Biosynthesis And Production Of Sophorolipids

Marcos Roberto de Oliveira, Doumit Camilios-Neto, Cristiani Baldo, Agnes Magri, Maria Antonia Pedrine Colabone Celligoi

Abstract: Sophorolipids are biosurfactants belonging to the class of the glycolipid, produced mainly by the osmophilic yeast *Candida bombicola*. Structurally they are composed by a disaccharide sophorose (2'-O-β-D-glucopyranosyl-β-D-glycopyranose) which is linked β -glycosidically to a long fatty acid chain with generally 16 to 18 atoms of carbon with one or more unsaturation. They are produced as a complex mix containing up to 40 molecules and associated isomers, depending on the species which produces it, the substrate used and the culture conditions. They present properties which are very similar or superior to the synthetic surfactants and other biosurfactants with the advantage of presenting low toxicity, higher biodegradability, better environmental compatibility, high selectivity and specific activity in a broad range of temperature, pH and salinity conditions. Its biological activities are directly related with its chemical structure. Sophorolipids possess a great potential for application in areas such as: food; bioremediation; cosmetics; pharmaceutical; biomedicine; nanotechnology and enhanced oil recovery.

Index Terms: biosurfactants, glycolipid, sophorolipids, sophorose, yeast, *Starmerella bombicola*, *Candida bombicola*.

1 INTRODUCTION

The sophorolipids (SLP) are biosurfactants belonging to the glycolipids class, produced by many non-pathogenic yeasts, being the yeast *Candida bombicola* the most important. Structurally they are composed by a disaccharide sophorose (2'-O-β-D-glucopyranosyl-β-D-glycopyranose) linked by a β-glycosidic bond to a long fatty acid chain [1], [2]. The SLP are one of the most promising known biosurfactants and offers several advantages over the synthetic surfactants such as: high selectivity and specific activity in a broad range of temperature, pH and salinity conditions [3], low toxicity, high biodegradability and ecological acceptance [4], [5], and they may be produced in great amounts [6]. They possess a low capacity of foam formation and high detergency which facilitates its application in several areas [7], and they may be produced from renewable resources [8] or industrial residues [9] and are of easy recovery [10].

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SLP can be applied in several areas such as: - agriculture (fungicide [11]); - food (anti-freezing [12], preservative [13]); - biomedicine (anti-tumor [14], anti-microbial [15], anti-viral and spermicide [16], immunomodulator [17]); - bioremediation (soil remediation [18], removal of heavy metals [19]); - cosmetics (dermatological applications [20], [21]); - nanotechnology (formation of nanoparticles [22], nanoparticles conjugated to drugs [23]) and oil (enhanced oil recovery [24]). In spite of the numerous applications which the SLP possess, the larger-scale production and the high cost are still great obstacles for its economic competitiveness. The high cost of production is mainly due to the synthetic culture medium and the downstream process which may come to represent 60% of the total cost of the fermentative process [25]. To overcome the obstacles of high production costs, two basic strategies may be used: (a) the use of low cost substrates for the formulation of the culture medium, (b) development of efficient and optimized bioprocesses of the culture conditions and recovery (high production with maximum recovery) [26], [27].

2 PRODUCER MICROORGANISMS

Several microorganisms have already been reported as producers of SLP. Gorin, Spencer and Tulloch (1961) [1] isolated from sow thistle petals the osmophylic yeast *Torulopsis magnoliae* which produced an extracellular glycolipid with an oily aspect, which has been identified as being composed by unities of a disaccharide sophorose (2'-O-β-D-glucopyranosyl-β-D-glycopyranose) partially acetylated, linked by a β-glycosidic bond to 17-L-hydroxyoctadecanoic and 17-L-hydroxy-9-octadecanoic acids [1]. In 1967, another species of yeast *Torulopsis gropengiesseri* was described by Jones [28] as a producer of extracellular glycolipids, not in the form of oil (acidic form) as described by Gorin, Spencer and Tulloch [1], but in the crystal form (lactonic form) where the sophorose was part of a macrocyclic ring. Tullcoch and Spencer, in 1968 [29] reclassified the yeast *Torulopsis magnoliae* as *Torulopsis apicola*. Nowadays, the *Torulopsis apicola* is classified and *Candida apicola*, once that the genus *Torulopsis* has become obsolete and is now classified under the genus *Candida* [30]. Also in 1968 Tulloch, Spencer and Deinema [31] have identified a new SLP producing yeast called *Candida bogoriensis* isolated from the leaf surface of the shrub (*Randia malleifera*), nowadays this yeast is known and *Rhodotorula bogoriensis*. Spencer, Gorin and Tulloch, in 1970 [32] have identified in several nectar samples collected from wild flowers from different regions in Canada, a SLP producing yeast identified *as Torulopsis bombicola*, nowadays known as *Candida bombicola*. In 1983 Cooper and Padock

