

# Oxygen Transfer Rate and Sophorose Lipid Production by *Candida bombicola*

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**Abstract:** Sophorose lipids (SLs) have applications as surfactants and are produced at high levels by several yeasts. We developed a fed-batch shake-flask method for the production of SLs by *Candida bombicola* ATCC 22214. Optimal aeration, expressed in terms of oxygen transfer rate, was between 50 and 80 mM O<sub>2</sub>/L h<sup>-1</sup> and resulted in maximum values for both volumetric product formation (1–1.5 g/L h<sup>-1</sup>) and SL yield (350 g/L). The lowest aeration levels resulted in the enrichment in saturated fatty acid SLs at the expense of unsaturated fatty acid SLs. © 2002 John Wiley & Sons, Inc. *Biotechnol Bioeng* 77: 489–494, 2002; DOI 10.1002/bit.10177

**Keywords:** sophorose lipids; *Candida bombicola*; oxygen transfer rate; optimal aeration; fed-batch culture; lactonic form; acidic form

## INTRODUCTION

Sophorolipids (SLs) are a family of structurally related extracellular glycolipids that contain the disaccharide sophorose and produced mainly by yeasts in the *Candida* genus (Asmer et al., 1988). The glycosidic carbon of sophorose is linked to the hydroxyl group at the penultimate carbon of predominantly C18 chain-length fatty acids (Gobbert et al., 1984). SLs have shown potential as: 1) primary surfactants for shampoos, body washes, and detergents (Hall et al., 1995; Inove et al., 1980); 2) emulsifiers for skin care products (Mager et al., 1987); 3) stable consumable encapsulants for food and food oils (Allingham, 1971); 4) products to reduce the interfacial tension at oil-water interfaces (Asmer et al., 1988); 5) hard surface cleaners and degreasing agents (Hall et al., 1995), and 6) additives for formulations to clean oil-contaminated containers (Hall et al., 1995). *Candida bombicola* produces 7 major and 15 minor SLs

(Davila et al., 1993). SL structure and production are probably related to the regulation of energy metabolism (Hommel and Huse, 1993; Davila et al., 1997; Lottermoser et al., 1996). Phosphate level (Albrecht et al., 1996) and nitrogen source also influence SL production (Hommel et al., 1994; Zhou et al., 1992). Some saccharides (glucose, fructose, sucrose, lactose), fatty acids, oils, alcohols, glycerol, and some alkanes are suitable carbon sources for SL production (Ito and Inoue, 1982). Lipophilic co-substrates, such as *n*-alkanes, fatty acid (FA) methyl or ethyl esters, and vegetable and animal oils, alter the SL acetylation pattern (Davila et al., 1992; Davila et al., 1994; Zhou et al., 1992). The biosynthesis of SLs by *C. bombicola* in fed-batch culture could produce up to 700 g of SL per 1 L of initial medium volume (Marchal et al., 1997; referred further in the text as SL yields). The co-substrates were fed at optimal pH and aeration and accumulated SLs removed to restore the productivity of the accumulated biomass and minimize inhibition of microbial growth in a fermentor. There exists a critical minimal level of oxygenation for high SL production, but this level has not been quantified (Asmer et al., 1988; Davila et al., 1997; Marchal et al., 1997), and its effect on the distribution of SLs produced remains undescribed (e.g., acidic versus lactonic).

Our objective in this study was to better understand the relationship between aeration and microbial growth, SL production, and SL distribution in shake flask cultures. Shake flask cultures are not as productive as fermentors but are essential for multi-parameter experiments for culture optimization and to obtain sufficient sample quantities from specialty carbon sources for structure–property studies. Studies with expensive or labeled (radioactive or heavy atom) substrates to identify metabolic pathways and SL structures in more detail also are commonly made in small-scale batch cultures.

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## MATERIAL AND METHODS

### SL Production

*C. bombicola* ATCC 22214 was grown aseptically on a medium containing 100 g/L glucose, 10 g/L yeast extract, and 1 g/L urea. 40 g of oleic acid (Sigma Chemical Co., St. Louis, MO, 99%, used as received) per L was supplied to the medium before inoculation at the stage of fermentation (Asmer et al., 1988). The portions of oleic acid and glucose powder were autoclaved and added as further described. The total oleic acid added over the course of the fermentations was 400 g/L. All cultures were performed in triplicate.

