BIODEGRADABILITY STUDY OF THE BIOSURFACTANT OBTAINED FROM CORN STEEP WATER UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

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ABSTRACT

Over the last few years, the global biosurfactants market has raised due to the increasing awareness among consumers for the use of biological or bio-based products. Because of its composition, it can be speculated that these are more biocompatible and more biodegradable than their chemical homologous. However, at the moment, there are no studies in the literature about the biodegradability of biosurfactants. In this work, a biosurfactant, obtained from a corn wet-milling industry stream, fermented spontaneously in presence of lactic acid bacteria, was subjected to a biodegradation study, without addition of external microbial biomass, under different conditions of temperature (5-45 ºC) and pH (5-7). The Box-Behnken factorial design allowed predicting the biodegradation percentage between the range of the dependent variables under study, obtaining values between 3 % and 80 %. Furthermore, it was also possible to predict the variation in $t_{1/2}$ (time to achieve the 50 % of biodegradation) under different conditions.

Keywords: surface-active compounds; biosurfactant; corn stream; fatty acids; biodegradability; environment.

INTRODUCTION

Surfactants are used in different areas such as pharmaceutical, chemical or environmental industries [1, 2]. This fact is caused by their typical structure, composed by a hydrophobic tail and a hydrophilic head, which improves the solubilization of oils, pigments and heavy metals, among others, in aqueous solutions [2–4]. Typical chemical detergents comprise sodium dodecyl sulfate (SDS), polysorbates (Tween 20, Tween 80) and other sorbitan derivatives (Span 20, Span 60). These synthetic surfactants are incorporated in detergents, house cleaning, shampoos and other cosmetic products related to personal care, leading to the presence of a high amount of surfactants in aquatic systems [5].

Some studies have demonstrated that chemical surfactants can remain in the environment, producing different effects in animal cells. For instance, Ying [6] have found toxicity associated with the presence of surfactants in marine media, due to the lack of efficiency in the secondary treatment of conventional wastewater plants [7]. As a consequence, the biodegradation of these chemical-based surfactants is one of the most critical steps for the reduction of surfactant concentration in wastewater. It must be mentioned that anionic and non-ionic surfactants produce chronic toxicity to aquatic animals at concentrations over 0.01 g/L, although cationic surfactants are more toxic than anionic and non-ionic ones [8]. Moreover, it should be noticed that compounds like alkyl benzene sulfonates (LAS), secondary alkane sulfonates (SAS) or cationic surfactants, such as quaternary ammonium-based compounds, are not biodegraded by microorganisms under anaerobic conditions [5, 9–11]. In order to avoid these problems, the use of biosurfactants instead of chemical detergents could be an interesting alternative.

Biosurfactants are biological or bio-based detergents, composed by a hydrophobic and hydrophilic side as their synthetic counterparts. The main difference is that biosurfactants are produced by microorganisms, and formed by natural biopolymers of lipids, proteins, peptides or sugars. Therefore, it can be speculated that they are more biocompatible and biodegradable than their synthetic homologous [12]. However, there has not been found any study in the literature regarding the evaluation of their biodegradability.

On the other hand, as it was mentioned, the presence of a fatty acid chain is very common in the structure of biosurfactants, specially in those produced by different strains of *Bacillus* [13, 14]. For instance, Surfactin and Fengycin, which are lipopeptides biosurfactants produced by *Bacillus subtilis*, are composed by C13-C16 fatty acids [15, 16]. Specifically, surfactin is composed by heptapeptides and β-hydroxy fatty acids of 13-15 carbons, whereas Fengycin is composed by C13 to C17 fatty acids, apart from a peptide chain [17]. Furthermore, the presence of a new biosurfactant in corn steep water, was recently discovered. This biosurfactant is composed by 41 % C16 and C18 fatty acids and 1.5 % of N, and its presence in corn steep water is related to the spontaneous growth of lactic acid bacteria and other microbial biomass. As a matter of fact, during the steeping process of corn, there can be produced different metabolites, being lactic acid the major compound [18] as well as other secondary metabolites including biosurfactants [19–21]. The biosurfactant extract obtained from corn steep water presents antioxidant properties [22]. Regarding the structure, it possesses an hydrophobic tail mainly composed by fatty acids [22, 23] and a hydrophilic head containing nitrogen, similarly to lipopeptides [20, 24].

In terms of biodegradation, it could be speculated that lipopeptides will be degraded in a similar way to fatty acids, as it is their predominant fraction. However, the presence of

fatty acids could limit the biodegradation of lipopeptide biosurfactants. For instance, Čipinytė and coworkers [25] have evaluated the biodegradability of different fatty acids. This study was carried out under the presence of 124 different microbial cultures capable of degrading lipid compounds, observing biodegradation rates between 65.5-95 % for oleic acid, 87.1-95.7 % for linoleic acid, 11.3-29.3 % for stearic acid and 12.7-31.2 % for palmitic acid, depending on the strain selected.

Additionally, it is also important to remark that most biosurfactants can act as antimicrobial agents [26–30]. Although this fact has many interesting applications, especially in the pharmaceutical industry, it could prevent their future biodegradation. In this sense, it would be essential that biosurfactants were not biodegraded immediately in order to develop their function in the formulations where they are included. However, from an environmental point of view, it could represent a problem, not only because of the changes in microbiota caused by the antimicrobial effect of biosurfactants, but also due to the costs that could imply their elimination [31]. Consequently, there is a need to study the biodegradation process of biosurfactants in order to establish their environmental impact as well as their optima formulation conditions and stability, due to they can be applied in different industrial sectors [15, 32, 41, 33–40].

Hence, the aim of this work was to evaluate the biodegradability of the biosurfactant extract obtained from a corn wet-milling industry stream, composed mainly by C16 and C18 fatty acids, similar to the lipopeptides produced by different *Bacillus strains,* under different environmental conditions, including different pH and temperatures.

