

Solid State Biosurfactant Production in a Fixed-Bed Column Bioreactor

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Biosurfactants are surface active substances which reduce interfacial tension and are produced or excreted at the microbial cell surface. We evaluated the biosurfactant production by *Aspergillus fumigatus* and *Phialemonium* sp. in solid state processes using fixed-bed column reactors. We evaluated two media, rice husks alone (simple support) and rice husks plus defatted rice bran (complex support), both enriched with either soy oil or diesel oil. The highest water-in-oil emulsifying activity ($EA_{w/o}$) obtained was 7.36 EU g^{-1} produced by *A. fumigatus* growing on complex support enriched with soy oil and supplied with air at a rate of $60 \text{ mL g}^{-1} \text{ h}^{-1}$, while *Phialemonium* sp. had a maximum production of 6.11 EU g^{-1} using the simple support with diesel oil and an aeration rate of $120 \text{ mL g}^{-1} \text{ h}^{-1}$. The highest oil-in-water emulsifying activity ($EA_{o/w}$) was 12.21 EU g^{-1} produced by *Phialemonium* sp. on the complex support enriched with diesel oil and at an aeration rate of $60 \text{ mL g}^{-1} \text{ h}^{-1}$, while *A. fumigatus* produced a maximum $EA_{o/w}$ of 10.98 EU g^{-1} when growing on the complex support with no additional carbon source and an aeration rate of $60 \text{ mL g}^{-1} \text{ h}^{-1}$.

Key words: Biosurfactant, Emulsifying Activity, Fixed-Bed Bioreactors, Solid State Processes

Introduction

The Brazilian economy is based on agriculture and is one of the largest producers of cassava, coffee, corn, rice, soybeans and sugarcane, all of which produce large quantities of wastes with the potential to cause environmental problems.

There have been many recent publications on the application of solid state processes involving agricultural products for the production of fine chemicals and enzymes (Smits *et al.*, 1996). Solid state processes are of special economic interest to countries with large quantities of biomass and agricultural/industrial wastes which can be used as a cheap source of raw materials. The current tendency in solid state fermentation is to focus on the development of bioprocesses such as bioremediation and biodegradation for the treatment of high risk, toxic products, biological detoxification, biotransformation of agricultural products and production of secondary metabolites, especially organic acids, enzymes, biosurfactants, biodiesel and aromatic compounds (Medeiros *et al.*, 2001; Brand *et al.*, 2000).

Because of their many advantages, solid state processes are one of the most efficient ways to produce biosurfactants but rarely receive attention for commercial exploitation (Veenanadig *et al.*, 2000). More than 13 billion dollars worth of chemical and biological surfactants are produced annually in the USA and in excess of 32 billion dollars worth in the rest of the world (Cameotra and Makkar, 2004).

Many microorganisms can grow on solid substrates but only the filamentous fungi can grow significantly in the absence of free water; bacteria and yeasts require a water content of 40 to 70% (w/v) and the presence of free water (Raghavarao *et al.*, 2003). Solid state processes have conventionally been used for the growth of filamentous fungi, which grow on surfaces and penetrate into the interstitial spaces between the particles of bedded material (Pandey, 2003).

Surfactants are used industrially as adhesives, flocculants, foaming and wetting agents, de-emulsifying agents and penetrating agents. These uses are based on the foaming action of surfactants and

their ability to reduce surface tension, increase solubility, and act as detergents and wetting agents. The petroleum industry has traditionally been the largest user of surfactants because such compounds increase the solubility of petroleum components and allow their more efficient recovery; a good surfactant is able to lower the surface tension of water from 72 to 35 mN m⁻¹ and the interfacial tension between water and *n*-hexadecane from 40 to 1 mN m⁻¹ (Mulligan, 2005).

Biosurfactants have several advantages in that they can resist 10% (w/v) sodium chloride whereas only 2–3% (w/v) of salt is sufficient to inhibit conventional surfactants (Bognolo, 1999). Another factor is that while conventional surfactants are resistant to degradation biosurfactants are readily biodegradable making them suitable for use in bioremediation and waste treatment processes (Nitschke and Pastore, 2002).

