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Production and characterization of sophorolipids from stearic acid by solid-state fermentation, a cleaner alternative to chemical surfactants

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Abstract

In this manuscript, we approach the production of biosurfactants as a cleaner alternative to the chemically-produced surfactants currently used in a wide range of industries. Sophorolipids are microbially produced biosurfactants of the glycolipid type that have entered the market in select applications such as detergent or cosmetic formulation ingredients. This study focuses on sophorolipid production by the yeast *Starmerella bombicola* from stearic acid (C18:0), a low-cost carbon source that is difficult to work with in submerged fermentation since it remains a solid due to its high melting temperature. Consequently, optimizations of solid-state fermentation inoculated with *Starmerella bombicola* were studied for conversions of stearic acid and molasses to sophorolipids. Polyurethane foam functioned as the inert support. The effect of polyurethane foam density and water holding capacity was assessed and the process was optimized in terms of substrate and inoculum ratio. The best conditions were: foam with a density of 32 kg m^{-3} at 75% water holding capacity, 1.17:1 molasses/stearic acid (w/w) and 5% (v/w) inoculum, to obtain a yield of 0.211 g sophorolipids per g of substrates. Mass spectrometry revealed that the sophorolipids produced herein had high concentrations of diacetylated acidic and lactonic C18:0 forms. The results of interfacial properties studies revealed that C18:0 sophorolipids had promising surface tension lowering capacity and emulsification behavior. This study describes a new strategy to produce biosurfactants using low environmental impact technologies as an alternative to traditional ways to produce chemical detergents.

Keywords: biosurfactant, polyurethane foam, *Starmerella bombicola,* sophorolipid, solid-state fermentation, stearic acid.

1. Introduction

Current research is focusing on replacing synthetic surfactants with other eco-friendlier molecules (Pradhan and Bhattacharyya, 2017; Muntaha and Khan, 2015). The key to success is finding biodegradable molecules with good surfactant properties and implementing consistent production processes for the successful replacement of petroleum-based surfactants. Sophorolipids (SLs) are glycolipid type microbial biosurfactants that provide an environmentally friendly alternative to chemically-produced surfactants used in a wide variety of applications. These molecules are produced extracellularly as secondary metabolites during the stationary growth phase and may be used as a carbon and energy reservoir for the yeast (Hommel et al., 1994). The biodegradability and low cytotoxicity of SLs has been reported by Hirata et al. (2009) determining that SLs show lower cytotoxicity than other surfactants (lipopeptide type biosurfactants, polyoxyethylene lauryl ether or sodium dodecyl sulfate). SLs can be classified as readily biodegradable chemicals (according to the OECD 301C method), showing a biodegradation pattern like sodium laurate, which is a good biodegradable natural surfactant. SLs are preferred over other biosurfactants since they can be produced in high volumetric yields (about 300 g L^{-1}) by fermentation of non-pathogenic yeast strains such as *Starmerella bombicola* (Van Bogaert et al., 2011). In fact, these molecules are currently being produced and used commercially by companies such as Ecover (Belgium), Soliance (France) and SyntheZyme (USA) for their application in detergents, cosmetics and more (Van Bogaert and Soetaert, 2011). SLs also have good anti-microbial, anti-inflammatory, anti-HIV and even anti-cancer effects, which allow these molecules to be used in the pharmaceutical industry (Chen et al., 2006; Díaz De Rienzo et al., 2015: Rashad et al., 2014).

SLs are conventionally produced by submerged fermentation (SmFs) using glucose as the hydrophilic carbon source and a high oleic acid feedstock (C18:1) as the hydrophobic carbon

source (Kim et al., 2009; Koh et al., 2016). Glucose can be replaced by sugarcane, sugar beet or soy molasses, which are substrates rich in sugars and nitrogen, allowing the exclusion of costly yeast extract and urea from fermentation media (Jiménez-Peñalver et al., 2016; Solaiman et al., 2004; Takahashi et al., 2011). SL molecules consist of a sophorose bound to a hydroxylated fatty acid (mainly C18:1) through a glycosidic linkage (Fig. 1) (Jiménez-Peñalver et al., 2016). It is well-known that SLs are produced as a mixture of slightly different molecules with two main points of variation: acetylation in the sophorose moiety and lactonization (Van Bogaert et al., 2011). Other variations that occur are the fatty acid chain length (generally 16 or 18 carbon atoms) and degree of unsaturation (0, 1, or 2 double bonds) (Fig. 1).

An approach to tune SLs properties is by their chemical modification. For example, esterification of lactonic sophorolipid with *n*-alkanols of varying lengths resulted in elongation of the hydrophobic tail (Zhang et al., 2004). This proved to be a useful strategy to improve their interfacial properties with oil phases (Koh et al., 2016; Koh et al., 2017). As would be anticipated, differences in the SLs structure impact their biological and physicochemical properties. This is especially important in improving their cost-performance relative to petroleum based surfactants in selected applications (Ashby et al., 2008; Koh et al., 2016). Modified SLs can be obtained either by post-fermentative modification (Peng et al., 2015; Zhang et al., 2004), or during the microbial synthesis. Examples of the latter are use of genetically modified strains (Roelants et al., 2016; Van Bogaert et al., 2016) and variation in the hydrophobic carbon source which can change the SL hydrophobic tail composition (Ashby et al., 2008; Hu and Ju, 2001). Some potential hydrophobic carbon sources are solid at the fermentation temperature (e.g. stearic acid, C18:0, melting point 69.3 ºC), which complicates their use as substrates in SmF processes. In such instances, solid-state fermentation (SSF) provides a potentially useful alternative fermentation approach. Also, SSF

avoids problems that typically occur during production of SLs by SmF such as foaming or high viscosity (Krieger et al., 2010).

There has been increased interest over the past decade in SSF for many processes including the production of biochemicals like enzymes (Abraham et al., 2014; Pitol et al., 2016) and the pretreatment of lignocellulose (de Barros et al., 2017). This has spurred work in SSF bioreactor design to increase efficiency (Mitchell et al., 2006). A key variable in SSF processes is the selection of a suitable inert support. Advantages of polyurethane foams (PUF) as an inert support is its high porosity, low density and high water holding capacity (Ramadas et al., 2013). The use of PUFs as inert support in SSF systems has been investigated for the production of bioplastics (Ramadas et al., 2013), enzymes (Hu et al., 2011; Martin del Campo et al., 2015; Subbalaxmi and Murty, 2016) and organic acids (Mata-Gómez et al., 2015). However, PUFs have not been explored for biosurfactant production.

Little research has been performed on the production of SLs by SSF. Jiménez-Peñalver et al. (2016) have recently used SSF for the production of SLs using a winterization oil cake from the oil refining industry and obtained 0.251 g of SLs per g of wet substrates at day 10. Mango kernel olein has also been used to produce SLs by SSF with a yield of 0.175 g g^{-1} substrates at day 10 (Parekh et al., 2012). These authors also produced SLs from SA under SmF and SSF and obtained 2.5 times higher substrate to SL conversion by SSF (0.085 g g^{-1} substrates, day 10). Research presented here is the only published work producing SLs from stearic acid by SSF, performed with 4 g of substrates (2 g glucose and 2 g SA) blended with wheat bran (6 g) and, as such, did not deal with scale-up issues such as compaction. Neither optimisation of the fermentation nor characterisation of the obtained biosurfactants was performed in this work.

This study approaches the production of biosurfactants as a lower impact alternative to current commercial, chemically produced surfactants. Specifically, this work focuses on the

biosynthesis of SLs by an SSF system using the yeast *Starmerella bombicola*, with stearic acid as the hydrophobic carbon source and sugar beet molasses as the hydrophilic carbon source. PUF was used as the inert support. The specific objectives were: (i) to optimize the SSF process to maximize SL yield and (ii) to characterize the SLs in terms of their structure and interfacial properties. To the authors' knowledge, this is the first report on C18:0 SLs production by SSF and characterization at a representative scale and with a significant production yield.

2. Materials and methods

2.1. Materials

All chemicals and reagents used were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA). The sugar company AB Azucarera Iberia S.L.U. (Madrid, Spain) provided sugar beet molasses with a water content of 12% (wet basis), a total sugar content of 78% (dry basis) and a nitrogen content of 1.9% (dry basis). Polyurethane foam of density 25 kg m^{-3} (PUF-25) was purchased from Servei Estació (Barcelona, Spain), while McMaster-Carr (Elmhurst, IL, USA) provided PUFs with densities of 32 and 45 kg m^{-3} (PUF-32 and PUF-45). PUF sheets were cut into cubes of 6x6x6 mm, were copiously washed with distilled water, and then dried at 65 ºC overnight.

2.2. Yeast strain and inoculum preparation

Starmerella bombicola ATCC 22214 was from the American Type Culture Collection (Manassas, USA). The microorganism was preserved as glycerol stocks at -80 ºC and for every experiment a new streak plate was cultivated. The strain, inoculated from a glycerol stock, was grown on agar streak plates for 48 h at 30 ºC that contained (in g L^{-1}): dextrose, 10;

peptone, 5; malt extract, 3; yeast extract, 3; agar, 20. To prepare the inoculum, a loop full of freshly grown yeast from agar plates was transferred to a 250-mL Erlenmeyer flask with 50 mL of sterile medium. The medium contained (in g L^{-1}): dextrose, 10; peptone, 5; malt extract, 3; yeast extract, 3. The culture was incubated in an orbital incubator shaker at 30 ºC, 180 rpm for 48 h. The final optical density (600 nm) of the inoculum was around 25 for all experiments, which corresponds to a cell density of 4 g L^{-1}.