[33] showed that the yeast *Torulopsis petrophilium*, isolated as a microorganism which degrades fractions of crude oil is capable of producing glycolipids identical to the ones of *Torulopsis bombicola*. Another yeast *Wickerhamiella domercqiae* Y2A, isolated from effluents contaminated with oil, was identified by Chen and colleagues (2006) [34], as a producer of SLP. In 2008 a strain of a thermo-tolerant yeast *Pichia anomala* PY1, was isolated from "Khao Mhak" (Thai fermented food) and described as a producer of SLP [35]. Konishi and colleagues, in 2008 [36], through of screening based on the phylogenetic information of a known SLP producer, described the yeast *Candida batistae* CBS 8550 as a producer of SLP. Imura and colleagues in 2010 [37] reported the production of SLP by yeast *Candida floricola* TM 1502. Kurtzman and colleagues (2010) [38] identified three more SLP producing species *Candida riodocensis*, *Candida stellata* and *Candida* sp. NRRL Y 27208. Kurtzman (2012) [39] reports that the *Candida* sp NRRL Y-27208 strain represents a new species *Candida kuoi*. Chandran and Das (2011) [4] isolated from hydrocarbon contaminated sites in India, two SLP producing yeasts *Candida rugosa* and *Rhodotorula muciliginosa*. The same authors in 2012 [3] also isolated from petroleum hydrocarbon-contaminated soil in India a new species of yeast which is an efficient degrading of diesel as well as a powerful SLP producer in the presence of diesel, the species was identified *as Candida tropicalis*. Poomtien and colleagues in 2013 [41] isolated from cosmetic industrial wastes in Thailand the yeast *Cyberlindnera samutprakarnensis* JP52 an efficient SLP producing. Basak and colleagues (2013) [42] isolated from common effluent treatment plant in India, the yeast *Cryptococcus* sp. VITGBN2 a potent producer of SLP.

2.1 *Candida bombicola* ATCC 22214

Candida bombicola ATCC 22214 is the most widely used SLP producing yeast, being initially identified through biochemical tests as belonging to the genus *Torulopsis*. The name *Torulopsis bombicola* is proposed because of its frequent occurrence in close association with bumble bees [32]. In 1998, based in phylogenetic analysis, the creation of the genus *Starmerella* was proposed to accommodate the teleomorphic state (sexual stage) of the *Candida bombicola*. The name *Starmerella* was given to the genus in honor to Willian T. Starmer, in recognition to its contribution to ecology and evolution of yeasts associated to plants and insects [43]. Nowadays the genus *Starmerella* contains approximately 30 species of yeast, most known by being associated to bees, nectar, pollen, or to its habitats and related substrates. The species belonging to the genus *Starmerell*a as *Candida magnoliae*, *Candida bombicola* and *Candida batistae* seem to be involved in mutualistic relations with bees. Physiologically similar to most yeasts of the genus *Starmerella*, are fermentative microorganisms which use few carbon sources, are osmotolerant, which points out relative specialization to the common niche and the possible association with nectar as its main habitats [43], [44]. Despite the *Candida bombicola* yeast carrying the name of the genus *Candida*, it finds itself phylogenetically far from the pathogenic yeasts, like *Candida albicans* [45]. Futhermore, *Starmerella bombicola* / *Candida bombicola* is incluided in microorganism with technological benifical use having frameworks as "the history of use", "traditional food" or "general recognition of safetty" [46].

3 Structures of the Sophorolipids

The SLP are amphipathic or amphiphilic molecules formed by a hydrophilic moiety and a hydrophobic moiety. The hydrophilic moiety is composed of a sophorose disaccharide (2'-O-β-D-glucopyranosyl-β-D-glycopyranose) linked to a hydrophobic moiety composed of a long chain of fatty acid linked by a glycosidic bond. The β-glycosidic bond occurs between the anomeric carbon of the sophorose (C1') and the ω carbon (terminal) or ω-1 (sub-terminal) hydroxilated from the fatty acid [2], [29]. They are typically produced in the form of a mix of molecules structurally related. This complex mix can be constituted by up to 40 different types of SLP with associated isomers [47]. They may also occur in the polymeric (dimeric and trimeric) form [48].This wide structural variation presented by the SLP is related to many factors: a) The hydroxyl grouping of C6' and C6'' of sophorose can be found deacetylated, monoacetylated or diacetylated [2], [49]; b) They may be produced in the lactonic and acidic form. In the lactonic form, the carboxyl grouping of the fatty acid is esterified to the C4'' of the sophorose, being also able to be esterified to the C6' or C6'', although this is less frequent [2]; c) The size of the carbonic chain of the fatty acid generally varies between C16 and C18 [50]; d) The fatty acids can be saturated, monounsaturated or polyunsaturated [49]; e) The fatty acids may be hydroxilated in the ω (terminal) or in the ω-1 (sub terminal) carbon [51]; f) Presence of stereoisomers [51]. The figure 1 shows the acidic, lactonic and trimeric forms of the SLP. Different microorganisms produce different forms of SLP, although these forms can also be altered varying the conditions of the fermentative process, such as the supplying of different lipophilic substrates during the fermentative process [52]. The table 1 shows different forms of SLP produced for several producer microorganisms. Since the SLP are produced as a mixing of the acidic and lactonic form, its properties and applications are directly related to these forms. The lactonic forms have better biocide activities [57], anticancer [14], spermicide, cytotoxic and proinflammatory [16] and are more hydrophobic [58]. The acidic forms are better foaming agents and have a higher solubility in water [7] and can be better used in the food industry, bioremediation and cosmetics [54].

