassava flour processing to obtain a shelf-stable product. After drying, the moisture content of the cassava chips should be less than 8%. The chips are then milled, and the flour is sieved through an 80-mesh sieve. Finally, the flour is packaged in plastic bags. Packaged in this way, the flour can be stored for at least eight months. The yield recovery of flour is about 20-40%, depending on the cultivar, the time of harvest, and the equipment used (HANSETHSUK, 2003; SHITTU et al., 2016).

Cassava starch extraction follows a similar flowchart of cassava flour production, excluding that rasping is conducted to enhance starch extraction from cassava roots, and starch milk is submitted to filtration of 150 microns to separate starch from fibers and other impurities. Decanters can be incorporated to remove proteins and others contaminants. The first stage of extraction is in coarse extractors with centrifugal perforated baskets (355-425 μm), repeatedly until minimal residual starch is reached. Starch milk is then directed to a fine extractor (150-125 μm), to remove fine fibers, and to a sieve of smaller aperture (140-200 mesh size).

Figure 7 - Flowchart of cassava flour and starch production processes and residue generation.



Source: authors (2021).

The next step is the water separation by a sedimentation channel. The surface of the starch is washed to remove the layers of dirt. Pneumatic conveying flash dryers are used to reduce the cassava starch moisture content to between 10 and 14%. The flash dryer reduces about 12% of the cassava starch cake moisture content within 6 sec, which prevents the modification of starch by exposure to high temperatures during long periods (SRIROTH et al., 2000). The right particle size according to the application is obtained by milling the dried cassava starch. The package is often made of polyethylene bags or linen cloth. And a low humid environment is indicated for storage (NDUELE; LUDWIG; VAN OOTEGHEM, 1993).

During the cassava processing chain, solid waste (bark, branches, crumb, discards, shavings, and leaf), liquid waste (cassava wastewater and washing water), and atmospheric emissions (atmospheric gases, dust, and smoke) from burning firewood are generated. In this context, the correct disposal of these residues must be explored, discussed and performed, considering the potential of valuable products generation, such as biosurfactants.

3.2.3 Process residues

3.2.3.1 Cassava solid wastes

The highest charge of solid residue produced by the cassava flour industry is from the peeling step, reaching 10-15% (w w^{-1}) of the total weight of the processed root (SRIROTH et al., 2001; UKWURU; EGBONU, 2013). About 20-670 kg of solid waste (peels and bagasse) are generated per ton of processed tuber (Fig. 3), according to the operation mode (manual or mechanical) (DE CARVALHO et al., 2018; EKOP; SIMONYAN; EVWIERHOMA, 2019). Considering the world cassava production, approximately 45.5 million tons of cassava solid waste were generated in 2019 (FAOstat 2019).

Cassava processing residues can cause serious contamination problems when released into the environment since cassava peels (e.g. have low degradation in water and soil) present a high organic load and lethal effects of the hydrocyanic acid content (OGHENEJOBOH; OTUAGOMA; OHIMOR, 2016; OGUNYINKA; OGUNTUASE, 2020). In addition to the environmental degradation caused by cassava peels, other solid waste such as leaves (≈10% w w^{-1}), stem (≈35% w w^{-1}), bagasse (≈15% w w^{-1}), and rhizome (between stem and tuber, ≈10% w w^{-1}) also represent a nuisance to the environment and need to be properly relocated (RAVINDRAN, 1993; SIVAMANI; BASKAR, 2018; SRIROTH et al., 2001; UKWURU; EGBONU, 2013). In this sense, Ezekiel et al. (2012) compared the growth rate of *Candida utilis* NRRL Y-1084 in submerged aerobic culture. Three different substrates were used: enzymatic and acid hydrolysates of cassava peels, and saline mineral medium containing glucose. In the enzymatic hydrolysate, its biomass yield coefficient ($Y_{x/s}$) value in sugar was 0.44 with a μ_{max} of 0.35 h^{-1}, while the corresponding values were 0.52 and 0.48 h^{-1} in the acid hydrolysate and 0.50 and 0.37 h^{-1} in mineral salts medium. The protein content of the produced biomass was 56.7% for the enzymatic hydrolysate of cassava peels against 49.1% and 47.5% for acid hydrolysate and glucose, respectively.

Another study using cassava peels was developed by Oghenejoboh et al. (2016). These authors carried out a comparative analysis of an activated carbon efficiency produced from fermented cassava peels and commercial activated carbon in the treatment of refinery wastewater. The activated carbon produced was more effective when compared to the commercial carbon. Fermented cassava peels was 18 and 14% more efficient in removing chemical oxygen demand (COD) and biochemical oxygen demand (BOD), respectively. An efficiency of 96 and 100% in phenol and trace metals removal from the wastewater was obtained. On the other hand, the commercial activated carbon efficiency was 57% trace metal and 83% phenol, respectively. The authors associated the better performance of fermented cassava peels with its higher pH from the removal of cyanide from the peels during the fermentation process.

Sivamani and Baskar (2018) performed the biological conversion of cassava stem into ethanol through enzymatic hydrolysis. The purpose of stem hydrolysis was to convert the cellulose into saccharin which was then fermented to convert the monomers into ethanol by distillation. The ethanol yield was 32% and the alcohol concentration produced was 1.622 g L^{-1}.

Summarily, the emergent prospects for these solid wastes recovery are concentrated in the following areas: (i) feed formulations and protein supplement for animals in partial or total replacement of cereals (e.g. corn, wheat, and barley), due to their energy value and its palatability, (ii) fine chemicals (e.g. organic acids, bio-surfactant, hydrogen gas, and bio-fertilizers), (iii) alcohol production and (iv) wastewater treatment (e.g. activated carbon production) (COSTA et al., 2020; SMAH et al., 2018).

3.2.3.2 Cassava liquid wastes

The liquid residues generated in cassava processing are composed of the process water - root washing and starch - and the water released from the drying step (pressing) (ACCHAR; DA SILVA, 2021; DE CARVALHO et al., 2018).

The highest volume of produced wastewater is from the washing step, which consumes 3-5 m^3 ton^{-1} of water to wash the peeled tubers and 12-20 m^3 ton^{-1} to wash the starch. Considering that cassava tubers contain more than 70% of water it is estimated that the steps of pressing and washing generate at least 25.3 m^3 of wastewater per ton of processed root (DE CARVALHO et al., 2018; EKOP; SIMONYAN; EVWIERHOMA, 2019).

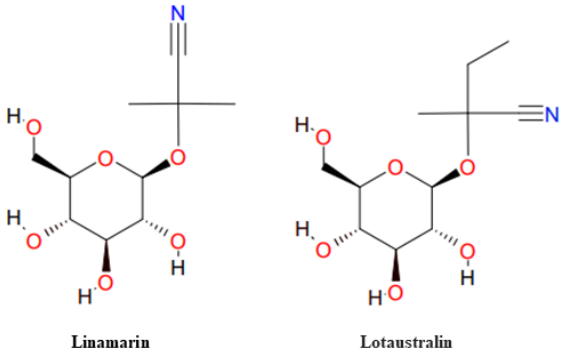
It is important to point out that the volume and characteristics effluent generated depends on the desired final product (e.g. starch production requires a large amount of water) (EKOP; SIMONYAN; EVWIERHOMA, 2019). Several substances are transported with the effluent, such as carbohydrates, nitrate, proteins, phosphate, potassium, and cyanide (Tables 8 and 9). Therefore, it must be treated prior disposal into the environment. Besides its usual treatment - anaerobic, facultative, and aerobic lagoons system, and a final polishing step - several alternatives for *in situ* valorization have been investigated over the years (e.g. production of biogas and ethanol, other fermentation products, microalgae biomass), with featured for the production of biosurfactants, the main subject of this chapter.

3.2.4 Cyanogenic glycosides (mechanism)

Cassava has high concentration of cyanogenic glycosides (CGs) when compared to others roots. The cyanogenic toxicity of cassava originates from the formation of HCN from the enzymatic hydrolysis of GCs derived from valine and isoleucine: linamarin (2-β-D-glucopyranosyloxy-2-methylpropanenitrile) and lotaustralin [(2R)-2-β-D-glucopyranosyloxy-2-

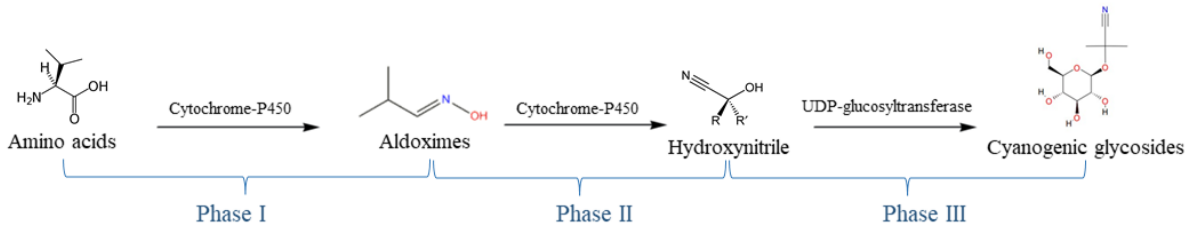
methylbutyronitrile] (Figure 8) (KRIEGER et al., 2004; WITTSTOCK; HALKIER, 2000). The generic biosynthetic pathway for the production of GCs from amino acids is elucidated in Figure 9.

Figure 8 - Structure of the cyanogenic glycosides of cassava.



Source: authors (2021).

Figure 9 - General biosynthetic pathway for cyanogenic glycosides from its precursor amino acid



Source: adapted from Zuk et al. (2020).

The first two phases of the biosynthetic production of GCs are catalyzed by a cytochrome P450 enzyme through two successive amino groups N-hydroxylations of the original amino acid – in the case of synthesis linamarin from Valine. α-hydroxynitrile is then generated after decarboxylation and dehydration of aldoxime and nitrile. The third step - which produces the GC - involves the glycosylation of the hydroxynitrile moiety, and the process is catalyzed by UDPG-glycosyltransferase (Ganjewala et al. 2010; Zuk et al. 2020). According to Krieger et al. (2004), the first and second are the limiting phases, and the substrate preference of the first cytochrome P450 is a determinant factor of the different GCs content accumulated in a tuber species.

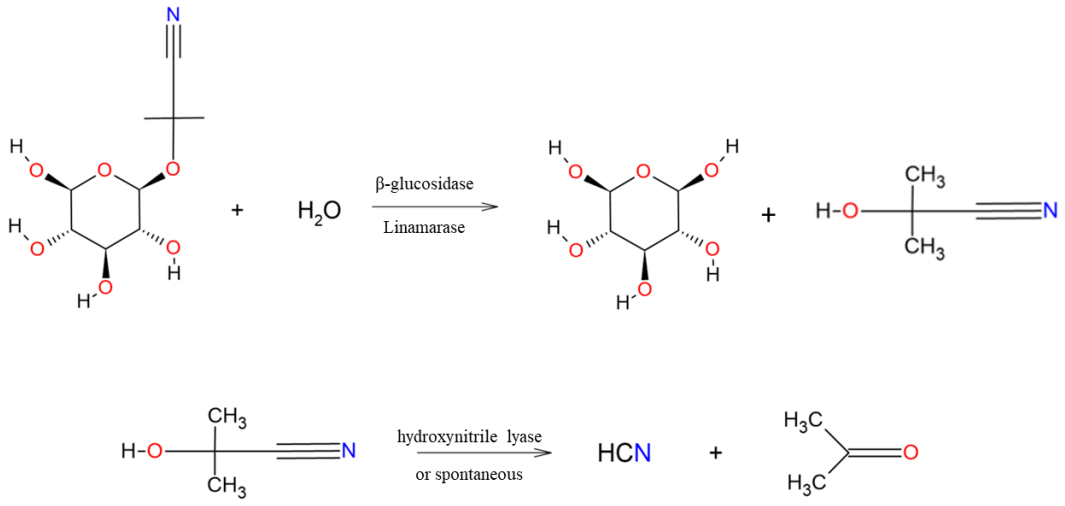
These glycosides are synthesized in cotyledons and are found in high proportions (Linamarin (92-98%) and lotaustralin (2-8%)) in all plant tissues, with greater concentration in leaves, stem, and root peels (DU et al., 1995; KRIEGER et al., 2004; NAMBISAN; SUNDARESAN, 1994). Environmental factors such as cultivation, soil types, growing conditions, and the different varieties of cassava result in a large variation in GCs content in the tuber (range 225-2000 mg kg^{-1}) (ELIAS; NAMBISAN; SUDHAKARAN, 1997).

3.2.5 Linamarin hydrolysis

In addition to CGs, cassava tissues are also composed of an endogenous hydrolyzing enzyme known as β-glucosidase (linamarase). Linamarin is non-toxic per se and is an unlikely source of cyanide exposure in humans (MLINGI; POULTER; ROSLING, 1992). However, the release of cyanide from the GCs starts when the cassava plant tissue is broken down – mainly by mechanical action (during the processing of the cassava flour) or microbial action (fermentation process or root deterioration) – favoring the action of these enzymes.