### Preparation of Inoculum

*C. bombicola* ATCC 22214 was stored in liquid nitrogen as inoculum aliquots ( $2-4 \cdot 10^{11}$  cells per mL in 10:90 glycerol:phosphate buffer, pH 7, containing 137 mM NaCl). Two sequential 100-mL precultures were grown in a rotary shaker at 100 rpm in 500 mL baffled Erlenmeyer flasks at  $30 \pm 1^\circ\text{C}$ . The first pre-culture was inoculated with 1 mL of the above inoculum aliquot (approximately 0.1 g of dry biomass). The cultures were passed to the next stage at the late exponential growth phase (about 24–36 h) estimated by  $A_{650}$  (Ito and Inoue, 1982). Final pre-cultures contained  $33 \pm 5$  g of dry biomass per liter.

### Biomass

Biomass was monitored by dry weight after preliminary removal of hydrophobic substrates and SL by methanol/chloroform treatment of 1-mL culture liquid aliquots (Zhou et al., 1992). The fed-batch culture method (Marchal et al., 1997) was adapted for shake flasks. To prevent potential end-product inhibition, accumulated SLs were removed every 24 h (see below).

### Fermentation

Studies were conducted in 0.5 L baffled Erlenmeyer flasks on a rotary shaker. The cultures were started at  $30 \pm 1^\circ\text{C}$  in 30, 70, 110, and 150 mL volumes of medium, and the agitation speed was increased from 100 to 360 rpm by 20 rpm per day for 13 days. Inoculum was introduced in the form of a wet biomass pellet after centrifugation (4500 g, 10 min) of culture liquid aliquots from the second preculture. The volume of these aliquots was 15% of the total volume of the culture medium. Thus, the initial biomass concentration was approximately 5 g/L. When the biomass content in the culture reached maximum (at 48 to 80 h, estimated by  $A_{650}$  readings), glucose (90 g/L) and oleic acid (40 g/L) were added. Subsequently, oleic acid (40 g/L) was added every 24 h until the end of the culture. Under optimal conditions, the cultures were continued to 14 days without retardation of SL production.

### Characterization of SLs

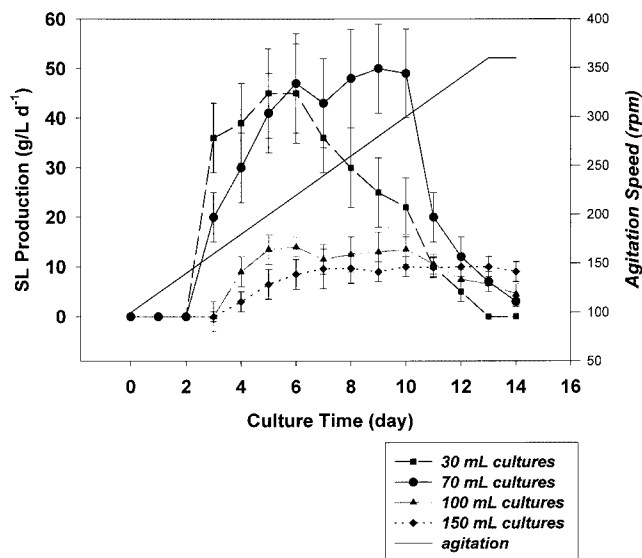
Qualitative and quantitative determinations of the individual SLs in the SL product mixture were performed by thin-layer chromatography (TLC) and by fast atom bombardment (FAB) mass spectrometry (MS) (Asmer et al., 1988; de Koster et al., 1995; Zhou and Kosaric, 1995). FAB mass spectra were recorded in the positive ion mode on a double-focusing Kratos MS 50 equipped with the Maspec2 data acquisition and processing system (Mass Spectrometry Services Ltd.) and a cesium ion source gun (20 KV). 3-Nitrobenzylalcohol (3-NBA) saturated with sodium chloride was used as a matrix. Cesium iodide (CsI) was used as a calibrant and the spectra were recorded at 10 s/decade from 60 to 2,000 m/z at resolution 2,000. The SLs were mixed with the matrix, and 2  $\mu\text{L}$  of the mixture was placed on the target of the direct insertion probe. Peak intensity values were averaged over three scans and were corrected for the contribution of the M+2 isotopic peak when appropriate. For instance, the intensity of m/z 713 peak corresponding to the  $\text{MNa}^+$  ion of the 1',4'-lactone-6',6'-diacetyl saturated SL was corrected for the contribution of the M+2 peak of m/z 711 that corresponds to the  $\text{MNa}^+$  ion of the 1',4'-lactone-6',6'-diacetyl monounsaturated SL. SL production was quantified by periodically harvesting the accumulated SLs. Starting after about 72–96 h, once per day the culture agitation was interrupted for 15 min, and a pipette was used to remove the dense viscous brown phase that had separated at the bottom of the flasks. The collected liquid (~45–50% w/w SLs) was washed twice with an equal volume of double distilled water ( $25^\circ\text{C}$ ). The first wash was returned to the culture flask to maintain the culture volume. The oily phase was extracted with an equal volume of ethyl acetate. After three successive extractions, the ethyl acetate extracts were combined and the solvent was removed under reduced pressure. The resulting yellow oily residue was treated three times with 150-mL portions of hot hexane that removed residual oleic acid, leaving a SL powder. The powder was dried in air at room temperature and then at reduced pressure in vacuo to constant weight to give a gray or pale cream-colored powder.