4 Physiological roles of the Sophorolipids

Even though the physiological roles of the biosurfactants are not completely elucidated, they perform several essential functions for the cell growth and maintenance. A generalization of such functions is extremely difficult, for the biosurfactants with different chemical structures and very distinct superficial properties. Therefore, each biosurfactant can perform different physiological functions, providing different advantages for the producing microorganisms in its ecological niches [59].

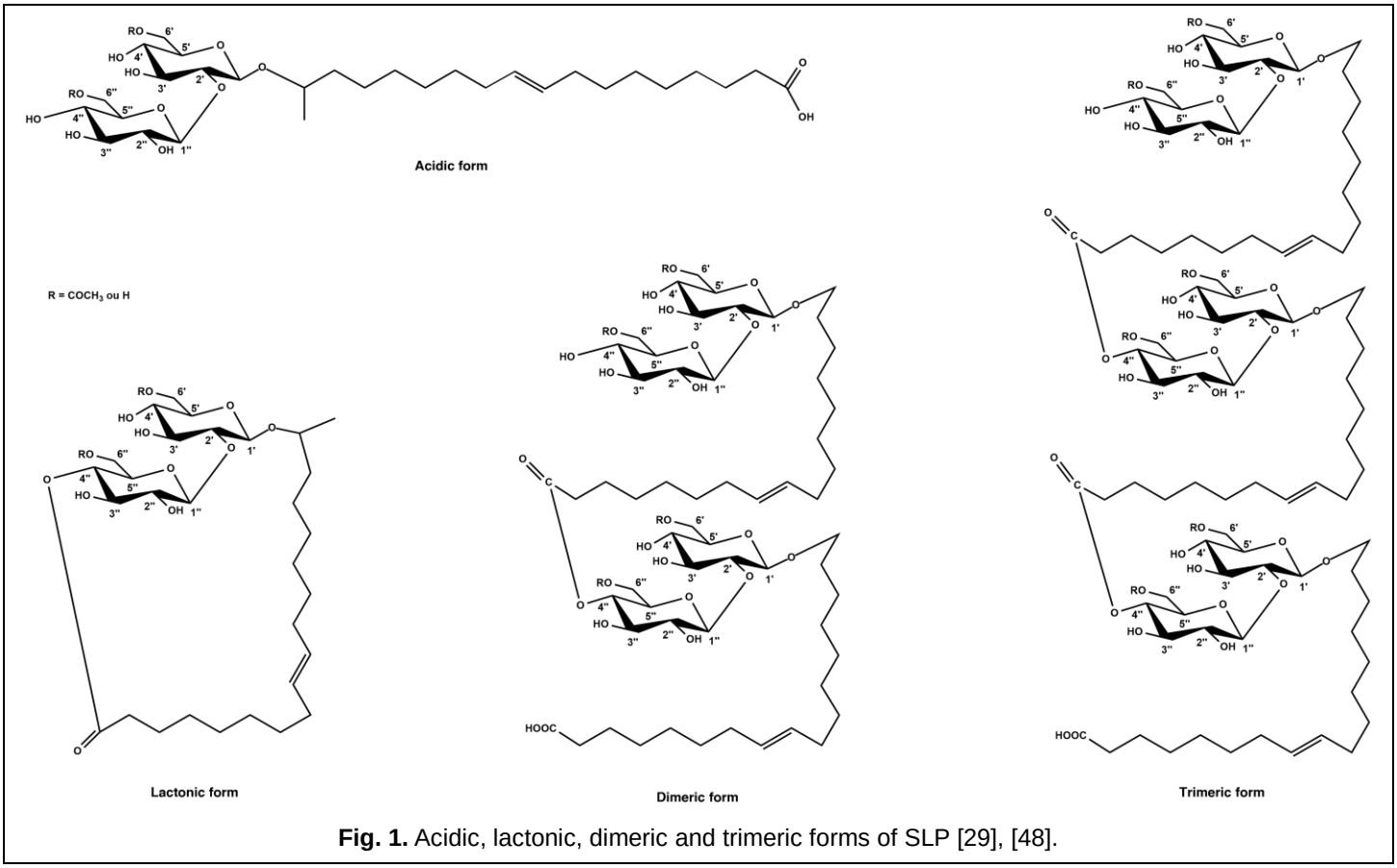

Fig. 1. Acidic, lactonic, dimeric and trimeric forms of SLP [29], [48].

