Mannosylerythritol lipids: Searching for production and downstream routes

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Abril 2017

Abstract

The present work aims the production, characterization, downstream and application of a biosurfactant produced by *Moesziomyces antarcticus*, mannosylerythritol lipids (MEL).

In the fermentation phase, different carbon sources were explored, including sugars (D-glucose and D-xylose) and a biological oil (soybean oil); also the influence of the nitrogen source addition was analysed. The highest MEL titre was achieved with soybean oil after 18 days of bioconversion, 19.4 g/l with 40 g/l of D-glucose as substrate and 21 g/l of soybean oil added at day 4 of culture.

A characterization of the product, MEL, was performed, in terms of surface tension and a critical micelle concentration (CMC) of 0.02 mg/ml was achieved. Following a perspective of an efficient product recovery from culture broth, with an increased percentage of removed MEL, a strategy of decreasing the amount of solvent used was followed. The best results were achieved using a combination of different steps, including firstly a cell disruption with a mechanical process, sonication, followed by solvent extraction (84.86 ± 14.45%). Lastly, one application was tested and the efficiency of MEL in the formulation of a detergent was evaluated. Soybean oil and chocolate were applied into cotton clothes and the biosurfactant addiction to a commercial detergent increased the removal percentage from 51.78 ± 2.49% to 68.18 ± 0.31% and 86.45 ± 0.28% to 91.73 ± 1.19%, respectively.

Keywords: Mannosylerythritol lipids, product characterization, downstream, application

1. Introduction

Surfactants are compounds with a huge industrial interest, especially due to the wide range of applications in which they can be used, high efficiency in lowering surface tension and very low CMC [1]. However, surfactants can be non-biodegradable and impact negatively the environment by their extend action and by their degradation products or some chemical petroleum accumulated by them, maybe toxic for the environment [2]. Biosurfactants appeared as an alternative way to replace the chemical synthesized compounds with biological products especially because of their low toxicity, high biodegradability, effectiveness at extreme temperatures or pH, and mild production conditions [3].

1.1. Mannosylerythritol Lipids

Mannosylerythritol lipid (MEL) is a glycolipid biosurfactant, mainly produced by *Moesziomyces sp* yeast strains but also by *Ustilago sp* strains, that contain a hydrophilic group, 4-O-β-D-mannopyranosyl-meso-erythritol or 1-O-β-D-mannopyranosylerythritol and a hydrophobic unit, the fatty acid and/or acetyl moiety [4].

According to the degree of acetylation of mannose and fatty acid length, there are different structures of MEL [4]. Therefore, MEL can be designated as MEL-A if it is diacetylated at C4 and C6 of the mannose ring, MEL-B or MEL-C if it is monoacetylated at C6 or C4, respectively and MEL-D if it is completely deacetylated [5]. MELs can be distinguished by thin layer chromatography (TLC) since the elution occurs in accordance with the chemical composition [6].

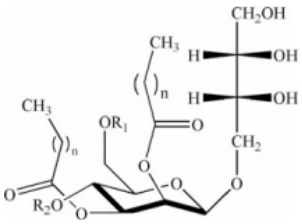

Figure 1 - MEL molecular structure. MEL-A (diacetylated): R1=Ac, R2=Ac; MEL-B (monoacetylated at C6): R1=Ac, R2=H; MEL-C (monoacetylated at C4): R1=H, R2=Ac; MEL-D (deacetylated): R1=R2=H; n=6-10 [7]

1.1.1. Cultivation conditions for MEL production

The composition of seed culture medium and substrate supply influence the production of MEL by the main culture [6]. The carbon source has significant influence on cell growth and MEL production. Soybean oil is identified as the best substrate for MEL production but, it can makes MEL isolation and purification more challenging [38] and, the use of water-soluble carbon sources instead of vegetable oil would thus be highly desirable with glucose and glycerol as the most successfully ones for bioproduction [7,8]. The nitrogen source used in the culture also plays an important role in the biosurfactant production medium as it is fundamental for microbial growth [9]. In the case of mannosylerythritol lipids, *Rau et al* 2005 reported that the highest yield is obtained when sodium nitrate is used [6].