### Oxygen Transfer Rate (OTR) Measurements

The values of OTR were measured for a model sodium sulfite solution as described by Freedman (Freedman, 1969).

### Statistical Analysis

The significance of differences for pairs was evaluated by Student's *t* tests. ANOVA was used for statistical tests of groups. Data are presented on graphs as the mean value with error bars representing the standard deviations.

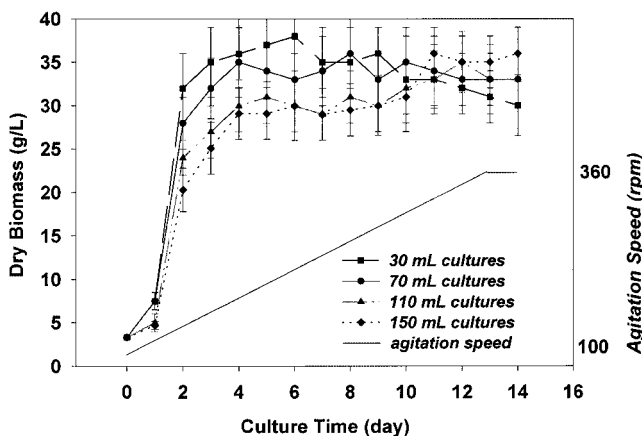


**Figure 1.** SL volumetric production as a function of the culture volume, agitation speed, and age.

## RESULTS

### Effects of Culture Volume and Agitation

Because the culture flasks were of identical size, cultures of higher medium volumes represented lower oxygenation and those with relatively smaller volumes represented higher oxygenation. Variation in agitation speed was used to further diversify of the oxygenation conditions (Figs. 1 and 2). For all of the medium volumes studied, a trend towards increased SL volumetric product formation was observed up to the sixth day of culture (220 rpm) (Fig. 1). The highest level of production was found for 30 and 70 mL cultures (~50 g SL/L day or ~350 g SL after a 1-week production period). The cultures of higher medium volume (110 and 150 mL) showed relatively lower levels of product formation (~10 g SL/L day) at equivalent agitation rates. The use of higher agitation between days 6 to 10 did not significantly influence SL production for the 70, 110, and



**Figure 2.** Growth of biomass as a function of culture volume, agitation speed, and age.

150 mL cultures. However, over the same time period, the increase in agitation for the 30 mL culture resulted in decreased product formation. The 70 mL cultures maintained high production up to day 10 but decreased sharply between days 10 and from 50 to 20 g SL/L day. By day 14 (360 rpm), the SL production for the 30 and 70 mL cultures had decreased to 1–2 g SL/L day.