Even as biosurfactants, the physiological role of the SLP isn't completely understood until now, but some hypothesis can be made. The SLP are secondary metabolites and do not seem to have an essential function for cellular growth, development and reproduction [2], [60]. They are probably formed and liberated to the extracellular medium as extracellular storage of carbon [61]. The SLP producer microorganisms such as *Candida bombicola* and *Candida apicola* occur in honey, nectar, pollen [43] and are involved in mutualistic relations with bees [44]. Since these habitats are of high osmotic pressure, the production of SLP may be a form of adaptation and protection to these high concentrations of sugar [61], besides being an adaptive advantage, where the yeast, converts and stores the carbohydrates and makes them less available to other microorganisms [62]. Many microorganisms are capable of growing in lipophilic substrates, like for example, hydrocarbons as an unique source of carbon. These microorganisms are capable of emulsifying hydrophobic carbon sources during growth. This emulsifying capacity is given by the production of extracellular biosurfactants, mainly of glycolipids, which allows the capture of the substrate to the periplasmic space [63]. However, this theory, isn't much accepted for the formation of SLP, especially because there can be produced without any hydrophobic substrate present and when present are produced in amounts which highly exceeds the necessary concentration for the emulsification of these substrates [62]. The SLP possess antimicrobial activity against algae [64], yeasts [57] and bacteria [65] through the mechanisms involving membrane destabilization and increased permeability [67]. The antimicrobial activity or biocide can serve as an interspecies competition mechanism as it occurs in other biosurfactants [68].

TABLE 1
STRUCTURAL PROFILE OF SLP PRODUCED FOR SEVERAL MICROORGANISMS

Producer microorganisms	Forms of SLP produced						Reference
	UnAc	MnAc	DiAc	UnLc	MnLc	DiLc	
Candida apicola NRRL Y-2481	•	•	•	●	●	●	[38]
Candida batistae CBS 8550			●		•	•	[36]
Candida bombicola ATCC 22214			•			●	[38], [52]
Candida floricola TM1502			●				[37]
Candida riodocensis	•	•	●		•		[38]
Candida rugosa					●		[40]
Candida sp. NRRL Y-27208 *(Candida kuoi)*	•	•	●		•		[38]
Candida stellata	•	•	●				[38]
Candida tropicalis					●		[3]
Cryptococcus sp. VITGBN2			●				[42]
Cyberlindnera samutprakarnensis JP52				●		●	[41]
Pichia anomala PY1			●			●	[35]
Rhodotorula muciliginosa			●				[40]
Rhodotorulla bogoriensis	•	•	●				[55], [56]
Wickerhamiella domercqiae Y2A	●	●	●		•	•	[54]

UnAc - Unacetylated acidic; MnAc - Monoacetylated acidic; DiAc - Diacetylated acidic; UnLc - Unacetylated lactonic; MnLc - Monoacetylated lactonic; DiLc - Diacetylated lactonic. - Large quantities; - Small quantities.

5 BIOSYNTHESIS OF THE SOPHOROLIPIDS

The biosynthesis of SLP occurs in the stationary phase under nitrogen limiting conditions [69], total phosphate exhaustion [70] and dissociated from cellular growth [71]. The figure 2 shows schematically the biosynthesis of the SLP. In the presence of hydrophobic carbon sources, such as the alkanes, alcohol, fatty acids and esters of fatty acids, the yeasts such as *Candida bombicola* synthetizes SLP. The biosynthesis process begins with a hydroxylation of the fatty acid present in the medium. This fatty acid can be of several origins: supplemented in the form of fatty acid; in the form of n-alkanes, alcohol, aldehyde, triglycerides or esters of fatty acids, which will be metabolized until its correspondent fatty acid; - in the case it isn't present, the fatty acid will be formed through de novo synthesis, from the acetyl-CoA. On the other hand, when the concentration of glucose is low, these hydrophobic carbon sources are metabolized through the β-oxidation and use for the cellular maintenance instead of the SLP synthesis [72]. The activation process of the fatty acids occur through hydroxylation of its terminal (ω) carbon or (ω-1) subterminal, this hydroxylation is mediated by the enzyme CYP52M1 (cytochrome P450 monooxygenase belonging to the CYP52 family) NADPH depending, bonded to the cellular membrane, leading to the formation of the activated hydroxilated correspondent fatty acid. It can be metabolized through β-oxidation or act as precursor for the synthesis of SLP [61]; [73]. The enzyme CYP52M1 is expressed exclusively in the stationary phase and possibly potentialized by a damage resistant protein (DAP1) which stabilizes and regulates the CYP450 protein and participates in the metabolism of the lipids and sterols [74], [75]. In the next two stages, two molecules of glucose will be linked to the activated fatty acid. The first glucose molecule is linked (position C1') to the ω hydroxyl grouping or ω-1 of the fatty acid by action of the Glucosyltransferase I (UgtA1). The reactions require that the glucose is activated in the form of UDP-glucose (Uridine diphosphate glucose) which acts as a donor of glucosyl grouping. In the next step, a second glucose is glycosidically linked to the first glucose (position C2') by Glucosyltransferase II (UgtA2). Both enzymes UgtA1 and UgtA2 are expressed in high amounts in the beginning of the stationary phase. The glucose supplied in the medium, isn't directly incorporated in

the SLP structure, great part of the present glucose in the medium is metabolized through the glycolytic pathway, being that the SLP glucose is formed from the gluconeogenesis [74], [75], [76]. The product of the second glycosylation reaction are SLP in its unacetylated acidic form, further acetylation and lactonization reactions promote structural variations. The acetylation of the sophorose in the C6' and C6'' positions is mediated by action of by acetyltransferase [62], [77]. The lactonization process occurs through an esterification reaction between the carboxyl grouping of the fatty acid and the hydroxyl C4'' grouping, being it possible to occur more rarely in the C6' or C6'', the enzyme responsible for the lactonization occurs by the action of a cell wall-bound lactonesterase [75]. The excretion process of the SLP was not still completely understood, this process may occur from the formation of vesicles, mediated possibly by passive or active transporters, like the ABC transporter protein (Mdr) [78].