2.3. Optimization of the SSF process

Fermentations were performed in 450-mL cylindrical polyvinylchloride packed-bed bioreactors (13 cm height and 7 cm diameter, Fig. 2) containing, unless otherwise indicated, 65 mL of media (substrates, water and inoculum) impregnated in PUF cubes. Substrates (total of 30 g dry wt.) consisted of a predetermined mixture of molasses (MOL) and stearic acid (SA). The final moisture content was set at 50%. The fermentation media was prepared by dissolving MOL in distilled water, and subsequently adjusting the pH to 6.0 ± 0.1 with 0.1 M HCl. Then SA was added to the media, and the mixture was heated at 80 ºC until stearic acid melting was complete. The PUF cubes were impregnated with the fermentation media. The resulting SSF mixture was sterilized (121 ºC, 30 min) and, after cooling, was inoculated with fresh inoculum (see above).

Reactors were placed in an external water bath at 30 ºC. Air was continuously supplied to the reactors by means of a mass flow controller (Bronkhorst, Spain) at a rate of 0.45 L air kg^{-1} total wet mass min^{-1} based on previous experiments (Jiménez-Peñalver et al., 2016). The air entered the bioreactor below the bed, passing through the solid bed and exiting at the top (Fig. 2). The oxygen concentration in the exhaust gases was recorded and used for the calculation of the Oxygen Uptake Rate (OUR) and the Cumulative Oxygen Consumption (COC) (Ponsá et al., 2010). The SSF was mixed every 2 days, unless otherwise indicated, as described in

Jiménez-Peñalver et al. (Jiménez-Peñalver et al., 2016). Intermittent mixing increases the bioavailability of substrates to the yeast favouring the production of SLs (Jiménez-Peñalver et al., 2016). At sampling times, one reactor was terminated and, prior to its analysis, the collected material was manually homogenized with a metal spatula. Results were analysed by one-way ANOVA followed by Duncan´s test ($p < 0.05$ confidence level) using SPSS 15.0 software for Windows.

2.3.1. Effect of PUF density

Water holding capacity (WHC) of PUF-25, PUF-32 and PUF-45 were determined in 14.5, 13.0 and 9.9 g of water per g of PUF cubes, respectively. Fermentations were carried out at 75% WHC for each PUF cube type based on the work of Haug et al. (Haug, 1993). Thus, 6, 6.7 and 8.8 g of PUF-25, PUF-32 and PUF-45, respectively, were impregnated with the 65-mL fermentation media. The ratio of the substrates was 1:1 (15 g each), the inoculum added was 10% (v/w), and experiments were performed in triplicate for 7 days.

2.3.2. Optimizing media composition

The ratio of MOL/SA and the inoculum size were optimized for maximum SL yield using a Central Composite Design (CCD). Each factor was evaluated at 3 levels with 5 replicates at the central point in 13 different trials (Table I). The ratio of MOL/SA (S, w/w) was fixed at 1:3, 1:1 and 3:1 (normalized values −1, 0, 1, respectively), and the inoculum size (I, % v/w) was fixed at 5, 10 and 15 (normalized values −1, 0, 1, respectively). SL yield (g g^{-1} substrates) at day 7 of fermentation was the response (Y). PUF cubes were saturated at the 75% WHC.

2.3.3. Effect of PUF saturation

In this experiment, PUF-32 cubes were saturated with 60, 75 and 90% WHC. The same amount of PUF-32 cubes were used in all the treatments to hold 52, 65 and 78 mL of media for fermentations with 60, 75 and 90% WHC, respectively. The media was prepared proportionally to the one optimized in the previous section. Experiments were performed in triplicate for 7 days.

2.3.4. Time course of the fermentation

The optimized process was monitored for 16 days. Eight reactors were prepared with the optimal conditions and one of the reactors was sacrificed for analysis at sampling points (0, 2, 3, 5, 7, 10, 13, and 16). In this experiment, reactor mixing was performed every day during the first week and then every 2 days until the end of the process. Analytical results are reported from triplicate samples taken from a single reactor. Samples collected at day 7 were used for characterization (see below). Scanning electron microscope (SEM) analyses were performed at days 0 (before inoculation), 2 and 7 using a Zeiss digital scanning microscope EVO (Germany). Samples were fixed according to the methodology described by Varesche et al. (Varesche et al., 1997).

2.4. Analytical methods

The pH, moisture content and WHC were determined according to standard methods (The US Department of Agriculture and The US Composting Council, 2001). To extract sugars from the fermentation, 4 g of material from the reactor was mixed with distilled water (1:10 w/v) at 200 rpm for 20 min in an orbital shaker. The extraction was repeated two more times to ensure maximum recovery of the sugars from PUF cubes. Consecutive extractions were pooled together, filtered through a 0.22-µm membrane filter and extracted twice with ethyl acetate in a ratio 1:1 (v/v) to isolate SLs which may be in the aqueous phase. Sugar content

determination of the aqueous phase was performed by the anthrone method (Scott and Melvin, 1953).

SA was extracted from the fermentation mixture by mixing 10 g of the material with hexane in a 1:10 (w/v) ratio at 200 rpm for 1 h. This extraction protocol on the 10 g sample was repeated three times. Then, SLs were extracted from the fermentation by mixing 10 g of the material with ethyl acetate in a 1:10 (w/v) ratio at 200 rpm for 1 h. This extraction protocol sample was repeated twice. During the time course experiment, a third extraction was carried out to ensure maximum SL recovery. Solvent extractions were pooled together, filtered through a Whatman paper Grade 1, and vacuum-dried at 40 ºC with a rotary evaporator. SA content and SL yield were determined gravimetrically. In this study, the SL yield is defined as grams of sophorolipid per gram (dry wt.) of initial substrates (MOL + SA). SL yield is also reported as grams of product per L. SLs obtained herein were lyophilized and stored at 4ºC to ensure their stability.

Viable cell numbers were quantified by mixing 1 g of material with 9 mL of culture solution (peptone 1 g L^{-1} and sodium chloride 8.5 g L^{-1}) in a vortex mixer for 1 min. Serial dilutions were prepared from this mixture, then plated on agar streak plates and incubated at 30 ºC for 72 h. Manual counting of viable cells was performed afterwards. Analyses were performed in triplicate and results were expressed as colony forming units (CFU) per gram (dry wt.) of initial substrates.

2.5. Characterization

2.5.1. Structural identification

A mass spectrometer (micrOTOF-Q^{TM}, Bruker Daltonics, Germany) was used to identify the structures of individual components of the natural SL mixture. SLs were dissolved in methanol (1 mg mL^{-1}) and passed through a 0.22-µm membrane filter. The sample was

directly infused into the mass spectrometer using a syringe pump to obtain the overall spectra. SL molecules were ionized by electrospray (negative ion mode) and further collected in an ion trap where their mass/charge (m/z) values were detected.

2.5.2. Surface tension

Minimum surface tension (mST) and critical micelle concentration (CMC) were estimated using a surface tensiometer (K100, Krüss, Germany) by the Wilhelmy plate method. The Wilhelmy platinum plate used was length: 10 mm, width: 19.9 mm and thickness: 0.2 mm (model PL01, Krüss, Germany). CMC was calculated from the relationship between the log of SL concentration and the corresponding surface tension. Measurements of surface tension were recorded at 25 ºC and reported as the average of triplicate analyses.

2.5.3. Emulsion properties

Emulsions were prepared and evaluated following a methodology previously described by Koh et al. (Koh et al., 2016). Briefly, emulsions were prepared with 10% almond oil by weight and 0.01 or 0.1% surfactant by total emulsion weight in deionized water and mixed using an IKA T25 shear homogenizer (24,000 rpm, 60 s). Analysis of emulsions was performed immediately after emulsification and after 1 and 7 days of aging at 25 ºC. Results were compared with those obtained for the commercial non-ionic surfactant Triton X-100. Average emulsion droplet sizes were determined with a Malvern Zetasizer ZSP (Worcestershire, UK) with a 173º backscatter angle detector. Droplet sizes are reported as the emulsion z-average diameter in nanometres. Emulsion phase stability was recorded by photographs with a Nikon D5200 camera fitted with a Nikon AF-S Micro Nikkor 60 mm lens. The same batch of almond oil was used herein for all the analysis and was stored at 4 ºC in an amber glass bottle to prevent degradation.

3. Results

3.1. Optimization of the SSF process

3.1.1. Effect of PUF density on SL yield

PUF density is a key parameter to assess prior to further optimization of a new SSF system (Baños et al., 2009). It affects the fermentation matrix mechanical strength, substrate distribution and oxygen transfer within PUF pores. Consequently, PUF density influences microbial growth and SL production. Three PUF densities (25, 32 and 45 kg m^{-3}; PUF-25, PUF-32 and PUF-45, respectively) were evaluated. Figure 3 shows that PUF-32 and PUF-45 resulted in higher SL yields (g L^{-1}) than PUF-25, with no significant differences between PUF-32 and PUF-45 based on Duncan´s test ($p < 0.05$). In other words, both foams would give the same SL volumetric yield. However, the highest substrate conversion was attained using PUF-32 (0.108 g g^{-1} substrates). Furthermore, PUF-45 has 25% lower WHC than PUF-32 so, consequently, more PUF-45 cubes are needed to hold the same volume of media. For these reasons, we selected PUF-32 for the additional studies discussed below.

3.1.2. Optimization of substrate ratio and inoculum size

To maximize sophorolipid production with respect to the parameters MOL/SA ratio and inoculum size, an experimental design was performed using Central Composite Design (CCD) (Table I). The maximum and minimum SL yields obtained were run 12 (0.133 g g^{-1} substrates) and run 2 (0.079 g g^{-1} substrates), respectively. The low variability (< 6%) of the central points (runs 5, 6, 7, 9, 13) is an indicator of the reproducibility of experimental data. The experimental data was evaluated using analysis of variance (ANOVA). The parameter inoculum size was non-significant in all the models studied indicating that this parameter is

not critical over the range studied in influencing SL production. The following quadratic equation was the best fit of the experimental data and represents SL yield (Y, g g^{-1} substrates) as a function of the ratio MOL/SA (S):

$$Y = 0.118 + 9.345 \cdot 10^{-3}S - 0.030S^2 \quad (1)$$

The proposed model is significant with an F value of 41.78, obtained by Fisher´s F-test, along with a very low probability value, which is significant at a 95% confidence interval (Table A.1). The R^2 value of 0.8931 indicates that the model explains 89.31% of total SL yield variability. The predicted R^2 of 0.8480 is in reasonable agreement with the adjusted R^2 (0.8717), which is close to R^2. Lack of fit was found to be non-significant ($p = 0.5039$) which indicates that Eq. 1 is adequate to predict SL yield under any combination of the studied variables. Coefficients S and S^2 were significant ($p < 0.05$) suggesting that the ratio MOL/SA is critical to SL production by SSF (Table A.1).