The hydrolysis of linamarin is a two-step reaction involving the formation of an intermediate, α-hydroxynitriles, which decomposes spontaneously or by the action of hydroxynitrile lyase to form ketone and HCN (Figure 10) (KUYUCAK; AKCIL, 2013; YEOH; TATSUMA; OYAMA, 1998).

Figure 10 - Enzymatic degradation of linamarin.



Source: Adapted from Zuk et al. (2020).

According to Zuk et al. (2020), the roles of GCs and CN− are diversified: they serve as factors in plant defense systems against insects and pathogens; act as nitrogen-containing precursors for amino acid and protein synthesis during plant development, playing an important role in primary metabolism; free cyanide (released from CGs) can act as a signaling molecule. Thus, while they bring benefits to plants by protecting them against infections, they can be toxic to vertebrates (Zuk et al. 2020).

3.2.6 Toxicity (human, animals, and environmental diseases)

Cassava toxicity in animals and humans is directly related to the absorption of free HCN, which lethal dose is 0.5-3.5 mg kg^{-1} of body weight by oral ingestion. The action route of this compound can promote some alterations in nerve cells. Combined with the blood hemoglobin, acts as an inhibitor of the respiratory chain (CEREDA, 1994). Some symptoms of sub-lethal doses are headache, muscle weakness, nausea palpitations, vertigo, and vomiting. Prolonged consumption of non-lethal doses of HCN might culminate in chronic toxicity (MONTGOMERY, 1969).

Tropical ataxic neuropathy is an example of chronic toxicity disease inherent to older adults which symptoms include loss of sensation in hands, deafness, vision loss, unsteady walking, and weakness. An upper motor neuron disorder, known as Konzo, causes irreversible legs paralysis and affects children and young women mostly (NZWALO; CLIFF, 2011). Iodine uptake by the thyroid gland was also hampered in some people that continuously consumed cyanide and its metabolites. This difficulty could aggravate to goiter and cretinism (SHIGAKI, 2016). Nevertheless, acute toxicity cases are rare and highlight the necessary caution in the processing of fresh roots. HCN boiling is at 26 °C being readily volatilized with heating promoted by boiling or cooking and consequently reducing HCN concentration to tolerable amounts (MONTGOMERY, 1969). According to the Codex Alimentarius (2019), the free HCN tolerable limits in cassava flour must not exceed 10 mg kg^{-1}, and raw cassava should not be consumed. Montagnac et al., (2009) emphasized soaking, fermenting, sun-drying, and roasting as a sequence of techniques with a capability to remove up to 99% out of the total cyanogenic glycosides.

Environmental pollution by cassava wastewater is from the high concentration of organic matter and linamarin. Once linamarin is highly soluble in water, this problem is hampered. An important compilation helps to understand the gravity of the improper discharge of cassava wastewater (SANTOS, 2009). Compared to domestic sewage, the wastewater from pressing cassava root and from the starch process have a polluting potential of 25 and 12.5 times larger, respectively. The natural renovation ability of a river is not enough to repair the damage caused by the direct discharge of cassava wastewater. Even with an efficiency of 90% in the effluent treatment process, the residual amount of contaminant has a hard influence on the aqueous

receptor system (BARANA, 2000). The impacts caused to the environment can be expressed in two types: eutrophication; and self-cleaning capacity modification. Concerning the effects on the fauna, the sweet taste of the cassava glucose attracts animals. There are some relates about the death of animals, mostly fish, after consumption of water from systems that received cassava wastewater discharges (FIORETTO, 1987). Environment, animals, and humans can be affected by the improper discharge of cassava wastewater. Therefore, an efficient treatment process is demanded to reduce the toxicological load of the effluent and ensure that the receptor system will not be damaged.

3.3 CASSAVA WASTEWATER

3.3.1 Composition

The liquid wastes generated by industrial processing of cassava are divided into cassava wastewater (pressing) and liquid residues (process water). Thus, Section 3.3 will focus on the main characteristics related to cassava wastewater, such as composition, treatments related to its high level of pollution and applications of cassava wastewater that have industrial and commercial importance.

Cassava wastewater is a yellowish liquid waste generated by the pressing process in the manufacture of cassava flour. This residual liquid is composed of starch, glucose, cyanide, carbohydrates, proteins, lipids, and minerals. Its composition varies according to environmental factors (such as edaphic, climatic, and cassava variety) and the knowledge of these physicochemical characteristics is essential to determine its future applications (Neves et al., 2014). According to Table 8, the levels of nitrogen and potassium presented the highest concentration values, with maximum of 3.3 and 5.9 g L^{-1}, respectively. Magnesium, phosphorus, calcium, iron and sodium were 0.62, 0.74, 0.38, 0.11 and 0.74 g L^{-1}, respectively. Copper, manganese and zinc presented very low concentration, not exceeding 0.02 g L^{-1}.

The cyanide content varied considerably among the authors (values between 0.002 and 0.09 g L^{-1}) (Table 9). Despite appearing to be a low value, free cyanide represents only 10% of all cyanide present in the residue (Cereda and Takahashi 1996). In studies such as Neves et al. (2014), the authors reported a cyanogenic potential of 257.5 mg L^{-1}, whereas Cereda 2001 cited 364 mg L^{-1} with 50% free cyanide, and Cereda and Takahashi (1996) quantified 444 mg L^{-1} of cyanide, the highest value ever mentioned.

The COD of cassava wastewater varies between 15.7 and 141 g L^{-1}; while the liquid residue has a maximum COD of 16 g L^{-1}. This significant difference is because the second one – the wastewater from the cassava starch - is diluted with the starch extraction water, reducing its concentration.

The BOD is very similar for both, with maximum of 8 and 12.21 g L^{-1}, for the wastewater from the flour and the starch, respectively. Considering the organic load of this agro-industrial residue, it is possible to infer the high source of pollution that it represents, requiring treatment or proper use (ACCHAR; DA SILVA, 2021; PINTO, 2008).

From another perspective, it is possible to note that cassava wastewater has a rich composition in micro and macronutrients, and has the potential to be used in several biotechnological processes. An example is using this residue as a culture medium for microorganisms in fermentation processes, reducing operating costs, and optimizing the process. According to Zanotto et al. (2019), the high amount of starch present in cassava wastewater ensures greater productivity than supplemented synthetic media. Other examples of the use of cassava wastewater will be further discussed in Section 3.3.

Carbon and nitrogen sources are of essential importance for biotechnological processes and although this residual liquid acts as a very complete carbon source, it can be supplemented with inducers to further enhance the process. Inducers can be hydrophilic (such as metals) or hydrophobic (such as vegetable oils) and should be added at very low concentration levels (BUENO, 2014; EHRHARDT; SECATO; TAMBOURGI, 2015).

The addition of hydrophilic inducers is currently more common, as they act as cofactor molecules, aiding in cell growth and, consequently, in the generation of the product of interest. Hydrophobic inducers also help the cell growth, acting as a secondary source of carbon, however, it has its great importance in the synthesis of the fatty acid portion of biosurfactants, being responsible for the large rate of homologous molecules produced. Meanwhile, studies are still needed to investigate its effects on productivity and the chemical structure of the molecules involved in the reaction (DE ARAUJO; FREIRE; NITSCHKE, 2013; DE OLIVEIRA SCHMIDT et al., 2021a; NIU et al., 2019; PATHANIA; JANA, 2020b).

3.4 WASTEWATER TREATMENT

Due to the complexity of the characteristics of the cassava wastewater, it can cause serious environmental problems if incorrectly discharged (PERES et al., 2019). The main challenge for the treatment of this effluent is the removal of the high COD , BOD , and the high cyanide content (POTIVICHAYANON et al., 2020). Usually, cassava wastewater is treated by the traditional anaerobic, facultative, and aerobic ponds, but can be used for the production of fermentation products (e.g. biohydrogen and biomethane) (DE CARVALHO et al., 2018).

The biodegradation of COD and cyanide was reported by several studies. Potivichayanon et al. (2020) achieved high levels of cyanide (78%) and COD (74%) removal from cassava wastewater by a fixed-film sequencing batch reactor, and 43 and 37% for a conventional sequencing batch reactor, respectively. Kaewkannetra et al. (2009) reported removing up to 90%

of the cyanide using strains of *Azotobactor vinelandii*, a nitrogen-fixing bacteria. Other microorganisms such as *Bacillus* and *Pseudomonas* have also been reported as cyanide biodegraders (AKCIL et al., 2003; EBBS, 2004; KAEWKANNETRA et al., 2009).

Anaerobic ponds are very efficient for the treatment of cassava wastewater, especially when this effluent is rich in suspended solids, which settle at the bottom of the ponds and are then digested by anaerobic microorganisms, reducing COD and TTS (total suspended solids) (RAJBHANDARI; ANNACHHATRE, 2004). Furthermore, anaerobic ponds can reduce cyanide concentrations, as reported by Rajbhandari and Annachhatre (2004), who investigated the removal of COD, TTS and cyanide of cassava wastewater by anaerobic ponds connected in series (3 ponds), this system achieved a removal of COD and TSS of more than 90%, and 51% cyanide removal.

There are several efficient strategies for cassava wastewater treatment, and there is potential for the development of multi-product biorefineries on an industrial scale for integration into cassava processing, enhancing the economic exploitation of waste and industrial development (PADI; CHIMPHANGO, 2021), which will be discussed in the next topic.

3.5 VALORIZATION OF CASSAVA WASTEWATER

Considering the composition and biochemical properties of cassava wastewater, it is clear that it requires several treatment processes before disposal since it can lead to an environmental imbalance. Thus, alternatives should be developed. The potential applications of cassava wastewater include the production of biogas, soil fertilizer, pesticide, and also as a culture medium for biotechnological products obtention (PINTO ZEVALLOS; PEREIRA QUEROL; AMBROGI, 2018), or even in civil construction, acting as a plasticizer in the production of ecological bricks (RAMOS FILHO, 2021).

Since the 1980s, cassava wastewater has been studied for application as fertilizers and pesticides, mainly driven by the increased demand for more organic products and more sustainable technologies (PINTO ZEVALLOS; PEREIRA QUEROL; AMBROGI, 2018). Vieites (1998) evaluated the use of cassava wastewater as a fertilizer in the cultivation of tomatoes, while Lima and Valente (2017) tested in the cultivation of bells peppers; in both works, when used in the proper proportion, this natural fertilizer provided an increase in productivity and size of the produced fruits. According to Costa et al. (2020), cassava wastewater can be used for partial or total replacement of mineral fertilizer - biquinho pepper (*Capsicum chinense*). The authors proved that at a dose ≈150 m^3 ha^{-1} was equivalent to mineral fertilizer (urea (40 kg ha^{-1}); potassium sulfate (180 kg ha^{-1}); single superphosphate (600 kg ha^{-1}); boric acid (1 kg ha^{-1}); and zinc sulfate (30 kg ha^{-1}). Bezerra et al. (2017) studied the use of cassava wastewater as a fertilizer in the production of 'Mandaru' grass pasture, and obtained an improvement in pasture characteristics, such as greater

forage mass and chlorophyll content, in addition to the pesticide action of cassava wastewater, which decreased unwanted plants in the crop.

In addition, cassava wastewater anaerobic digestion is efficient in biogas production (MARI et al., 2020; MONTORO et al., 2019). Jiraprasertwong, Maitriwong, and Chavadej (2019) observed the generation of 328 mL of CH_4 g^{-1} COD (92% COD reduction) applying a three-stage UASB reactor. The biogas production was maximized by Andrade et al. (2020), who pre-treated the CW by alkalinization and photocatalysis to reduce the cyanide concentration; they obtained 27.6% more methane compared to AD from raw CW.

Researchers have reported the potential of H_2 production by cassava wastewater. Wadjeam et al., (2019) indicated that the co-digestion of cassava wastewater with buffalo dung improved the H_2 production, once mixing these two substrates provided a balance of carbon, nitrogen, and other nutrients for indigenous hydrogen producers. Meier et al. (2020) obtained a 4.5-fold increase in H_2 production when applying a process of co-digestion of cassava wastewater with 3% glycerol, codigestion produced 1106.7 mL H_2 while cassava wastewater digestion without glycerol produced only 243.5 mL H_2. According to the authors, the increase in hydrogen production when glycerol is added is due to an alteration in the metabolic pathways of butyric acid production (MEIER et al., 2020). Thanwised, Wirojanagud, and Reungsang (2012) revealed an optimized anaerobic baffled reactor for H_2 production; they obtained 883 mL H_2 $(L\ d)^{-1}$, nevertheless only about 29% of the COD was removed since a significant portion of COD was converted to liquid intermediate products (e.g. ethanol, butyrate, and propionate) and remained in the effluent. Therefore, the anaerobic digestion processes of cassava wastewater is interesting from the point of view of the formation of a product with added value (H_2), however, in some cases it may require a subsequent complementary treatment of the effluent to increase the efficiency of COD removal (Rodrigues al., 2021).