6 Production of Sophorolipids

The production of SLP is strongly stimulated when two sources of carbon (lipophilic and hydrophilic) are present in the medium [2], in the absence of hydrophobic sources low productions can be observed [79]. The hydrophobic carbon source is normally incorporated in the hydrophobic faction of the SLP, mainly those with C16 and C18 [51]. In the case of absence, it can be synthetized from other sources of carbon from the synthesis de novo, however, the production of SLP will be smaller [70]. The production, type and proportion of the acidic/lactonic forms of SLP produced depend on several factors such as: producer strain, the composition of the environment (hydrophilic and hydrophobic carbon sources, nitrogen and salt source), environmental conditions (temperature, pH, agitation, aeration and time) and the kind of cultivation process employed (batch, feed batch and continuous) [80], [69].

6.1 Carbon sources

The SLP can be produced from a single source of hydrophilic carbon (carbohydrates), however the production will considerably increase if a second hydrophobic source of carbon (lipids, hydrocarbon, vegetal oil and animal fat) is added [69]. The main source of hydrophilic carbon used in the production of the SLP is glucose, which may be used for cellular growth and the production of SLP [81]. Other mono or disaccharides can be used as a hydrophilic substrate, among these carbohydrates we have sucrose, fructose, xylose [81], lactose, galactose [82], [8], mannose, maltose and raffinose [83]. The use of other hydrophilic carbon sources doesn't change the structure of the hydrophilic portion of the SLP (sophorose), which means the sophorose structure isn't determined by the sugar used. The different mono and disaccharides are metabolized, the glucose, supplying substrate for the intracellular synthesis of sophorose [83]. However, when these other carbohydrates are used as a source of hydrophilic carbon, smaller levels of production are obtained [84], [85]. The lactose, among the several carbohydrates already evaluated for the production of SLP can't be metabolized by *Candida bombicola*, when it is used, an absence of cellular growth can be observed [82]. The absence of capacity to metabolize lactose is due to the deficiency of a transporter system of lactose or of the enzyme galactosidase [86]. With the goal of decreasing the production cost, several sources of complex hydrophilic carbon sources, such as sugar cane molasses [87], soy molasses [53] and deproteinized whey [10] were evaluated. However, the observed production levels are smaller when compared to the levels obtained in the synthetic medium under the same experimental conditions. This way, the cost reduction may end up counter the smaller production obtained.