By solving the fitted regression model (Eq. 1) for maximum SL yield, the optimum MOL/SA (w/w) level is 0.16, which corresponds to the ratio 1.17:1 (16.2 g of MOL and 13.8 g of SA, dry basis). Since non-significant differences were observed between levels for the inoculum size (%, v/w), the level -1, which corresponds with 5 % (v/w) of inoculum, was selected for practical and economic reasons. Under these conditions, the optimal predicted SL yield is 0.120 g g^{-1} substrates.

3.1.3. Effect of PUF saturation on SL yield

The saturation of the inert support can also influence the SSF process. The more saturated the inert support, the more concentrated the substrates and the inoculum in the SSF mixture and, therefore, the better bioavailability of nutrients to the yeast. This would favour biomass

growth and SL synthesis, improving the process volumetric yield. The effect of PUF saturation on SL production was determined at 60, 75 and 90% of PUF-32 WHC. Figure 4 shows that increasing WHC to 90% did not improve SL yield in g L^{-1}, with no significant differences with 75% WHC according to Duncan´s test ($p < 0.05$). SSF mixtures that are too water saturated may block PUF pores obstructing oxygen diffusion through the matrix. This phenomenon hindered the fermentation process and led to lower production yields in the mixture at 90% WHC. The excess of saturation was confirmed by the observed leachate produced during the fermentation. Decreasing WHC to 60% gave significantly similar results as 75% in terms of g g^{-1} substrates, but worse performance in terms of g L^{-1} (Fig. 4). Based on the above results PUF-32 was adjusted to 75% WHC for the remaining experiments.

3.1.4. Time course

Process monitoring was performed as a function of culture time (16 days) under the optimized conditions described above (Fig. 5). Analyses included SL production, substrate consumption, viable cell numbers by respirometric methods. Substantial SL formation occurred by day 2 (0.079 g g^{-1}) and the maximum SL yield was on day 13 (0.211 g g^{-1}). Parekh et al. (Parekh et al., 2012) produced SLs from a SSF mixture consisting of glucose and stearic acid blended with wheat bran and reported a SL yield of 0.085 g g^{-1} substrates at day 10, which is 2.2 times lower than the yield obtained in this study at the same time point.

Gradual sugar consumption occurred until its depletion on day 7 (Fig. 5A). In contrast, SA consumption occurred gradually such that, by day 16, 30% SA was utilized (Fig. 5A). Remaining SA may not be easily accessible due to, for example, attachment within PUF pores. In addition, since sugar depletion occurred on day 7, further utilization of SA for SL production should slow or even stop. Interestingly, SL yield (g g^{-1} substrates) was directly proportional to SA consumed (g g^{-1} substrates), with a proportionality constant of 1.438 (g SL

per g of SA consumed) (n=8, $R^2 = 0.985$, $p < 0.001$). Jiménez-Peñalver et al. (Jiménez-Peñalver et al., 2016) found that SL yield correlated with fats consumed from a winterization oil cake, the hydrophobic carbon source for SL synthesis, with a proportionality constant of 0.805 (g SL per g of fat consumed). This indicates that, in the present work, there is a better conversion of the hydrophobic carbon source into SLs, probably due to higher substrate purity herein. Winterization oil cake consists of waxes and triglycerides that require metabolic conversion to fatty acids prior to use for SL formation. In contrast, stearic acid can be directly incorporated into SL molecules.

S. bombicola colonized the solid mixture within the first two days of fermentation. Viable cell numbers increased by one order of magnitude at day 2 (2.8×10^9 compared to 2.4×10^8 CFU g^{-1} substrates), and no significant growth was observed after that (Fig. 5B). This behaviour has also been observed in SmFs with *S. bombicola* (Hu and Ju, 2001; Maddikeri et al., 2015). Figure 5B also shows the oxygen uptake rate (OUR) and cumulative oxygen consumption (COC) profiles of the reactor sampled at day 16. OUR reached a value of about 0.9 by day 2, and between days 2 and 7 OUR fluctuated between 0.8 to 1 mg O_2 g^{-1} substrates h^{-1}. With the depletion of sugars at day 7, OUR generally decreases to day 16. Similar OUR profiles were obtained from the rest of the reactors with a variability below 6%. The increase in the biological activity (viable cell numbers and OUR) in the first two days of fermentation agrees with the initial drop in sugar content (Fig. 5). The oxygen consumed (COC) at the end of the fermentation was 0.280 g O_2 g^{-1} substrates. The COC values of the experiments performed with PUF-32 (optimization, effect of PUF saturation and time course until day 13) were compiled in the plot displayed in Figure 6. Data from the time course study (Fig. 5) gave values that are consistent with other experiments performed herein. The data correlated linearly with a proportionality constant of 0.973 (n=27, $R^2 = 0.985$, $p < 0.001$). These results show that the higher the SL production, the higher the total oxygen consumed (COC), which

could be explained by the higher microbial activity needed to synthetize the SLs (Fig. 6). In the work with winterization oil cake previously mentioned, correlation of SL yield with COC gave a proportionality constant of 0.502 (g SL per g O_2) (Jiménez-Peñalver et al., 2016). Therefore, the production of SLs per g of O_2 consumed is almost the double in the present work.

The SEM images taken after the media was impregnated in PUF cubes confirmed that SA is well-distributed over the surface of the PUF cubes (Fig. 7). The SEM images taken after 2 and 7 days of fermentation showed that *S. bombicola* grows on the surface of the foam as dispersed individual cells (Fig. 7). No granules or biofilm formation was observed.

3.2. Characterization of the SLs

3.2.1. Identification of SL molecules

Mass spectrometry was used to identify SL molecules contained in the SL natural mixture produced during SSFs. The mass spectrum shown in Figure 8 revealed that SLs consist of a complex mixture containing both diacetylated lactonic SLs (C18:0 and C18:1 with m/z = 689.4 and 687.4, respectively) and diacetylated acidic SLs (C18:0, C18:1 and C16:0 with m/z = 707.4, 705.4 and 679.4, respectively). Also, the mono-acetylated acidic C18:0 SL is observed in the peak m/z = 665.4 but with lower intensity than the corresponding diacetylated structures. The rest of the peaks illustrated in Figure 8 correspond to isotopes of the above SLs. Assignation of the peaks from the mass spectra to the corresponding SLs is in agreement with other studies (Ashby et al., 2008; Hu and Ju, 2001).

Two peaks stand out from the others in the mass spectra: m/z = 689.4 and 707.4, which correspond to the diacetylated lactonic and acidic C18:0 SLs, respectively. The abundance of these two SLs is consistent with stearic acid as the predominant fatty acid in SSF fermentations. The significant presence of C18:1 SLs is due to operative fatty acid metabolic

pathways of the yeast that include desaturation prior to the SL synthesis (Brett et al., 1971). Ashby et al. (Ashby et al., 2008) also produced SLs from stearic acid but by SmF instead of SSF. They also reported formation of SLs with both C18:0 and C18:1 fatty acid substituents where the latter was most abundant. Based on the work herein that uses mass spectrometry to identify the products formed, quantification of the relative amount of SLs with C18:0 and C18:1 fatty acid substituents is not possible.

3.2.2. Surface tension

Surfactants, both chemical and biological, are well-known for their surface tension lowering capacities at oil-water or water-air interfaces (Pradhan and Bhattacharyya, 2017). In general, biosurfactants have a comparable performance to chemically derived surfactants while still being non-toxic, which makes them attractive for environmental and household surfactant applications. For example, natural SLs decrease the surface tension at the water-air interface from 72.0 mN m^{-1} (25 ºC) to values ranging from 40 to 30 mN m^{-1} with critical micelle concentration (CMC) values from 11 to 250 mg L^{-1} (Develter and Lauryssen, 2010). The SLs synthesized by SSF herein decreased the surface tension to 33.8 mN m^{-1} with a CMC of 37.9 mg L^{-1} (Fig. A.1). These values are within the lower range reported in the literature for natural SLs and are very similar to those reported for SLs produced using stearic acid (Ashby et al., 2008).

Values of minimal surface tension (mST) and CMC reported in literature are mainly dependent on the composition of SLs. For example, it has been reported that a mixture of SLs displays better interfacial properties than a SL alone suggesting a natural synergism between molecules (Hirata et al., 2009). Also, it is well-known that the higher the hydrophobicity of the SL hydrophobic tail, the lower the mST and the CMC (Zhang et al., 2004). In this sense, C18:0 SLs display better interfacial properties than the most common C18:1 SLs because of

the higher hydrophobicity of the tail (Ashby et al., 2008). As previously shown in Figure 8, SLs produced in this work are a mixture of molecules containing mainly acidic and lactonic C18:0 SLs, which would explain the low mST and CMC values obtained. Surfactants with low mST and CMC values are desirable to industry because this improves their cost-performance profile that determines their commercial viability.