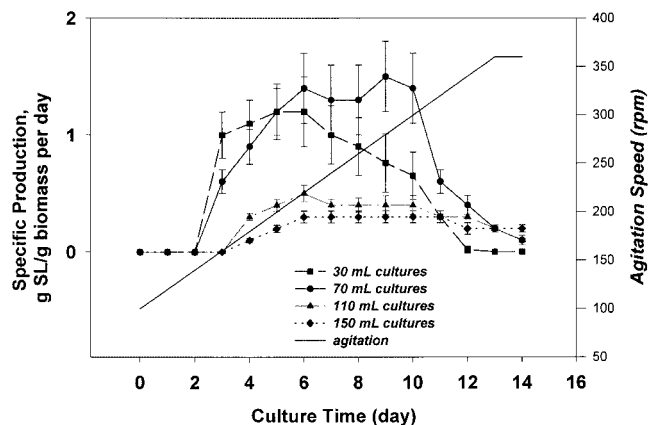
The profile of SL volumetric production for the 110- and 150-mL cultures was very different than that of the corresponding 30 and 70 mL cultures. From days 3–6, the 110 and 150 mL cultures had a slow increase in SL volumetric production, and then stabilized, subsequently, at about 10 g SL/L day.

The profiles of biomass concentrations obtained at increasing agitation speed (see “Fermentation” section for details) were similar for all of the cultures and showed maximum values of between 33–37 g/L (Fig. 2). The time/oxygenation required to reach maximum biomass concentrations was 3–6 days (lower agitation) for the 30 and 70 mL cultures but was 10–13 days (higher agitation) for the 110 and 150 mL cultures.

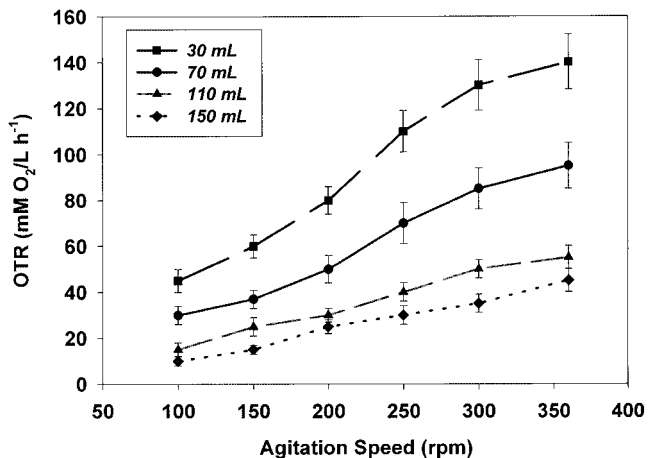
Specific productivity (g SL/g biomass day) is shown in Figure 3. By day 4, the 30 and 70 mL cultures had reached specific productivity values of up to 1–1.2 g SL/g biomass day. In comparison, the specific productivity values of the 110 and 150 mL cultures were modest (0.5 g SL/g biomass day by day 6). The specific productivity of the 70 mL cultures reached ~1.5 g SL/g BM day by day 6 and then remained at this value up to day 10. In contrast, the higher aeration experienced by the 30 mL cultures led to decreased specific productivity from day 6 to 12. By day 6, the specific productivity of biomass for the 110 and 150 mL cultures reached values of only ~0.5 g SL/g biomass day.

### Control Cultures

The possibility that prolonged culture time might lead to a loss in culture productivity was examined. Thus, to assure the biosynthetic capabilities of the inoculum used were not lost, reference cultures were studied over 14 days using a moderate or medium volume and constant