Besides, cassava wastewater was already evaluated as a low-cost culture medium to produce carotenoids and fatty acids by *Rhodotorula glutinis* (SANTOS RIBEIRO et al., 2019), and, mainly biosurfactants. Nitschke & Pastore (2004) used cassava wastewater for the production of surfactin using two different strains of *Bacillus subtilis*. As a result, it was produced ≈3.0 g of crude surfactin.L^{-1}. Recently, cassava wastewater was used to produce others biosurfactants as mannosylerythritol lipids (≈1 g L^{-1}) (Andrade et al., 2017).

According to de Oliveira Schmidt et al. (2021) biosurfactants have a wide range of chemical structures, even within sub-classes (homologues). The production of specific homologues is correlated with the culture medium, the biosurfactant producing microorganism and the mode of operation, among others. Based on this, Section 3.3.1 has the main objective of discussing the focus and a more specific approach to the feasibility of using cassava wastewater as an alternative substrate in the production of biosurfactants.

Table 8 - Micronutrients composition (g L^{-1}) of cassava wastewater from starch and flour industries.

P	N	Na	K	Ca	Mg	Cu	Fe	Mn	Zn	Reference
0.38	0.88	n.a.(*)	3.90	2.03	0.57	n.a.	0.093	0.002	n.a.	Nasu et al. (2010)
0.74	0.98	0.460	1.97	0.24	0.36	0.02	0.010	0.003	0.002	Duarte et al. (2012)
0.67	1.59	0.126	5.90	0.38	1.52	n.a.	n.a.	n.a.	n.a.	Barreto (2011)
0.28	n.a.	0.74	4.79	0.24	1.59	n.a.	n.a.	n.a.	n.a.	Magalhães et al. (2014)
0.40	3.30	n.a.	2.80	0.20	0.60	0.001	0.001	0.0008	0.001	Mesquita (2016)
0.35	1.54	0.44	2.94	0.2	0.38	0.0005	0.022	0.004	0.005	Bezerra et al. (2017)
0.03	0.95	n.a.	0.35	0.02	0.01	0.0004	n.a.	n.a.	n.a.	Rodrigues et al. (2021)
0.16	1.14	n.a.	0.95	0.03	0.007	0.0002	n.a.	n.a.	n.a.	Rodrigues et al. (2021)
0.08	0.95	n.a.	0.46	0.02	0.01	0.0002	n.a.	n.a.	n.a.	Rodrigues et al. (2021)
0.13	1.12	n.a.	1.45	0.09	0.21	0.009	0.11	0.001	0.002	Acchar and Monteiro (2021)

(*) n.a.: not analyzed.

Table 9 - Composition of cassava wastewater and liquid residues from flour and starch industries, respectively.

Product	pH	COD (g L^{-1})	BOD (g L^{-1})	TA [1]	VFA[2]	TN [3]	TP [4]	TS [5]	CN [6]	Reference
Flour	4.6	65	n.a.[7]	3.41	n.a.	1.73	0.70	58	n.a.	Silva (2009)
	4.5	141	n.a.	1.62	0.01	2.05	0.27	n.a.	n.a.	Araújo et al. (2012)
	6.63	14.3	12.21	n.a.	n.a.	0.36	0.04	0.007	0.012	Pinto (2008)
	4.0	15.7	10.3	n.a.	n.a.	n.a.	n.a.	3.45	0.04	Agarry et al. (2016)
	5.1	89.75	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.09	Rodrigues et al. (2021)
	6.2	79.48	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.02	Rodrigues et al. (2021)
	6.2	92.44	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.07	Rodrigues et al. (2021)
Starch	n.a.	8	6	n.a.	n.a.	0.17	0.04	6.02	<0.05	Cardoso et al. (2009)
	4.9	11	n.a.	n.a.	n.a.	0.53	0.09	n.a.	0.002	Sun et al. (2012)
	4.5	16	8	n.a.	0.07	n.a.	n.a.	14.34	n.a.	Thanwised et al. (2012)
	4.8	2	2	n.a.	n.a.	0.17	0.09	7.67	0.023	Lied (2012)
	5.7	10	n.a.	0.21	n.a.	0.07	0.02	n.a.	n.a.	Zempulski (2013)
	4.8	2.24	1.45	n.a.	n.a.	0.02	0.09	7.66	0.023	Trevisan et al. (2019)
	4.5	14.7	6	n.a.	n.a.	n.a.	n.a.	n.a.	0.005	Acchar; Monteiro (2021)

[1]Total alkalinity ($gCaCO_3$ L^{-1}); [2]Volatile fatty acids in g acetic acid per liter (gHAc L^{-1}); [3]Total nitrogen (g L^{-1}); [4]Total phosphorus (g L^{-1}); [5]Total solids (g L^{-1}); [6]Total cyanide (g L^{-1}); [7]not analyzed

3.5.1 Biosurfactants

Surfactants are synthetic (originated from petroleum) or bio-based (produced mainly by microorganisms) compounds, with a molecular structure that presents amphiphilic characteristics, which makes them capable of interacting with polar and non-polar compounds, reducing surface and interfacial tensions between immiscible fluids. Thus, surfactants have a wide range of industrial applications as detergents, emulsifiers, defoamers, biopesticides, among others (JAUREGI; KOURMENTZA, 2019; JOY; RAHMAN; SHARMA, 2017).

The market of bio-based surfactants (biosurfactants) has been increasing yearly, very likely due to the consumer demand for eco-friendly products and processes. In addition, environmental laws favor the biosurfactants market (AHUJA; SINGH, 2020; VICENTE et al., 2021).

Recently, with the coronavirus pandemic, there has been a drop in the production of biosurfactants due to shortages of raw material supplies, caused by travel prohibition and manufacture units closure impositions around the world. This fact can reduce its use in agricultural defensives, oil fields, or textile industries until everything stabilizes again. However, to contain the virus outbreak, the lockdown strategy was adopted. As a result, people were spending more time at home, increasing the use of hygiene and personal care, and household cleaning products. Therefore, the search for greener versions of products also increased considerably (AHUJA; SINGH, 2020).

Although biosurfactants are promising, the high production cost, compared to analogs of synthetic origin, still makes it difficult for this market to grow. As a strategy to reduce production costs, the use of residues as substrates to produce these natural surfactants has been investigated since these synthetic substrates usually represent approximately 30% out of the total production costs (DE OLIVEIRA SCHMIDT et al., 2021a; MOHANTY et al., 2021; TAN; LI, 2018). This strategy, in addition to transforming a waste into a product with high added value, reducing waste treatment and biosurfactant production costs, makes the product even more environmentally friendly (MOHANTY et al., 2021).

Research involving the replacement of synthetic substrates by agro-industrial residues such as vegetable oils, lignocellulosic residues, residues from the dairy industry, or even residues rich in starch, as cassava wastewater in the production of biosurfactants have been frequent (MOHANTY et al., 2021; ZANOTTO et al., 2019).

As well as their synthetic analogs, biosurfactants reduce surface and interfacial tension as their concentration is increased in the medium until the maximum possible reduction (variable according to the type of surfactant or even the producing strain) is obtained when reaching the critical micelle concentration (CMC). Above CMC, these surfactants form self-aggregating structures that can be micellar, hexagonal, cubic, or lamellar (diameter 2 to 3 orders of magnitude

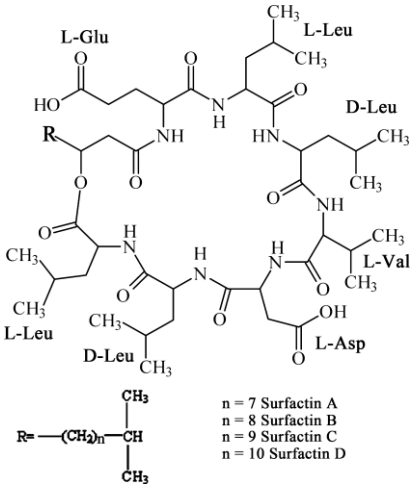
larger than the isolated molecule) (JAUREGI; KOURMENTZA, 2019; VICENTE et al., 2021; ZANOTTO et al., 2019).

It is also important to point out that a wide range of microorganisms can produce biosurfactants (RON; ROSENBERG, 2001). In addition, the same strain can produce more than one type of biosurfactant; for example, *Bacillus subtilis* can produce surfactin, iturine, fengycin, and subtilisin (VICENTE et al., 2021). Natural surfactants can be classified into glycolipids, lipopeptides, lipoproteins, phospholipids, polymeric biosurfactants, and particulate surfactants. Among the classifications cited, glycolipids (mainly rhamnolipids, sophorolipids, and mannosylerythritol lipid) and lipopeptides (mainly surfactin) are the most known and studied due to their great efficiency in reducing surface and interfacial tension (Jauregi and Kourmentza, 2019). Besides, lipopeptides are also highlighted due to their biological activities (Maier, 2003; Vicente et al., 2021).

3.5.1.1 Surfactin

Surfactin is a lipopeptide-type biosurfactant and was identified as a secondary metabolism product of *Bacillus subtilis* in 1968. Surfactin was initially described as a lipopeptide composed of L-aspartic acid, L-glutamic acid, L-valine, L-leucine, D-leucine (1:1:1:2:2) and unidentified fatty acids with a long chain of hydrophobic fatty acid (ARIMA; KAKINUMA; TAMURA, 1968). Currently, the structure of surfactin and its four isomers (Surfactin A–D) is known (Figure 11). Structural variation in the chain length of the fatty acid component or substitution of the amino acid components of the peptide ring may result in a change in the physicochemical properties of surfactin (JAHAN et al., 2020).

Figure 11 - Chemical structures of surfactins



Source: Chen, Juang, and Wei, (2015)

Surfactin is one of the most powerful biosurfactants, as it can reduce the surface tension of water from 72 mN m^{-1} to 27 mN m^{-1} (FARIAS et al., 2021). It is produced by various strains (e.g., *Bacillus pumilus, Bacillus mojavensis, Bacillus licheniformis, Bacillus amyloliquefaciens*), and mainly *Bacillus subtilis* (CHEN; JUANG; WEI, 2015). Metabolically, the production of surfactin is correlated to microbial sporulation, which can lead to low surfactin production yield. Thus, often the optimization of surfactin production is evaluated (DRAKONTIS; AMIN, 2020; MARKANDE; PATEL; VARJANI, 2021).

Surfactin has potential application in several areas, such as therapeutic, environmental, and agricultural. The therapeutic applications of surfactin are antibacterial, antiviral, antifungal and antimycoplasma, due to ion-conducting channel formation in bacterial lipid bi-layer membranes (CHEN; JUANG; WEI, 2015; SAŁEK; EUSTON, 2019). It is worth noting that surfactin is a potent immunosuppressive agent and may serve as an alternative to immunosuppressive drugs used in autoimmune diseases (allergy, asthma, arthritis, and diabetes) and organ transplantation (SAJID et al., 2020). In addition, acts as an anti-inflammatory, one it inhibits the activity of phospholipase-A_2 (SAJID et al., 2020). There are reports of anticancer activity of surfactin, acting as an inhibitor of proliferation and inducing apoptosis breast cancer cells (CAO et al., 2010) and lung carcinoma cells (ROUTHU et al., 2019).

The ability of surfactin to reduce surface tension makes it an interesting product for personal care and detergent formulations, as it has low toxicity and non-irritating properties (MARKANDE; PATEL; VARJANI, 2021). Moreover, surfactin has anti-aging, anti-photoaging, anti-wrinkle, anti-oxidation, and collagen inducer properties - skin restorative functions (SAŁEK; EUSTON, 2019).

Regarding environmental applications, Mulligan (2009) highlighted the use of surfactin as a stimulating agent in bioremediation processes of soils contaminated with petroleum hydrocarbons; nevertheless, it was emphasized that high concentrations can inhibit the biological process, due to the antimicrobial characteristics of surfactin. Furthermore, surfactin is reported as a stabilizing agent for the production of nanoparticles (MARKANDE; PATEL; VARJANI, 2021; MULLIGAN, 2009). In agriculture, surfactin has been reported to be effective against phytopathogens, including those that have already acquired resistance to other products (SAŁEK; EUSTON, 2019).

A major challenge of large-scale surfactin production is the production cost. The culture medium can represente from 30 to 50% out of the total cost of production (DE OLIVEIRA SCHMIDT et al., 2021a). The use of agro-indutrial residues is a interesting alternative for culture medium. Several agro-industrial residues rich in carbon have been successfully tested as substrate for surfactin production (BARROS; PONEZI; PASTORE, 2008), as potato waste (FOX; BALA, 2000), whey poder (CAGRI-MEHMETOGLU; KUSAKLI; VAN DE VENTER, 2012), rice straw (ZHU et al., 2013), brewery waste (NAZARETH et al., 2021; PARASZKIEWICZ et al., 2018).