3.2.3. Emulsification properties

An emulsion is the mixture of two immiscible liquids. Such systems are thermodynamically unstable and require a surfactant to reduce the system´s free energy and stabilize the interfacial area between the two phases. The emulsion properties of SLs synthesized herein were assessed at a water-almond oil interface in order to observe the average emulsion droplet size and the emulsion phase stability. Both parameters are important to determine the stability of an emulsion. Emulsions with stable droplet sizes do not have necessarily a stable emulsion phase, and vice versa (Koh et al., 2016). Results were compared with the commercial surfactant Triton X-100 due to its non-ionic nature and its wide use in the industry.

Emulsion droplet size is an indicator of system stability. The smaller the emulsion droplet size, the more kinetically stable the emulsion and, therefore, the better the efficiency in combating thermodynamic instability (Solans et al., 2005). Mean droplet sizes of SL emulsions at the immediate time point (time of formation) had values ranging from 3000 to 4000 nm, which were similar to those prepared using the commercial surfactant Triton X-100 (Fig. 9). All emulsion droplet sizes were stable for 7 days as is evident from the small change in emulsion droplet size from the immediate time after emulsification to 1 and 7 days of aging. Interestingly, the average droplet sizes of 0.01% SL emulsions were similar to 0.1% SL emulsions, which indicates excellent emulsification capacity even at low concentrations. This agrees with the low CMC value of the SLs of this work.

Emulsion phase stability is also an indicator of how a surfactant will perform as an emulsifier. Ideally, the emulsion phase should be homogeneous without oil separation or surfactant precipitation. Although none of the emulsions of this work showed oil separation or surfactant precipitation, they all show creaming (Fig. A.2), which is expected due to the large particle sizes of the emulsions (Koh et al., 2016). All the emulsion phases were stable for 7 days, even for emulsions where the oil concentration was 1000 times that of surfactant by weight (Fig. A.2). No visual differences in the emulsification performance of SLs and Triton X-100 were observed. Koh et al. (2016) reported lower average emulsion droplet sizes ranging from 2000 to 3000 nm for a mixture of pure acidic and lactonic C18:1 SLs (50:50, w/w). However, those emulsions showed oil separation and precipitation at 1 and 7 days contrary to the SL natural mixture produced in this work.

4. Conclusions

This work demonstrates the huge potential of solid state fermentation to produce biosurfactants as a cleaner alternative to commercial, petroleum-derived surfactants. Sophorolipids were produced by solid-state fermentation using stearic acid (C18:0) as the fatty acid carbon source and polyurethane foam as the inert support. The media cost was reduced by replacing glucose and nitrogen source (yeast extract and urea) with sugar beet molasses. Optimization of the fermentation process resulted in a sophorolipid yield of 0.211 g g^{-1} substrates. The sophorolipids produced were mainly composed of the diacetylated acidic and lactonic C18:0 sophorolipids, which reflects a preference to form C18:0 sophorolipids when stearic acid is used as the substrate. These sophorolipids exhibited interesting interfacial properties. They decreased the surface tension at an air-water interface to 33.8 mN m^{-1} with a

CMC of 37.9 mg L^{-1}, and showed emulsification properties at water-almond oil interfaces similar to the commercial surfactant Triton X-100.

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Conflicts of interest: none

References

Abraham, J., Gea, T., Sánchez, A., 2014. Substitution of chemical dehairing by proteases from solid-state fermentation of hair wastes. J. Clean. Prod. 74, 191-198.

Ashby RD, Solaiman DKY, Foglia TA. 2008. Property control of sophorolipids: Influence of fatty acid substrate and blending. *Biotechnol. Lett.* **30**:1093–1100.

Baños JG, Tomasini A, Szakács G, Barrios-González J. 2009. High lovastatin production by Aspergillus terreus in solid-state fermentation on polyurethane foam: An artificial inert support. *J. Biosci. Bioeng.* **108**:105–110. http://dx.doi.org/10.1016/j.jbiosc.2009.03.006.

de Barros EM, Carvalho VM, Rodrigues THS, Rocha MVP, Gonçalves LRB. 2017. Comparison of strategies for the simultaneous saccharification and fermentation of cashew apple bagasse using a thermotolerant Kluyveromyces marxianus to enhance cellulosic ethanol production. *Chem. Eng. J.* **307**:939–947. http://www.sciencedirect.com/science/article/pii/S1385894716312426.

Van Bogaert INA, Soetaert W. 2011. Sophorolipids. In: Soberón-Chávez, G, editors. *Biosurfactants, Microbiol. Monogr. 20*. Berlin, Germany: Springer-Verlag, pp. 179–210. doi:10.1007/978-3-642-.

Van Bogaert INA, Zhang J, Soetaert W. 2011. Microbial synthesis of sophorolipids. *Process Biochem.* **46**:821–833. http://linkinghub.elsevier.com/retrieve/pii/S135951131100016X.

Van Bogaert INA, Buyst D, Martins JC, Roelants SLKW, Soetaert WK. 2016. Synthesis of bolaform biosurfactants by an engineered *Starmerella bombicola* yeast. *Biotechnol. Bioeng.* **113**:2644–2651.

Brett D, Howling D, Morris LJ, James AT. 1971. Specificity of the fatty acid desaturases: The conversion of saturated to monoenoic acids. *Arch. Biochem. Biophys.* **143**:535–547.

Cerda A, Gea T, Vargas-García MC, Sánchez A. 2017. Towards a competitive solid state fermentation: Cellulases production from coffee husk by sequential batch operation and role of microbial diversity. *Sci. Total Environ.* **589**:56–65. http://www.sciencedirect.com/science/article/pii/S0048969717304412.

Chen J, Song X, Zhang H, Qu Y. 2006. Production, structure elucidation and anticancer properties of sophorolipid from *Wickerhamiella domercqiae*. *Enzyme Microb. Technol.* **39**:501–506.

Develter DWG, Lauryssen LML. 2010. Properties and industrial applications of sophorolipids. *Eur. J. Lipid Sci. Technol.* **112**:628–638. http://doi.wiley.com/10.1002/ejlt.200900153.

Díaz De Rienzo MA, Banat IM, Dolman B, Winterburn J, Martin PJ. 2015. Sophorolipid biosurfactants: Possible uses as antibacterial and antibiofilm agent. *N. Biotechnol.* **32**:720–726.

Haug RT. 1993. The Practical Handbook of Compost Engineering. Boca Raton, Florida: Lewis Publishers.

Hirata Y, Ryu M, Igarashi K, Nagatsuka A, Furuta T, Kanaya S, Sugiura M. 2009. Natural synergism of acid and lactone type mixed sophorolipids in interfacial activities and cytotoxicities. *J. Oleo Sci.* **58**:565–572. http://www.ncbi.nlm.nih.gov/pubmed/19844071.

Hommel RK, Stegner S, Kleber HP, Weber L. 1994. Effect of ammonium ions on glycolipid production by *Candida (Torulopsis) apicola*. *Appl. Microbiol.* **42**:192-197.

Hu T, Zhou Y, Dai L, Wang Y, Liu D, Zhang J, Liu H. 2011. Enhanced cellulase production by solid state fermentation with polyurethane foam as inert supports. *Procedia Eng.* **18**:335–340.

Hu Y, Ju L-K. 2001. Sophorolipid production from different lipid precursors observed with LC-MS. *Enzyme Microb. Technol.* **29**:593–601. http://linkinghub.elsevier.com/retrieve/pii/S0141022901004392.

Jiménez-Peñalver P, Gea T, Sánchez A, Font X. 2016. Production of sophorolipids from winterization oil cake by solid-state fermentation: Optimization, monitoring and effect of mixing. *Biochem. Eng. J.* **115**:93–100.

Kim YB, Yun HS, Kim EK. 2009. Enhanced sophorolipid production by feeding-rate-controlled fed-batch culture. *Bioresour. Technol.* **100**:6028–6032. http://dx.doi.org/10.1016/j.biortech.2009.06.053.

Koh A, Linhardt RJ, Gross R. 2016. Effect of Sophorolipid n-Alkyl Ester Chain Length on Its Interfacial Properties at the Almond Oil-Water Interface. *Langmuir* **32**:5562–5572.

Koh A, Wong A, Quinteros A, Desplat C, Gross R. 2017. Influence of Sophorolipid Structure on Interfacial Properties of Aqueous ‐ Arabian Light Crude and Related Constituent Emulsions. *J. Am. Oil Chem. Soc.* **94**:107–119.

Krieger N, Neto DC, Mitchell DA. 2010. Production of Microbial Biosurfactants by Solid-State Cultivation. In: . *Biosurfactants*, pp. 203–210.

Maddikeri GL, Gogate PR, Pandit AB. 2015. Improved synthesis of sophorolipids from waste cooking oil using fed batch approach in the presence of ultrasound. *Chem. Eng. J.* **263**:479–487. http://linkinghub.elsevier.com/retrieve/pii/S1385894714014533.

Martin del Campo M, Camacho RM, Mateos-Díaz JC, Müller-Santos M, Córdova J, Rodríguez JA. 2015. Solid-state fermentation as a potential technique for esterase/lipase production by halophilic archaea. *Extremophiles* **19**:1121–1132.

Mata-Gómez M, Mussatto SI, Rodríguez R, Teixeira JA, Martinez JL, Hernandez A, Aguilar

CN. 2015. Gallic Acid Production with Mouldy Polyurethane Particles Obtained from Solid State Culture of Aspergillus niger GH1. *Appl. Biochem. Biotechnol.* **176**:1131–1140.

Mitchell DA, Berovič M, Krieger N. 2006. Solid-State Fermentation Bioreactor Fundamentals: Introduction and Overview. In: Mitchell, DA, Berovič, M, Krieger, N, editors. *Solid-State Ferment. Bioreact. Fundam. Des. Oper.* Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 1–12. http://dx.doi.org/10.1007/3-540-31286-2_1.

Parekh VJ, Patravale VB, Pandit AB. 2012. Mango kernel fat : A novel lipid source for the fermentative production of sophorolipid biosurfactant using Starmerella Bombicola NRRL -Y 17069. *Ann. Biol. Res.* **3**:1798–1803.