MATERIALS AND METHODS

Extraction of biosurfactant extract from CSW

Corn steep water (CSW) was provided by Santa Cruz Biotechnology with a solid content of 50 % (LOT D2814) and diluted in distilled water up to 50 g/L. This solution was extracted with chloroform (CSW:CHCl$_3$ 1:2 v/v), at 56 ºC for 1 h, following the protocol established by Vecino and collaborators [21]. After that, the organic solvent was eliminated by vacuum distillation, using a rotatory evaporator R-210 (Buchi, Switzerland) and the biosurfactant extract was obtained.

Characterization of the biosurfactant extract from CSW

After extraction, the biosurfactant was dissolved in water up to concentration of 1 g/L, measuring the surface tension of the biosurfactant solution by triplicate using a tensiometer EasyDyne (Kruss, Germany), following the Wilhelmy plate method. Moreover, the pH was measured with a pH-meter Basic 20 (Crison, Spain).

In order to determine the critical micellar concentration (CMC) of the biosurfactant extract, different solutions of biosurfactant between 0.100 g/L and 1 g/L were prepared and filtered through a 0.45 μm pore and 13 mm diameter membrane of polytetrafluoroethylene (PTFE). Following, the surface tension of these solutions was measured. As negative control, the surface tension of water was also measured, obtaining a value of 72 mN/m. Below the CMC, the concentration of biosurfactant is directly related with the surface tension of the aqueous solution in which it is dissolved. Moreover, the ionic charge of the biosurfactant was evaluated by using cationic and anionic resins. The anionic resin consisted of Amberlite IRA 400 charged with Cl$^-$; whereas the cationic resin consisted of Amberlite IR 120 charged with H$^+$. Before applying any resin, both were washed with HCl 1 mol/L, followed by deionize water up to pH 7, in order to ensure their activation. For the evaluation of the ionic charge, biosurfactant was dissolved in water at a concentration of 0.241 g/L, close to their CMC [24], and 50 mL of these solutions were passed trough a cartridge containing 1.2 g of

Amberlite IRA 400 or Amberlite IR 120. In order to study if the resin entrapped the biosurfactant, the surface tension of the eluted phase was measured.

Purification and Mass spectrophotometry analysis of biosurfactant extract

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was used to characterize the different masses present in the biosurfactant extract under evaluation, and compared with two commercial lipopeptide biosurfactants: Surfactin (Sigma-Aldrich, USA) and Fengycin (Sigma-Aldrich, USA). In order to obtain a purer biosurfactant extract from CSW, once extracted with chloroform, the biosurfactant was dissolved in water at concentration 1g/L, and 30 mL were passed through a cartridge containing 1.22 g of Amberlite IR 120. Following, the biosurfactant entrapped in the resin was desorbed using an aqueous solution of NaCl 0.5 mol/L. Next, the biosurfactant solution was dialyzed during 48 h, at 4 ºC, against demineralized water in a Spectra/Por ® dialysis membrane (molecular weight cut-off 6000-8000 Da; Spectrum Laboratories, Inc., USA) and lyophilized using a LyoQuest HT40 (Telstar, USA) prior to MALDI-TOF-MS analysis.

The matrix used for crystalizing the sample before the analysis by MALDI-TOF-MS was α-Cyano-4-hydroxycinnamic acid (HCCA), and the analysis were carried out with a Bruker FTMS APEXIII Mass spectrometer (Bruker, USA) using a voltage of 70 eV in positive mode.

Fatty acid characterization

Complementary to MALDI-TOF-MS, a fatty acid analysis was also done for determining the type of fatty acids present in the sample. The fatty acid composition of the biosurfactant extract studied during this work was determined by gas chromatography (Trace GC Ultra, Thermo Scientific) coupled to a mass spectrometer

(Trace DSQ, Thermo Finnigan). First of all, the extract was methylated and trans-esterificated, tranforming them into fatty acid methyl esters (FAMEs), according to the method described in the European Standard, ISO 12966- 2:2011. FAMEs separation was performed on a ZB-WAX column (60 m x 0.25 mm i.d. x 0.25 μm film thickness) with an oven temperature gradient of 60 ºC for 2 min. Then, the temperature was increased firstly until reaching 150 ºC at 3 ºC min^{-1}, and subsequently up to 200 ºC at 10 ºC min^{-1} and held for 27 min. Finally, the temperature was increased again to 240 ºC at 5 ºC min^{-1}, and held for 30 min. Helium was used as carrier gas at a flow rate of 1 mL min^{-1}, and the temperature of both injector inlet and the transfer line of detector was set at 250 ºC. The mass spectra were obtained using a mass selective detector under electron impact ionization at a voltage of 70 eV, and data were acquired over an m/z range of 40–400. FAMEs were identified from a mass spectra library supplied with the GC-MS system and by comparison of retention times and mass spectra of a FAME standard mix, injected under the same conditions.

Biodegradability study

Biodegradability study was carried out in 50 mL Erlenmeyer flasks, without stirring, with 10 mL of an aqueous solution containing 1 g/L of biosurfactant extract. The independent variables fixed in the study were temperature (5, 25 or 45 ºC), biodegradation time (15, 35 or 55 days) and pH (5, 6 or 7). Before carrying out the experiments, the pH of samples was adjusted using NaOH 1 mol/L or HCl 1 mol/L. These range of pH was chosen due to most microorganisms need neutral growth conditions, and because these conditions are the most common in real biodegradation processes [42].

The dependent variable evaluated was the amount of biosurfactant biodegraded at the time established in the design. For calculating this parameter, samples were filtered

through a 0.45 μm pore, and 13 mm diameter membrane of PTFE. Following, their surface tension was measured.