In this paper we report the production of biosurfactants by filamentous fungi in fixed-bed column bioreactors under different aeration conditions, using three different carbon sources and two media.

Materials and Methods

Microorganisms

We used the filamentous fungi *Aspergillus fumigatus* and *Phialemonium* sp.; both organisms had been isolated from a hydrocarbon-contaminated site and were kindly supplied by the Food Microbiology Laboratory of the Food Engineering Faculty, State University of Campinas (FEA/UNICAMP), Campinas, São Paulo, Brazil. Both fungi were maintained at 4 °C on slopes of potato-dextrose agar (PDA) containing 1% (v/v) glycerol.

Solid state cultures

Spores were scraped from the slopes into 5 mL of 0.2% (v/v) aqueous Tween 80 and 0.5 mL of the suspension were transferred to each of two Roux flasks containing PDA, the flasks being incubated at 30 °C for 7 d to allow complete covering of the surface and sporulation of the fungi. Spores were scraped off from the PDA, suspended in 0.2% (v/v) aqueous Tween 80 and enumerated, *A. fumigatus* by using a Neubauer counting chamber and *Phialemonium* sp. by plating onto Dichloran Rose Bengal Chloramphenicol (DRBC) agar and incubating at 25 °C for 3 d until colony counts could be made. For the experimental runs appropriate

spore suspensions were made in 0.2% (v/v) aqueous Tween 80 and appropriate dilutions added to the support medium such that the final spore content was 4 × 10⁶ g⁻¹.

Solid state process

Two supports were used, a simple support consisting of degreased rice husks only and a complex support consisting of 15% (w/w) degreased rice husks plus 85% (w/w) defatted rice bran, both supports being ground and sieved to pass through a 0.42 to 0.5 mm mesh. To each support a nutrient solution was added containing (g L⁻¹) MgSO₄ · 7H₂O (0.5); NaNO₃ (3.0); KH₂PO₄ (1.0); yeast extract (1.0); peptone (0.3). For the experiments, 1% (w/v) diesel or soy oil was added to the support (final pH 4.5) which was inoculated with appropriate dilutions of spore suspension such that the final spore content in the support substrate was 4 × 10⁶ g⁻¹ and then packed into jacketed columns (50 mm diameter and 250 mm high), each column containing about 270 g for complex support and 130 g for simple support. Each support was produced with a final water content of about 50% (w/v). Control columns were prepared in the same way except that the supports were not supplemented with diesel or soy oil. During each run, water was passed through the column jackets to maintain the temperature at 30 °C and each column was connected to a rotary pump which supplied filtered and re-humidified air at, depending on the experiment, 60 or 120 mL g⁻¹ h⁻¹. Each run continued for up to 144 h (*t*₀ – *t*₁₄₄) and equal triplicate samples were taken from each column every 24 h as described (Costa *et al.*, 1998).

Analyses determination

The pH value and water content of the samples were assessed by standard methods (AOAC, 1995). Biosurfactant was extracted from the solid phase by adding 3 parts (w/v) of 90 °C water and shaking the mixture at 160 rpm, 50 °C, for 30 min in a shaker (BRAUN CERTOMAT BS-1, Melsungen, Germany) followed by vacuum filtration and extraction. The oil-in-water emulsifying activity (*EA*_{o/w}) of the crude biosurfactant was determined using 2 mL soy oil and 3.5 mL of extract which was vortexed for 1 min at 700 rpm and then allowed to stand for 1 h before reading the absorbance of the emulsion at 610 nm using a model 700 Plus spectrophotometer (Femto, São Paulo, Brazil) and cal-

culating the $EA_{o/w}$ value (Johnson *et al.*, 1992). One $EA_{o/w}$ unit was defined as the quantity of biosurfactant necessary to increase the absorbance at 610 nm by one unit above that of the control and was expressed in emulsifying units per gram ($EU\ g^{-1}$). The water-in-oil emulsifying activity ($EA_{w/o}$) was estimated after 24 h by calculating the ratio between the total height of oil and the height of emulsified oil (Broderick and Cooney, 1982) and expressed as $EU\ g^{-1}$. The following equations were used to calculate the different emulsifying activities:

