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Lipopeptide Surfactant Production by *Bacillus subtilis* Grown on Low-Cost Raw Materials

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Abstract

The production of biosurfactant by *Bacillus subtilis* ATCC 6633 was investigated using commercial sugar, sugarcane juice and cane molasses, sugarcane juice alcohol stillage, glycerol, mannitol, and soybean oil. Commercial sugar generated the minimum values of surface tension, with the best results (28.7 mN/m, (relative critical micelle concentration [CMC^{-1}] of 78.6) being achieved with 10 g of substrate/L in 48 h. At a pH between 7.0 and 8.0, a higher production of surface-active compounds and a greater emulsifier activity was also observed. Enrichment of the culture medium with trace minerals and EDTA showed maximum yields, whereas supplementation with yeast extract stimulated only cell growth. The kinetic studies revealed that biosurfactant production is a cell growth–associated process; surface tension, CMC, and emulsification index values of 29.6 dyn/cm, 82.3, and 57%, respectively, were achieved, thus indicating that it is feasible to produce biosurfactants from a renewable and low-cost carbon source.

Index Entries: Biosurfactant; *Bacillus subtilis;* lipopeptide; surfactin; raw materials; commercial sugar.

Introduction

Surfactants are amphiphilic molecules widely used for different purposes in industrial processes, with a worldwide annual demand of about $10 billion *(1,2)*. The most used surfactants are produced from petrochemical sources *(3)*; however, compounds having surface activity characteristics may be synthetized by a wide variety of microorganisms *(4,5)*. Such compounds, called biosurfactants, when compared with the syn-

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thetic ones offer important advantages such as biodegradability, low toxicity, and high specificity *(6,7)*. Furthermore, because some biosurfactants also present good thermal and chemical (pH, salinity) characteristics, they may be suited for environmental applications such as bioremediation, dispersion of oil spills, transportation of crude oil, heavy metal removal, and *in situ* microbial oil recovery *(7–14)*. In addition, biosurfactants can be used in a variety of food-processing, cosmetic, agricultural and pharmacologic applications *(15,16)*.

Among the microbial surfactant producers, *Bacillus subtilis* strains generate a lipopeptide called surfactin, one of the most effective biosurfactants known. This biomolecule is usually a cyclic compound consisting of seven amino acids bonded to a lipid moiety. Surfactin is effective in lowering the surface tension of water to <30 dyn/cm *(17)*, which is comparable with the values obtained by conventional synthetic surfactants. Additionally, surfactin preparations have other interesting characteristics, including antibiotic and antiviral properties *(18)*. In fact, surfactin is one of the few biosurfactants that has found commercial use *(19)*.

Despite the advantages of biosurfactants, they will not replace the synthetic ones unless there is a great improvement in biosurfactant production technology in order to reduce its costs. Thus, the use of renewable, readily available, and relatively inexpensive raw materials, considering that *B. subtilis* may synthesize biosurfactants from different carbon sources *(20,21)*, may account for the feasibility of this bioprocess.

Moreover, the establishment of fermentation conditions to maximize biosurfactant yield and productivity is essential for cost reduction and the large-scale production. Some previous works have evidenced some peculiarities about biosurfactant production. For instance, reports have appeared dealing with the use of locally available agricultural and food-processing residuals for promoting biosurfactant synthesis by different microorganisms *(21–24)*. Cooper et al. *(20)* have demonstrated that certain metals enhanced surfactant production by *B. subtilis*. In addition, the yield of the lipopeptide herbicolina A, produced by *Erwinia herbicola* A111 strain, was affected by the addition of amino acids or fatty acids but the structure of the molecule was not *(25)*. The aim of the present study was to evaluate the production of biosurfactant by *B. subtilis* ATCC 6633 using low-cost substrates in different culture conditions.

Materials and Methods

Microorganism

B. subtilis 6633, obtained from American Type Culture Collection (ATCC), provided by Dr. Leon Rabinovitch (Instituto Oswaldo Cruz/FIOCRUZ), was maintained on nutrient agar (Difco 0003) slants at 4ºC.