The production of MEL is also influenced by environmental factors that can affect yield and titres and a pH between 4 and 8 as well as a temperature between 25 and 30 ºC should be considered as the optimum values. [3,6] .

1.2. Lignocellulosic materials

In a search for more carbon source sustainable alternatives, there has been an increase in lignocellulosic biomass processing research [48], considered a substrate of enormous biotechnological value [10] and that can be converted in value-add products such as sources for microbial fermentations and chemicals [11]. Lignocellulosic materials are wood, agricultural and forest residues, agro-industrial and municipal solid wastes and comprises about 40-50% cellulose, 25-30% hemicellulose and 15-20% lignin and other extractable components [12,13].

This substrate can be a good alternative since its use has a clear benefit for the environmental impact and recover MEL become easier and cheaper without compromising the final product [12].

1.3. Applications

The wide potential range of applications of biosurfactants in bioremediation, health care, oil and food processing industries make these molecules interesting objects of studies since they possess wetting, emulsifying, detergency and dispersing properties [15]. Among biosurfactants, MEL can be used for cosmetic purposes because of its moisturizing properties [8], in processes of biotreatment, by enhancing the emulsification of hydrocarbon in water and it can also be used for the degradation of petroleum compounds instead of chemical synthetic surfactants and thus reduce the environmental pollution [5, 14].

This biosurfactant also can be used for medical purposes, showing antitumor and antioxidant activity [4] and its application in formulations of laundry detergents, due to its detergency properties can also be explored.

1.4. Extraction and purification of biosurfactants

Every step involved in the production of a biosurfactant has influence on the obtained final product. Beside a high yield and productivity of the production process, the subsequent downstream of the product is economically crucial. The

downstream processing involves steps of recovery and/or purification that represent around 60% of the total production costs, which makes it quite expensive [17].

Apart from the problems with costs, some recovery techniques require the use of solvents and that can be a problem, because if on one hand some applications need a high level of purity, on the other hand, some of these solvents are toxic and harmful to the environment [18]. The most common methods used for biosurfactant recovery include solvent extraction, adsorption followed by solvent extraction, precipitation, foam fractionation, ultrafiltration and adsorption-desorption [19]. A strategy that includes a combination of several steps may be desirable in order to obtain a high degree of purity.

In case of MEL, its isolation and purification is difficult when hydrophobic substrates, such as vegetable oils, are used due to the several complex extraction and purification steps that result in a cost increase of the overall process to obtain pure MEL and with low yields [20] which can be overcome with the use of hydrophilic substrates as sugars [7]. The use of different organic solvents in liquid-liquid extractions achieved a purity level of 100% w/w however with a reduced recovery yield (around 8% w/w); also adsorption on commercial resins and a heat treatment to the cell suspension were tested [21]. The use of a physical method before solvent extraction is also reported, including centrifugation and extraction with ethyl acetate [23]. Another method reported [21] combines the use of ethyl acetate and a preparative chromatography column filled with silica gel using chloroform and acetone as eluents and a yield of 79% w/w for a purity level of 100% w/w was reached.

2. Materials and methods

2.1. MEL cultivation conditions and extraction

MEL was produced , using previously description [3] from *Moesziomyces antarcticus* PYCC 5048^T, cultivated for 3 days at 30ºC on yeast malt agar medium that consists on yeast extract (3 g/l), malt extract (3 g/l), peptone (5 g/l), D-glucose (10 g/l) and agar (20 g/l). Inoculum was prepared (3 g/l $NaNO_3$, 0.3 g/l $MgSO_4$, 0.3 g/l KH_2PO_4, 1 g/l yeast extract, 40 g/l D-glucose) and incubated at 27°C and 250 rpm for 48 hours. The inoculum was prepared in Erlenmeyer flasks with 1/5 working volume of the medium described above. To start the fermentation, 10% (v/v) of the inoculum was transferred and incubated (Aralab, Agitorb 200) for 14 days at 27°C and 250 rpm. To follow cell growth, samples of 1 mL were taken to quantify the biomass (cell dry weight). After centrifugation (Sigma, Sartorius 1-15P) at 13000 rpm for 5 minutes, the supernatant was collected and the pellet, after being washed twice with deionized water, was dried at 60 °C for 48 hours before being weighed. The final product is isolated from the culture broth by a liquid-liquid extraction with ethyl acetate.