It is known that below the CMC exists a linear relationship between the surface tension and the biosurfactant concentration in water. Therefore, it is possible to convert values of surface tension in concentration of biosurfactant, when the concentration is below the CMC [43, 44]. However, it is important to remark that CMC can vary depending on the pH [45–47]. For that, three different calibration curves were established measuring the surface tension of water at different concentrations of biosurfactant, below the CMC, at the three chosen pH in the experimental design. When the amount of biosurfactant was over the CMC, samples were diluted, and the dilution factor was taken into consideration for calculating the amount of biosurfactant that was not biodegraded.

The experimental design developed consisted of a Box-Behnken factorial design [48, 49], establishing a theoretical equation that relates the independent variables under study with the percentage of biosurfactant removed (see **Equation 1**). Using **Equation 1,** it is possible to predict the time needed to biodegrade the biosurfactant subject of study, under different environmental conditions. The standardized dimensionless independent variables used, with limits of variation (-1,1) were defined as: X_1 (temperature), X_2 (time), and X_3 (pH), whereas the dependent variable consisted of the percentage of biodegraded biosurfactant extract was named as Y_1.

$$Y = \beta_0 + \beta_1 X_1 + \beta_{11} X_1^2 + \beta_2 X_2 + \beta_{22} X_2^2 + \beta_3 X_3 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3$$
$$+ \beta_{23} X_2 X_3 \qquad (1)$$

Table 1 includes the range of the independent and dependent variables under study. The relationship between coded and un-coded variables was established by linear equations, also included in **Table 1**, and deduced from their respective variation limits.

Microbial screening after biodegradability study

A screening of microbial biomass was carried out by counting the number of colonies forming units (CFU), in some selected samples. For that, samples subjected to 55 days of biodegradation were diluted several times depending on the case, and plated in Columbia Blood Agar (CBA), for testing the presence of aerobic bacteria, and Sabouraud Dextrose Agar (SDA), for testing the presence of yeast and fungi [50], until the amount of colonies were countable. All plates were incubated for 3 days, and the temperature was the same as the experimental design established for the biodegradation test in each case (5, 25 or 45 ºC).

RESULTS AND DISCUSSION

Characterization of the biosurfactant extract from CSW

The biosurfactant evaluated in this work was produced in a corn milling industry stream, fermented spontaneously in presence of lactic acid bacteria. During the extraction and characterization of the natural extract the pH was not modified. However, as the CMC and surface activity can be modified by the pH, these two particular properties were determined at the range of pH established in the biodegradation study. As a matter of fact, this biosurfactant was able to reduce the surface tension of water from 18 to 33 units, with a CMC between 0.119 to 0.237 g/L, depending on the pH. **Table 2** also includes the equations that relate the concentration of biosurfactant, below the CMC, with the surface tension, at different pH, where y is the surface tension value and x is the biosurfactant concentration.

Additionally, the biosurfactant was entrapped by anionic (Amberlite IRA 400) and cationic resins (Amberlite IR 120), as the aqueous solution eluted from both resins gave surface tensions around 68-70 mN/m, similar to water. Thus, it can be said that the

biosurfactant is amphoteric, which is in agreement with the data published in previous works [24].

Before performing the mass spectrophotometry analysis, in order to obtain a purer extract free of fatty acids or glycerides, the biosurfactant extract obtained from CSW was passed through the cationic exchange resin Amberlite IR 120. The compounds entrapped in the resin were desorbed with a saline aqueous solution. After desorption, the presence of biosurfactant was corroborated by measuring the surface tension of the eluted fraction. Following, biosurfactant solution was dialyzed, lyophilized and analyzed by MALDI-TOF-MS. This technique was developed during the past decade into a versatile tool for biopolymer analysis, which is similar to electrospray ionization (ESI). Both techniques consist of analyzing the fragmentation pattern of biomolecules, when they are ionized through matrix-assisted laser desorption/ionization (MALDI), although MALDI produces far fewer multi-charged ions in comparison with ESI [51– 53].

Fig. 1 shows the MALDI-TOF-MS spectrum acquired for the biosurfactant extract obtained from CSW, as well as for the two commercial lipopeptides biosurfactants used as controls (Surfactin and Fengycin). It can be observed in the biosurfactant extract under evaluation, the presence of compounds with molecular weights between 800 Da, and 1482 Da. These m/z signals are similar to those found in lipopeptide biosurfactants. Although, most of Surfactin isomers gave higher intensity peaks, about 1000 m/z, some authors have reported the presence of intense signals between 887 and 1527 m/z. Therefore, DimKic et al. [52] analyzed different biosurfactant extracts obtained from *Bacillus* strains fermentation, using MALDI-TOF-MS. These authors extracted the biosurfactant with ethyl acetate, followed by acid precipitation and methanol extraction, observing in their mass spectra high intense signals at 861, 882, 900, 912, 934, 950,

956, 1016, 1024, 1042, 1048, 1064, 1070, 1102, 1123, 1145, 1174, 1196, 1508, and 1527 m/z, similarly to those peaks observed in the biosurfactant extract obtained from corn steep water.

The fragmentation of masses corresponding to the signals at 1066, 1277 and 1482 m/z, of the biosurfactant under evaluation, gave similar peaks than those produced by the fragmentation of peaks at about 1000 and 1500 m/z of Surfactin and Fengycin, respectively. In **Fig. 2** and **Fig. 3** are highlighted those peaks with similar mass to Surfactin and Fengycin, produced by the fragmentation of the mass at about 1000 m/z and 1500 m/z respectively. Rincón-Fontán and coworkers [54], also reported the presence of masses about 1500 Da in the biosurfactant extract obtained from CSW, compatible with one similar to Fengycin. Additionally, the mass found at 1277 m/z in the biosurfactant extract under evaluation, was fragmented, producing the following fragments: 1259, 1088, 899, 773, 704, 498, and 74 m/z.