$$EA_{w/o} = \frac{E * D}{[m(1 - U)]}, \quad (1)$$

$$EA_{o/w} = \frac{Abs * D}{[m(1 - U)]}, \quad (2)$$

where $EA_{w/o}$ is the water-in-oil emulsifying activity ($EU\ g^{-1}$); $EA_{o/w}$ is the oil-in-water emulsifying activity ($EU\ g^{-1}$); E is the percentage ration between height of emulsion and total height; Abs is the absorbance of the oil-in-water suspension; D is the dilution of the sample in water; m is the wet mass (g); and U is the water content of the fermented medium.

The $EA_{w/o}$ is defined as the quantity of fermented bran needed to produce an 1% hydrophobic phase emulsion stable for 24 h, and is expressed in $EU\ g^{-1}$ of fermented medium. $EA_{o/w}$ is defined as the quantity of biosurfactant required to increase the 610 nm absorbance by one unit compared to the control, and is also expressed in $EU\ g^{-1}$.

Statistical analysis

A mixed $2^3 \times 3^1$ factorial design (Bruns *et al.*, 2003) was used to test for significant differences between solid support (simple and complex), fungus (*Aspergillus fumigatus* and *Phialemonium* sp.),

aeration rates (60 and 120 $mL\ g^{-1}\ h^{-1}$) and carbon sources (soy oil, diesel oil, or neither). A significance level of 5% was used (Table I).

Results and Discussion

The moisture of the complex support remained stable at about 50% (w/w) throughout the process but the humidity of the simple support decreased at t_{96} and reached only 20% (w/w) in some cases, possibly due to the more hydrophobic nature of pure rice husks even when ground. Pokorny *et al.* (1997) have stated that humidity has very important effects on the physical properties of solid state processes, and Mahadik *et al.* (2002) have pointed out that high humidity can decrease porosity and oxygen transfer while low humidity can not only reduce the solubility of solid substrates and their degree of hydration but can also alter surface tension. In our study, the pH value of both types of support remained stable at about pH 5 throughout the process.

The effects and significance of the different variables on EA are shown in Table II, from which it can be seen that for $EA_{o/w}$ there was a significant interaction ($p < 0.05$) between type of support, showing that the complex support resulted in a $2.54\ EU\ g^{-1}$ increase in $EA_{o/w}$ as compared to the simple support. The $EA_{w/o}$ values presented significant ($p < 0.05$) differences in relation not only to the support but also to the interaction support and fungal strain, with the $EA_{w/o}$ value for the complex support being $0.8\ EU\ g^{-1}$ higher than that for the simple support. Aeration was not a significant factor ($p > 0.05$), although our highest $EA_{o/w}$ value was $12.21\ EU\ g^{-1}$ produced by *Phialemonium* sp. growing on the complex support supplemented with diesel oil and aerated at a rate of $60\ mL\ g^{-1}\ h^{-1}$. The highest $EA_{w/o}$ value was $7.36\ EU\ g^{-1}$ pro-

Table I. Factors and levels of mixed $2^3 \times 3^1$ factorial design.

Factor	Level		
	-1	0	+1
Fungus	<i>A. fumigatus</i>	–	<i>Phialemonium</i> sp.
Medium	Simple	–	Complex
Aeration	60 $mL\ g^{-1}\ h^{-1}$	–	120 $mL\ g^{-1}\ h^{-1}$
Carbon source	Soy oil	Diesel oil	Neither

Table II. Factorial analysis and significance level of emulsifying activities (EA) for oil-in-water ($EA_{o/w}$) and water-in-oil ($EA_{w/o}$) biosurfactants [in emulsifying units (EU) per gram].

Variable	$EA_{o/w}$ [$EU\ g^{-1}$]	p	$EA_{w/o}$ [$EU\ g^{-1}$]	p
Mean	8.76	0.00	5.43	0.00
Fungus	0.96	0.07	-0.28	0.20
Support	2.54	0.01	0.80	0.01
Carbon	-0.13	0.78	0.28	0.20
Aeration	1.12	0.08	-0.25	0.34

p , Significance level.

duced by *A. fumigatus* on the complex support supplemented with soy oil and aerated at $60 \text{ mL g}^{-1} \text{ h}^{-1}$.