Cassava wastewater has been used successfully as susbtrate for surfactin production in several studies. Nitschke and Pastore, 2006 produced a lipopeptide surfactant with characteristics similar to surfactin synthesized by a *Bacillus subtilis* strain using cassava as substrate. Andrade et al., 2016 used a mixture of whey, activated carbon and cassava (27.7-34 g L^{-1}, 25 g L^{-1} e 74 g L^{-1}, respectively) as substrate for *B. subtilis* and obtained ~27.07 mg L^{-1} of surfactin. Cassava wastewater stands out as a agro-industrial residue rich in carbon and essential nutrients to production of surfactin, in addition, it has advantages over other agro-industrial for being produced all year round and with low soil fertility requirements (DE OLIVEIRA SCHMIDT et al., 2021a).

Surfactin purification is a stage in the production process that remains a challenge, mainly due to the subtle variations that occur in the surfactin molecules (isomers), as the similarities between these molecules make the separation and purification process difficult. The presence of isomers makes it difficult to purify the product when a single isomer is needed, as in the case of application in the pharmaceutical industry. On the other hand, it facilitates the separation of a family of molecules from the other impurities; thus, a partially purified surfactin mixture suitable for applications that require a lower degree of purity (e.g. environmental applications) can be obtained directly from the culture supernatant (RANGARAJAN; CLARKE, 2016). Several levels of purification can be achieved: a partial purification (when impurities such as proteins, amino acids, and unconsumed substrate remain); a purified product without the impurities, but with a mixture of isoforms; and an ultra-purified product containing only one isoform (RANGARAJAN; CLARKE, 2016).

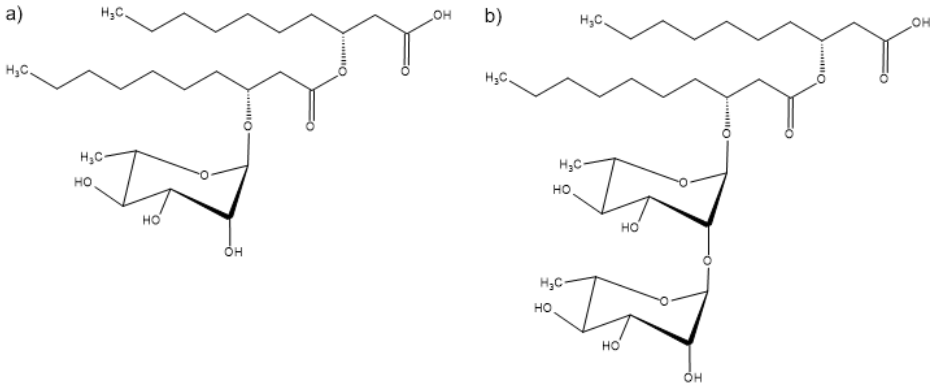
Surfactin is an excellent alternative to chemical surfactants, and the characteristics of surfactin make it highly interesting for industries related to human health, the environment, and agriculture (SAŁEK; EUSTON, 2019). Nevertheless, studies are needed to enable its production on an industrial scale, especially in terms of reducing the costs of the process and purification technologies.

3.5.1.2 Rhamnolipids

Belonging to the glycolipids group, rhamnolipids (RLs) are anionic biosurfactants, mainly produced by *Pseudomonas aeruginosa* (ELAKKIYA et al., 2020). A RL molecule is composed of a lipophilic part (β-hydroxy fatty acids chains) and a hydrophilic part (rhamnoses), which can vary between mono-rhamnose and di-rhamnose, with one and two rhamnose residues in the molecule (Figure 12) (de Oliveira Schmidt et al., 2021). Due to their physicochemical and biological properties such as surface-active, emulsifying capacity, antimicrobial activity, among others, rhamnolipids can be applied for bioremediation, advanced oil recovery, pharmacology and cosmetology, and food production (LIU et al., 2018) .

The main carbon sources for RLs production are glucose and glycerol (JI et al., 2016). However, the characteristics of the carbon source directly affect the RL production yield (VARJANI et al., 2021). Thus, hydrophobic carbon sources such as soy, olive, and sunflower oils, and petroleum-derived hydrocarbons such as kerosene and diesel have been evaluated for RL production (JI et al., 2016; RADZUAN; BANAT; WINTERBURN, 2017; WEI; CHOU; CHANG, 2005).

Figure 12 - Chemical structure of a) mono-rhamnolipid and b) di-rhamnolipid



Adapted from de Oliveira Schmidt et al. (2021).

The use of agro-industrial residues such as cassava residues and cassava wastewater (alternative culture medium) has been drawing attention due to its high production potential, low production cost, and inherent reduction discarded into the environment. Costa et al. (2009) used cassava wastewater as a carbon source and frying oil as an inducer for the production of RLs and polyhydroxyalkanoates with different strains of *Pseudomonas aeruginosa*. After 120 h of fermentation, it was noted production of 0.660 g L^{-1}, with a reduction in surface tension of 30 mN m^{-1} and the CMC of 0.0265 g L^{-1}.

Costa et al. (2010) studied the properties and applications of commercial RLs (JBR599 mixture) in comparison to those produced by *P. aeruginosa* L2-1 by using cassava wastewater and waste cooking oil as a substrate. 16 different rhamnolipids (homologues) were produced, in which their surfactant properties were very similar to commercial ones. Regarding RLs applications, the removal of 69% of the oil in contaminated sand was observed, as well as microbial activity against different bacteria (such as *Bacillus cereus*, *Micrococcus luteus*, *Staphylococcus aureus*). Therefore, it was proved that the use of this agro-industrial residue as a substrate has a high productive potential for RLs and is an interesting alternative aimed at reducing the environmental impact.

Suryanti et al. (2013) evaluated the *P. flourescens* sp. as a potential RLs producer by using in a culture medium containing cassava wastewater and other growth nutrients. The RLs production was confirmed by FT-IR and UV-vis spectral analyses. In addition, a reduction in the surface tension of water from 80 to 59 mN m^{-1} and a CMC of 0.715 g L^{-1} was observed. Concerning application, stability tests were carried out for the emulsion, formed until 30 days, using water-immiscible compounds such as kerosene, soybean oil, and lubricating oil. As well as the interfacial tension reduction test of 51-70% when using water-immiscible compounds such as kerosene, palm oil, and benzyl chloride. Similarly, Elakkiya et al., (2020) determined 0.34 g L^{-1} of RLs using *P. aeruginosa* TEN01. -

Based on the potential application of RLs in several industrial sectors, it is essential to investigate the use of alternative substrates such as the cassava wastewater and the optimization of the production steps.

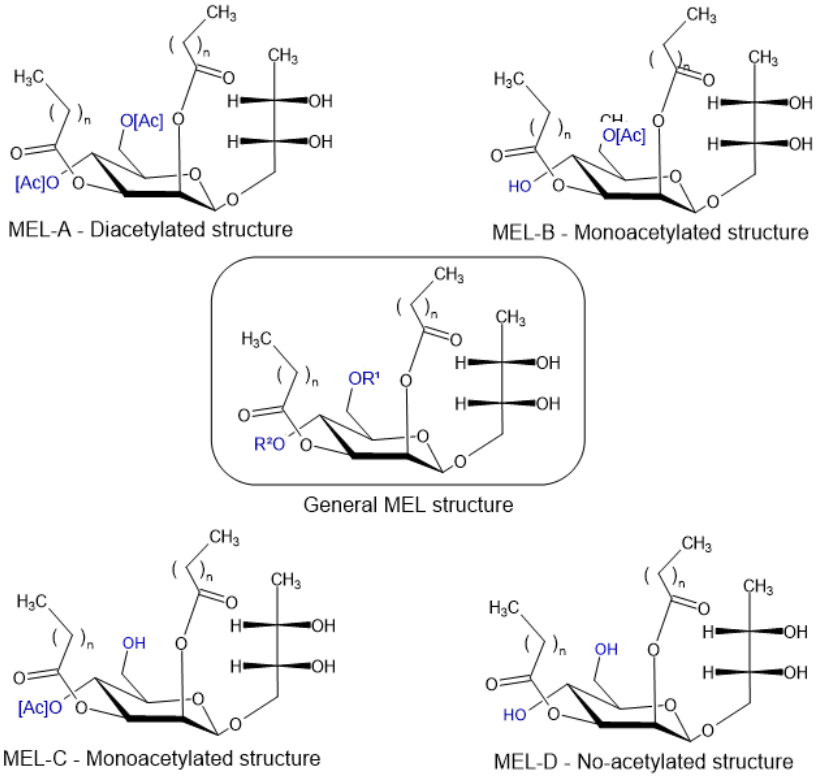
3.5.1.3 Mannosylerythriol lipids

The mannosylerythriol lipids (MELs) are one of the most promising classes of biosurfactants - glycolipids. The chemical structures of MELs vary according to the fatty acid chains and the degree of acetylation, named MEL-A, MEL-B, MEL-C, and MEL-D (COELHO et al., 2020; SHU et al., 2020; SIMIQUELI; DE ANDRADE; FAI, 2017) as shown in Figure 13.

Due to the chemical structure variation, MELs have different properties that can be applied in several areas of interest, such as antimicrobial activity against Gram-positive bacteria and pathogens, antioxidant for cell protection in cosmetic products aimed at skincare and other pharmaceutical and industrial products, as well as biological activity for medicinal purposes and human health (ARUTCHELVI et al., 2008; CERESA et al., 2020; COELHO et al., 2020; MORITA et al., 2015; NASHIDA et al., 2018).

Boothroyd et al. (1956) was the first reporting MEL production. The author used a submerged fermentation system with the fungus *Ustilado* sp.. More recent studies have investigated the production of MELs using a wide range of microorganisms such as *Schizonella melanogramma*, *Kurtzmanomyces* sp., and *Pseudozyma* sp. (SIMIQUELI; DE ANDRADE; FAI, 2017). In this sense, *Pseudozyma tsukubaenis* has gained great prominence for the production of MEL-B as a major component, since the species of *Pseudozyma* produce a mixture of different MEL molecules (ANDRADE et al., 2017; ARUTCHELVI et al., 2008; FUKUOKA et al., 2008).

Figure 13 - Variety of chemical structures of MELs according to acetyl substitution.



Adapted from Coelho et al. (2020).

Fermentation processes for the production of MELs in general use a hydrophobic carbon source (for example, soy oil), nevertheless the use of water-solubilized carbohydrates (cassava wastewater) has been drawing attention since there is an advantage on purification process (ANDRADE et al., 2017; FUKUOKA et al., 2008; KONISHI et al., 2008; SIMIQUELI; DE ANDRADE; FAI, 2017).

It is worth noting that the use of cassava wastewater as a cultivation medium for the production of MELs has been poorly investigated. Fai et al. (2015), described that the use of cassava wastewater for the production of MEL-B is a sustainable alternative. It was shown that the surface tension was reduced to 26.9 mN m^{-1}, approaching the value of 33.8 mN m^{-1} found by Fukuoka et al (2008), in the critical micelle concentration (CMC). Fai et al. (2015) speculate that the production of mono-acetylated MEL (MEL-B) is more correlated with the strain and the culture medium than the microorganism species itself. Fukuoka et al. (2008) studied 11 different strains, including *P. tsukubaenis* with glucose as a carbon source solubilized in the culture medium, showing the production of mono-acetylated MEL for *P. antarctica* and *P. parantarctica.* Thus, the use of cassava wastewater is a sustainable alternative, with low-cost and with a carbon source available in an aqueous medium for the production of MELs. Andrade et al. (2017), used the culture medium with cassava wastewater to scale the process in a bioreactor, for the production

of MEL-B. After 84 h of fermentation, the bioprocess obtained a production of 1.26 g L^{-1} of MEL-B and the surface tension was in the range of 50 mN m^{-1}. Therefore, it is evident that cassava wastewater may provide all essential nutrients to microbial biosurfactant producers.

MELs have wide industrial applicability, mainly pharmaceutical and medicinal industries, but more research is needed to comprehend the specific characteristics of the use of cassava waswater as an alternative culture medium, as well as process scale-up, and optimization.

3.6 CONCLUSION

This chapter aimed to explore the current state of the art and future trends for the production of biosurfactants from cassava wastewater. Both residues, liquid and solid, that are generated during the steps of cassava processing have some toxic compounds that need pre-treatment before being eliminated in the environment, others can also serve as a source of nutrients for the production of valuable products aggregate. Despite the limitations in its nutritional composition, cassava has a wide applicability potential, as presented here. Starch makes up about 80% of cassava, it has great gelatinization ability, and greater water binding capacity and viscosity compared to different starch sources. These technological properties allow its use in different biotechnological processes. Surfactin, ramnolipides and mannosylerythriol lipids can be produced using cassava wastewater residues, however they were reported at laboratory scale, only. The great potential of biosurfactants produced from liquid cassava husk residue, via fermentation, lead to a wide range of innovations in the area of food, medicine, personal care products, among others with biological activity. The use of cassava residues impact on the economy and also minimize the environmental damage. Biosurfactants have potent antimicrobial applications including antifungal, antibacterial, antimycoplasmal and antiviral activities. In recent years, the trend in using the biorefinery concept has made processes increasingly more challenging and innovative. It is essential to develop techniques for reusing the waste generated in the food processing stages.