Media

Experiments on growth and biosurfactant production were performed on a mineral salt medium *(26)* containing 2.0 g/L of $NaNO_3$, 0.1 g/L of KCl,

0.5 g/L of KH_2PO_4, 1.0 g/L of K_2HPO_4, 0.01 g/L of $CaCl_2$, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, and 5 mL of a trace metals solution *(27)*.

The initial pH of the medium was adjusted to 7.0. All chemicals were of analytical grade. The carbon sources (commercial sugar–sucrose, sugarcane juice and molasses, sugarcane juice alcohol stillage, glycerol, mannitol, soybean oil) were added in order to establish an initial substrate concentration of 20 g/L. In some cases, the medium was also aseptically supplemented with 0.1% (w/v) yeast extract and 0.001% (w/v) Na EDTA previously and separately sterilized by filtration through a Millipore membrane with a pore size of 0.22 μm.

Culture Conditions

The experiments were carried out using two types of vessels. Most experiments used 500-mL Erlenmeyer flasks, each containing 100 mL of the mineral salt medium, just described. Incubation was carried out in a rotary shaker at 150 rpm and 30°C for 48 h. For kinetics experiments, a 2-L MultiGen Fermentor (New Brunswick, Scientific) containing 1 L of medium was used. Temperature was controlled at 30°C, and aeration and agitation were set at 1 vvm and 300 rpm, respectively. For quantification analysis, culture samples were first centrifuged (10,000*g* for 15 min at 4°C) to remove cells. The culture supernatants obtained were stored at 4°C until monitored for surface tension, emulsification index, (E_{24}) and biosurfactant concentration, which was determined by relative critical micelle concentration (CMC^{-1}). The data presented represent the average of at least four measurements.

Inocula were prepared by adding a loopful of cells from the stock culture to 100 mL of Difco nutrient broth and incubating at 30°C for 16 h on a rotatory shaker at 150 rpm. Inoculation volumes corresponding to about 0.04 g/L of exponential-phase cells were used.

Analytical Methods

Biomass

The cell concentration was determined by dry weight by filtering through a 0.22-μm previously weighted Millipore membrane. Filtered samples of 5 mL culture broth under vaccuum, after being washed with 3 vol of distilled water (to remove broth components), were dried in a microwave until reaching constant weight *(7)*. The dry weights of sugarcane juice, molasses, and stillage media were also determined in the same manner, and the respective values accounted for biomass obtained in those nutritional conditions.

Sugar

Substrate concentration was measured enzymatically by the glucose oxidase method (Glucose Method God-Pap-Merck), based on the oxidation of glucose to gluconic acid and hydrogen peroxide. Samples of fermented broth were first centrifuged for cell removal and then hydrolyzed, after adequate dilution, by treating with 2 N HCl (1:1) and heating at 65–70°C for 10 min *(28)*.

Surface Tension

The changes in the culture's surface tension were evaluated by the ring Du Nouy method *(29)* using a SIGMA70 system unit (KSV Instruments, Trumbull, CT) tensiometer. Measurements were performed at 25ºC. The decrease in surface tension was used as a qualitative measurement of surfactant concentration and a quantitative indicator of efficiency.

Emulsification Index

The emulsifier activity of culture broth was determined according to Cooper and Goldenberg *(30)*. Four milliliters of cell-free culture samples was added to 6 mL of kerosene, vigorously mixed with a vortex for 2 min, and left undisturbed to stand for 24 h at room temperature. The E_{24} is given as a percentage of the height of emulsified layer (cm) divided by the total height of the liquid column *(31)*.

Critical Micelle Concentration

The biosurfactant yield was evaluated by CMC^{-1}. The CMC values were determined by measuring the surface tension for varying dilutions of cell-free broth (after centrifugation). The logarithms of the dilutions were plotted as a function of surface tension. The CMC is the point of abrupt increase in surface tension *(15)*. The surfactant concentration is a function of the inverse of CMC, i.e., CMC^{-1}.