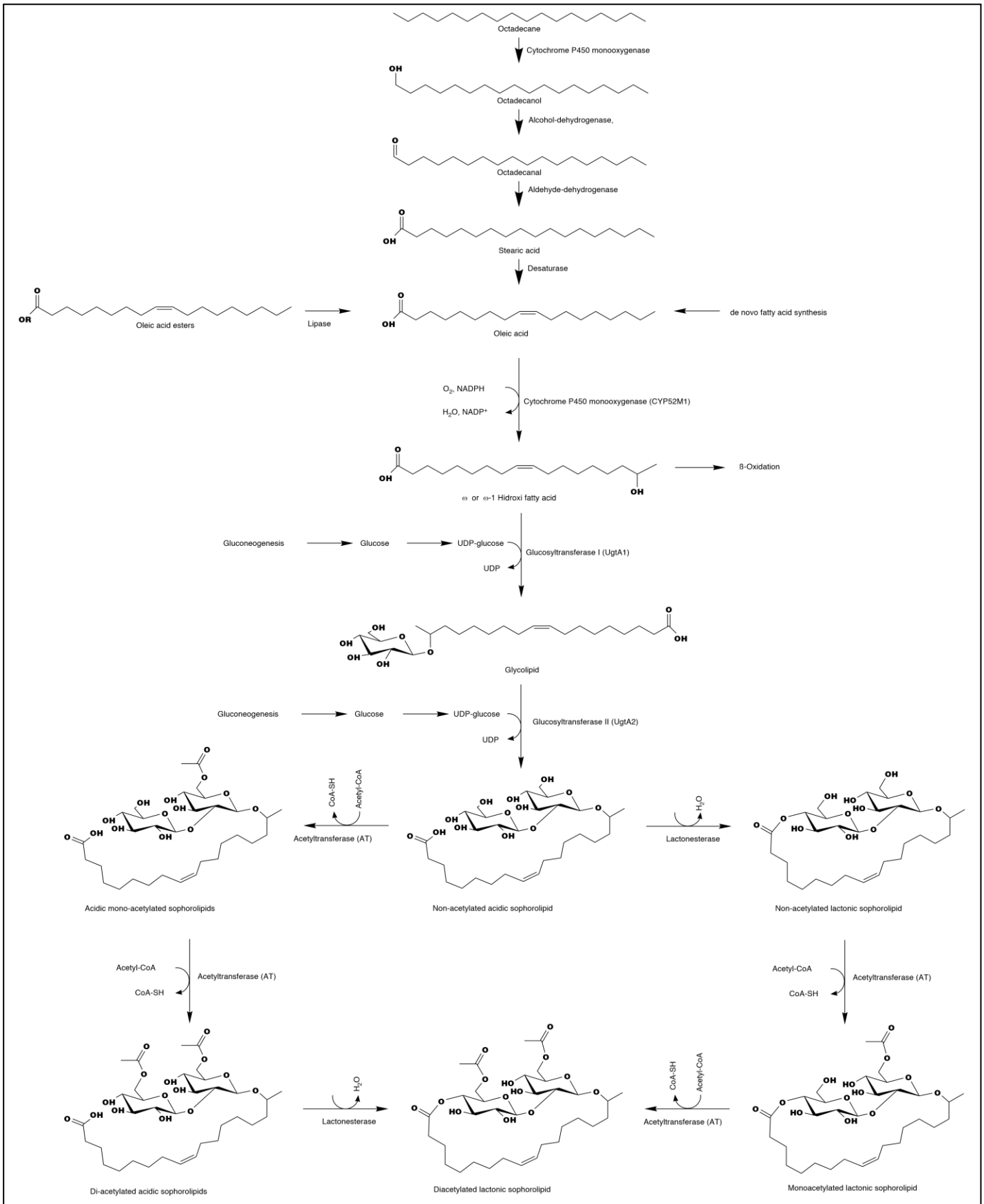

Fig. 2. Biosynthesis of the SLP [62], [73], [74], [75].

The hydrophobic carbon source influences in the production and final composition of the SLP mix. The structural profile commonly observed are SLP with a hydrophobic portion composed by chains with C16 and C18, due to the specificity of the cytochrome P450 monooxygenase, which promotes the terminal hydroxylation (ω) or subterminal (ω-1) and further covalent linked to the glucose molecule by the glucosyltransferase. The enzymatic affinity by certain substrates depends on its absolute length, fatty acids with a length between 22,55 and 25,0 Å, as the stearic acid (C18:0) and the oleic acid (C18:1) are easily incorporated directly to the SLP structure, while the myristic acid (C14) or lauric acid (C12) are shorter fatty acids which difficult the direct incorporation. The addition of hydroxilated substrates or the addition of substrates with similar structures to the stearic and oleic acids facilitates the direct incorporation [88]. Several sources of hydrophobic carbon were evaluated for the production of SLP, such as the alkanes (C12, C14, C15, C16, C17, C18 and C20), saturated fatty acids (C18:0), unsaturated fatty acids (C16:1; C18:1 and C20:4) [51], alcohols (1,12-dodecanodiol), ethyl-ester dodecanoic acid, 12-hydroxydecanoic acid, 1,12-dodecanodiol [69] and single cell-oil (*Cryptococcus curvatus*) [10], vegetal oils such as coconut oil, corn oil, grape seed oil, olive oil, sunflower oil [89], rapeseed oil, palm plant oil [69], Turkish corn oil [6], safflower oil [90] and soy oil [91]. Also several residues were used as substrate for the production of SLP as restaurant waste oil [66], soybean dark oil [71], dairy industry wastewater [92], oil refinery residue [93] and animal fat such as pig tallow [94] and fish oil [69]. When the alkanes are used as a hydrophobic source, they need to be oxidates into its corresponding fatty acids therefore the substrates which are similar to the fatty acids are preferred over the substrates which are similar with the alkanes, like the alkil esters. The production of SLP from the n-alkanes increases with the increase of the chain in the following order C18>C16>C14>C12. Alkanes C16 and C18 are directly incorporated e for the shortest a stretching can occur in the chain with the addiction of 2, 4 or 6 carbons, being that a direct incorporation can also occur, but in a smaller proportion [69]. The production of SLP from canola, sunflower and palm oil is smaller when compared to its respective esters, once the esters can be easily hydrolyzed, supplying the previous fatty acids and a source of energy. In the case of the oils to the hydrolysis of the glycerides seems to be a limiting stage for the production of SLP. The composition of the fatty acids of the SLP obtained from its oils and esters are similar. As in the case of the alkanes, the fatty acids with C16 and C18 show a direct incorporation. SLP produced from oils always exhibit a higher level of the diacetylated lactonic forms than the ones produced from its esters, even though its esters present a higher production rate. A higher content of polyunsaturated fatty acids promotes the production of SLP in the acidic form [69]. The production of SLP from several fatty acids such as the myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3) and eicasonoic acid (C20:0). It can be seen that the increase in the chain's length from C14 to C16 and C18 results in a significant increase in the production of SLP, with a drop in production from C20:0. The degree unsaturation affects the production of SLP, the absence of unsaturation C18:0, leads to a small decrease in the production of SLP. Monounsaturated fatty acids C18:1 result in a high production of SLP, while a decrease is observed with the increase of the amount of unsaturation (C18:1 > C18:0 > C18:2 > C18:3) [95]. Hydrophobic substrates with a short chain (C10 to C14) such as fatty acids and its esters and primary alcohols are not directly incorporated in the SLP structure but degraded and used by synthesis de novo of the C16 and C18 fatty acids and further incorporation to the structure of the SLP. Secondary alcohols (β-1 hydroxilated) as 2-dodecanol (C12), 2–tetradecanol (C14) and 2-hexadecanol (C16), allows the direct incorporation. With the increase of secondary alcohol chain (C12 – C16) the production of SLP increase too [96, 97]. Substrates with the terminal carboxylic grouping, like dodecyl, glutarate, and malonate ester are substrates with a higher degree of incorporation over substrates with groupings alkil terminal with pentenyl dodecanate and dodecyl pentanoate [98]. The addition of hydrophobic esterified substrates with longer chains, such as the methyl ester of erucic acid (54% of docosenoic acid C22:1 and docosadienoic acid C22:2) leads to the consequent production of SLP of the longest chain, showing the direct incorporation of these fatty acids in the SLP structure. The methyl esterification of these substrates promotes the production of SLP mainly in the acidic form [99].