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CHAPTER 4

This chapter presents the methodologies and results on the effect of specific hydrophobic inducers on surfactin production. Screening on the effect of inducers (yields of surfactin production and their kinetics), the surface tension properties, and the identification of the potential production of new surfactin homologues by HPLC analysis were studied and carefully described. This study was written in an article format to be further submitted.

4 HYDROPHOBIC INDUCERS TO ENHANCED SURFACTIN PRODUCTION USING CASSAVA WASTEWATER AS LOW-COST CULTURE MEDIUM: A PROSPECTION ON NEW HOMOLOGUES

Abstract

Biosurfactants are amphipathic molecules synthesized by living cells, with numerous potential applications in the areas of health and the environment, among others. However, the high cost of production limits the massive applications of biosurfactants. A recent review on the subject showed that biosurfactant productivity can be easily increased by adding inducers to the culture medium, which stimulate microbial growth and also trigger the metabolism of biosurfactant production. In this study, it was investigated the effect of different hydrophobic inducer concentrations (1, 2, 5, and 10% - soybean oil, palmitic acid, and oleic acid) in the production of surfactin by *B. subtilis* ATTCC 6633 using cassava as carbon source. The cultivation was carried out under submerged fermentation at 30 °C, 150 rpm for 72 h. The main fermentation parameters analyzed were bacterial growth, pH, surface tension and consumption of major sugars. Regarding the surfactin production, the main parameters analyzed were the influence of acid concentrations in increasing surfactin production and in inducing homologues. No major changes in pH were observed. The surface tension decreased by 40% after 12 h, indicating surfactin production. The total consumption of soluble sugars (sucrose, glucose, and fructose) was observed to all experiments. Palmitic acid, at 5%, presented the highest yield in terms of surfactin production, since it reached a maximum yield of ≈1.3 $g.L^{-1}$ (pure surfactin). The inducers led to, very likely, to the production of different surfactin homologues, in particular the oleic acid - high diversity. Thus, the new structures of surfactin, problably, will show new(s) biological activities.

Keywords: Lipopeptides, palmitic acid, oleic acid, biosurfactant inducers.

4.1 INTRODUCTION

Surfactin is a natural biosurfactant, commonly produced by bacteria of the genus *Bacillus* (e.g. *B. pumilus, B. licheniformis, B. amyloliquefacien*, and mainly by *B. subtilis*) (CHEN; JUANG; WEI, 2015; DING et al., 2021). It has numerous bioactivities (e.g. anti-inflammatory, antifungal, antiviral, and biostimulant) and exceptional surfactant properties that show its potential use in several areas such as environmental, agricultural, petroleum and pharmaceutical, among others (DING et al., 2021).

However, the high cost of production and purification of surfactin limits the scale-up and its consequent industrial application, mainly due to its raw material - which represents more than 50% of the total cost of production of the biosurfactant (GAUR et al., 2022; VICENTE et

al., 2021). Thus, using techniques with high yield and low cost in research related to biosurfactants has become imperative.

A promising alternative for reducing the cost of surfactin production is the use of agro-industrial residues, such as liquid residue generated in the production of cassava flour. According to Acchar & Da Silva, (2021), considering the concentration of organic load and the toxic effect of this agro-industrial residue, it is possible to infer the high source of pollution that it represents. In this sense, Nitschke and Pastore (2006) reported that cassava wastewater stands out as an agro-industrial waste. In its composition, high levels of carbon and essential nutrients are identified that benefit surfactin production. Nitschke and Pastore, (2004) reported using cassava wastewater for the production of surfactin, using two different strains of *B. subtilis*, as a result ≈3.0 g of crude surfactin/L was produced. Similarly, Andrade et al., (2016) reported the surfactin production by *B. subtilis* LB5a using cassava wastewater as an alternative substrate, in which ≈1.01 $g.L^{-1}$ of pure surfactin was achieved.

Despite the complexity in the use of agro-industrial residues such as the standardization of the substrate due to the natural variation of the composition, costs of storage, transport, and purification, and/or previous residual treatment, among others, it is possible to establish controlled processes for the production of surfactin with pre-existing chemical characteristics - established, and enhance their products with the use of hydrophobic inducers (DE OLIVEIRA SCHMIDT et al., 2021a). Biochemically, the productivity of biosurfactants can be easily increased by adding inducers to the culture medium, which stimulate microbial growth and also trigger the metabolism to produce this biosurfactant (PATHANIA; JANA, 2020a). The inducers are related to the size of hydrophobic chains of biosurfactants and, consequently, greater stability and efficiency.

Thus, the effect of complex hydrophobic sources on yeasts was described by Zinjarde and Pant, (2002). They observed that surfactant biosynthesis using soluble substrates as carbon sources is not enabled by *Yarrowia lipolytica* NCIM 3589. However, in mixed media containing crude oil and alkanes (C_{10} - C_{18}) the authors described a significant increase in bioemulsifier production. Decesaro et al., (2013) described the production of biosurfactant by *Pseudomonas aeruginosa* and *B. pumilus* using mineral medium supplemented with diesel oil and soybean oil as hydrophobic inducers at 1% and 2%. The lowest surface tension was obtained with 1% soybean oil for both treatments.

Biosurfactant inducers are mainly composed of hydrophobic molecules (e.g. soybean oil and olive oil - a pool of saturated and unsaturated fatty acids, proteins, and vitamins) (DE OLIVEIRA SCHMIDT et al., 2021a). However, there is little information on the effect of these specific molecules (e.g. oleic acid and palmitic acid) on the surfactin production and, mainly its chemical structure.

Therefore, soybean oil (SO), palmitic (PA), and oleic (OA) fatty acids were evaluated as hydrophobic inducers in the production of surfactin by *B. subtilis* ATCC 6633 using cassava

wastewater as alternative culture medium. To the best of our knowledge, this report is the first study that details surfactin production using cassava wastewater associated with hydrophobic inducers.

4.2 MATERIALS AND METHODS

4.2.1 Generals

Brain and Heart Infusion Broth (BHIB) (Kasvi, Brasil), glucose (Sigma-Aldrich, USA), fructose (Sigma-Aldrich, USA), sucrose (Sigma-Aldrich, St. Louis, USA), oleic acid (Neon, Brazil), palmític acid (Neon, Brazil), soybean oil (Soya, Brazil), acetonitrile (J.T. Baker, Brazil), trifluoroacetic acid (Êxodo Científica, Brazil), sulfuric acid (H_2SO_4) (Synth, Brazil), surfactin (Sigma-Aldrich ≥ 98%, Brazil), hydrochloric acid (HCl) (Neon, Brazil), sodium hydroxide (NaOH) (Lafan, Brazil), phosphoric acid (H_3PO_4) (Synth, Brazil), hydrofluoric acid (HF) (Sigma-Aldrich, USA), nitric acid (HNO_3) (Sigma-Aldrich, USA), boric acid (H_3BO_3) (Sigma-Aldrich, USA), cellulose acetate membrane 0.22 μm (Sartorius Stedim Biotech, Germany).

4.2.2 Collection, transport, pretreatment, storage, and characterization of substrate

Approximately 50 L of effluents from cassava flour processing were collected in a flour industry (Rocha & Filho Ltda - SC - Brazil) (28.558105 latitude, 49.5560082 longitude) and transported under refrigeration. The cassava wastewater was boiled at 100 °C for 3 min to facilitate the removal of insoluble solid material and centrifuged at 10^4 *g* for 10 min (Jouan B4, USA). The supernatant was stored at -22 °C (DE ANDRADE et al., 2016). Natural pH of the medium was 5.8 and was not adjusted. The substrate was characterized by mineral fraction analysis by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), major sugars by High-Performance Liquid Chromatography (HPLC), and physical-chemical characterization. The same cassava wastewater batch was used for all experiments.

4.2.2.1 Inductively Coupled Plasma Optical Emission Spectrometry

The samples of the cassava wastewater (flour) were pre-digested (mixture of 4 acids, 2.5 mL of H_2SO_4, 2.5 mL of H_3PO_4, 3.0 mL of HNO_3, and 2 mL of HF) for 30 min at room temperature. Then the samples were microwaved (model MARS6, CEM Corporation) 600 W, ramp of 1-5 min, hold for 45 min, from 50 to 210 °C, and as the guard temperature 250 °C. After the digestion, the samples were cooled at room temperature and 0.9 g of H_3BO_3 was added to neutralize the HF. The samples were microwaved again (neutralization). After cooling at room

temperature, the samples were filtered on medium filter paper and swelled with HNO_3 5% in 25 mL flasks. The samples were analyzed in triplicate in ICP-OES (Agilent Technologies 710 Series ICP-OES; ICP Expert 2 software), identifying and quantifying the composition of metals, semi-metals, and non-metals present in the sample (BARROS; PONEZI; PASTORE, 2008).

4.2.2.2 Quantification of sugars by HPLC

The quantification of glucose, fructose, and sucrose were carried out by HPLC (Shimadzu, Japan), equipped with a refractive index detector and Bio-Rad HPX-87H column (300 mm×7.8 mm) using 5 mM sulfuric acid as eluent at 35 ºC, flow rate of 0.4 $mL.min^{-1}$ and 10 µL sample. Samples were centrifuged (10^4 *g*, 25 °C for 10 min). The supernatant was diluted 1:3 and filtered with membrane pore size 0.22 µm and calculated from standard glucose, fructose, and sucrose curves (LEONARSKI et al., 2021).

4.2.2.3 Physico-chemical characterization cassava wastewater

The physicochemical characterization of the cassava wastewater was performed by the analysis laboratory of the Department of Food Sciences and Technology - Florianópolis - UFSC. Resistant starch (Method: AOAC, n°. 991.16), total acidity (Method: IAL, 016/IV:2005), total sugars (Method: IAL, 040/IV:2005), phosphorus (Method: IAL, 031/IV:2005), total nitrogen (Method: AOAC, n°. 991.20) and total solids (Method: IAL, 012/IV:2005).

4.2.3 Fermentative process

4.2.3.1 Microorganism and inoculum

The culture of *Bacillus subtilis* ATCC 6633 was periodically replicated in BHIB broth and kept under refrigeration (10 °C). The inoculum was prepared from the culture inoculated in a Petri dish with BHIB, transferred to Erlenmeyer flasks containing 50 mL BHIB and incubated in a shaker (Tecnal, TE-424, Brasil) at 30 ºC, 150 RPM for 12 h. Before incubation, 0.5 optical density was read in the 600 nm range for inoculum standardization, considering a previously constructed standard curve (BARROS; PONEZI; PASTORE, 2008; DE ANDRADE et al., 2016).

4.2.3.2 Fermentation parameters and sampling

The assays were carried out in Erlenmeyer flasks of 250 mL containing 150 mL of culture medium consisting of cassava wastewater (pH 5.8) supplemented with different concentrations (1, 2, 5, and 10% ($m.v^{-1}$)) of inducers (palmitic acid (PA), oleic acid (OA) and soybean oil (SO), separately) and previously sterilized at 120 ºC, 1 atm for 20 min. The flasks were inoculated with the culture standardized at a concentration of 7% ($v.v^{-1}$). For each inducer, three experiments (validation) were performed: 150 RPM 30 °C for 72 h (BARROS; PONEZI; PASTORE, 2008). Samples of the culture medium were initially collected at intervals of 4 h, during the first 12 h of fermentation. The next 60 h of the process were followed with sampling every 24 h. To subsequently perform the analytical procedures.

4.2.3.3 Bacterial growth

Microbial growth was followed by turbidimetry analysis at 600 nm using a UV/Visible spectrophotometer (Femto, Cirrus 80, Brazil) and pH variation (AZ 86505, Taiwan).

4.2.3.4 Measurement of surface activity

The samples from the culture medium were initially centrifuged at 10^4 *g* for 20 min (Andrade et al. 2016). Afterward, the surface tension was measured in the supernatant phase using a goniometer (ramé-Hart 250, USA) by the pendant drop method (PDM) (BERRY et al., 2015; HANSEN; RØDSRUD, 1991). The software (Drop Image) performed 10 repetitions of measurements for each analysis in triplicate.

4.2.4 Surfactin characterization

4.2.4.1 Surfactin extraction - cellular withdrawal and acid precipitation

At the end of cultivation, the samples were centrifuged (10^4 *g* for 10 min) for the withdrawal of cells. The pH of the supernatant was adjusted to 2 using HCl solution (3 and 1 M) and kept under resting decantation (24 h). After that, it was centrifuged (10^4 g for 10 min at 5 °C), the precipitate was collected, neutralized (pH 7) with NaOH solution (3 and 1 M), and lyophilized (Liotop L101, Brazil). The solid obtained was called crude surfactin and their final yield was analyzed. It was crushed and stored under refrigeration (-22 °C) (DE ANDRADE et al., 2016).