Bhardwaj et al., [51], isolated a dark brown honey colored biosurfactant, produced by *Fusarium proliferatum* growing on rice bran. This biosurfactant was able to reduce the surface tension of the distilled water from 71.2 to 36.6 mN/m with a CMC of 0.33 g/L, similarly to the biosurfactant evaluated in this work. The biosurfactant studied by Bhardwaj and coworkers [51] was composed by 3 chains of long fatty acids and linked to 3 amides with a molecular weight of 1236 Da. The fragmentation of the main peak of this biosurfactant using ESI techniques, gave fragments of 871, 701, 634, 605, 475 and 453 m/z, which are also compatible with the fragments observed in the biosurfactant extract obtained from corn steep water. Bhardwaj et al. [51] also found that the biosurfactant obtained from rice showed antioxidant properties, which is also a characteristic of the biosurfactant extract obtained from corn steep water, as it was demonstrated in a previous work [22]. Therefore, further analysis should be carried out

in order to characterize the nitrogen chain of the biosurfactant under evaluation. However, in a first approximation, it can be speculated that the biosurfactant under evaluation is similar to Fengycin and Surfactin, due to the mass spectrum fragmentation showed peaks at 74 and 72 m/z which are compatible with the mass of amino acids, not observed by Bhardwaj et al. [51]. Some authors [55] observed that the mass fragmentation of amino acids such as valine, leucine, aspartic acid and glutamic acid, gave a fragmented mass of 74 m/z present in the fragmentation of lipopeptides.

On the other hand, the fatty acid analysis of the biosurfactant extract under evaluation revealed the presence of C16 and C18 fatty acids such as palmitic, stearic, oleic and linoleaidic acids (see supplementary material **Fig. 1),** which is in concordance with the data reported in previous works [20, 22, 56].

Biodegradability study

The biosurfactant extract under evaluation, obtained after its extraction with chloroform from corn steep water, has shown interesting properties for its applications in the environmental [37], nanotechnology [57] and cosmetic industries [22, 39, 54, 56]. However, before studying its industrial application, it would be interesting to evaluate its biodegradability.

In order to study the biodegradability of the biosurfactant extract, an incomplete Box-Behnken factorial design was carried out, establishing different temperatures, pH and biodegradation times. **Fig. 4** shows the variation on the biosurfactant biodegradation predicted by the model with the most significant variables, temperature and pH after 15 days (**Fig. 4a**), 35 days (**Fig. 4b**) and 55 days (**Fig. 4c**), which is in concordance with the experimental data included in **Table 3**.

The biodegradation process of biosurfactant is strongly influenced by the pH, observing a big difference between low and high pH, keeping constant the other variables (time

and temperature). In this regard, it was observed that low pH produced lower degradation. For instance, the percentage of biodegradation values achieved after 15 days at 25 ºC were 29.83 % and 59.66 % for pH 5 and 7, respectively. Moreover, after 25 days of biodegradation process and 45 ºC, the values obtained were 0 % and 41.33 % for pH 5 and 7, respectively.

As a matter of fact, Katam et al. [58] obtained percentages of LAS biodegradation between 53.9 and 99.7 % after 10 days at pH 8. On the other hand, Menzies et al. [59] also observed rapid degradation, achieving 50 % of LAS biodegradation after 36 hours. However, in these experiments, seed sludge and wastewater with microorganisms were used as inoculum, so the biodegradation time was drastically reduced in comparison with the experiments performed in absence of inoculum.

On the other hand, **Table 3** shows the coded values of the dependent variables included in this study, as well as the experimental results obtained for the dependent variable selected (Y_1). The difference between the theoretical and experimental data, based on the percentage of biosurfactant biodegraded, gave a r^2 of 0.966. Additionally, **Table 3** also includes the coefficients and their statistical significance obtained after data treatment. Those coefficients with p values higher than 0.05 were not considered significant. Therefore, it was possible to establish a theoretical equation (**Equation 2**) that allowed predicting the percentage of biodegraded biosurfactant, in the work range established for the independent variables selected.

$$Y_1 = 66.48 - 5.25X_1 - 39.45X_1^2 + 10.36X_2 - 6.51X_2^2 + 15.06X_3 - 1.43X_3^2$$
$$+ 2.19X_1X_3 - 3.27X_2X_3 \qquad (2)$$

The most significant variable in the biodegradation of this biosurfactant extract was the temperature, followed by the pH. The theoretical equation obtained predicts maximum

biodegradation of biosurfactant extract at intermediate temperatures (25 ℃) and pH higher than 6. Under these conditions, 30 days will be enough to biodegrade about 80 % of biosurfactant extract.

Moreover, **Fig. 5** shows the variation of $t_{1/2}$ predicted by **Equation 3**, with the temperature under pH 5 (**Fig. 5a**) and 6 (**Fig. 5b**). The variation of $t_{1/2}$ predicted under pH 7 was included as **Figure 2** in Supplementary material. It followed the same behavior than the variation of $t_{1/2}$ predicted by **Equation 3** at pH 5 and 6. However, at pH 7 were obtained lower $t_{1/2}$ values, more distant from the range of time included in the experimental design. The **Equation 3** was obtained from **Equation 2**, taking into account only those significant coefficients and the lineal term corresponding to variable X_2. Therefore, **Equation 2** was converted in **Equation 3**, where Y_1 was replaced by a constant value of 50, corresponding with the % of biodegradation expected at $t_{1/2}$, and X_2 was renamed as $t_{1/2}$, corresponding with the time where is achieved 50 % of biosurfactant biodegradation, as follows:

$$t_{1/2} = (66.48 - 39.45X_1^2 + 15.06X_3 - 50)/-10.36 \qquad (3)$$

In **Equation 3,** $t_{1/2}$ can be predicted at different pH values and temperatures. Thus, when the pH was fixed at 5, the lowest $t_{1/2}$ was predicted at 25 ℃. In general, it was observed that $t_{1/2}$ decreased between 14 ℃ and 25 ℃ and increased between 25 ℃ and 36 ℃. Below 14 ℃ and above 36 ℃ and at pH 5, **Equation 3** predicts biodegradation times higher than 60 days. When the pH was increased up to 6, the $t_{1/2}$ values were reduced to 16 days and lower. In this way, between 18 ℃ and 32 ℃ for pH 6 and between 11 ℃ and 39 ℃ for pH 7, the time predicted by **Equation 3** was lower than 15 days.