6.2 Nitrogen Sources

The SLP producing microorganisms may metabolize organic and inorganic sources of nitrogen. Several authors have already evaluated inorganic sources such as NH_4NO_3 [100], $NaNO_3$ [79], [55], $(NH_4)2SO_4$ [101], [55], NH_4Cl [38], and organic sources such as yeast extract [60], malt extract, peptone extract, soytone [102], corn steep liquor [103]. Among the evaluated nitrogen sources, the yeast extract is the best nitrogen source used for the production of biomass and SLP [60], being a source of nitrogen, vitamins and trace elements such as zinc, magnesium and iron. The values found in literature for the yeast extract are of 1 $g.L^{-1}$ [89], [81]; 2 $g.L^{-1}$ [104]); 3 $g.L^{-1}$ [90]; 4 $g.L^{-1}$ [8] e 5 $g.L^{-1}$ [79]. The variation observed in these values are given by the different experimental conditions tested, as well as different carbon sources, like those of complex carbon [105] or the addition of sources of inorganic nitrogen [100]. In low concentrations ($1g.L^{-1}$) of yeast extract the production of SLP is stimulated and in higher concentrations ($5g.L^{-1}$) cellular growth is stimulated [60] [89]. Nitrogen limitation is one of the requirements for the production of SLP by *Candida bombicola*. Several complex nitrogen sources such as yeast extract, malt extract and soytone are sources with high in nitrogen/carbohydrate ratios; therefore any increase in the concentration of these sources can decrease the production of SLP. However the malt extract has high proportions of carbohydrates decreasing the nitrogen/carbohydrate ratio, increasing the production of SLP [102]. When urea is used as the only source of nitrogen, the cellular growth becomes limited, for other essential elements for the cellular growth, such as the pantothenic acid, thiamine, pyridoxine, which are supplied with the yeast extract, aren't present [90], [79]. The utilization of the low-value soy molasses as a combined nitrogen and carbon-source for SL production, results in lower production, but could be justified by the cost reduction of the fermentative process [106]. When used, the sugar cane molasses and soybean oil, the production of SLP was higher without the addition of yeast extract, showing that it is possible to eliminate unnecessary nitrogen sources (yeast extract, urea), decreasing costs and increase the production,

depending on the type of substrate used in the fermentative process [104].The type of nitrogen source and its concentration influences in the structure of the SLP obtained and in the ratio of acidic/lactonic forms. Concentrations of yeast extract smaller than 5 $g.L^{-1}$ favors the formation of lactonic forms and in higher concentrations the acidic fractions increase, smaller concentrations of yeast extract and long fermentation periods increase the rates of produced lactonic forms, independently of the fermentation method used [89]. The absence of yeast extract and urea decrease the production of lactonic forms [106], in contrast, when to the ones produced in the presence of it [52]. The production of acidic diacetylated forms are favored by the substitution of urea by $NaNO_3$, possibly by the delay of the stationary phase, for the acidic forms are precursors of the lactonic forms [55].

6.3 Minerals

In literature, there are few studies which evaluate the influence of the minerals in the production of SLP. Some minerals are essential for the microbial growth and metabolism, generally the culture used for the production of SLP, when supplemented with minerals containing KH_2PO_4, $MgSO_4$, $MgCl_2$, NaCl and $CaCl_2$. The supplementation of the medium with KH_2PO_4, beyond the nitrogen source, possesses great influence in the production of biomass. For the production of SLP, the K_2HPO_4 is a better source of phosphorus than KH_2PO4. On the contrary $MgCl_2$ doesn't influence the production of biomass [60] and influences negatively the production of SLP and $CaCl_2$ influences positively the production of SLP [102].