4.2.4.2 Surfactin concentration

To quantify surfactin production, 20 mg of sample were diluted with 1.0 mL methanol, filtered (0.22 μm pore filter), diluted to a 1:5 ratio, and subjected to HPLC analysis. The system consisted of an LCMS-2020 (Shimadzu Corp., Japan), equipped with a binary pump, autosampler, oven, and PDA detector. Stationary phase was a Kromasil® C18 (100A, 300 mm x 4.6 mm i.d.), whereas mobile phase consisted of ultrapure water (A) and 0.1% ($v.v^{-1}$) TFA-MeCN solution (B). Gradient elution was used as reported previously, with a modified total flow of 0.9 $mL.min^{-1}$ due to backpressure constraints. Volume injection was 50 μL, whist oven temperature remained at 30 °C throughout the analysis. PDA acquisition was set to 190-560 nm and monitored at 214 nm. Chromatogram analysis and area calculation was performed with LabSolutions software (v.5.75, Shimadzu Corp.). System calibration was done through external standard calibration with newly-acquired surfactin. The n-level standard curve ranged from 25 $mg.L^{-1}$ to 1600 $mg.L^{-1}$, with a deviation of 0.999.

4.2.5 Statistical analysis

Statistical analysis was performed using OriginPro® 8.5 software. Analysis of variance (ANOVA) was employed to verify the adequacy of the experiment.

4.3 RESULTS AND DISCUSSION

4.3.1 Characterization of substrate

The composition of the cassava wastewater varies according to environmental factors (such as edaphic, climatic, and cassava variety) (NEVES et al., 2014). The identification of these physicochemical characteristics is essential to determine its future applications. Therefore, in this study, it was investigated the profile of major sugars and micronutrients obtained in cassava wastewater of the cultivar SCS254 Sambaqui (Epagri), to produce surfactin (Table 10). Sucrose (27.08 ± 1.08 $g.L^{-1}$) is the main sugar present in cassava, although fructose (8.43 ± 0.39 $g.L^{-1}$) and glucose (8.94 ± 0.48 $g.L^{-1}$) were also present in significant concentrations. These values are expressive compared to the average composition of these sugars reported in the literature 4.51-14.90 $g.L^{-1}$ of fructose, 6.82-22.34 $g.L^{-1}$ of glucose and 18.7-22.0 $g.L^{-1}$ of sucrose, respectively (Nitschke and Pastore, 2006).

Table 10 - Composition of cassava wastewater of the cultivar SCS254 Sambaqui (Epagri)

Components	**Concentration**
Resistant starch	0.50 g/100 g
Total acidity	1.56 mL de sol. N/100 g
Total sugars	3.00 g/100 g
Total nitrogen	<0.30 g/100 g
Total solids	4.92 g/100 g
Major sugars	
Sucrose	27.08 ± 1.08 g/L
Glucose	8.94 ± 0.48 g/L
Fructose	8.43 ± 0.39 g/L
Mineral fraction	
Phosphorous	33.82 mg/100 g
Potassium	2381 mg/L
Calcium	489 mg/L
Magnesium	519 mg/L
Manganese	3.54 mg/L
Zinc	3.02 mg/L
Iron	0.447 mg/L
Sodium	95.4 mg/L
Silver	0.129 mg/L
Aluminum	0.870 mg/L
Cadmium	0.035 mg/L
Chrome	0.021 mg/L
Chumbo	0.093 mg/L
Copper	0.559 mg/L
pH	5.8 ± 0.08

Source: authors

The analyzed cassava wastewater proved to be a very rich residue in nutrients. In addition to Fe^{2+}, Mn^{2+} and Mg^{2+}, cassava wastewater has other micronutrients (e.g. phosphorus, potassium and zinc) essential for microbial cell growth. These components make cassava wastewater a promising substrate, since it is composed of a significant amount of nutrients and does not require supplementation. These nutrients act as cofactors of important enzymes in microbial metabolism, for example, the manganese ion may be the most important cofactor of glutamine synthetase. This enzyme is very important for organisms' assimilation of organic nitrogen (de Oliveira Schmidt et al., 2021; Deshpande et al., 1981; Zanotto et al., 2019). The Mn^{2+} concentration was 3.54 $mg.L^{-1}$, a significant value compared to the supplemented synthetic medium used by Wei and Chu (2002) of 1.1 $mg.L^{-1}$. According to the authors, Mn^{2+} significantly increased cell growth and surfactin production. Similarly, Gudiña et al. (2015) reported that the production of surfactin by *B. subtilis* using corn liquor, when supplemented with Mg^{2+}, Mn^{2+} and Fe^{2+} (1 mM) showed a higher production of the biosurfactant 4.829 ± 0.193 $g.L^{-1}$ (3.6 times). Fe^{2+} is also considered an important micronutrient for the production of biosurfactants. The analyzed concentration of Fe^{2+} in cassava was 0.447 $mg.L^{-1}$. According Wei and Chu (1998) an iron-enriched substrate results in increased biomass concentration and surfactin production can

be increased. Recently Tsipa et al. (2021) reported the use of $FeSO_4$, $FeCl_3$ and $Fe(NO_3)_3$ at various concentration levels to evaluate the possible improvement of biosurfactant production. The threshold concentration of Fe^{2+} compounds between increased biosurfactant formation and microbial toxicity was 0.1 mM. $FeCl_3$ improved the biodegradation of oil well drilling residues and more than doubled the yield of biosurfactant formation, determining an optimization strategy for its production.

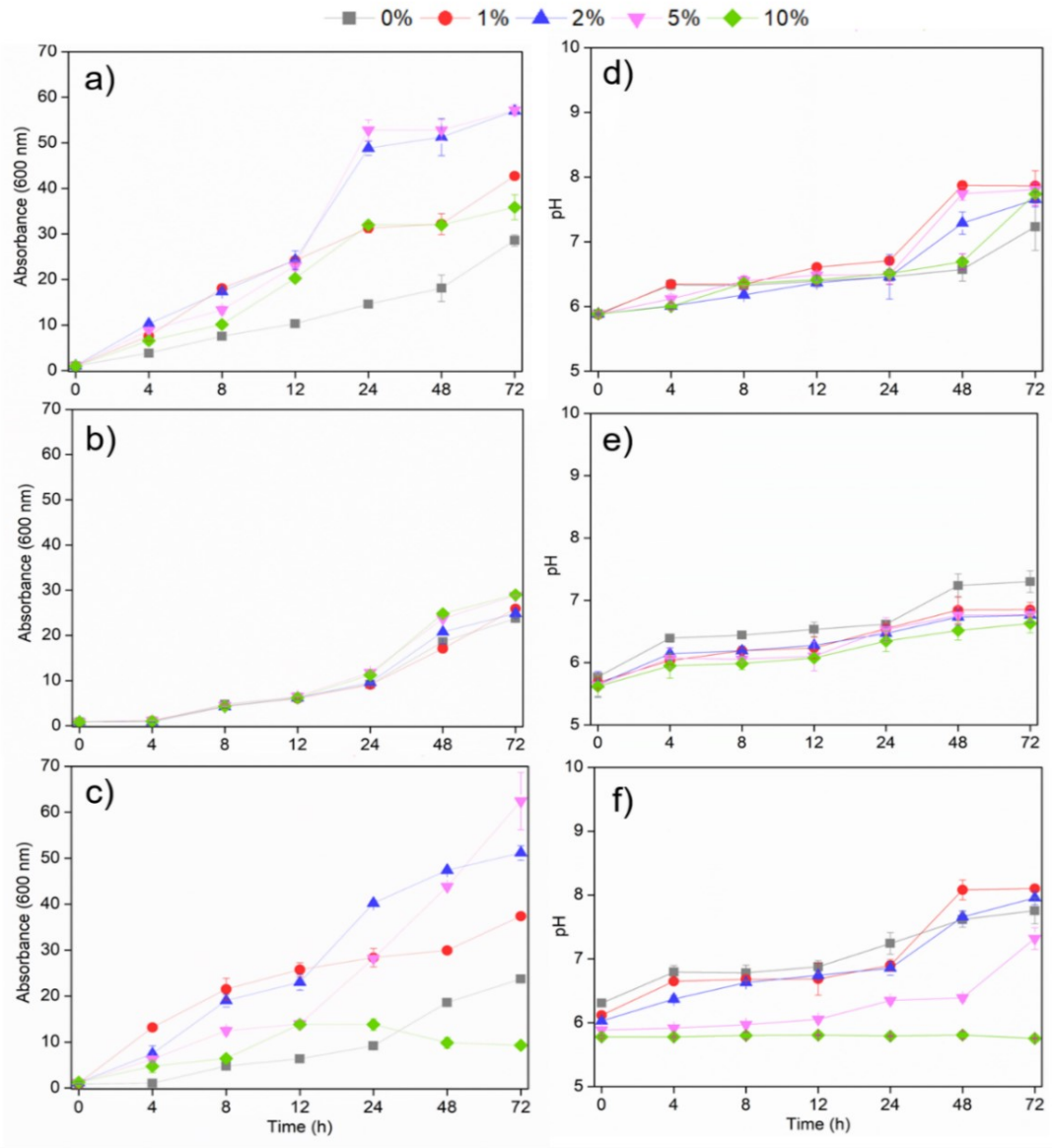
Cassava can be considered a potential carbon source used as a substrate in biotechnological processes, mainly by different microorganisms producing biosurfactants (ACCHAR; DA SILVA, 2021; NITSCHKE; PASTORE, 2006). Becoming a viable alternative for reducing operating costs and optimizing industrial processes.

4.3.2 Fermentative process

Hydrophobic inducers can easily increase cell growth and consequently the production of biosurfactants. However, there is little information correlating biosurfactant inducers and metabolic pathways. Therefore, reports as Ramírez et al. (2015) that detailed the efficiency of different inducers in the production of rhamnolipid and surfactin from *P. aeruginosa* and *B. subtilis*, respectively are fundamental. The authors report that the concentration (2, 5 and 10% $w.v^{-1}$) of inducer (residual olive oil and glycerol) directly affected cell growth, excepting treatments with 2%, the *P. aeruginosa* fermentation with glycerol yielded the highest values of dry biomass (2 and 4.5 $g.L^{-1}$) at 5 and 10% concentrations. However, in the case of *B. subtilis*, the biomass increased significantly (2.1, 3.1 and 3.8 $g.L^{-1}$) with the use of glycerol, whereas the residual olive oil produced led to lower biomass (1, 0.8 and 0.5 $g.L^{-1}$). The authors concluded that higher concentrations of inducers produced high biomass values for all experiments, in particular for glycerol.

The growth kinetics of *B. subtilis* ATCC 6633, related to pH and growth, using cassava as a substrate associated with hydrophobic inducers, are illustrated in Figure 14. The presence of hydrophobic inducers (SO and OA) at concentrations of 2% and 5% increased cell growth (Figure 14a and 14c). After 24 h of the fermentation process, the increase of biomass was ≈3.5 and ≈3.7 times using soybean oil as inducer and ≈4.5 and ≈3.2 times for oleic acid, respectively.

Figure 14 - Cell growth over time (a) soybean oil, (b) palmitic acid, and (c) oleic acid. pH variation (d) soybean oil, (e) palmitic acid, and (f) oleic acid.



In general, the production of biosurfactants is triggered by *quorum sensing* - small signaling molecules (e.g. intra and extracellular autoinducers) - that induce or repress metabolic pathways, including gene expression correlated with surfactin production (Zhao et al., 2020). The presence of changes in the chemical structure of autoinducers and similar molecules that compete for the sensor protein binding site can have a negative effect on *quorum sensing* communication (KALAMARA et al., 2018). According to Galloway et al. (2011) several *quorum sensing* are antagonist and agonist molecules. Among the natural products capable of inhibiting *quorum sensing*, flavonoids and some long-chain fatty acids are considered potent inhibitors (Favero 2017).

This phenomenon may be related to substrate supplementation with 10% OA. A reduction in cell growth (≈3 times) was observed at 72 h (Figure 14c). This improvement proved that this

inducer can have a suppressive effect on bacterial growth and consequently on the production of surfactin (Figure 16c). Pepi et al. (2013) reported that OA at a concentration of ≈11% associated with alternative substrates could also inhibit the production of biosurfactants by *Bacillu*s spp.. Therefore, specific compounds can impair *quorum sensing* detection, corroborating the decrease in the concentration of these autoinducer molecules due to the reduction in cell density.

The inhibition of cell growth was not observed when using soybean oil at this concentration (10%) (Fig. 14a). For palmitic acid, there was no significant difference between concentrations in 24 h. However, at the end of the experiment (72 h) it was observed a significant increase (≈1.2 times) in cell growth using concentrations of 5% and 10% (Figure 14b).

Fermentations take place over a wide range of pH values. The literature reports acidic pH values for effluents from cassava flour production in the range of 3.6-4.4, respectively (Barros et al., 2008; Santos et al., 2017). Low pH values support the loss of nutrients (e.g. potassium and nitrogen), resulting in reduced cell growth (AMARAL, 2009). Segundo Brenner et al., (2005) for *B. subtilis* the ideal growth pH is in the range of 5.5 and 8.5. A pH of 5.80 ± 0.08 was determined in the investigated effluent, ideal for cultivating this microorganism. In all the tests, the pH was stable, in all the conditions studied, varying in the range of 6.5 and 8 (Figure 14). Therefore, it was observed that the use of different concentrations of inducers did not negatively influence the pH variation of the medium since all the tests presented pH close to neutrality. Table 11 shows the consumption of sugars in 72 h of fermentation.