Results and Discussion

Effect of Carbon Source

Table 1 presents the average values of cell growth and reduction in surface tension produced by *B. subtilis* ATCC 6633 using different carbon sources (commercial sugar, sugarcane juice and molasses, sugarcane juice alcohol stillage, glycerol, mannitol and soybean oil). When saccharine raw materials were used (commercial sugar and sugarcane juice), cell growth was favored similarly to that observed with glucose. However, in the presence of sugarcane molasses, biomass production doubled, demonstrating that such raw materials stimulate the microorganism's activity. The lipidic raw material (soybean oil) enhanced cell growth, reaching a value 37% greater than when glucose was used. Stillage, however, was not able to support growth of the *B. subtilis* strain, probably because of the presence of inhibitors to the bacterial metabolism. On the other hand, separate addition of polyalcohols such as glycerol and mannitol, which are stillage components, has caused less harmful effects on cell growth.

All carbon sources tested have favored extracellular production of active surface agent by *B. subtilis* ATCC 6633, which was estimated by the reduction in surface tension of the fermented broth. However, except for stillage, there was no relationship between cell growth and biosurfactant production. Similar results were reported by other investigators *(21,32,33)* not only with *B. subtilis* strains, but also with other species of bacteria. The

Table 1
Effect of Different Substrates on Growth
and Biosurfactant Production by *B. subtilis* ATCC 6633 [a]

Carbon source	Final pH	Biomass (g/L) [b]	Surface tension (dyn/cm) [c]
Glucose	8.0	2.4 ± 0.2	32.1 ± 0.6 (68.3)
Commercial sugar	8.0	2.0 ± 0.1	30.6 ± .09 (68.8)
Sugarcane juice	8.0	2.5 ± 0.2	34.8 ± 0.6 (42.4)
Sugarcane molasses	7.5	4.4 ± 0.6	34.4 ± 0.8 (52.5)
Stillage	7.6	0.6 ± 0.1	35.3 ± 1.3 (45.5)
Glycerol	7.7	1.3 ± 0.0	32.0 ± .07 (49.0)
Mannitol	7.9	1.9 ± 0.1	34.1 ± 0.9 (49.9)
Soybean oil	7.3	3.3 ± 0.5	35.3 ± 0.6 (50.4)

[a] Initial conditions: 20 g/L substrate concentration, 0.03 g/L cellular concentration; batch fermentation conditions: 100 mL of medium, pH 7.0, 150 rpm, 48 h.
[b] Mean ± SD.
[c] Values given in parentheses represent surface tension in culture broth before inoculation.

surface tension values differed concerning the raw material used, with the best results obtained with commercial sugar. The other sucrose raw materials (sugarcane juice and molasses) promoted different microorganism behavior regarding biosurfactant production. The probable presence of mineral salts in those carbon sources, mainly for molasses, may have favored cell growth to the detriment of biosurfactant production. Previous studies stated that biosurfactant is better produced in scarce quantities of certain elements such as amino acids *(34,35)*.

Makkar and Cameotra *(21)* comparing biomass and biosurfactant production by thermophilic strains of *B. subtilis* MTCC 2423 grown on different carbon sources, both soluble and insoluble, evidenced great variations in final concentrations of cells and lipopeptides. The maximum biomass values were reached for sucrose and hexadecane, 2.6 and 2.4 g/L respectively, whereas the bacterial growth on glucose resulted in only 1.85 g of cells/L. Low cell growth was also observed with the same bacteria growing on dodecane, decane, kerosene and sodium citrate, with values remaining between 0.34 and 0.75 g/L. The greatest reductions in surface tension—28, 29, and 30 dyn/cm—were achieved, respectively, when glucose, sucrose, and cow meat extract were used as carbon sources. In addition, although hexane favored cell growth, there was no evidence of biosurfactant production.

Other studies evidenced that *B. subtilis* ATCC 21332 was able to reduce the surface tension of potato and potato process effluents media below 36 dyn/cm, indicating surface activity from the production of surfactin *(23,24)*.