A reference condition (here named α) uses an initial concentration of 40 g/l of D-glucose as carbon source with feeding at day 4 of 40 g/l (also D-glucose) and addition of 3 g/l of $NaNO_3$ at day 0 [3] was considered for *M. antarcticus*.

2.2. MEL analysis through GC-FID and sugar quantification by HPLC

A procedure for fatty acid analyses based on a transesterification reaction with a mixture of methanol/acetyl chloride, followed by extraction with hexane and water was implemented [3].

Pure methanol (20 ml) was cooled down to 0◦C under nitrogen atmosphere and 1 ml acetyl chloride was added under stirring over 10 min, which generated a water-free HCl/methanol solution. Culture broth samples (3 ml) were lyophilized (Christ, Alpha 1-2 LD plus) for 48 hours, weighted and mixed with 2 ml HCl/methanol solution and incubated for 1 h at 80◦C for reaction into methyl esters. Heptanoic acid was used as internal standard. The resulting product was extracted with hexane (1 ml) and 1 µl of the organic phase was injected in a GC system (Hewlett-Packard, HP5890), equipped with a FID detector and a HP-Ultra 2 column. The oven was programmed from 140ºC and temperature raised to 170ºC at 15ºC/min to 210 °C at 40 °C/min and to 310 °C at 50 °C/min;

nitrogen gas was used at a flow rate of 25 ml/min. MELs were quantified through the amount of C8, C10 and C12 fatty acids.

To follow the sugar profile, more specifically, to quantify D-glucose and D-xylose, samples of supernatant were measured in a high liquid performance chromatography (HPLC) system equipped with an auto sampler (Hitachi LaChrom Elite L-2200) and with a UV detector (Hitachi LaChrom Elite L-2400) coupled to a (Chromolith Performance RP-18) endcapped column. The supernatants were diluted in a proportion of 1:2 with a solution of sulphuric acid (0.05 M) and centrifuged (Sigma, Sartorius 1-15P) at 13000 rpm for 1 minute to remove some protein that may have precipitated. The supernatant was collected and diluted (1:10) once more in the same sulphuric acid solution (0.05 M). Samples with a total dilution of 1:20 were then analysed and sulfuric acid was used as mobile phase.

2.3. Surface tension and Critical Micelle Concentration

To determine the surface tension, aqueous solutions of MEL were prepared (0.0001 mg/ml, 0.001 mg/ml, 0.01 mg/ml, 0.02 mg/ml, 0.05 mg/ml, and 0.1 mg/ml).

The surface tension was measured in a tensiometer (Kruss, Reagente 5), using the ring method. The values of surface tension decrease with the increase of concentration until stabilize, which corresponds to the critical micelle concentration. Each assay was repeated three times with a difference between them at least of 1 mN/m, above or below, and the final value of surface tension corresponds to the average of them. Also the surface tension of the supernatant was measured.

2.4 Downstream processes

For all the processes described, samples of 10 ml of a 14 days fermentation were taken and centrifuged (Eppendorf, centrifuge 5810 R) at 10000 rpm and 4ºC for 5 minutes in order to separate the cells from the supernatant. The cells were used as described below and the supernatant was frozen. Cells viability was calculated using the following expression:

$$\frac{CFU}{ml} = Nº\ of\ cells\ (average\ of\ 3\ drops) \times Dilution\ factor \times V_{inoculated} \quad (1)$$

2.4.1. Solvent extraction without cell disruption

Cells were re-suspended in 10 ml of solvent aqueous solution. One aliquot was taken and diluted to measure CFU and the remaining mixture was vortexed for 2 minutes and centrifuged (Eppendorf, centrifuge 5810 R) to separate the organic fraction from the cells. After evaporate the solvents, MEL was analysed through GC-FID (Hewlett-Packard, HP5890) after transesterification.

2.4.2. Solvent extraction with cell disruption

Cells were exposed to different disruption techniques, heated in autoclave (10 minutes at 100ºC) (AJC, Uniclave 88), sonicated (3 cycles of 30 seconds interleaved with breaks of 60 seconds) (Bandelin, Sonopuls) and mixed with glass beads (5 ml of NaOH (1M) and 1 ml of glass beads, with a diameter of 0.5 mm, were added to the cells and vortexed for 2 minutes followed by centrifugation to separate the phases). All the phases described earlier were extracted with ethyl acetate and after evaporate the solvent, the samples were lyophilized (Christ, Alpha 1-2 LD plus), transesterified and analysed through GC-FID (Hewlett-Packard, HP5890) to quantify MEL.