In general, it was observed that at pH 6, $t_{1/2}$ has been reduced at all the temperatures assayed, in comparison with the results predicted at pH 5. For pH 6, the $t_{1/2}$ values between 3 days, at intermediate temperatures, and 56.6 days at 9 ℃ were observed.

However, those $t_{1/2}$ lower than 15 days or higher than 55 days, should not be taken into consideration, as they are out of the time range studied (15-55 days). For this reason, **Fig. 5**, shows the covered range of the study, between 2 lines, in order to point out which values are in and out of it. The same behavior was observed at pH 7, but in this case **Equation 3** predicts $t_{1/2}$ values lower than 15 days between 11 and 39 ºC, even achieving negative values between 14 and 36 ºC, what suggest a strong biodegradation of this biosurfactant extract in this temperature range. Therefore, in order to use this biosurfactant for the bioremediation of contaminated sites as solubilizing agent of hydrophobic contaminants, pH should be controlled, fixing its value between 5 and 6, depending on the temperature, to avoid its immediate biodegradation.

On the other hand, **Fig. 6** shows a picture of the microbial biomass isolated after 55 days from experiments 2, 4, 6 and 8. The main objective of this screening is to determine the kind of microorganisms responsible for the biosurfactant biodegradation. It was observed that at pH 5 and 25 ºC (experiment 2) aerobic bacteria were the predominant microorganisms, with a concentration of 1.1×10^{32} CFU/mL, whereas the concentration of fungi and yeast was 1.7×10^5 CFU/mL. Moreover, at this temperature (25 ºC), but at a higher pH, pH 7 (experiment 4), the concentration of yeast and fungi, and aerobic bacteria 1.0×10^5 CFU/mL and 1.8×10^{32} CFU/mL, respectively. On the other hand, at 45 ºC and pH 6 (experiment 8) it was only detected the presence of yeast and fungi, with a concentration of 6.3×10^3 CFU/mL, which is consistent with the low biodegradation rates of biosurfactant obtained in the experimental assay (22 %). Furthermore, at lower pH the concentration of yeast and fungi detected was higher, which is in agreement with the optima pH growth conditions of these microorganisms, considering those at pH between 5 and 6 [60]. On the other hand, at low temperatures, 5 ºC, and pH 6 (experiment 6) was detected only the presence of aerobic bacteria, $3.4 \times$

10^6 CFU/mL, which is consistent with the biodegradation rate of biosurfactant observed under this experimental condition (33 %). The higher concentration of microorganisms detected at 25 ºC is in concordance with higher biodegradation rates, observed at intermediate temperature conditions. The more favorable biodegradation conditions were observed at 25 ºC and pH 7, leading to 80.7 % of biosurfactant biodegradation. These results are in agreement with other works [61], where it was found that biodegradability can be improved using basic conditions.

CONCLUSIONS

In general, it was demonstrated that the biodegradation of the biosurfactant, extracted from a residual stream of corn milling industry, in absence of inoculum addition is linked to the environmental conditions, observing a $t_{1/2}$, no lower than 35 days at pH 5. This is very interesting regarding the use of this biosurfactant extract in different applications, being possible to strike the balance between their permanence and biodegradability in the environment. An immediate biodegradation of biosurfactant would not be desirable because there would be a risk of being biodegraded before realizing its task.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Table 1. Dependent and independent variables used in the Box-Behnken factorial design.

a) Independent variables			
Variable	Nomenclature	Units	Range of variation
Temperature	T	ºC	5-45
Biodegradation time	t	days	15-55
pH	pH	-	5-7
b) Dimensionless, coded independent variables			
Variable	Nomenclature	Definition	Range of variation
Temperature	X_1	$(T - 25)/20$	(-1,1)
Biodegradation time	X_2	$(t - 35)/20$	(-1,1)
pH	X_3	$(pH - 6)/1$	(-1,1)
c) Dependent variables			
Variable	Nomenclature		Units
Biodegraded biosurfactant	Y_1		%

Table 2. Equations used for calculating the concentration of biosurfactant (x) based on surface tension values (y) at different pH.

pH	Equation	r^2
7	$y = -0.0797x + 74.369$	0.809
6	$y = -0.1847x + 73.640$	0.928
5	$y = -0.2455x + 74.182$	0.938

Table 3. Uncoded independent variable values assayed, experimental results (Y_1) achieved for dependent variables, and theoretical data predicted by **Equation 1.**

Experiment	Independent variables			Dependent variables	
	X_1	X_2	X_3	Y_1	Predicted Data
1	25	15	5	29.83	29.85
2	25	55	5	63.95	57.11
3	25	15	7	59.66	66.51
4	25	55	7	80.72	80.70
5	5	15	6	19.13	15.40
6	5	55	6	33.00	36.13
7	45	15	6	8.04	4.91
8	45	55	6	21.91	25.64
9	5	35	5	14.26	17.97
10	5	35	7	46.84	43.73
11	45	35	5	0.00	3.11
12	45	35	7	41.33	37.61
13	25	35	6	57.76	66.48
14	25	35	6	73.02	66.48
15	25	35	6	68.66	66.48

Regression coefficients	Statistical significance	
	Y_1	p_{Y_1}
b_0	66.48*	0.004624
b_1	-5.25	0.199611
b_{11}	-39.45*	0.010574
b_2	10.36	0.064932
b_{22}	-6.51	0.252224
b_3	15.06*	0.032364
b_{33}	-1.43	0.76042
b_{12}	0.00	1.000000
b_{13}	2.19	0.633677
b_{23}	-3.27	0.493338

* Significant variable ($p < 0.05$)

FIGURE CAPTIONS

Fig. 1 MALDI spectra of biosurfactant extract from CSW (a) in comparison with Surfactin (b) and Fengycin (c).