Peng Y, Totsingan F, Meier MAR, Steinmann M, Wurm F, Koh A, Gross RA, Kim YB, Yun HS, Kim EK. 2015. Sophorolipids: Expanding structural diversity by ring-opening cross-metathesis. *Eur. J. Lipid Sci. Technol.* **117**:6028–6032. http://dx.doi.org/10.1016/j.biortech.2009.06.053.

Pitol LO, Biz A, Mallmann E, Krieger N, Mitchell DA. 2016. Production of pectinases by solid-state fermentation in a pilot-scale packed-bed bioreactor. *Chem. Eng. J.* **283**:1009–1018. http://linkinghub.elsevier.com/retrieve/pii/S1385894715011286.

Ponsá S, Gea T, Sánchez A. 2010. Different indices to express biodegradability in organic solid wastes. *J. Environ. Qual.* **39**:706–12. http://www.ncbi.nlm.nih.gov/pubmed/20176843.

Ramadas N V., Sindhu R, Binod P, Pandey A. 2013. Development of a novel solid-state fermentation strategy for the production of poly-3-hydroxybutyrate using polyurethane foams by Bacillus sphaericus NII 0838. *Ann. Microbiol.* **63**:1265–1274.

Rashad MM, Nooman MU, Ali MM, Mahmoud AE. 2014. Production, characterization and anticancer activity of *Candida bombicola* sophorolipids by means of solid state fermentation of sunflower oil cake and soybean oil. *Grasas Aceites* **65(2)**:e017.

Roelants SLKW, Ciesielska K, De Maeseneire SL, Moens H, Everaert B, Verweire S, Denon Q, Vanlerberghe B, Van Bogaert INA, Van der Meeren P, Devreese B, Soetaert W. 2016. Towards the industrialization of new biosurfactants: Biotechnological opportunities for the lactone esterase gene from Starmerella bombicola. *Biotechnol. Bioeng.* **113**:550–559.

Scott TA, Melvin EH. 1953. Determination of Dextran with Anthrone. *Anal. Chem.* **25**:1656–1661. http://pubs.acs.org/doi/abs/10.1021/ac60083a023.

Solaiman DKY, Ashby RD, Nuñez A, Foglia TA. 2004. Production of sophorolipids by Candida bombicola grown on soy molasses. *Biotechnol. Lett.* **26**:1241–1245.

Solans C, Izquierdo P, Nolla J, Azemar N, Garcia-Celma MJ. 2005. Nano-emulsions. *Curr. Opin. Colloid Interface Sci.* **10**:102–110. http://www.sciencedirect.com/science/article/pii/S1359029405000348.

Subbalaxmi S, Murty VR. 2016. Process optimization for tannase production by Bacillus gottheilii M2S2 on inert polyurethane foam support. *Biocatal. Agric. Biotechnol.* **7**:48–55. http://dx.doi.org/10.1016/j.bcab.2016.05.004.

Takahashi M, Morita T, Wada K, Hirose N, Fukuoka T, Imura T, Kitamoto D. 2011. Production of sophorolipid glycolipid biosurfactants from sugarcane molasses using Starmerella bombicola NBRC 10243. *J. Oleo Sci.* **60**:267–273. http://www.ncbi.nlm.nih.gov/pubmed/21737925.

The US Department of Agriculture and The US Composting Council. 2001. Test Methods for

the Examination of Composting and Compost. Houston: Edaphos International.

Varesche MB, Zaiat M, Vieira RF, Vazoller RF, Foresti E. 1997. Microbial colonization of polyurethane foam matrices in horizontal- flow anaerobic immobilized-sludge reactor:534–538.

Zhang L, Somasundaran P, Singh SK, Felse AP, Gross R. 2004. Synthesis and interfacial properties of sophorolipid derivatives. *Colloids Surfaces A Physicochem. Eng. Asp.* **240**:75–82.

Tables

Table I

Central Composite Design (CCD) matrix for the optimization of the ratio of substrates and inoculum size to produce SLs with the coded and real values for the variables and the experimental and predicted values for the response.

Run	S: Ratio MOL/SA (w/w)		I: Inoculum size (%, v/w)		Y: SL yield (g g^{-1} substrates)	
	Coded	Real	Coded	Real	Experimental	Predicted
1	1	3:1	-1	5	0.102	0.099
2	-1	1:3	1	15	0.079	0.080
3	1	3:1	0	10	0.096	0.099
4	-1	1:3	0	10	0.079	0.080
5	0	1:1	0	10	0.119	0.120
6	0	1:1	0	10	0.125	0.120
7	0	1:1	0	10	0.107	0.120
8	0	1:1	-1	5	0.114	0.120
9	0	1:1	0	10	0.117	0.120
10	1	3:1	1	15	0.097	0.099
11	-1	1:3	-1	5	0.082	0.080
12	0	1:1	1	15	0.133	0.120
13	0	1:1	0	10	0.119	0.120

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Figures

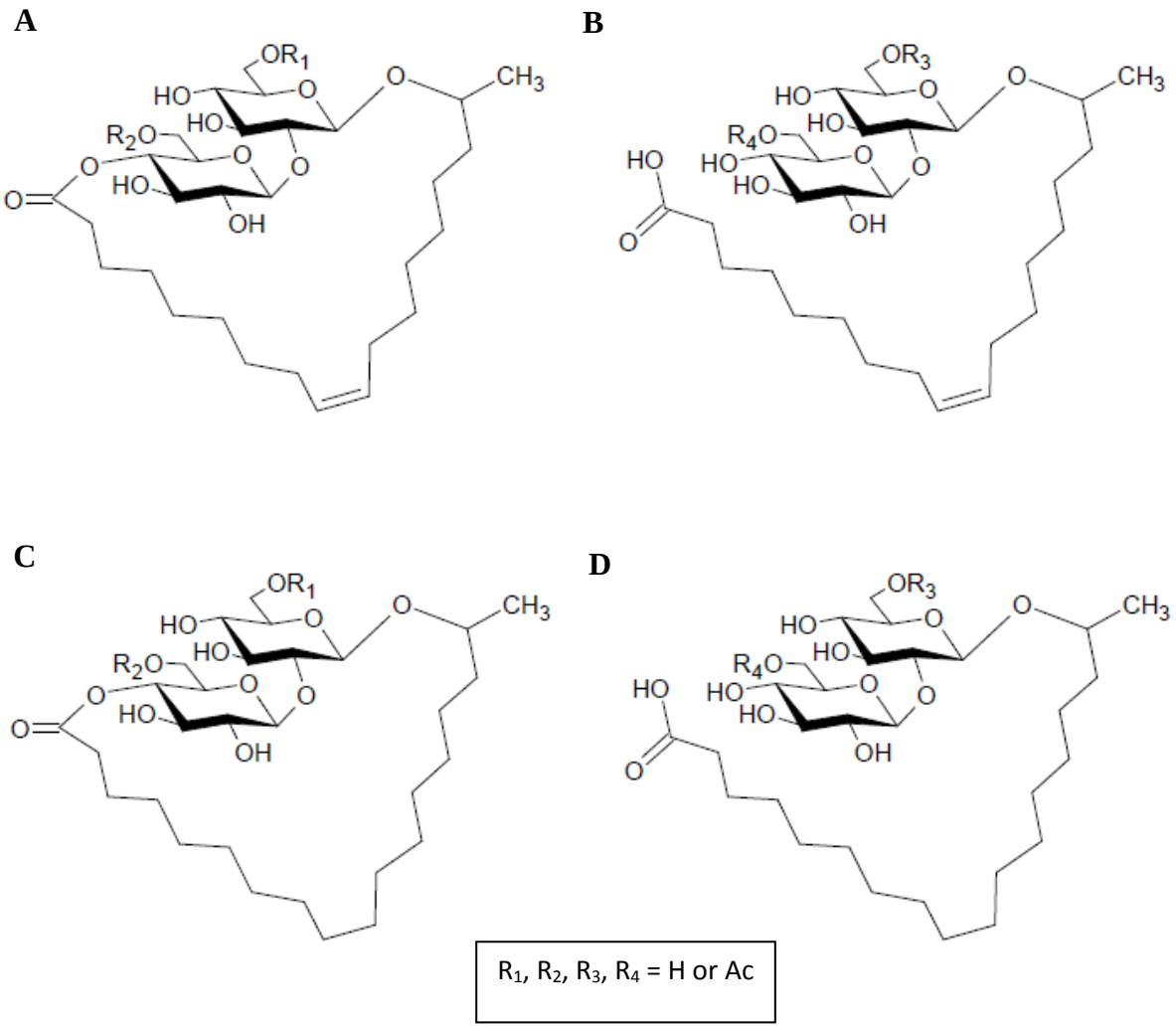

Figure 1. Structures of C18:1 sophorolipids (A) lactonic form and (B) free acid form, and C18:0 sophorolipids (C) lactonic and (D) free form.