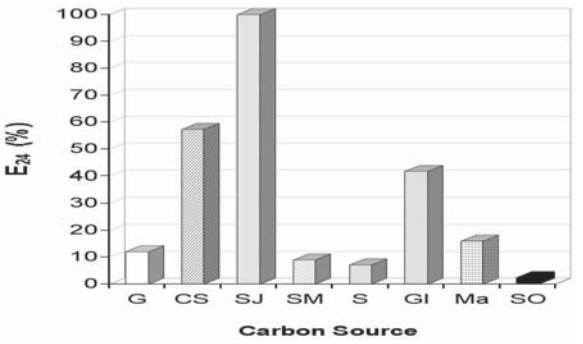

Fig. 1. E_{24} for surface-active compounds produced by *B. subtilis* ATCC 6633 in different substrates. G, glucose; CS, commercial sugar; SJ, sugarcane juice; SM, sugarcane molasses; S, sugarcane juice alcohol stillage; Gl, glycerol; Ma, mannitol; SO, soybean oil.

It was also found that potato alone could support growth, reducing the surface tension below 30 dyn/cm *(23)*.

As shown in Table 1 an increase in pH values was also observed. Fox and Bala *(23)* also noticed an increase in pH of different potato nonbuffered media from 6.2 to 8.5. Carvalho et al. *(36)* reported a similar behavior for *Planococcus citreus* growing on toluene, xylene, and olive oil.

A practical measurement of a surface-active compound utility is its ability to turn immiscible liquids into stable emulsions. Figure 1, shows that all samples were able to form stable emulsions with kerosene for 24 h. However, samples originated from glycerol, commercial sugar, and sugarcane juice were the most successful regarding emulsifying activity. Bacterial growth in mineral medium supplemented with commercial sugar yielded a biosurfactant with an E_{24} of 57.4%. This is a promising result compared with the values reported from biosurfactants produced by different microbial species. It should be emphasized that the best E_{24} was obtained with sugarcane juice as the raw material.

Effect of Initial pH

Certain microorganism have the ability to grow and produce biosurfactants in a wide pH range, rendering possible the microbial production of surfactant for *in situ* applications, such as bioremediation and enhanced oil recovery. According to Claus and Berkeley *(37)*, the active growth of *B. subtilis* was detected mainly in a pH range of 5.5–8.5, although this bacteria is able to grow at pH values lower or higher than these.

Figure 2 presents surface tension and E_{24} values obtained for fermented broth at different initial pH values in CS media. For all the pH values tested, a decrease in surface tension of the medium was noted before inoculation (62.5 dyn/cm) by the microorganism activity. These results indicate that the tested bacteria have the ability to produce biosurfactant at pH values ranging from 5.0 to 8.0 The minimum surface tension values of 30.1 and 31.5 dyn/cm were obtained when the medium's initial pH was 7.0 and 8.0, respectively.

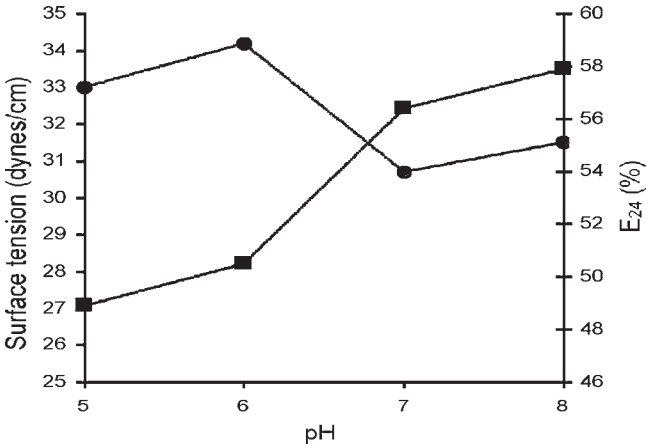

Fig. 2. Surface tension (●) and E_{24} (■) of *B. subtilis* ATCC 6633 fermented broth at various initial pH values, (20 g/L of commercial sugar, 0.03 g/L of initial cell concentration, 30ºC, 200 rpm, 48 h).

Regarding the emulsification index, it was observed the same behavior that is, all the fermented broth investigated, at all initial pH values tested, could emulsify kerosene forming stable emulsions. It has been also observed a relation between emulsification activity and initial pH, the best results been achieved for E_{24} at pH 7 (56,4%) and 8 (57,9%).