Fig. 2 MALDI fragmentation pattern of 1066 m/z signal for biosurfactant extract obtained from CSW (a) in comparison with the fragmentation of 1060 m/z signal (b) and 1030 m/z signal (c) for Surfactin.

Fig. 3 MALDI fragmentation pattern of 1482 m/z signal for biosurfactant extract obtained from CSW (a) in comparison with 1516 m/z signal (b) and 1544 m/z signal (c) for Fengycin.

Fig. 4 Variation of percentage of biodegradation (Y_1) in aqueous solution with the temperature (X_1) and the pH (X_3), fixing the time (X_2) at the lowest (a), intermediate (b), and highest (c) value respectively.

Fig. 5 Variation of $t_{1/2}$ with temperature determined using **Equation 3** at pH 5 (a) and pH 6 (b).

Fig. 6 General screening of microorganisms isolated after 55 days from experiments 2, 4, 6 and 8, which allowed classifying them in bacteria, yeast or fungi.
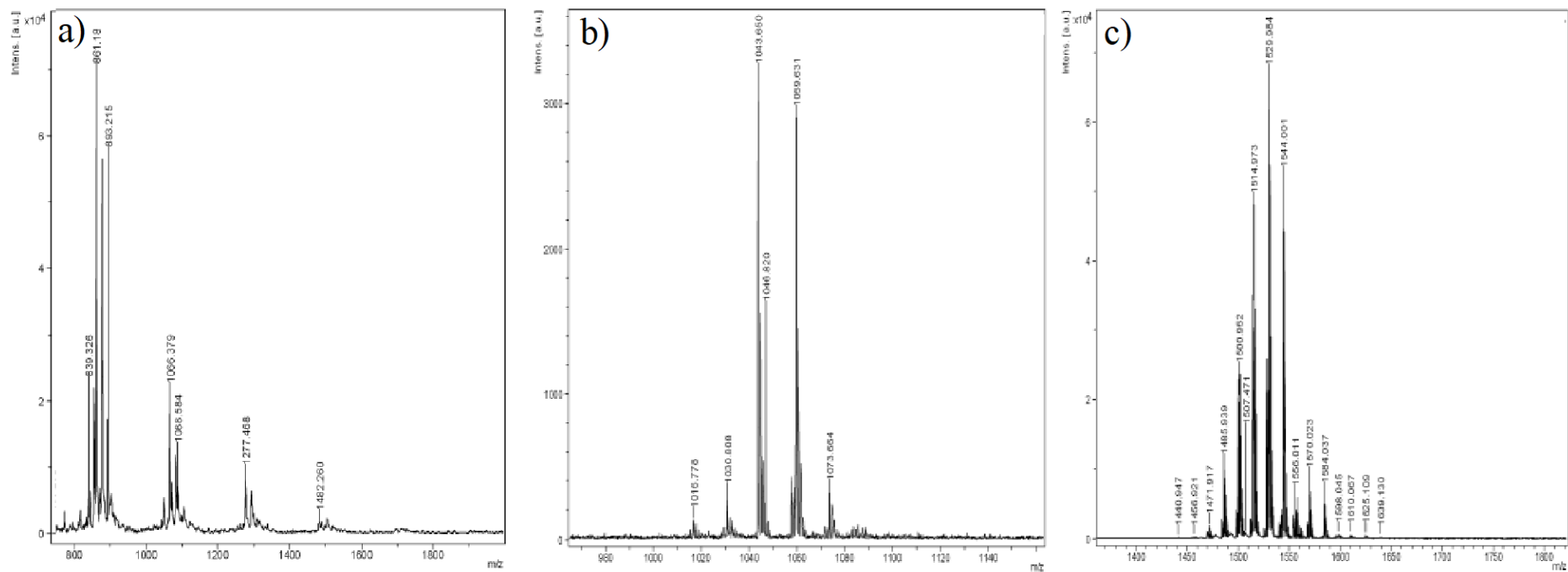
Figure 1
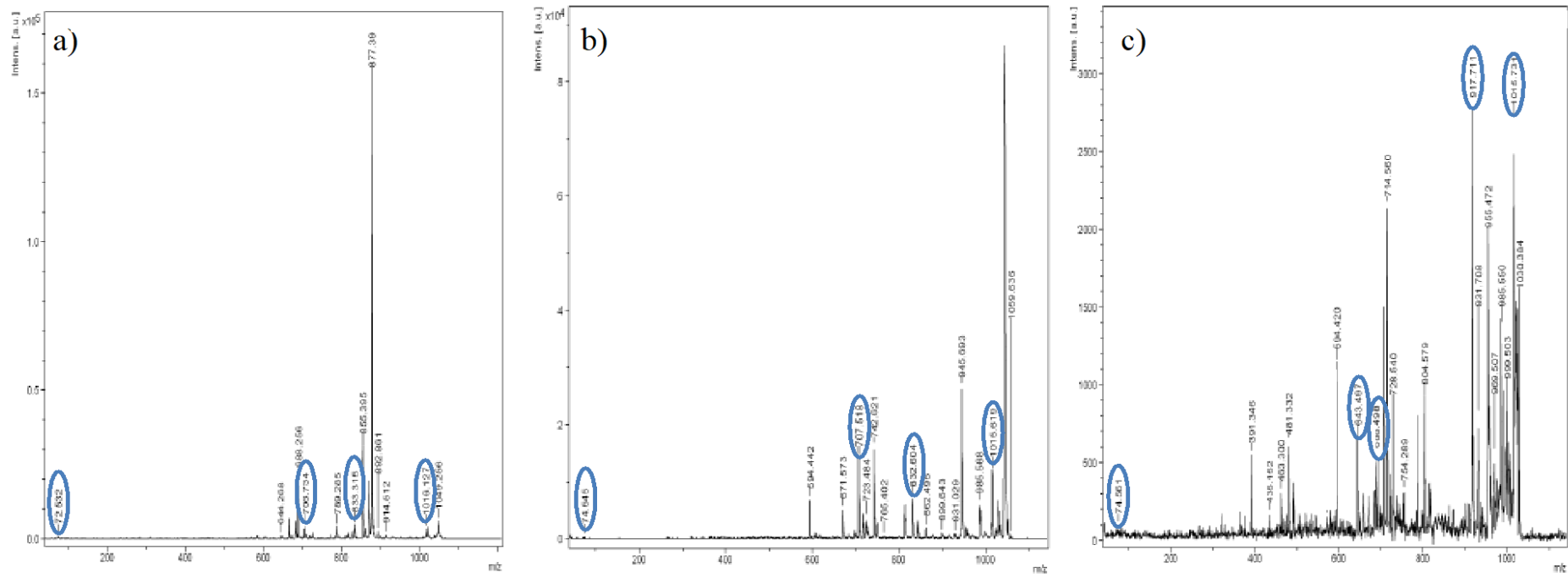
Figure 2
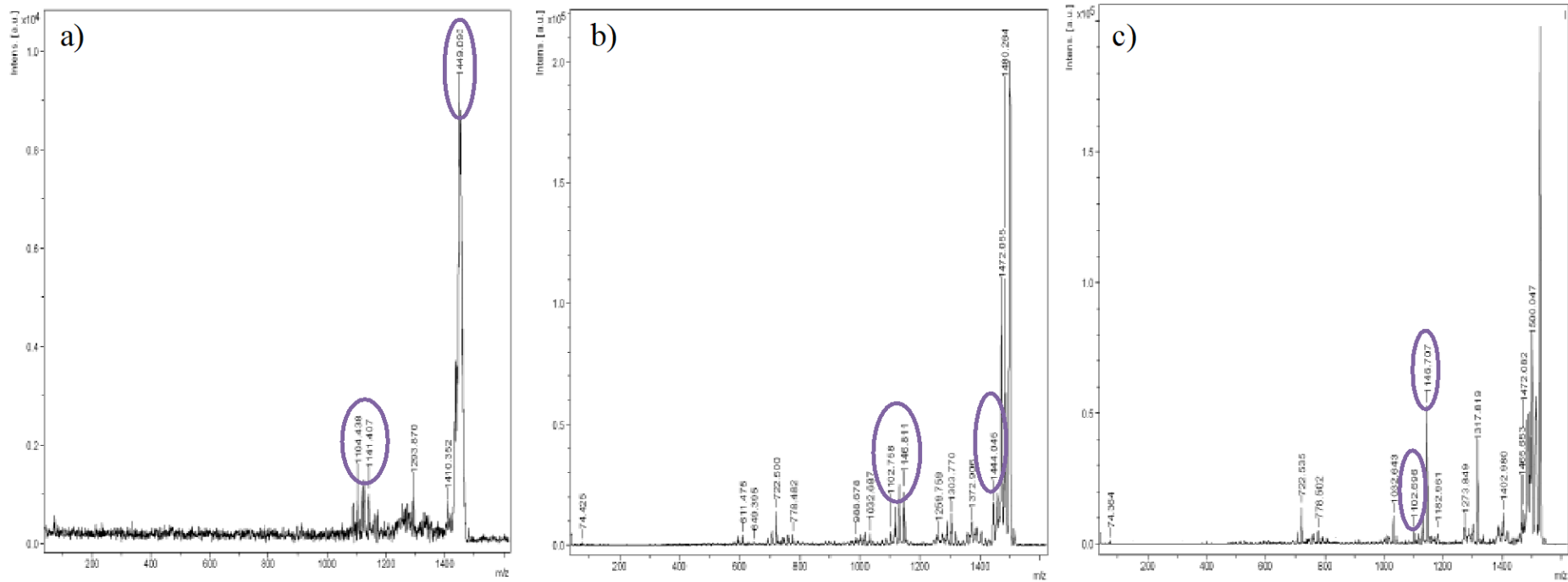
Figure 3