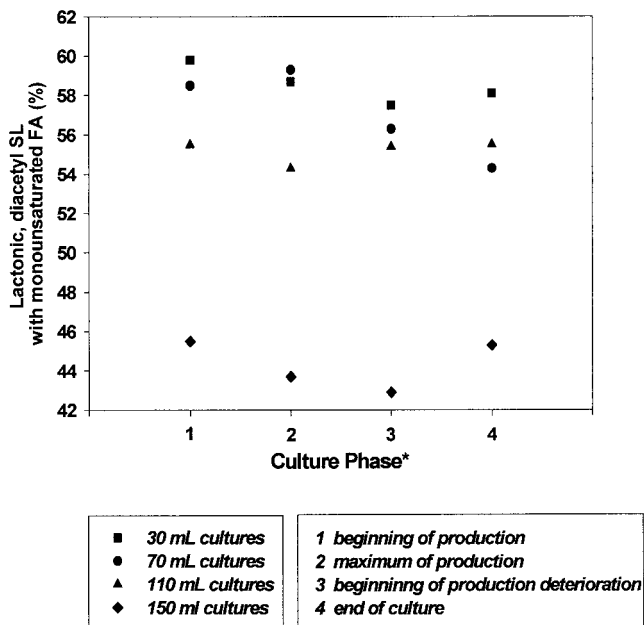


**Figure 3.** Specific productivity of biomass as a function of culture volume, agitation speed, and age.



**Figure 4.** OTR in a model sodium sulfite solution in flasks as a function of medium volume and agitation speed.

agitation (70 mL, 250 rpm). The biomass concentration and specific productivity values were equal to those of the experimental 70 mL cultures during their period of 240–280 rpm agitation. However, in contrast to the experimental cultures, the reference cultures did not show any deterioration in SL production during the 14 days of culture (data not shown).



**Figure 5.** Changes in lactonic diacetyl monounsaturated SL for different culture volumes as a function of culture time.

\*The culture phases correspond the following culture times (day):

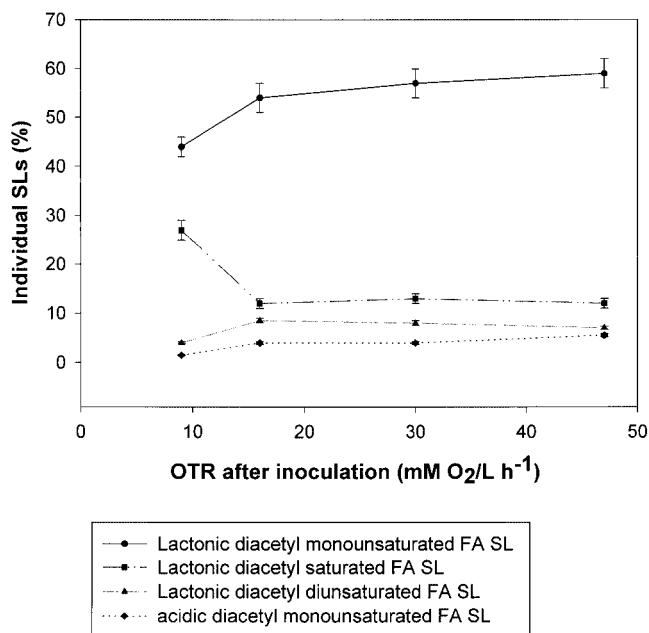
Culture volume (mL)	Culture phase (number)			
	1	2	3	4
30	2–3	5–6	6–7	13–14
70	2–3	5–6	6–7	13–14
110	3–4	5–6	10–11	14
150	3–4	7–8	10–13	14

### OTR Values at Different Agitation Speeds

Figure 4 shows a plot of the OTR values as a function of the agitation speed and the liquid volume. As expected, the plot shows a pronounced increase in oxygenation with decreased medium volume.

### Profiles of Individual SLs

Monitoring profiles of individual SLs was performed by FAB MS. FAB mass spectra were recorded in a positive ion mode using NaCl saturated 3-NBA to obtain a single pseudomolecular ion for each molecular species. In contrast, when neat 3-NBA was used, the ion current is split into  $MH^+$  and  $MNa^+$  ions, complicating the spectrum and making quantitation difficult. Furthermore, fragmentation of SLs  $MNa^+$  ions is greatly reduced compared to  $MH^+$  ions. The methodology that was ultimately adopted by us for the FAB MA studies allowed the identification and quantitation of several SL structures, including the following: 1',4'-lactone-6',6'-diacetyl and the corresponding 6'-monoacetyl form; 1',4'-lactone-SL with saturated, monounsaturated, and diunsaturated C18 FA SLs; and 6',6'-diacetyl ring-opened or acidic SLs. Also, with high probability, the presence of 6',6'-diacetyl and 6'-monoacetyl acidic di- and tri-unsaturated C18 SLs was determined. Some difficulty in assignment of the relevant m/z ratios to the latter three SLs was due to the possibility of assignment of these ratios to C19 FAs of different degree of unsaturation. However, based on the tendency of this organism to incorporate predominantly C18 chain-length FAs in SLs (Gobbert et al., 1984), and the use of high purity oleic acid as the sole carbon source of FAs (Sigma, 99%,