6.4 Parameters of the Fermentative Process

6.4.1 Temperature

The fermentative processes for the production of the SLP occur between temperatures of 21 ˚C to 30˚C. The best temperature for the production of SLP varies accordingly to the experimental conditions employed and established by several authors. The best temperature for the production of SLP by resting-cell is of 21˚C, but because of technical matters (better sample-taking and better analysis at lower glycolipid concentration) the temperature of 30˚C is used [83]. For processes in a bioreactor in a batch and fed-batch, temperatures of 25°C and 30°C show very similar results for the production of SLP [89].The production of SLP in shake flasks decreases quickly with the increase of temperature from 27 ˚C (129,8 $g.L^{-1}$) > 30 ˚C (93,2 $g.L^{-1}$) > 34 ˚C (3,0 $g.L^{-1}$) >37 ˚C (0,3 $g.L^{-1}$) [94]. In an industrial process, temperatures of 25 / 30 ˚C are preferred over 21˚C, once the high costs are associated to processes of refrigeration/cooling processes and another factor is that fats and oils do not mix well at lower temperatures like 21°C.

6.4.2 pH

The pH plays an important role in the enzymatic efficiency and the production of SLP can be affected as a result of the specificity differences of the enzyme-substrate. The growth of *Candida bombicola* and the production of SLP are associated to a strong decrease of the pH. During the exponential phase of growth, the pH decreased from values of 6,0 to more acid values between 2,6 and 4,0. This descent of pH is promoted due to the consumption of the nitrogen source and generation of fatty acids [70], [80]. For the production of SLP initially the pH must be adjusted for values between 5,0 and 6,0 to promote cellular growth and further during the stationary phase keep it stable in 3,5 for the production of SLP [83], [49]. Although some authors do not control the conditions of pH for the production of SLP [2], [84] the production under controlled conditions of pH may promote and increase of up to 27,6% in the production of SLP [104]. The production of SLP in acid conditions (3,5) and the antimicrobial effect of the SLP protects the culture of the contamination process, even more, where the SLP are produced in too long fermentative processes (200 hours of fermentation) [62].

6.4.3 Aeration and Oxygenation

The oxygen is essential not only during the exponential phase, but also good aeration conditions are essential for the biosynthesis of SLP, mainly due to the cytochrome P450 monooxygenase needing molecular oxygen for its activity. Generally high oxygenation levels result in increase of the SLP production, this increase is stronger when lower volumes of culture are used. However, high levels of agitation with lower volumes of culture medium decrease the formation of SLP. Therefore, there is a good rate of oxygenation for the production of SLP, which can be defined in terms of transfer rate of oxygen. In fed-batch in shake flasks the best aeration is between 50 and 80 mm O_2. L^{-1}. h^{-1} [107].The aeration can influence in the structure of the SLP, high levels of aeration during the fermentative process lead to the absence of the deacetylated forms and the formation of high proportions of diacetylated forms, also increasing the fraction of lactonic forms [108]. The adjustment in the oxygenation conditions in the initial periods can control the degree of unsaturation of the SLP, low levels of oxygenation can increase the proportion of saturated SLP [107].

6.5 Fermentation Process

The different types of fermentation process (batch, fed-batch, continuous), influences in the production of SLP. The production in batch in shake flasks is used for the production in small scale, ideal for the initial studies of production, with an initial environment volume of 20% of the nominal volume of the flask [71], [105] or in large scales in bioreactors [49], [66]. The biggest productions described in literature are obtained in bioreactor working in the fed-batch form, productions of 300 $g.L^{-1}$ [101], 365 $g.L^{-1}$ [71], 400 g.L-1 [6] and bigger than 422 $g.L^{-1}$ [48]. The production in bioreactor by batch, fed-batch and resting cell allows productions up to six times higher if compared to batch in shake flasks, mainly due to a better aeration promoted by the bioreactor [89]. The controlled addition of the hydrophobic substrate prevents the inhibitory effect of the fatty acids in cellular growth, the production of SLP increases from 20 $g.L^{-1}$ to 317 $g.L^{-1}$ with the controlled addition of hydrophobic substrates (ethyl esters of rapeseed) to the fermentation medium, also influencing in the form of the mix of the SLP produced, which means, in the distribution of the acid and lactonic forms and in the degree of acetylation [109]. The addition of hydrophobic substrates in excess leads to the formation of SLP with a viscous aspect (lactonic forms) which require additional washings, while the controlled addition of the lipid substrate produces SLP in the form of microcrystalline precipitates (acidic forms), relatively simpler to collect and purify [110].

7 Conclusion

Despite numerous advantages and applications that the SLP possess over the synthetic surfactants, the large scale production and the relatively high cost are still a great obstacle for the economic competition. To overcome the barrier of high costs in the production of SLP, many strategies can be used: the use of substrates of low cost and renewable agro industrial alternative substrates for the formulation of medium of culture, development of efficient and optimized bioprocesses of the cultivation conditions and downstream (maximum production with maximum recovery). Therefore, in the industrial fermentative processes the optimization of the medium and culture conditions are essential and critical factors which affect directly the concentration, production, productivity and the costs of the metabolite wished to produce the SLP.

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