a)
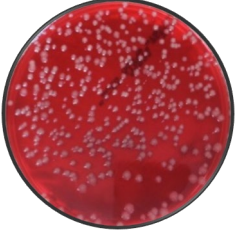

b)

c)

Figure 4

a)

b)
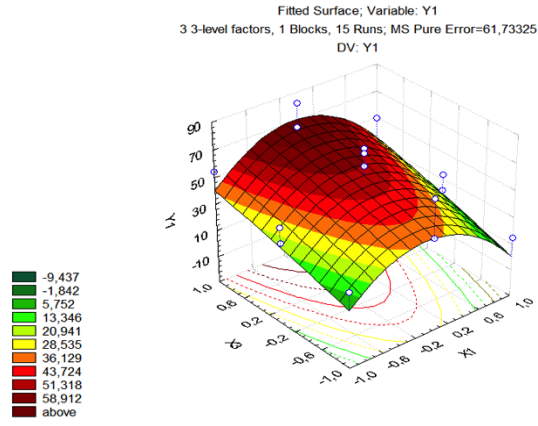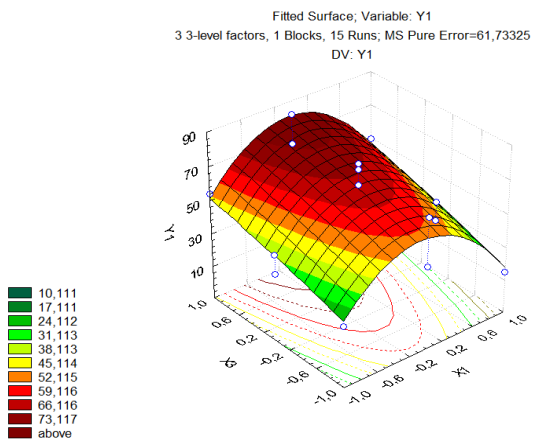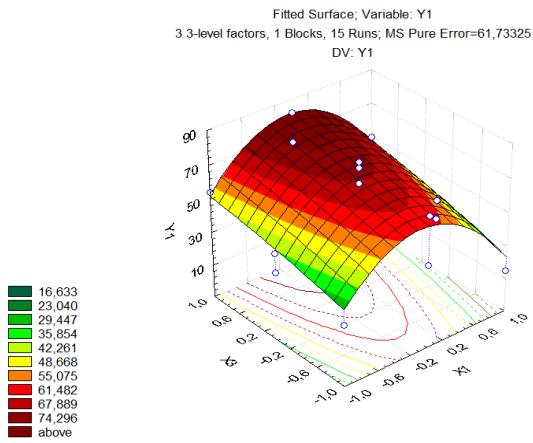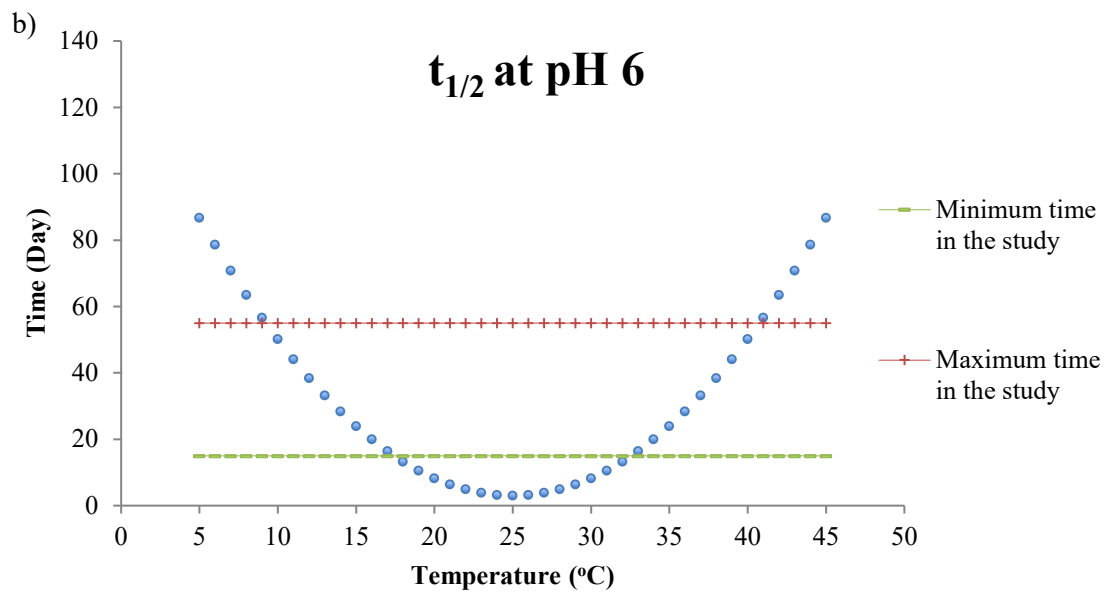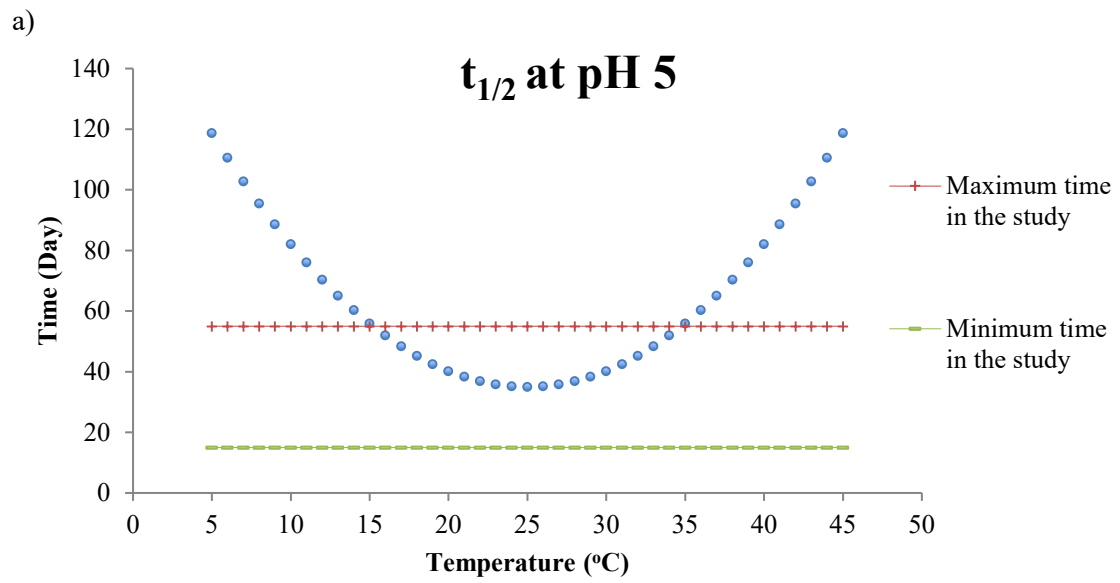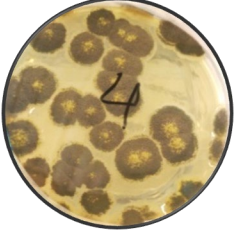
Figure 5

Experiment number	CBA: aerobic bacteria	SDA: Fungi and Yeast
2		
4		
6		
8		

Figure 6