**Figure 6.** Changes in the content of individual SLs as a function of OTR.

produced by hydrolysis of olive and sunflower oils that usually do not contain does not contain C19 FAs; Gurr and Harwood, 1991), the assignment of the m/z ratios to the C18 SLs is likely correct. Changes in the most abundant individual SLs are presented in Figures 5 and 6. For a specific culture volume, the concentrations of individual SLs did not substantially vary with culture time and/or the agitation rate. As an example, the profile of lactonic diacetyl monounsaturated FA SL is shown on the graph in Table I. However, comparison of the proportions of individual SLs for the different culture volumes used showed a remarkable difference for the 150 mL culture that had an OTR after inoculation of 10 mM O<sub>2</sub>/L h<sup>-1</sup> (Fig. 6). Under these conditions, the concentration of unsaturated FA SLs declined whereas the content of saturated FAs increased in the product. For example, the fraction of lactonic diacetyl monounsaturated FA SL was 44% of total SLs for 150 mL cultures but accounted for 54 to 60% in the products received at smaller culture volumes. Conversely, the content of lactonic diacetyl saturated FA SLs was more than twice as high in the 150 mL cultures (27%) in comparison with the cultures of smaller volumes (from 10 to 16%). All of the cultures of smaller volume correspond to cultures that had OTR values greater than 10 mM O<sub>2</sub>/L h<sup>-1</sup> (Fig. 6). The concentration of acidic monoacetyl SLs with di- and tri-unsaturated FAs was twice as high for the 150 mL cultures (~11% in total, data not shown).

## DISCUSSION

Studies using *C. bombicola* were conducted to determine the relationship between oxygen conditions, SL production, and SL composition in small shake flask cultures. The underlying motivation for this study was to be able to apply these conditions to the development of media components for SL production by using experimental design strategies in shake flasks. It is noteworthy that efforts to conduct improvements in product formation in small cultures may not be applicable sometimes to high yield experiments due to lower productivity in flasks. Hence, it is very useful to establish high-yield conditions in shake flasks where numerous experimental conditions, and replicate experiments are easily performed. Previous to this study, medium optimization in shake flasks had been performed with lower levels of productivity and with no attempt to correlate the culture conditions with the distribution of SL structures (Kle-  
kner et al., 1991; Zhou and Kosaric, 1995).

Generally, higher levels of oxygenation resulted in increased SL formation. This was concluded based on increases in SL formation in all cultures with increased agitation speed. This increase in SL productivity was more profound when lower culture volumes were used. However, critical levels of agitation at low culture volumes were found where a further increase in oxygenation resulted in a decrease in product formation. For

example, the 30 and 70 mL cultures showed a decline in product formation when agitation was increased above 220 and 280 rpm, respectively (Fig. 1). For the 110 and 150 mL cultures, the same decline was also recorded. However, for these higher volume cultures, this decline may be attributed to an accumulation of oleic acid in the medium (Kim et al., 1997). A decline in product formation often results from a reduction in biomass or of specific biomass productivity. These two factors may work synergistically or independently. The rates of biomass growth and of specific productivity are shown in Figures 2 and 3, respectively. In the case of higher oxygenation levels (30 and 70 mL cultures, agitation 200 and 280 rpm, respectively), the deterioration of product formation was due to the sharp decrease of biomass specific productivity. The biomass of these cultures remained unchanged. In the case of cultures of lower oxygenation levels (110 and 150 mL cultures), the decrease in SL formation was not observed at the same elevated agitation speeds. In general, within a certain agitation range, the production of SLs was higher in the lower, rather than the higher, medium volumes. However, excessive oxygenation observed in the lowest volume (30 mL) at higher agitation speeds (>200 rpm) led to an inhibition of production. Thus, it is concluded that there exists a window of oxygenation levels that is optimal for SL biosynthesis. This optimal window of oxygenation will be defined below in terms of oxygen transfer rates.