High aeration rates result in high oxygen transfer rates which can increase biosurfactant production and thereby lower surface tension, although high rates can also reduce the humidity of the bed in packed reactors. Veenanadig *et al.* (2000) have shown that in a packed column reactor (15 cm diameter and 24.5 cm high) an aeration rate of 10 L min^{-1} was insufficient to transfer the oxygen required for the biomass to produce high levels of surfactants but when the rate was increased to 20 L min^{-1} more biosurfactant was produced as indicated by a surface tension value of 24 dynes cm^{-1} .

The complex support appeared to be more efficient for biosurfactant production, although this might have been due to the low humidity of the simple support.

The $EA_{o/w}$ values were higher than the $EA_{w/o}$ values, indicating that the biosurfactants produced by the strains tested would be more suitable for use in bioremediation or effluent treatment because oil-in-water emulsions are generally formed in these situations. The $EA_{o/w}$ and $EA_{w/o}$ profiles in function of the variables studied are shown in Fig. 1.

The highest $EA_{o/w}$ value (12.21 EU g^{-1}) was produced by *Phialemonium* sp. growing on the complex support, using diesel oil as carbon source and aerated at $60 \text{ mL g}^{-1} \text{ h}^{-1}$, indicating that this strain can use hydrocarbons as substrates. *Phialemonium* sp. growing on the complex support, using soy oil as carbon source and aerated at $120 \text{ mL g}^{-1} \text{ h}^{-1}$ produced a maximum $EA_{o/w}$ value of 11.54 EU g^{-1} , while under the same conditions *A. fumigatus* produced a maximum $EA_{o/w}$ value of 8.5 EU g^{-1} . The maximum $EA_{o/w}$ value for *Phialemonium* sp. was 12.21 EU g^{-1} while that for *A. fumigatus* was 10.98 EU g^{-1} , both using the complex support and an aeration rate of $60 \text{ mL g}^{-1} \text{ h}^{-1}$ but different carbon supplements. The $EA_{o/w}$ values produced under different conditions during the 144 h of the process are shown in Fig. 2. Mulligan (2005) has noted that the production and composition of biosurfactants depend on many factors, including the type of reactor, pH value, nutrients and temperature.

Veenanadig *et al.* (2000) produced biosurfactants in column reactors using *Bacillus subtilis* but only reached a maximum $EA_{o/w}$ value of 1.9 EU g^{-1} at t_{31} . Toren *et al.* (2001) used *Acinetobacter radioresistens* strain KA 53 to produce the biosurfactant 'Alasan' which was found to be made up of a high molecular mass complex of