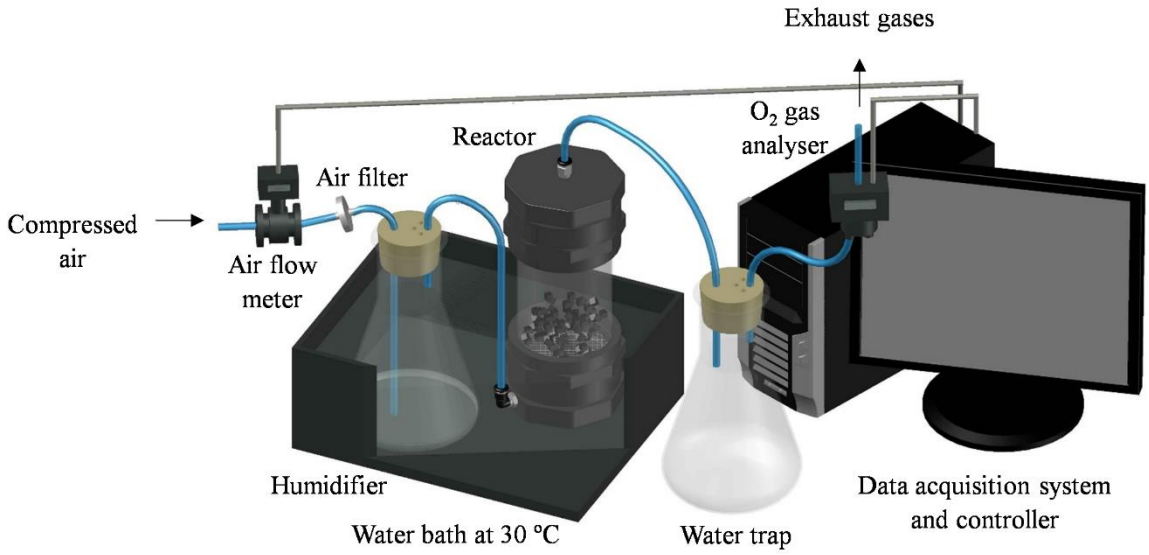

Figure 2. Experimental setup for SSF experiments.

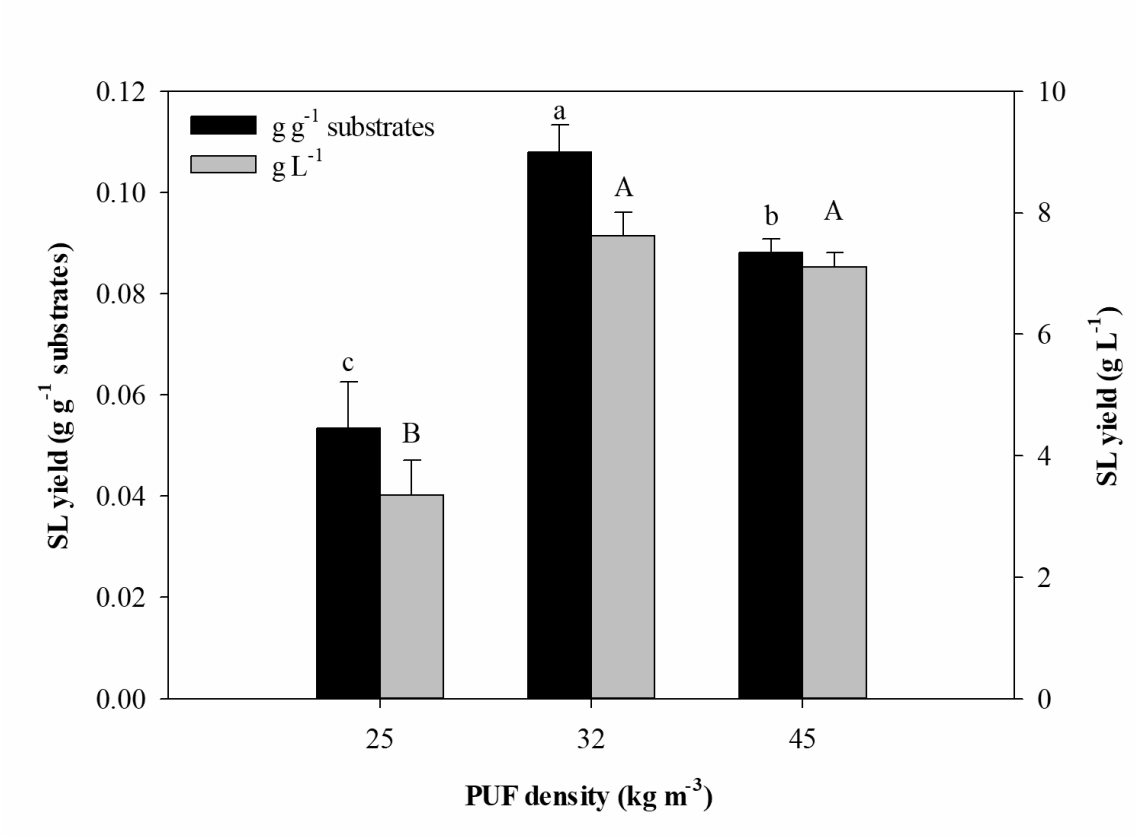

Figure 3. SL yield obtained for 3 different polyurethane foam (PUF) densities. Results are expressed as mean ± standard deviation of three replicates. Means with the same letters are statistically equal per Duncan´s test ($p < 0.05$).

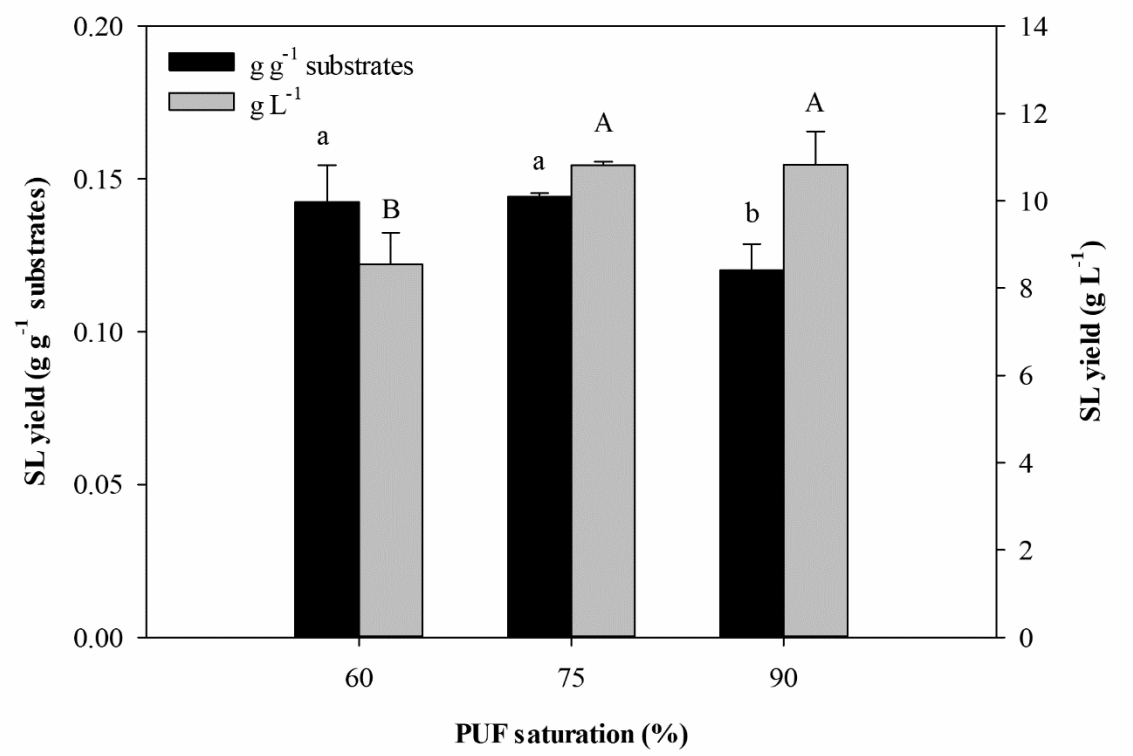

Figure 4. Effect of PUF saturation in reference to water holding capacity (WHC) on SL yield. Results are expressed as mean ± standard deviation of three replicates. Means with the same letters are statistically equal per Duncan´s test ($p < 0.05$).