Variation in the proportions of individual SLs, depending on aeration conditions, was observed. A decline in the content of unsaturated FA SLs at the expense of saturated FA SLs for the conditions of lowest aeration (150 mL cultures) was found in comparison with the other cultures (30, 70, 110 mL). This increase in saturated FA SLs under low aeration conditions may be explained by a decrease in activity or expression of the microbial desaturase under such conditions (Gurr and Harwood, 1991). It is noteworthy that the composition of SLs was influenced only at the lowest studied oxygenation levels. In other words, the SL composition was highly conserved over a wide range of aeration conditions that did influence product yield. Also, no significant changes in the proportions of individual SLs were measured within a specific culture volume, despite increased agitation rates (Figs. 5 and 6).

Both the number of revolutions per minute of an orbital shaker and medium volumes in flasks do not adequately describe the hydro-aerodynamics of a fermentation process (Wang et al., 1979). This is also true for information on the rpm values and air flow rates reported for fermentors. Hence, a representation of the oxygenation/aeration conditions is needed. The power absorption, or specific input of power per unit of liquid volume, P, and the oxygen mass transfer coefficient, k<sub>L</sub> a, are often used (Wang et al., 1979). OTR, an analog of the latter, was applied to the present experimental data on oxygenation/aeration.

Correlation of the results in Figures 1, 3, and 4 provided more insight into the culture behavior. Thus, both 30 and 70 mL cultures showed a decrease in SL production (Fig. 1) and specific biomass productivity (Fig. 3) when the OTR values exceeded  $80 \text{ mM O}_2/\text{L h}^{-1}$  (Fig. 4). The slope of the decrease in SL production was steeper for the 70 mL cultures. This result can be explained from the higher values of the power absorption for the 30 mL cultures. As long as the 110 and 150 mL cultures did not exceed the  $80 \text{ mM O}_2/\text{L h}^{-1}$  limit, the pronounced decline in product formation was not observed. It is also interesting to compare the kinetics of biomass accumulation (Fig. 3) with the OTR (Fig. 4). In contrast to product formation, the maximum of biomass was not dependent on the conditions of aeration within the limits studied. The difference in the content of biomass between the cultures was negligible as the 110 and 150 mL cultures approached  $20\text{--}30 \text{ mM O}_2/\text{L h}^{-1}$  values. Only a small decrease in biomass was found at the highest aeration values studied. It is noteworthy that the distribution of the hydrophobic substrate at the air-water interface may compensate to some extent for the increase of oxygenation caused by higher agitation. This suggests that the values of OTR may be slightly less than that determined by the model sodium sulfite solution.

OTR is easily varied by both changes of agitation rate or culture volume. However, significant changes in the proportions of individual SLs were found for variations of culture volume rather than agitation rate. Thus, OTR could not be directly used to predict the composition of the SL mixture. It is possible that prolonged exposure (3–4 days) to the conditions of low oxygen supply ( $10\text{--}15 \text{ mM O}_2/\text{L h}^{-1}$ ) during the initial period of the 150 mL culture (Figs. 4 and 5) established a particular physiological state of the cells that resulted in the decrease in desaturase activity or expression of this enzyme, or the expression of a hydrogenase type enzyme occurred.

The effect of agitation and aeration on SL production was explored for SL-producing flask cultures of *C. bombicola*. A window of optimal aeration for SL production in flasks was determined. This window, using OTR measured by model sulfite solution experiments, was  $50\text{--}80 \text{ mM O}_2/\text{L h}^{-1}$ . SL formation and specific productivity were highest in this range. For OTR values above  $20\text{--}30 \text{ mM O}_2/\text{L h}^{-1}$ , biomass accumulation was maximal.

SL formation at optimum conditions ( $1\text{--}1.5 \text{ g/L h}^{-1}$ ) permitted harvesting more than  $350 \text{ g/L}$  of sophorolipids during 7 days. This was about two times higher than previously reported. These results will facilitate investigation of physiological changes in small culture volumes and assure better significance of computed fermentation kinetics in scale-up studies.

Adjusting the conditions of oxygenation at the initial periods of SL production can control the degree of SL FA unsaturation. Lower oxygenation contributed to the increase of saturated SL FAs.

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