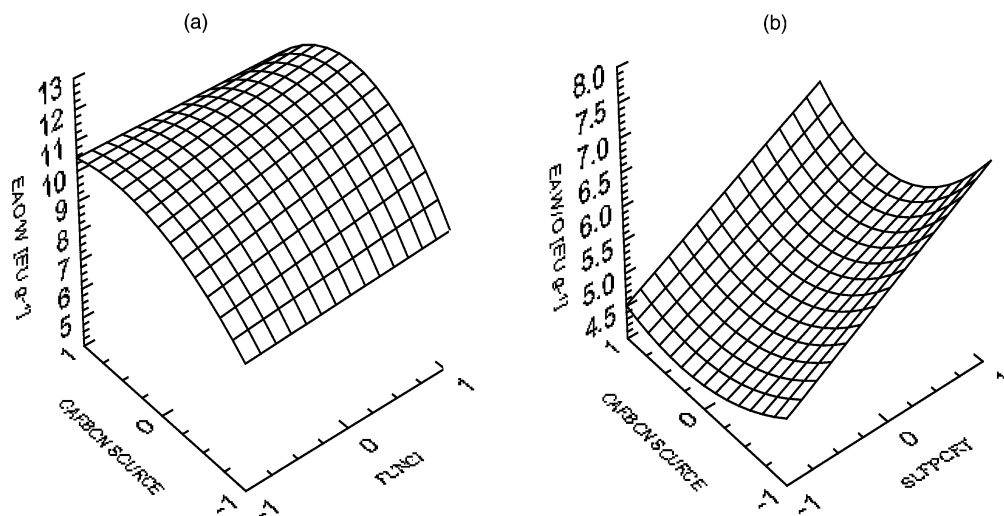


Fig. 1. Maximum production of oil-in-water and water-in-oil biosurfactants by *Aspergillus fumigatus* growing on a complex support of rice husk plus rice bran moistened with nutrient solution. (a) Oil-in-water emulsifying activity ($EA_{o/w}$) in emulsifying units per gram of substrate (EU g^{-1}) produced using an air flow rate of $60 \text{ mL g}^{-1} \text{ h}^{-1}$; (b) water-in-oil emulsifying activity ($EA_{w/o}$) in EU g^{-1} produced at an aeration rate of $60 \text{ mL g}^{-1} \text{ h}^{-1}$. (Level values of the axes are according to Table I.)

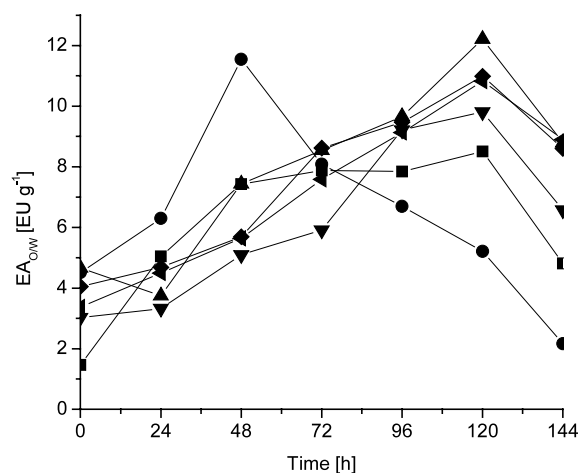


Fig. 2. Emulsifying activity oil-in-water during the solid state process in complex support in emulsifying units per gram of substrate (EU g^{-1}). (■) *Aspergillus fumigatus* plus soy oil and an aeration rate of $120 \text{ mL g}^{-1} \text{ h}^{-1}$; (●) *Phialemonium* sp. plus soy oil and an aeration rate of $120 \text{ mL g}^{-1} \text{ h}^{-1}$; (▲) *Phialemonium* sp. plus diesel oil and an aeration rate of $60 \text{ mL g}^{-1} \text{ h}^{-1}$; (▼) *A. fumigatus* plus diesel oil and an aeration rate of $120 \text{ mL g}^{-1} \text{ h}^{-1}$; (◆) *A. fumigatus* without oil and an aeration rate of $60 \text{ mL g}^{-1} \text{ h}^{-1}$; (◄) *A. fumigatus* without oil and an aeration rate of $120 \text{ mL g}^{-1} \text{ h}^{-1}$.

polysaccharides and proteins. The emulsifying power of the purified Alasan components was very low, being 0.35 ODU_{600} for the polysaccharide component and 0.39 ODU_{600} units for the protein fraction. Sarubbo *et al.* (2001) isolated an extracellular bipolymeric biosurfactant (consisting of carbohydrates, proteins and lipids) from *Candida lipolytica* strain IA 1055 growing in media containing various concentrations of glucose, the maximum emulsification activity being 1.6 ODU_{540} during the stationary phase when pH had reached a minimum.

In our study, when soy oil was added to either of the supports, biosurfactant production by both fungi increased when the aeration was increased to $120 \text{ mL g}^{-1} \text{ h}^{-1}$. These findings contrast with those of Adamczak and Bednarski (2000) who found that when the yeast *Candida antarctica* was growing in a medium containing soy oil increased aeration resulted in slower growth and reduced biosurfactant production. Our results for the support substrates without any additional hydrocarbon (*i.e.* the controls which received nutrient solution only) showed a maximum $EA_{o/w}$ value of 10.98 EU g^{-1} for *A. fumigatus* growing on the