On the other hand, cell growth was not influence much by the initial pH value. For an initial pH of 5.0, the cell concentration attained was 3.6 g/L, and for the other initial pH values tested, cell growth remained about 2.5 g/L (data not shown).

Effect of Addition of Yeast Extract, Microsalts, and EDTA

The culture medium employed for biosurfactant production by *B. subtilis* generally presents similar chemical composition, being normally supplemented with microsalts varying, however, relative to the presence or absence of yeast extract and/or EDTA *(20,30,38)*. The addition of yeast extract is used in order to stimulate cell growth because it contains complex B vitamins, which are necessary to form enzymes and coenzymes, and also amino acids and other cell growth–stimulating compounds *(39,40)*. Furthermore, the addition of EDTA also may contribute to biosurfactant production *(41)*. EDTA acts at the cell wall, altering the cell wall's permeability or promoting biosurfactant removal when EDTA is attached to the wall, and/or can act as a metal-complexing agent to favor the metal's assimilation by the microorganism.

The addition of trace minerals (medium 2 and 3) considerably stimulated cell growth and had a stronger effect when yeast extract (medium 1) was added simultaneously, resulting in an increase of biomass in 40%, compared with medium 2 (Table 2). However, EDTA supplementation (medium 4) had practically no affect on cell growth, chiefly when it was

Table 2
Effect of Addition of Yeast Extract, Microsalts, and EDTA on Growth and Biosurfactant Production by *B. subtilis* ATCC 6633 [a]

Medium	Addition [b]			Final pH	Biomass (g/L) [c]	Surface tension (dyn/cm)	E_{24} (%)
	Yeast extract	Microsalts	EDTA				
1	Yes	Yes	Yes	8.4	3.5 ± 0.2	32.8 ± 0.7	49.4
2	No	Yes	Yes	8.2	2.3 ± 0.1	27.1 ± 0.8	51.2
3	No	Yes	No	8.2	2.6 ± 0.1	28.9 ± 0.8	51.8
4	No	No	Yes	8.2	1.4 ± 0.1	29.0 ± 0.9	39.8
5	No	No	No	8.3	0.9 ± 0.1	28.7 ± 1.0	47.8

[a] Initial conditions: (20 g/L substrate concentration, 0.05 g/L cellular concentration, 30°C, pH 7.0, 150 rpm, 48 h. Medium control: surface tension = 65.2 dyn/cm; E_{24} = 0.

[b] Yeast extract (1g/L); trace minerals (5 mL/L) (0.116 g/L of $FeSO.7H_2O$, 0.232 g/L of H_3BO_3, 0.41 g/L of $CoCl_26H_2O$, 0.008 g/L of $CuSO_45H_2O$, 0.008 g/L of $MnSO_4H_2O$, 0.022 g/L of $([NH_4]_6Mo_7O_{24}$, 0.174 g/L of $ZnSO_4$), EDTA (0.01 g/L).

[c] Mean ± SD.

done simultaneously with yeast extract and/or microsalts. In these experiments, the alkalinization of the final fermented broth was detected again.

In Table 2, the surface tension data of *B. subtilis* ATCC 6633 fermented broth according to different nutritional conditions are also given. There was expressive reduction in surface tension values, evidencing biosurfactant synthesis by this bacterial strain. Medium 1 (with the addition of yeast extract, trace minerals, and EDTA) showed the highest surface tension, indicating that nutritional supplementation does not favor biosurfactant production although it has stimulated cell growth. In the presence of microsalts (medium 3) or EDTA (medium 4), or even in the absence of those compounds (medium 5), surface tension values were similar. However, these results cannot be considered as an evaluation of the quantity of biosurfactant produced, because larger concentrations of that biosurfactant do not alter surface tension values, after having reached the minimum value, which is related to a maximum surface activity.

Furthermore, Fox and Bala *(23)* verified that although the addition of yeast extract could stimulate growth of a *B. subtilis* strain in an established potato medium, it did not decrease surface tensions. In fact, it has previously been related that the higher growth did not translate into lower surface tensions *(24)*. In reality, the reduction in surface tension is inversely related to cell growth.