2.5. Application of MEL in the formulation of a detergent

Pieces of dry cotton cloth (Piriuki) were cut into 5 x 10 cm and each piece was stained with 0.25 ml soybean oil or 0.5 ml chocolate. The pieces were stored at room temperature overnight and weighted precisely before washing. The stained cotton cloths were washed in an Erlenmeyer containing approximately 1 g of detergent in 50 ml of tap water under stirring (Aralab, Agitorb 200) (300 rpm) and with a set temperature of 30ºC for 30 minutes. After

washing, the pieces were rinsed in 100 ml of distilled water twice, and dried at room temperature to a constant weight.

The same procedure was used for washing the pieces with a commercial detergent and a mixture of biosurfactant in commercial detergent.

It was used a set of formulation containing 0.7 g of sodium tripolyphosphate (STPP), 0.3 g of sodium sulfate (SS) and 0.025 g of MEL in a total volume of 50 mL diluted (solution A) with an aqueous solution of sodium tripolyphosphate and sodium sulfate (solution B) until final concentrations of 0.01 mg/ml, 0.02 mg/ml and 0.1 mg/ml.

$$Oil\ or\ choc\ removal(\%) = \frac{m_{after\ overnight}(g) - m_{after\ dried}(g)}{m_{ater\ overnight}(g)} \times 100 \quad (2)$$

3. Results and discussion

3.1. Fermentation

In this section, the overall goal is to improve MEL titres in sugar-based fermentations, specifically testing alternatives to improve titres achieved with condition α. To do so, the effect of carbon (hydrophilic and hydrophobic) and nitrogen sources were tested to try to increase MEL final concentration.

Figure 2 shows the titres of MEL obtained, after 18 days of fermentation, for all the conditions tested and generally it is possible to conclude that the addition of the nitrate source in different days does not influence the production of the biosurfactant. The results shown also allow the comparison between the carbon sources and some conclusions can be taken such as the fact that using high concentrations of glucose does not increase the concentration of MEL in a significant way which means that with the conditions used, the yeast cannot consume all the substrate introduced in the medium and that results in an expenditure of this component without achieving better results. However, if the comparison is made between different carbon sources, the results are different because it is proved that a boost of soybean oil increases the concentration of MEL when compared to an exclusive use of hydrophilic carbon sources. At this point, it is interesting to analyse these two kinds of substrate because in one way, soybean oil is described as the substrate leading to higher MEL titres and productivities, in other way it has the disadvantage to be hardly sustainable due to the increasing prices of this hydrophobic substrate and the recovery of MEL from oil-containing broth is hindered when vegetable oils are used.

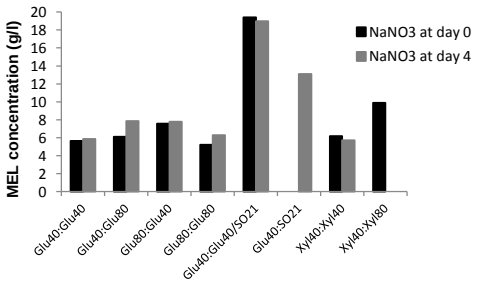

Figure 2 - MEL titre at day 18 of all conditions tested

It was also evaluated MEL yields and productivities for the different conditions tested (Table 1). Analysing the yield results it is possible to conclude that increasing the concentration of the hydrophilic substrates (D-glucose or D-xylose) did not lead to an improve of the yield (for example, for condition α it was achieved a yield of 0.09 g/g which was the same value obtained with the same starting conditions but a feed of 80 g/l at day 4) which means that some of the carbon source introduced in the fermentation medium was not even consumed and, consequently, an unnecessary expense of resources happened. The addition of $NaNO_3$, as the nitrogen source, in different fermentation moments did not also result in differences for the obtained yield. The highest values were reached when a feed of a hydrophobic vegetable oil (soybean oil) occurred with yields of around 0.20 g/g, the double of the ones obtained for D-glucose and D-xylose.