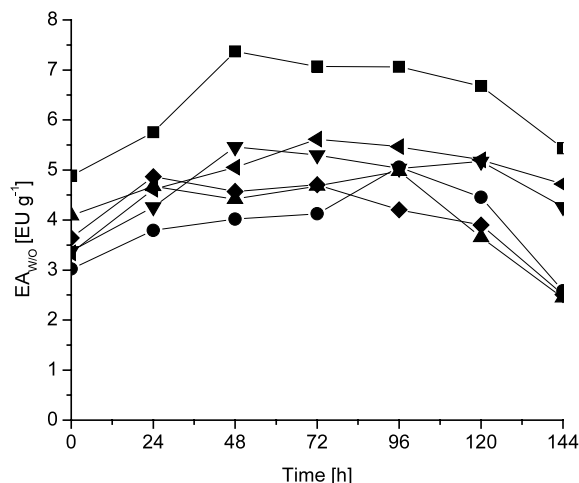


Fig. 3. Emulsifying activity water-in-oil during the solid state process in emulsifying units per gram of substrate (EU g^{-1}) produced by fungi growing on a simple support (SS) or a complex support (CS). (■) *Aspergillus fumigatus* growing on CS plus soy oil and an aeration rate of $60 \text{ mL g}^{-1} \text{ h}^{-1}$; (●) *Phialemonium* sp. growing on CS plus soy oil at an aeration rate of $120 \text{ mL g}^{-1} \text{ h}^{-1}$; (▲) *Phialemonium* sp. growing on SS plus diesel oil at an aeration rate of $60 \text{ mL g}^{-1} \text{ h}^{-1}$; (▼) *A. fumigatus* growing on CS plus diesel oil at an aeration rate of $120 \text{ mL g}^{-1} \text{ h}^{-1}$; (◆) *Phialemonium* sp. growing on SS without oil at an aeration rate of $120 \text{ mL g}^{-1} \text{ h}^{-1}$; (◄) *Phialemonium* sp. growing on CS without oil at an aeration rate of $120 \text{ mL g}^{-1} \text{ h}^{-1}$.

complex support with $60 \text{ mL g}^{-1} \text{ h}^{-1}$ aeration while *Phialemonium* sp. growing under the same conditions produced a maximum $EA_{o/w}$ value of 10.54 EU g^{-1} . A plot of the $EA_{w/o}$ values for different production conditions is shown in Fig 3, from which it can be seen that the $EA_{w/o}$ values ranged from 4.45 to 7.36 EU g^{-1} , indicating that the $EA_{w/o}$ values are more homogenous than the $EA_{o/w}$ values, which ranged from 5.87 a 12.21 EU g^{-1} .

Soy oil added to the complex support and inoculated with *A. fumigatus* aerated at $60 \text{ mL g}^{-1} \text{ h}^{-1}$ gave the highest $EA_{w/o}$ value (7.36 EU g^{-1}), *Phialemonium* sp. having a maximum $EA_{w/o}$ value of only 5.07 EU g^{-1} under the same conditions. However, for diesel oil it was the simple support inoculated with *Phialemonium* sp. and aerated at $120 \text{ mL g}^{-1} \text{ h}^{-1}$ which showed the highest $EA_{w/o}$ value (6.11 EU g^{-1}) as compared to *A. fumigatus* which, under the same conditions, produced 5.02 EU g^{-1} .

We also found that when the complex support without added oil was inoculated with *A. fumiga-*

tus and aerated at $120 \text{ mL g}^{-1} \text{ h}^{-1}$ the highest $EA_{w/o}$ value was 6.32 EU g^{-1} , while under the same conditions *Phialemonium* sp. produced a maximum $EA_{w/o}$ value of 5.61 EU g^{-1} .

The fact that *Phialemonium* sp. growing on the simple support produced 6.11 EU g^{-1} of $EA_{w/o}$ means that this system is a viable alternative for the production of biosurfactants with water-in-oil activity. The relatively inert simple substrate facili-

tated the separation of the biosurfactant, relatively inert substrates being favored for solid state cultivation because they contain little soluble material and simplify product purification (Pandey, 2003). In our study, maximum emulsifying activity for both oil-in-water and water-in-oil emulsions was determined using unpurified extract and it is reasonable to assume that the activity of the purified biosurfactant would be higher.

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