All tested media produced a biosurfactant capable of emulsifying kerosene. However, except when EDTA was added alone (medium 4), the E_{24} values were satisfactory in comparison with those related to other emulsifiers produced by different microorganisms *(30)*.

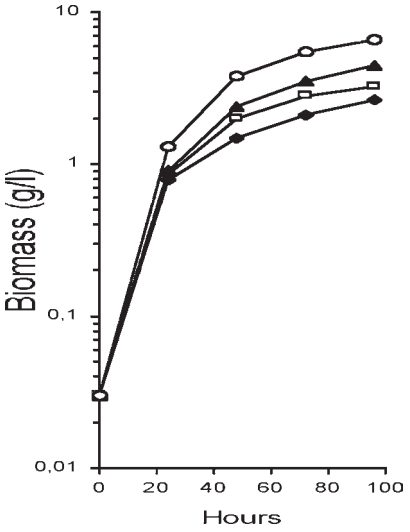

Fig. 3. Effect of initial substrate concentration on cellular growth of strain *B. subtilis* ATCC 6633: (▲) 5 g/L; (□) 10 g/L; (▲) 20 g/L; (○) 40 g/L.

Effect of Substrate Concentration

Normally, biosurfactant accumulation occurs when the microorganism is grown on a medium containing carbon source in excess, especially in relation to a limiting nutrient *(23,41)*. In most cases, the limiting nutrient is the nitrogen source, although limiting magnesium, iron, and phosphates can induce similar responses.

Cell growth of *B. subtilis* ATCC 6633 was proportional to the initial concentration of commercial sugar (Fig. 3). Regarding the kinetic profiles obtained, it can be concluded that cell growth rates were similar to those attained when 5 and 10 g/L of substrate were used, although the metabolic activity reached its peak at an initial concentration of 40 g/L. However, the values referring to specific cell growth velocities showed little variation, from 0.13 to 0.16 h^{-1}. Different media having potato process effluents as carbon source presented specific growth rates varying from 0.1 to 0.529 h^{-1} *(24)*. Substrate concentration had little effect owing to incomplete conversion (data not shown).

Biosurfactant production, expressed in terms of CMC^{-1}, reached maximum values within 48 h, practically the same for all substrate initial concentrations tested (Fig. 4); the corresponding values of surface tension were 28.7 and 29.3 dyn/cm (data not shown). These results are quite satisfactory when compared to values reported for more active biosurfactants, indicating a good performance of the product formed.

By contrast, Fox and Bala *(23)* observed that the degree of surface tension reduction was dependent on the amount of carbon source (potato) initially present in the medium.

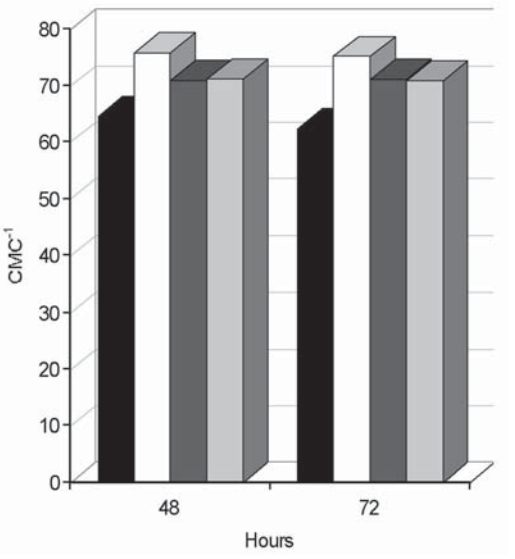

Fig. 4. Effect of initial substrate concentration on biosurfactant produced by *B. subtilis* ATCC 6633: (■) 5 g/L; (□) 10 g/L; (■) 20 g/L; (■) 40 g/L).

Analysis of the results obtained so far shows that substrate concentrations of 10, 20, and 40 g/L were quite adequate to produce biosurfactant by *B. subtilis* ATCC 6633, although the maximum concentration of the product was observed for 10 g/L of commercial sugar. Therefore, it should be concluded that the carbon/nitrogen relation in the range of 6.4–25.6 g/g had no affect on the biosurfactant synthesis.