In terms of productivity, this parameter did not rise significantly with an increase of the substrate concentration and, once more, only the feeding of soybean oil brought better results, corresponding to a maximum value of 1.24 to a feed of 21 g/l to α condition.

Table 1 - MEL maximum concentrations, yields and productivities in 18 days cultivation of *M. antarcticus*

		MEL maximum concentration (g/l)	$Y_{MEL/S}$ (g/g)	Productivity (day 14) (g/l.dia)
Addition of $NaNO_3$ at day 0 / Addition of $NaNO_3$ at day 4	Glu40:Glu40	5.66 / 5.92	0.09 / 0.10	0.39 / 0.42
	Glu40:Glu80	6.10 / 7.84	0.09 / 0.12	0.39 / 0.43
	Glu80:Glu40	7.56 / 7.78	0.09 / 0.10	0.48 / 0.47
	Glu80:Glu80	5.20 / 6.67	0.08 / 0.06	0.31 / 0.48
	Glu40:Glu40/SO21	19.39 / 18.99	0.20 / 0.21	1.24 / 1.29
	Glu40:SO21	- / 15.06	- / 0.24	- / 1.08
	Xyl40:Xyl40	6.17 / 6.23	0.11 / 0.11	0.43 / 0.45
	Xyl40:Xyl80	9.90 / -	0.11 / -	0.59 / -

It was observed that the addition of nitrate does not influence the production of MEL (Figure 2); however, the results showed in Table 2 suggest that the addition of nitrogen source at day 4 improve sugar consumption rate, using D-glucose or D-xylose as substrate, being higher in the last case.

When nitrate was added at the beginning the sugar consumption rate decreases after 4 days of fermentation, most probably due to the lack of $NaNO_3$. Rather, when nitrate is only supplied at day 4, the sugar consumption rate increases from here, supporting what is written above. The simultaneous sugar feeding and supply of $NaNO_3$ at day 4 allow higher consumption rates proving that the presence of nitrate favour D-glucose or D-xylose assimilation.

Table 2 - Sugar consumption rate before and after feeding of hydrophilic or/and hydrophobic substrate at day 4 for all conditions tested

	$NaNO_3$ at day 0		$NaNO_3$ at day 4	
	Before feed at day 4 ($g.l^{-1}dia^{-1}$)	After feed at day 4 ($g.l^{-1}dia^{-1}$)	Before feed at day 4 ($g.l^{-1}dia^{-1}$)	After feed at day 4 ($g.l^{-1}dia^{-1}$)
Glu40:Glu40	8.79	7.23	4.70	7.22
Glu40:Glu80	9.67	6.11	4.85	6.60
Glu80:Glu40	11.03	8.46	4.66	8.68
Glu80:Glu80	8.47	6.30	5.02	10.53
Glu40:Glu40/SO21	9.42	5.22	5.26	7.13
Glu40:SO21			5.25	6.02
Xyl40:Xyl40	9.20	8.55	5.08	8.63
Xyl40:Xyl80	9.21	8.52		

3.2. Surface tension and critical micelle concentration (CMC)

Increasing biosurfactant concentration decreases the surface tension until a minimum value of 27.4 mN/m being constant hereafter which means that at this point the surface became fully loaded with surfactant. This value corresponds to a concentration of 0.02 mg/ml, the critical micelle concentration (Figure 3). These values are in accordance with the ones mentioned in the literature since it is known that this biosurfactant can reduce the surface tension of water from 72 mN/m [23] to less than 30 mN/m [4]. The surface tension obtained for the supernatant corresponds to a value of 25 mN/m, also lower when compared to water.