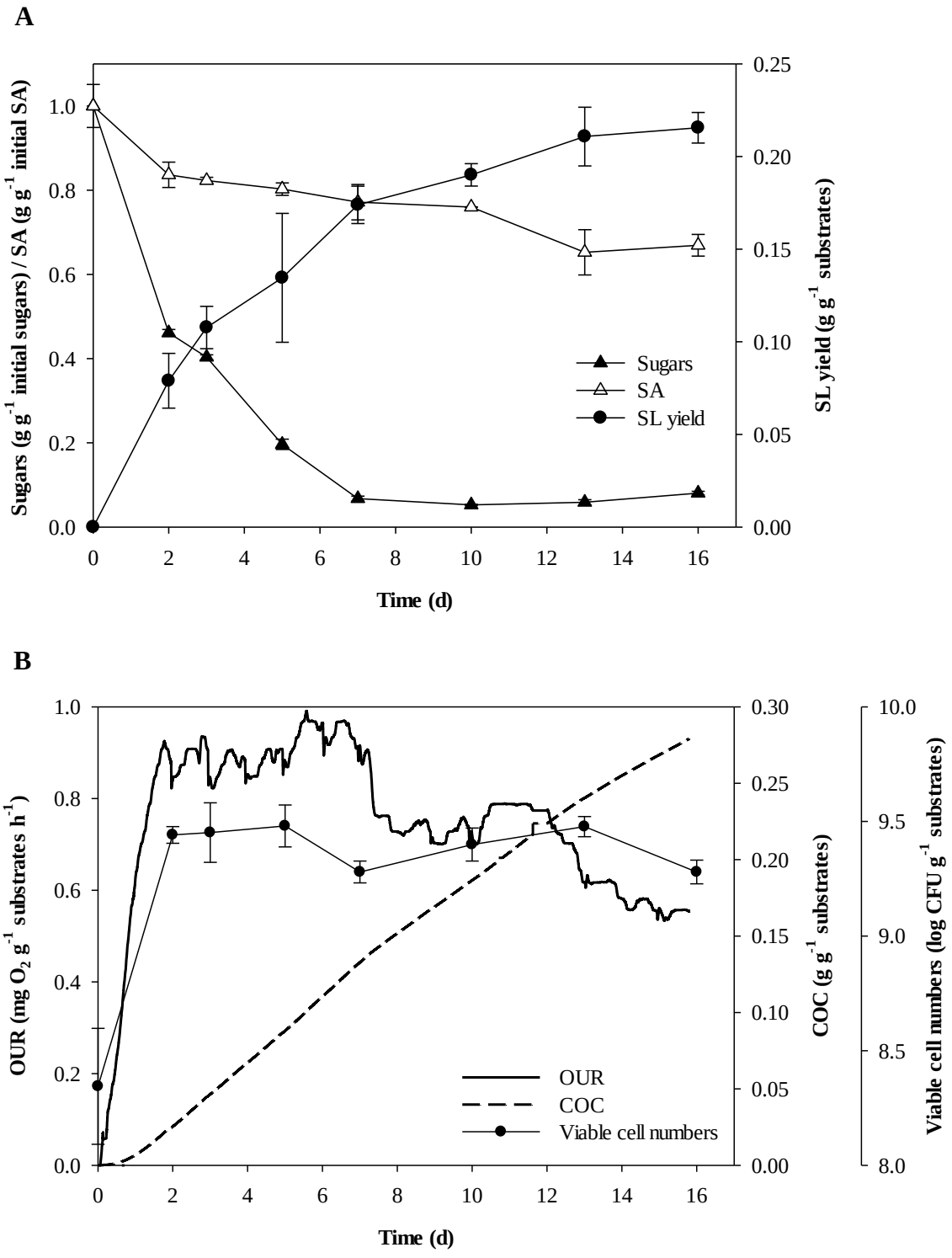

Figure 5. Solid-state fermentation profile under optimized conditions: (A) time course of SL yield, sugars and SA content; and (B) time course of viable cell numbers, Oxygen Uptake Rate (OUR) and Cumulative Oxygen Consumption (COC) profiles.

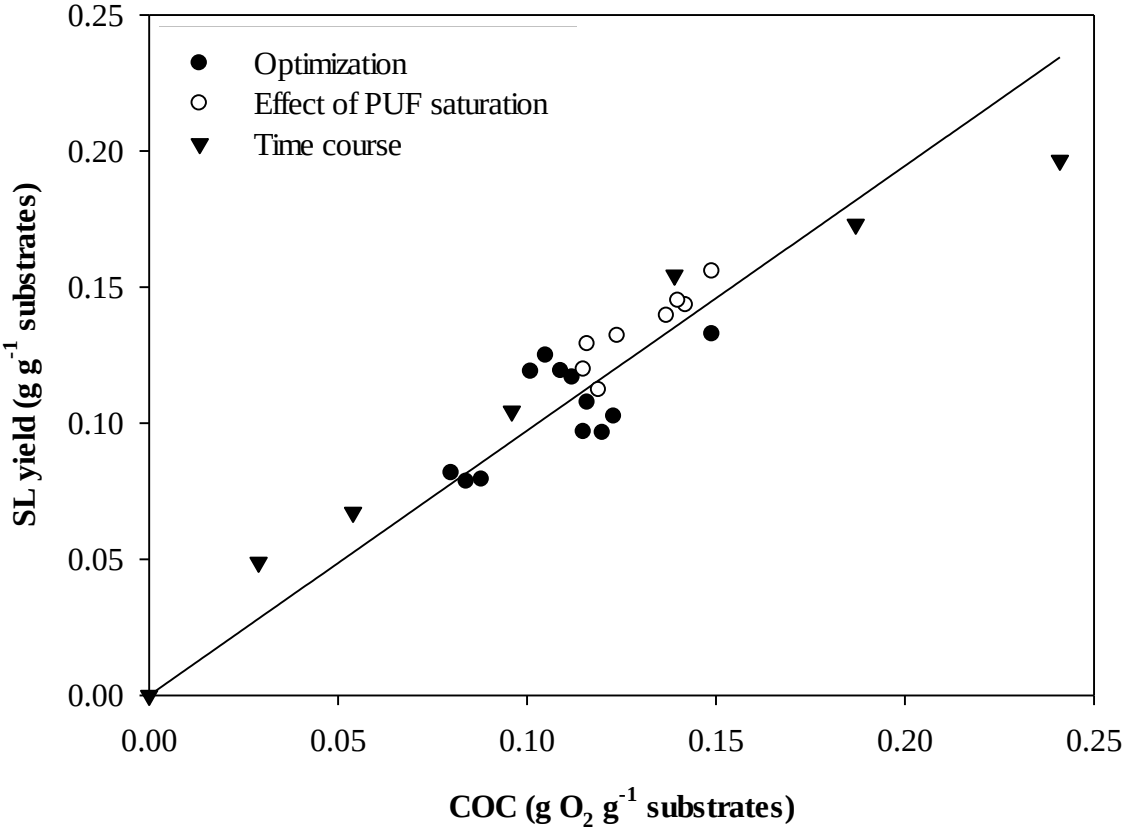

Figure 6. Correlation between SL yield and Cumulative Oxygen Consumption (COC).

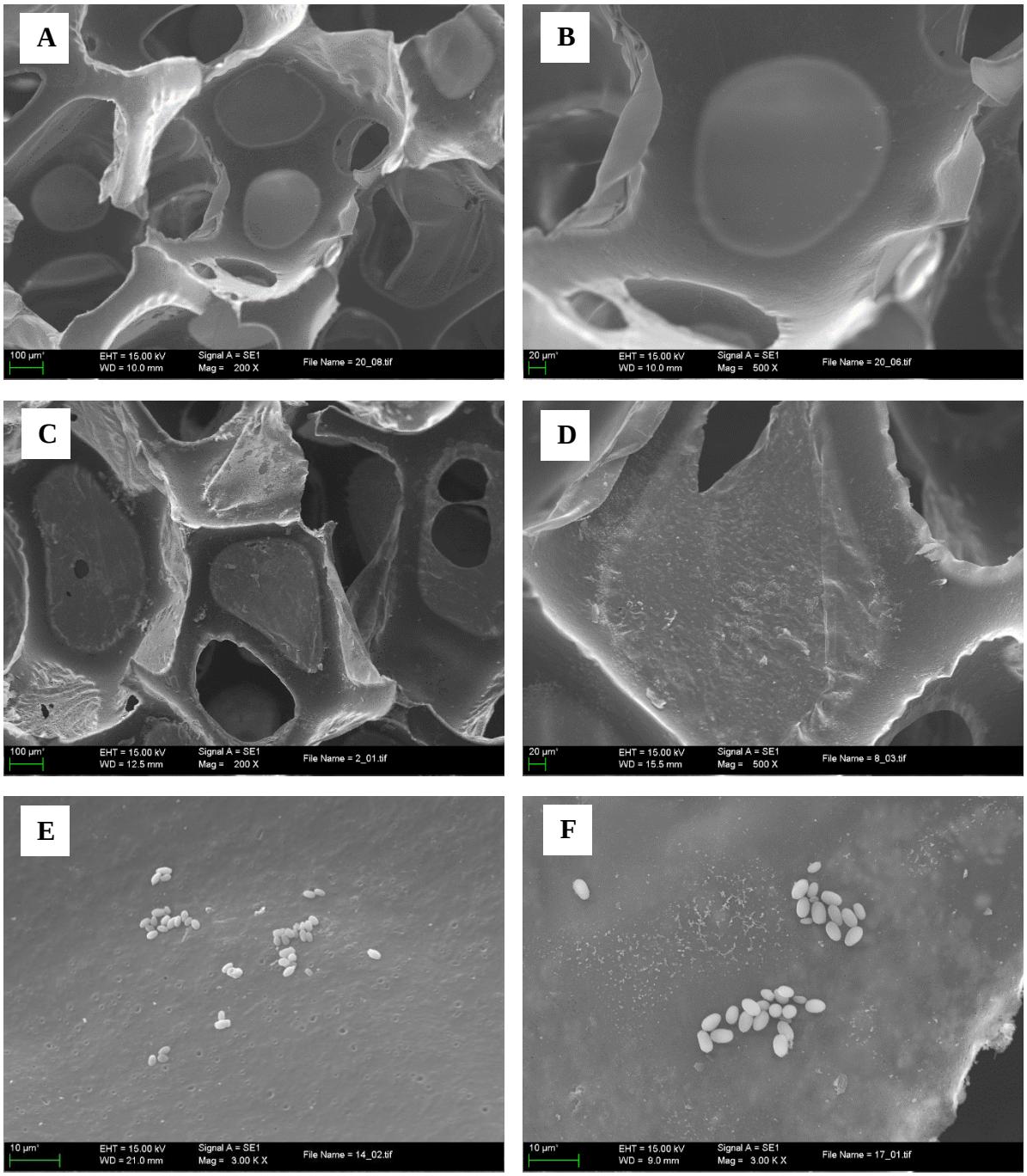

Figure 7. SEM pictures of PUF-32 (A, B), PUF-32 with the fermentation media (C, D), and PUF-32 after 2 (E) and 7 (F) days of fermentation with dispersed cells attached.