Table 11 - Sugars consumption by *B. subtilis* ATCC 6633 growing in cassava wastewater

Inducer	***Palmitic acid***				***Oleic acid***				***Soybean oil***			
	Carbohydrates consumption at 72 h (%)											
	S[1]	G[2]	F[3]	T[4]	S	G	F	T	S	G	F	T
0%	100	100	96.67	99.37	100	99.34	91.71	98.36	100	100	95.87	99.27
1%	100	100	9568	99.17	100	100	86.88	97.87	100	100	97.67	99.59
2%	100	100	99.93	99.99	100	100	91.64	98.62	100	100	98.61	99.75
5%	100	100	99.37	99.87	97.90	100	99,39	98.54	100	100	99.69	99.94
10%	100	100	100	100	57.07	18.02	75.85	45.78	100	100	92.90	98.74

[1]Sucrose; [2]Glucose; [3]Fructose; [4]Total major sugars.

The total consumption/conversion of major sugars determined at the end of the process was ≈100% ($m.v^{-1}$) in most treatments. Similarly Nitschke and Pastore, (2006) reported sucrose exhaustion after 48 h of cultivation. However, the total carbohydrate content was not completely consumed after 72 h. Thus, it was possible to conclude that a decrease in sucrose levels and, consequently, an increase in glucose and fructose, observed after 12 h, is due to the hydrolysis of

sucrose. The authors also correlated the presence of maltose with the degradation of soluble starch, resulting in glucose and maltose.

Therefore, there is no negative evidence on carbohydrate consumption and surfactin production when the culture medium was supplemented with hydrophobic inducers at ideal concentrations, considering the depletion of major sugars in 72 h of fermentation. A promising alternative for optimizing surfactin production is to investigate the technical feasibility of fed-batch and chemostatic mode of operation that can be conducted with the formulated medium (e.g. cassava associated with inducers).

On the other hand, when the culture medium is optimized with OA (10%), the decrease in sugar levels is not evident at 72 h. Most carbohydrates were not completely metabolized in this treatment, reaching a lower conversion percentage (45%). According Dusane et al. (2010) the presence of hydrophobic inducers at high concentrations can lead to low oxygen transfer, alteration in metabolism and consequently reduced cell growth. Notably, there was a decrease in cell growth and surfactin synthesis in all treatments using the maximum concentration (10%) of inducers, which was aligned to Sheppard and Cooper, (1990) which reported that oxygen transfer is one of the key parameters for the production of biosurfactants by *B. subtilis*.

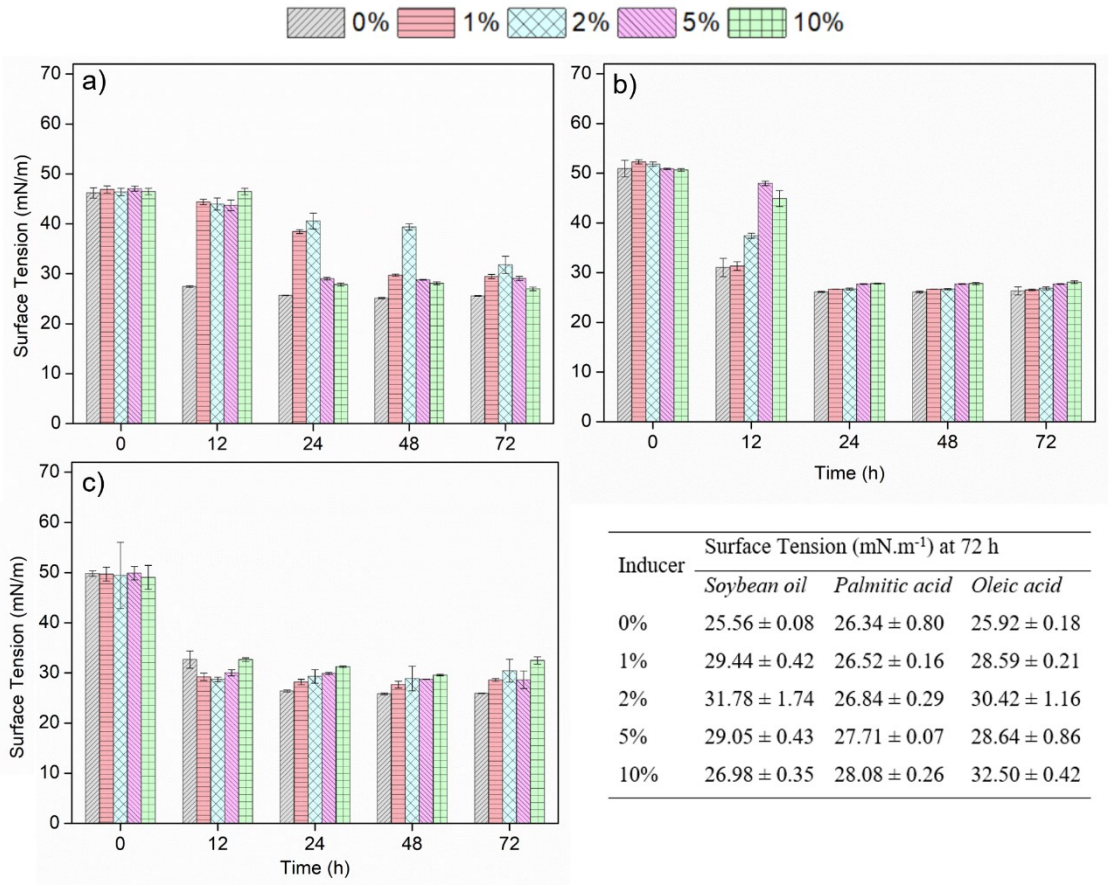
When using a substrate enriched with different carbohydrates, the microorganism first sequentially uses the preferred carbohydrates in the presence of non-preferred sources with significant amounts (e.g. hydrophobic inducers) (CHEN et al., 2020). However, there are reports on the ability of some microorganisms to co-use mixed substrates simultaneously as a carbon source. According Pathania and Jana (2020) cell growth by *P. aeruginosa* was enhanced using mixed carbon sources (glucose and fried soybean oil 2% $m.v^{-1}$). They obtained an accumulation of 0.638, 1.1 and 1.48 $g.L^{-1}$ using fried soybean oil (2% $m.v^{-1}$), glucose (2% $m.v^{-1}$) and a mixture of glucose and fried soybean oil 2% $m.v^{-1}$. In this sense, the authors reported that when multiple carbon sources are used, both are consumed, that is, the two metabolic pathways (glycolytic and lipogenic pathways) are activated to assist the increase cell growth and formation of the hydrophilic and hydrophobic moiety of the biosurfactant, instantly.

The results clearly indicate that the feasible concentration and profile of inducers would have greater resulting effects on cell growth or even the possible change in bacterial metabolism for surfactin production. Therefore, it is evident that an interesting alternative to reduce the cost of biosurfactant production is to use cassava wastewater supplemented with hydrophobic inducers. However, it is essential to carefully investigate all the effects promoted by the inducers in relation to bacterial metabolism.

4.3.3 Surface activity measurements

Figure 15 presents the results of the evolution of surface tension (ST) along the fermentation process time. Considering that the ST reduction occurred between 12 h and 24 h (Figure 15a, 15b and 15c).

Figure 15 - Variation of surface tension over time (a) soybean oil, (b) palmitic acid and (c) oleic acid.



Inducer	Surface Tension ($mN.m^{-1}$) at 72 h		
	Soybean oil	*Palmitic acid*	*Oleic acid*
0%	25.56 ± 0.08	26.34 ± 0.80	25.92 ± 0.18
1%	29.44 ± 0.42	26.52 ± 0.16	28.59 ± 0.21
2%	31.78 ± 1.74	26.84 ± 0.29	30.42 ± 1.16
5%	29.05 ± 0.43	27.71 ± 0.07	28.64 ± 0.86
10%	26.98 ± 0.35	28.08 ± 0.26	32.50 ± 0.42

It can be speculated that the surfactin production by *B. subtilis* ATCC 6633 occurred during the transition between the exponential and stationary phases of growth.

The broth ST (≈50 $mN.m^{-1}$) decreased during the fermentation period, reaching a minimum value of ≈26.5 and 28.6 $mN.m^{-1}$ using PA (1 and 2%) and OA (1 and 5%) as inducers (Fig. 15b and 15c). Using SO as an inducer, ST reduction was ≈29 and 27 $mN.m^{-1}$ at concentrations of 5 and 10% (Fig. 15a). According to Fooladi et al. (2016) biosurfactants were, mostly, secreted into the culture medium during the exponential growth phase, which is aligned to the obtained results, where the highest reduction of STs occurred during the microbial growth

phase between 12 (PA and OA) and 24 h (SO). There was no significant difference in ST reduction using PA as a hydrophobic inducer at different concentrations.

When taking into account only the surface tension reduction of the fermentation with PA (≈26,5 $mN.m^{-1}$) and OA (≈28.6 $mN.m^{-1}$) (Fig. 15b and 15c), it is noticed that this result is promising to what has already been reported in the literature for other microorganisms. Decesaro et al. (2013) achieved the highest surface tension reduction for both *P. aeruginosa* (20%) and *B. pumilus* (33%) using soybean oil as inducer (1%) and no supplementation with ammonium sulfate. The authors reported a final surface tension of 35.67 $mN.m^{-1}$ after 4 days of fermentation. The minimum ST obtained using palmitic acid as a hydrophobic inducer was ≈26.5 $mN.m^{-1}$, very close to the ST obtained using cassava wastewater without the addition of inducers (≈26 $mN.m^{-1}$). Thus, the fatty acids in their pure form, very likely, induced the surfactin production in a shorter fermentation time - higher productivity.

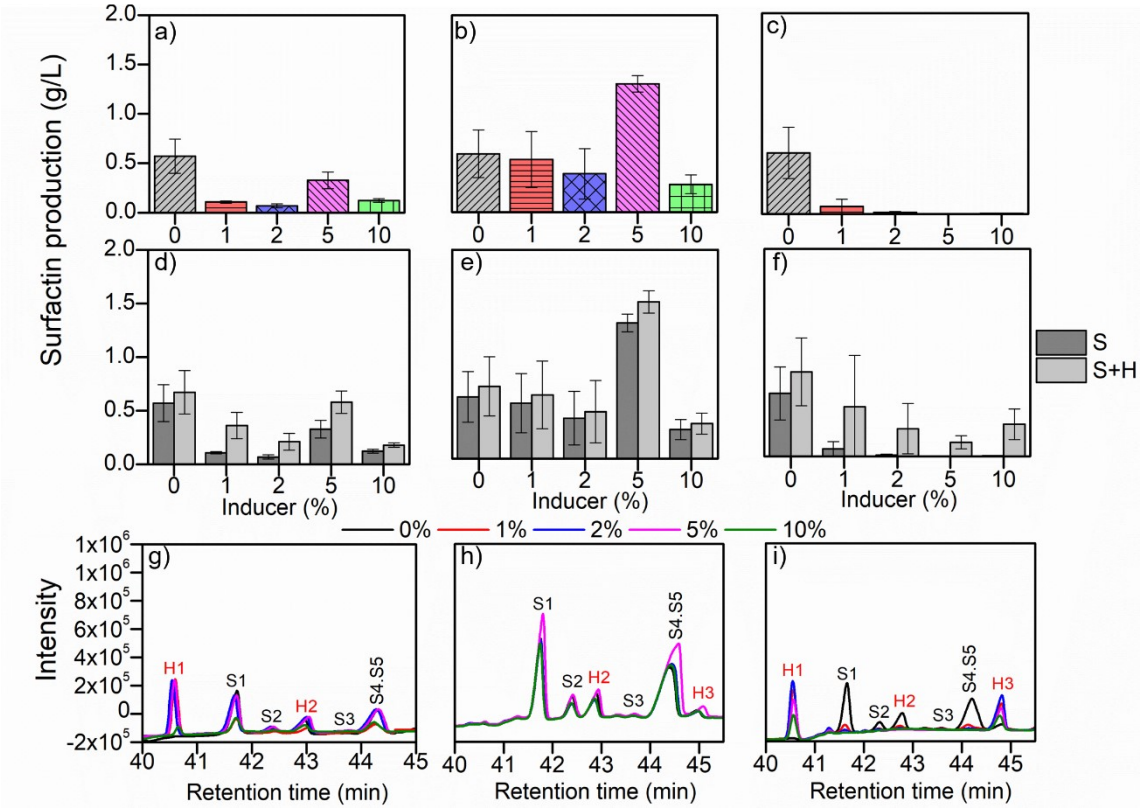
4.3.4 Effects of hydrophobic inducers in surfactin production

In general, biosurfactants are produced in the form of a mixture of homologues, which the composition depends on the strain used, cultivation conditions and substrate (KRIEGER; NETO; MITCHELL, 2010). Surfactin is composed of a heptapeptide ring (generally described by the following amino acid sequence: L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu) linked to a of β-hydroxylated fatty acid containing 12-16 carbon atoms (de Oliveira Schmidt et al., 2021; Ding et al., 2021). Amino acid residues at positions 1, 3, 5 and 6 in the heptapeptide are highly conserved. However, positions 2, 4 and 7 are capable of accepting various L-aliphatic amino acid residues (Leu, Ile and Val) as substrates (DING et al., 2021). These structural variations due to substitution of amino acid components of the peptide ring can result in changes in the physicochemical properties of surfactin that directly depend on the composition of the mixture of homologues (DING et al., 2021; JAHAN et al., 2020; KRIEGER; NETO; MITCHELL, 2010). These factors corroborate to explore unknown structures and bioactivities.