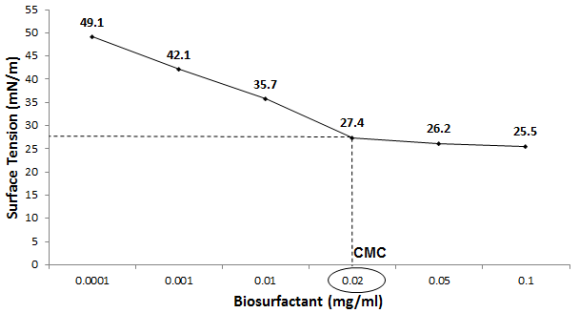

Figure 3 - Surface tension of aqueous solutions of MEL (mN/m) as function of concentration (mg/ml) and the correspondent critical micelle concentration

3.3. Downstream processes

3.3.1. MEL extraction with different solvents

By analysis of Figure 4 it is possible to conclude that most of the systems of water and solvent used showed low capacity to extract MEL from cells; however, ethyl acetate, which is the most common solvent used in extraction due to its favourable characteristics, and isopropanol were the best ones, reaching removal percentages of, 42.27% and 28.80%, respectively, of the total MEL determined in cell pellet fractions.

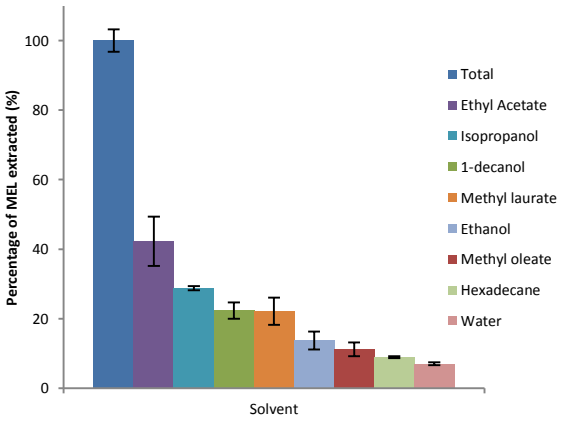

Figure 4 - Percentage of MEL extracted by an aqueous solution of different solvents, 5ml of each solvent and around 5ml of aqueous phase. The solutions are all biphasic except with ethanol and isopropanol that are miscible in water

Although the well-known effectiveness of ethyl acetate in biosurfactants extraction, it was studied, in parallel, the viability of cells after a solvent extraction procedure.

Based on the solvents tested and on the results showed, in a 2-D goal of extract MEL with less cell viability affectation (Figure 5), isopropanol showed the best results because (after the ethyl acetate) is the one that is capable of removing MEL (despite the removal percentage being lower than ethyl acetate) and does not break the cells which are capable to grow in a plate with agar medium. Comparing the two solvents, the ethyl acetate has the advantage of behaving like a biphasic system and that means that all the debris go directly to the aqueous phase which does not occur when isopropanol is used. That fact can represent a disadvantage to use isopropanol because it requires an additional step to remove the debris and cannot be considered, for instance, for a byphasic cultivation directly with use of solvent for in situ product recovery. In this regard, methyl laurate showed to be capable of extracting MEL without breaking cells however with a low percentage of biosurfactant removal when compared with the other two, representing a total percentage of 22, but generating a two-phase system.

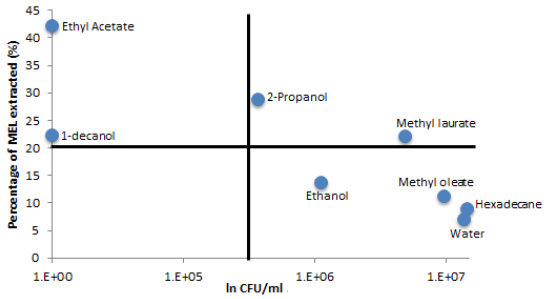

Figure 5 - Comparison between the cells' resistance to the solvents and MEL extraction. The upper left quadrant concerns to solvents that are capable of extracting MEL but affects cells viability; the upper right quadrant concerns to solvents that are capable of extracting MEL without break cells; the lower left quadrant concerns to solvents that affect cells viability but do not extract MEL and the lower right quadrant concerns to solvents that do not affect cells viability and do not extract MEL

3.3.2. MEL extraction with ethyl acetate

The results present in Figure 6 show that when the concentration of ethyl acetate is lower, the extraction of MEL decreases drastically from around 95% to around 50%, which means that the extraction is not as efficient as when higher quantities of solvent are used. Many references use ethyl acetate to extract MEL using equal amounts of solvent and fermentation broth because of its efficiency, as proved in this assay, and more recently as a substitute for tert-butyl methyl ether since this solvent is more toxic [12, 21, 24].