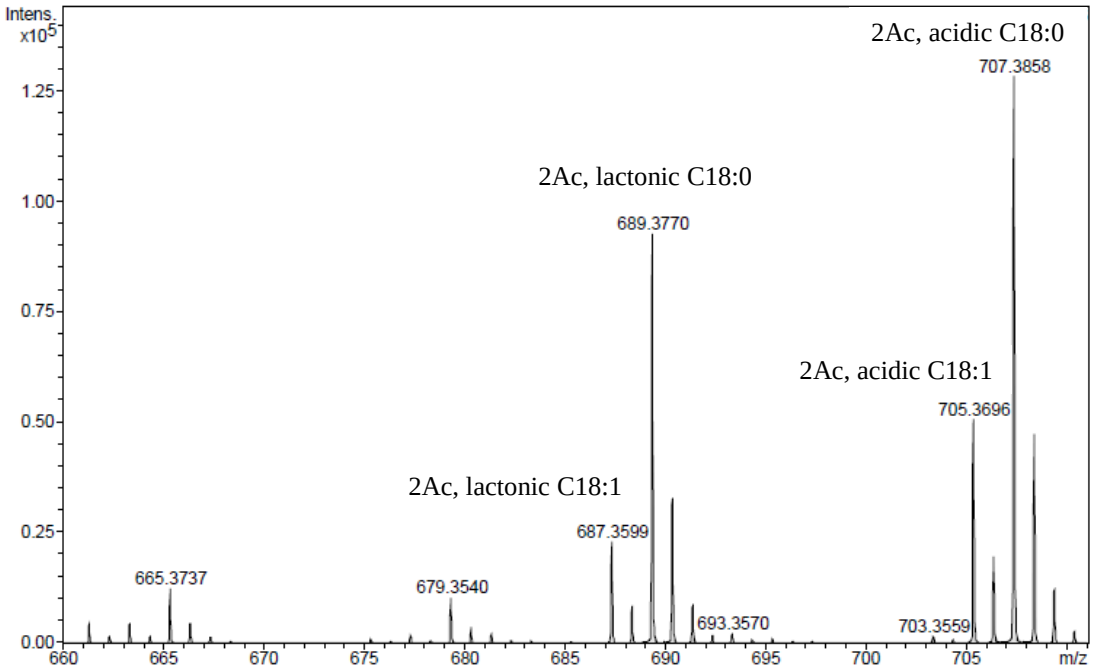

Figure 8. Mass spectrometry spectra of the obtained SLs.

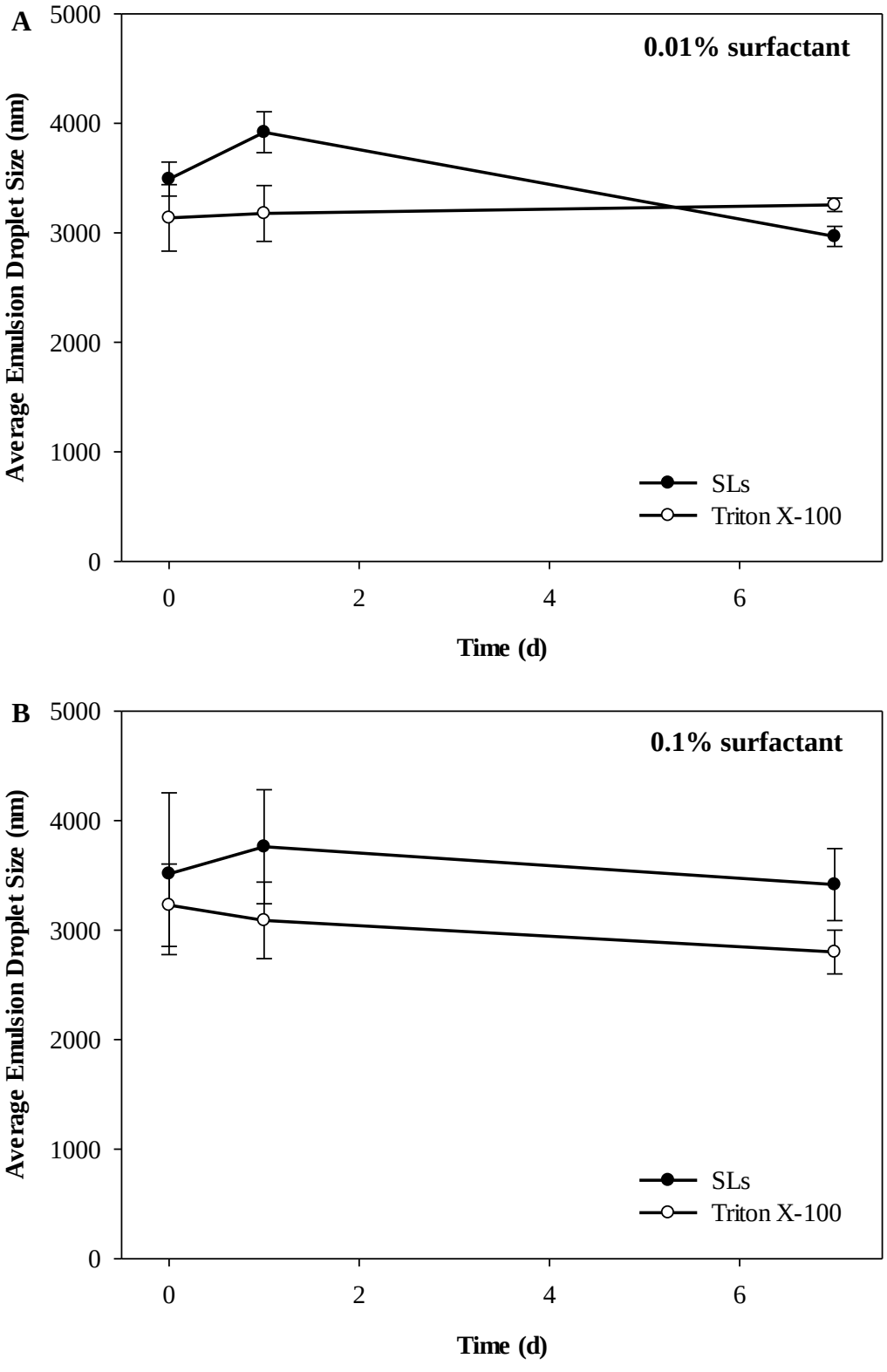

Figure 9. Average emulsion droplet sizes immediately after homogenization and after 1 and 7 days aging using 0.01% (A) and 0.1% (B) surfactant. Mean ± standard deviation of three triplicates.

Appendix

Table A.1

Analysis of variance of the fitted model obtained from the CCD for optimal SL yield (g g^{-1} substrates).

Source	Sum of squares	Degrees of freedom	Mean squares	F-value	p-value	
Model	$3.421 \cdot 10^{-3}$	2	$1.710 \cdot 10^{-3}$	41.78	< 0.0001	*
A	$5.240 \cdot 10^{-4}$	1	$5.240 \cdot 10^{-4}$	12.80	0.0050	*
A^2	$2.897 \cdot 10^{-3}$	1	$2.897 \cdot 10^{-3}$	70.76	< 0.0001	*
Residual	$4.093 \cdot 10^{-4}$	10	$4.093 \cdot 10^{-5}$			
Lack of fit	$2.505 \cdot 10^{-4}$	6	$4.175 \cdot 10^{-5}$	1.05	0.5039	NS
Pure error	$1.588 \cdot 10^{-4}$	4	$3.970 \cdot 10^{-5}$			
Total	$3.830 \cdot 10^{-3}$	12				

R^2: 0.8931; adj R^2: 0.8717; pre R^2: 0.8480; C.V.: 6.08; adequate precision: 12.78.

*Statistically significant (95% confident interval).

NS=Statistically not significant (95% confident interval).

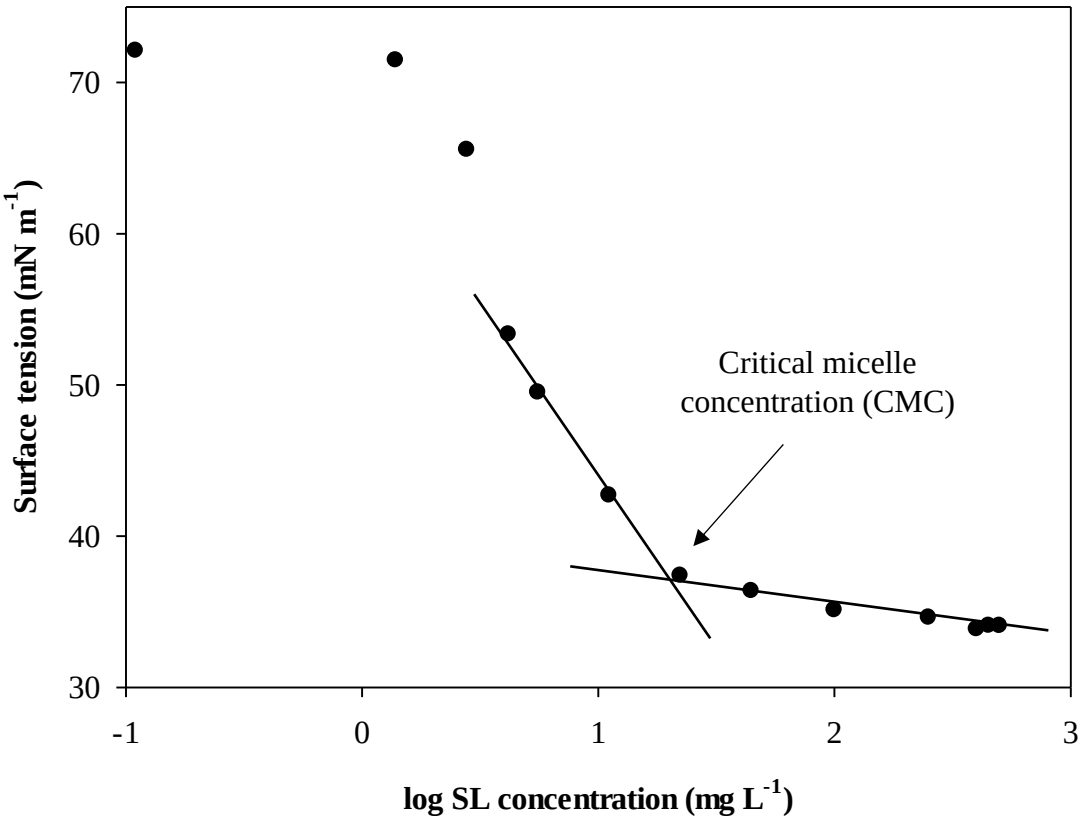

Figure A.1. Surface tension curve of the SLs.

	Triton X-100		Sophorolipid	
	1 day	**7 days**	**1 day**	**7 days**
0.01% surfactant				
0.1% surfactant				

Figure A.2. Photographs of water-almond oil emulsions after 1 and 7 days aging.