Thus, Liu et al., (2015) reported new surfactin molecules, the C_{12} lipopeptide, with a shorter fatty acid chain length, and the C_{15}-surfactin-O-methyl ester, an esterified lipopeptide, were isolated and identified by *B. subtilis* HSO 121 was cultivated. The fatty acid moieties of C_{15}-surfactin-*O*-methyl were identified as iso C_{15} and anteiso C_{15} β-hydroxy. The peptide ring was analyzed, and the amino acid sequence of N-Glu(OMe)-Leu-Leu-Val-Asp-Leu-Leu-C was identified for the first time (LIU et al., 2015; XIANGYANG LIU et al., 2007). These studies prove that different bacterial strains can produce the same type of surfactin. The same strains can simultaneously synthesize different lipopeptides (e.g. surfactin, iturinin and fengicin) (LIU et al., 2015).

Therefore, using hydrophobic inducers can directly affect the production and application of these homologues. Recently, a study by Zhao et al. (2020) compared two culture media (hydrophilic and hydrophobic) to produce rhamnolipids by *P. aeruginosa*. The authors achieved 7.06 and 10.32 $g.L^{-1}$ of rhamnolipids using glucose and soybean oil. Furthermore, rhamnolipids produced from glucose showed higher surface activity (26.3 $mN.m^{-1}$) than rhamnolipids produced from soybean oil (28.1 $mN.m^{-1}$). On the other hand, rhamnolipids produced from soybean oil showed a higher index of petroleum emulsifying activity (76.1%) than rhamnolipids produced from glucose (65.5%). These results are most likely correlated with the rates of rhamnolipid homologues produced. Figure 16 elucidates surfactin production using hydrophobic inducers.

Figure 16 - Total surfactin yield (a) soybean oil, (b) palmitic acid and (c) oleic acid. Total yield of crude surfactin plus homologues (d) soybean oil, (e) palmitic acid and (f) oleic acid. HPLC cromatograms for the produced surfactin product (g) soybean oil, (h) palmitic acid and (i) oleic acid.



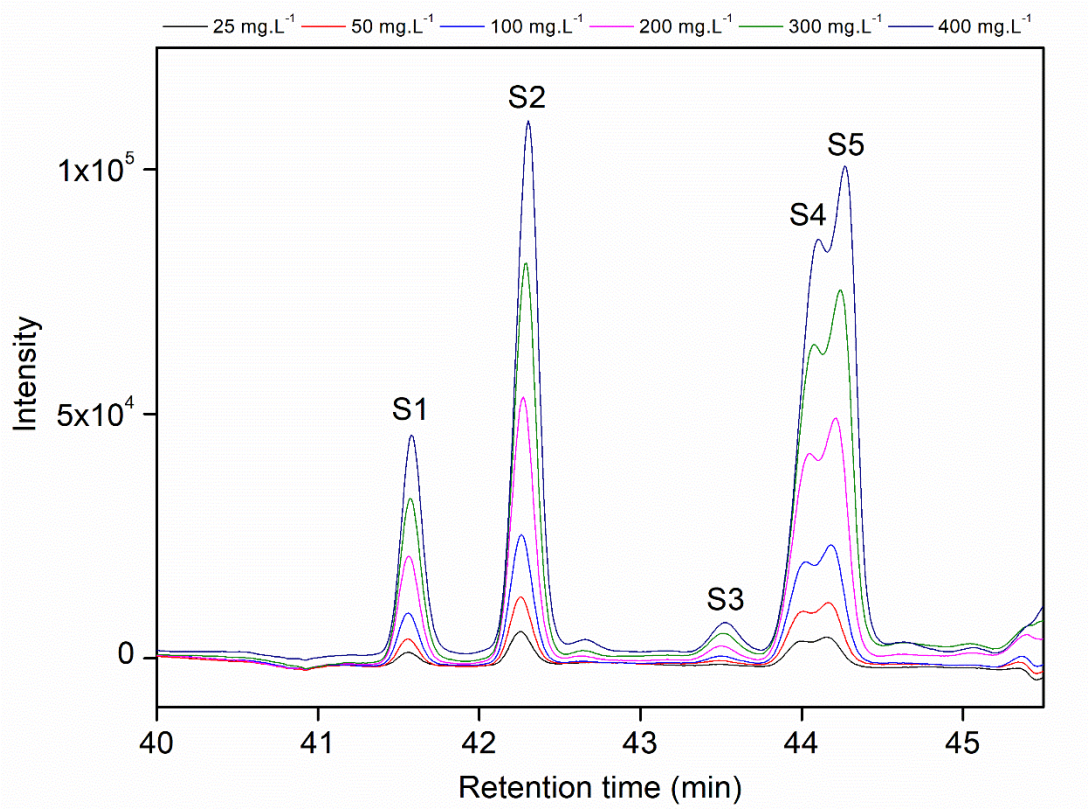
*Five peaks were used to calculate the amount of surfactin. The retention times (min) of the five peaks are: 41.58, 42.20, 43.53, 44.09 and 44.27.

The results show the production of surfactin and the possible formation of different homologues. The production of specific homologues is directly related to the substrate, microorganism and mode of operation (DE OLIVEIRA SCHMIDT et al., 2021). Usually, the literature presents soybean oil as an alternative co-substrate and/or hydrophobic inducer that is easy to assimilate (ZHAO et al., 2020). Regarding the concentration of produced surfactin, the results were similar to Ghribi and Ellouze-Chaabouni (2011). The authors clearly showed that supplementation of the optimized medium with 2% of oils such as olive oil, sunflower oil, corn oil increased (1 $g.L^{-1}$) the production of biosurfactants by the strain *B. subtilis* SPB1. Similarly Pathania and Jana (2020) reported that rhamnolipid production by *P. aeruginosa* was enhanced using multiples carbon sources (glucose and fried soybean oil 2% $w.v^{-1}$). They obtained a production of biosurfactant ≈3.3 $g.g^{-1}$ after 96 h of fermentation.

However, considering the complexity of the composition of soybean oil and the results obtained in this study, it is possible to observe that this inducer had no significant influence on the final yield of surfactin production, compared to treatments using cassava wastewater (pure) and AP as inducer. Notably, the supplementation of the culture medium with different concentrations of PA (1, 2, 5 and 10%) significantly increased the yield of surfactin production. The highest production was ≈1.3 $g.L^{-1}$ at a concentration of 5% (Fig. 16b), compared with cassava control (no inducer) (≈0.6 $g.L^{-1}$).

On the other hand, it was found with the isolated analysis of OA, a reduction of ≈9, 27, 38 and 67 times in the production of surfactin isomers, for concentrations of 1, 2, 5 and 10%, respectively. This inducer considerably inhibited the production of isomers (peaks S1, S2, S3, S4 and S5) of surfactin compared to treatments using pure cassava (0%). The production of homologues was estimated from the comparison with the surfactin isomers obtained by the HPLC standard (Sigma-Aldrich) (Fig. 17).

Figure 17 - Chromatogram of surfactin standard (from *B. subtilis*, ≥ 98% HPLC, Sigma-Aldrich)



Adding these concentrations to the values obtained from pure surfactin, it is possible to suggest a significant increase in the final yield of surfactin. Therefore, it was observed a significant increase in surfactin yield when using SO (Fig.16d). Soybean oil induced the production of "homologous H1 and H2" peaks, unlike PA and OA. At concentrations 1, 2 and 5% the production of H1 increased ≈18 times.

When PA was used as an inducer, there was a considerable increase in the production of surfactin isomers (Fig. 16h). In addition to stimulating the formation of two additional peaks, "homologues H2 and H3" (Fig. 16e and 16h). For all concentrations, when it was added PA as an inducer, there was a ≈2 times increase in the production of H1 and H2. The addition of the OA inducer led to the production of the homologues H1, H2 and H3 (Fig. 16i). When it was added the inducer at 1 and 2% there was an increase of ≈4 times in the production of H3. However, using higher concentrations, we obtained an increase of ≈1 times.

The production of surfactin homologues (H1 and H3) from OA increased inversely proportional to the concentrations of inducers. However, at the highest concentrations (5 and 10%) it was obtained a significant production of homologues (Table 12).

Table 12 - Estimated increase (%) in the total yield of surfactin production, taking into account the produced surfactin homologues (S+H).

	% S+H		
Inducer	***Soybean oil***	***Palmitic acid***	***Oleic acid***
1%	70.16	12.58	84.41
2%	67.66	13,62	94.50
5%	43.56	13.39	97.60
10%	30.82	17.12	98.08

Notably, there was a significant increase in H2 production using only cassava as a substrate. Taking into account the decrease in surfactin production (Fig. 16c), it is worth noting that the production of homologues using OA effectively influenced the reduction of ST, which is probably related to the new homologues.

4.4 CONCLUSION

Cassava wastewater (flour) associated with hydrophobic inducers can be used as an efficient and renewable carbon source for the production of surfactin by *B. subtilis* ATCC 6633. The surfactin produced by the inducers reduced the surface tension (26.5 $mN.m^{-1}$) and reached a yield maximum of ≈1.3 ± 0.08 $g.L^{-1}$ (5% PA). The HPLC analyzes of all fermentations using the inducers soybean oil, palmitic acid and oleic acid, and at all concentrations, indicated the possible formation of different surfactin homologues, differing from the commercially available surfactin obtained from Sigma-Aldrich. Increasing their yields by up to ≈70, 17 and 98% respectively. A significant (≈2 times) increase of biosurfactant production was observed. The surface tension decreased by 40% after 12 h fermentation using the inducers (PA and OA). The fermentable sugars were depleted for all experiments. The oleic acid has induced to the greatest diversity of produced homologues. However, more studies are needed to prospecting for new surfactin homologues. It is necessary to evaluate its functional properties, toxicity and suitability for industrial application.

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5 CONCLUSION

5.1 CONSIDERATIONS REGARDING THE STATE OF THE ART

CHAPTER 2

In chapter 2, through the systematic review of the literature, it was possible to observe that the supplementation of the culture medium with hydrophobic inducers can directly affect the productivity of the biosurfactant. And that differences in physicochemical properties in homologous series and isoforms of these compounds can affect the application and effectiveness of these biomolecules. However, there are no reports on the influence of different compositions of inducers in the optimization of biosurfactants production (e.g. chemical structure modification and metabolism associated the production of biosurfactants) using hydrophobic inducers in their pure form. It was concluded in a concise way that new strategies to increase the yield of biosurfactant production need to be developed, as well as an in-depth study of the functioning of these molecules associated with low-cost alternative substrates.

CHAPTER 3

According to the literature, the liquid waste generated during the cassava flour processing steps have some toxic compounds that need pre-treatment before being disposed of in the environment. However, from another perspective, this chapter clarified that some studies helped to synthesize the evidence on the potential use of cassava wastewater in biotechnological processes, mainly by different microorganisms. Evidencing the cassava wastewater as a viable alternative for reducing operating costs and optimizing industrial processes.

5.2 CONCLUSION AND PERSPECTIVES

Regarding the experimental results, in chapter 4 it was possible to conclude that the culture medium composed of cassava wastewater (flour) and hydrophobic inducers increased surfactin production by *Bacillus subtilis* ATCC 6633. For the first time, fatty acids were used in their pure form to produce surfactin. Surfactin produced by inducers reduced the surface tension ($\approx$26.5 $mN.m^{-1}$) and reached a maximum yield of $\approx 1.3 \pm 0.08$ $g.L^{-1}$ (5% PA). The analysis of the results of all fermentations using the inducers soybean oil, palmitic acid and oleic acid, and in all concentrations, indicated the possible formation of different surfactin homologues, increasing their yield up to ≈70, 17 and 98%, respectively. A significant (≈2 times) increase in biosurfactant

production was observed. No major changes in pH were observed. The surface tension decreased by 40% in 12 h, indicating surfactin production. The consumption of soluble sugars was almost total for all study conditions. And oleic acid showed greater diversity of homologues. These results refer to the valorization of agro-industrial residues, such as cassava, through new production strategies. Other suggestions to improve the production of surfactin: i) investigate different origins of cassava wastewater and ii) the use of hydrophilic inducers associated with hydrophobic inducers. However, further investigation should elucidate its correlation with biosurfactant metabolic pathways. The next step will be to characterize these possible homologues produced and their surfactant properties produced in order to define possible applications. These advances will be of great interest for a circular economy in the cassava processing industries, given the wide applicability of this biosurfactant.