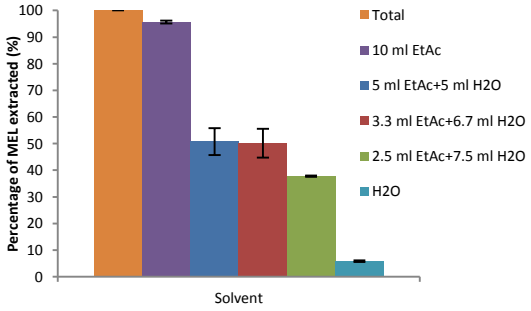

Figure 6 - Percentage of MEL extracted as function of the different concentrations of ethyl acetate used

3.3.3. Cells disruption followed by extraction with ethyl acetate

Three different methods of cells disruption were tested including the use of an autoclave, the sonication the use of glass beads. The results are shown in Figure 7.

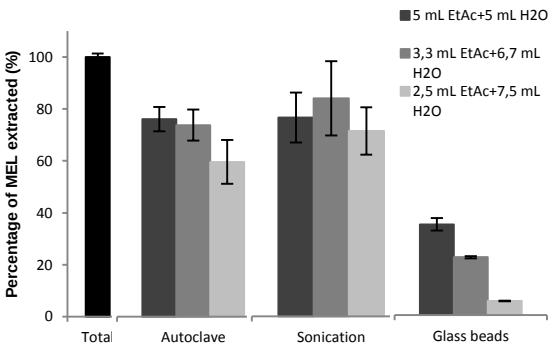

Figure 7 - Percentage of MEL extracted as function of the different concentrations of ethyl acetate used after cell's disruption by autoclave, sonication and the action of glass beads

Comparing the results obtained for the three different methods of disruption, the use of the autoclave or the ultra sounds increased the removal percentage of MEL; however, the use of glass beads showed not to be efficient. Possibly the mechanical action that the glass beads are exerting on the cells are not strong enough to destabilize the medium and it does not promote such an efficient mobilization of MEL to the aqueous phase. On the other hand, and assuming that the glass beads

action was efficient, the lower percentage of MEL removed using this method can due to the fact that ethyl acetate is not the adequate solvent to disadsorb MEL from the glass beads.

So, it is possible to verify that two steps of downstream, including cells disruption followed by solvent extraction, perform better results than just one step. The values showed in Figure 7 concern to a total volume of MEL however before sonication the cells were re-suspended in water or in NaOH before the addition of the glass beads. The aqueous and cells phases were then separated by centrifugation and both of them were extracted with ethyl acetate.

3.4. Applications

3.4.1. Application of MEL in the formulation of a detergent

One of the main applications of biosurfactants is its addition in the formulation of a detergent. For this work, small pieces of cotton cloth were stained with chocolate and soybean oil and then washed following the method described on 2.5. The percentage of oil or chocolate removed from each cotton cloth was calculated in accordance with equation (2). The results obtained are present in Figure 8.

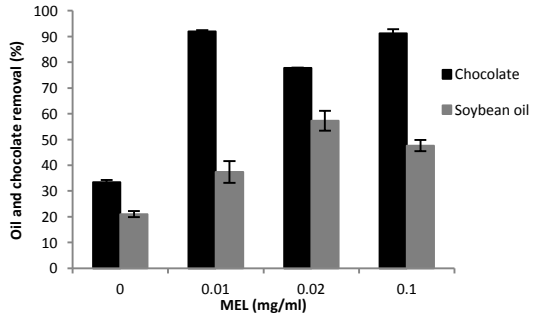

Figure 8 - Weight percentage of oil and chocolate removed in accordance with the different concentration of biosurfactant used in the set of formulation; the dark blue bars correspond to chocolate and the light blue bars to soybean oil

The results indicate clearly the effectiveness of biosurfactant in oil and chocolate removal.

On the one hand, washing the cotton cloths stained with soybean oil, using MEL at CMC (0,02 mg/ml) improves rapidly the removal when compared with its absent; however, this removal is not improved by concentrations above this value which was selected as the optimum value.

On the other hand, use concentrations of MEL above or below the CMC to wash the cotton cloths stained with chocolate is greater as the weight percentage removal is higher when compared with the washing solution that contains MEL at critical micelle concentration. Without biosurfactant, the removal was lower as it happened with soybean oil.

Comparing the removal of chocolate with the removal of soybean oil, the first is higher which could happen because chocolate is more hydrophilic and that result in a higher dissolution in water, instead of soybean oil that is more hydrophobic.

Also the comparison between a commercial detergent and a mixture of commercial detergent with MEL was investigated. The obtained results are described in Table 3.

Table 3 - Soybean oil and chocolate weight percentage removal (%) by commercial detergent and by commercial detergent supplemented with MEL

	Soybean oil (%)	Chocolate (%)
Commercial Detergent	51.78±2.49	86.45±0.28
Commercial Detergent + MEL	68.18±0.31	91.73±1.19

The results mentioned in Table 3 show that the biosurfactant had a significantly positive effect on the performance of the commercial detergent. Once more the removal percentage of chocolate was higher than the removal percentage of soybean oil, in support of what was written before that the removal is easier for hydrophilic contents.

4. Conclusions and future work

All the experiences performed have been built based on the same fermentation condition, previously nominated as condition α, a sustainable condition based on the use of sugars as carbon source, with titres of 5.7 g/l after 18 days of fermentation.

It was first tested the influence of different concentrations of D-glucose as well as other carbon sources, such as xylose and a boost of a soybean oil. In parallel, the influence of the nitrogen source was assessed with additions of $NaNO_3$ at day 0 or

at day 4. With a condition that mixes glucose and soybean oil, better results were obtained and maximum value of 19.39 g/l was reached after 18 days of fermentation. Even a condition with initial 40 g/l and 21 g/l of soybean oil added at day 4, with total molar carbon equivalent to condition α, resulted in 13.11 g/l of MEL, an improvement of 131.63% in MEL production, and maintaining a low concentration of residual oils in the end of fermentation.

In order to characterize the product the surface tension was measured, as one of the main importants properties in the biosurfactants' class. The surface tension and, consequently, the critical micelle concentration have never been analysed in this kind of fermentation and a CMC of 0.02 mg/ml, corresponding to a surface tension of 27.4 mN/m was obtained. This value is in accordance with others obtained for fermentations using soybean oil as carbon source [23, 26]. Lower surface tension is a characteristic common to biosurfactants and it is responsible for the potential of MEL for commercial applications.

To increase the concentration and purity of MEL extracted, different downstream processes were assessed, including solvent extraction with different solvents and cells disruption with mechanical processes followed by solvent extraction. The use of solvents demonstrated the lack of efficiency of this method, with exception to the ethyl acetate and the isopropanol. The first one reaching the highest percentage of MEL removal and the second one, although decreases the removal percentages, has proven to be capable of maintaining the cells integrity. The best results were achieved when disruption methods followed by solvent extraction was performed with a maximum removal percentage of 84.86 ± 14.45% using sonication followed by extraction with ethyl acetate. Using high temperatures (autoclave) as disruption method also good values were obtained (76.09 ± 4.72%). Never before, this kind of downstream processes had been tested with *Moesziomyces antarcticus* and using sugars as the only carbon source.

Finally, the application of MEL as a detergent was tested, firstly using different concentrations of MEL in the formulation and secondly comparing its efficiency with a commercial detergent. The removal of chocolate and soybean oil from a cotton cloth was performed and for chocolate, cleaning percentages of around 90% were obtained, even for a concentration of MEL below the CMC. The commercial detergent used was capable of removing around 51.78 ± 2.49% of soybean oil and 86.45 ± 0.28% of chocolate. The addition of 10% of MEL in the commercial detergent solution increased the values in 33 and 6.1%, respectively.

Regarding the production of MEL, it would be important to increase the titres of MEL without using or reducing biological oils, and using lignocellulosic materials as carbon source due to the benefits for the environment that this kind of materials has.

Also downstream processes need to be developed, especially the disruption methods that showed to be efficient and can be an alternative the use of solvents, especially ethyl acetate.

Also the supernatant disclosed to have many interesting properties that need to be developed in order to increase its use in different situations. One of these situations, which also need to be further explored, is the application of MEL in the formulation of a detergent and since the supernatant also has lower surface tension would be an interesting possibility to explore. Other applications, such as the use of this biosurfactant in bioremediation assays should be performed in the future.

5. References

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