Figure 5 shows some data referring to the ability of biosurfactant to emulsify kerosene produced by *B. subtilis* ATCC 6633 at the different substrate concentrations tested (5, 10, 20, and 40 g/L). Besides a decrease in surface tension, stabilization of hydrocarbon/water is frequently used as an indicator of surface activity. Note, however, that the quantity of biosurfactant produced should not be related to the E_{24} because that is an intrinsic property of the molecule. A similar behavior of the emulsifying activity in relation to the carbon source concentration and to the incubation period has been observed. The diverse initial concentrations of commercial sugar studied favor the formation of a surface-active compound, with an emulsifying activity >50% in a 48-h process. The maximum values for emulsion activity of 57.9 and 56.9% were determined for 10 and 20 g/L of substrate, respectively. It should be emphasized that there was a reduction in the E_{24} after a 96-h period of incubation. Carvalho et al. *(36)* reported similar results for cell-free fermented broth by *Bacillus sp*. emulsified in kerosene.

Kinetic Studies

The results of kinetic studies related to biosurfactant synthesis by *B. subtilis* ATCC 6633 cultivation in a fermentor containing a medium

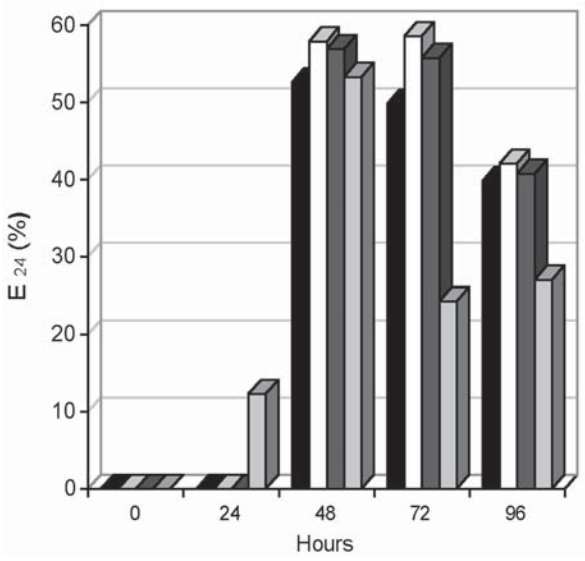

Fig. 5. Emulsification index (E_{24}) for biosurfactant produced by *B. subtilis* ATCC 6633 cultured in different initial substrate concentrations: (■) 5 g/L; (□) 10 g/L; (■) 20 g/L; (■) 40 g/L).

supplemented with microsalts and EDTA are shown in Fig. 6A. In these conditions, the growth rate and biosurfactant production profiles were similar to the ones obtained when the medium was not supplemented (Fig. 6B). However, the supplementation resulted in greater biomass production and product (CMC^{-1}), 1.5 g/L and 82.3, respectively.

By contrast, growth studies of *B. subtilis* ATCC 21332 using established potato media evidenced cells reaching early stationary phase whithin 12 h *(23)*. The addition of mineral salts to potato media resulted in a prolongation of the lag phase, the stationary phase being reached only 48 h after inoculation.

In the present work, the addition of microsalts and EDTA in the medium retarded biosurfactant synthesis, which was initiated only at the exponential phase of growth. The critical micelle concentration (CMC^{-1}) was reached at the onset of the stationary phase (after 50 h), although it is unknown whether surfactant production continued stationary phase.

The accumulation of the surface-active compound in the fermentation broth during the process was a function of the relative CMC^{-1} (Fig. 6A,B). Biosurfactant synthesis only started after 24 h, reaching its maximum after 50 h, thus confirming the kinetic profile of surface tension.

As shown in Fig. 6 (A,B), the synthesis of the surface-active agent took place in the late-exponential phase, achieving its maximum value at the beginning of the stationary growth phase. Therefore, it can be concluded that the biosurfactant produced by *B. subtilis* ATCC 6633 is a primary

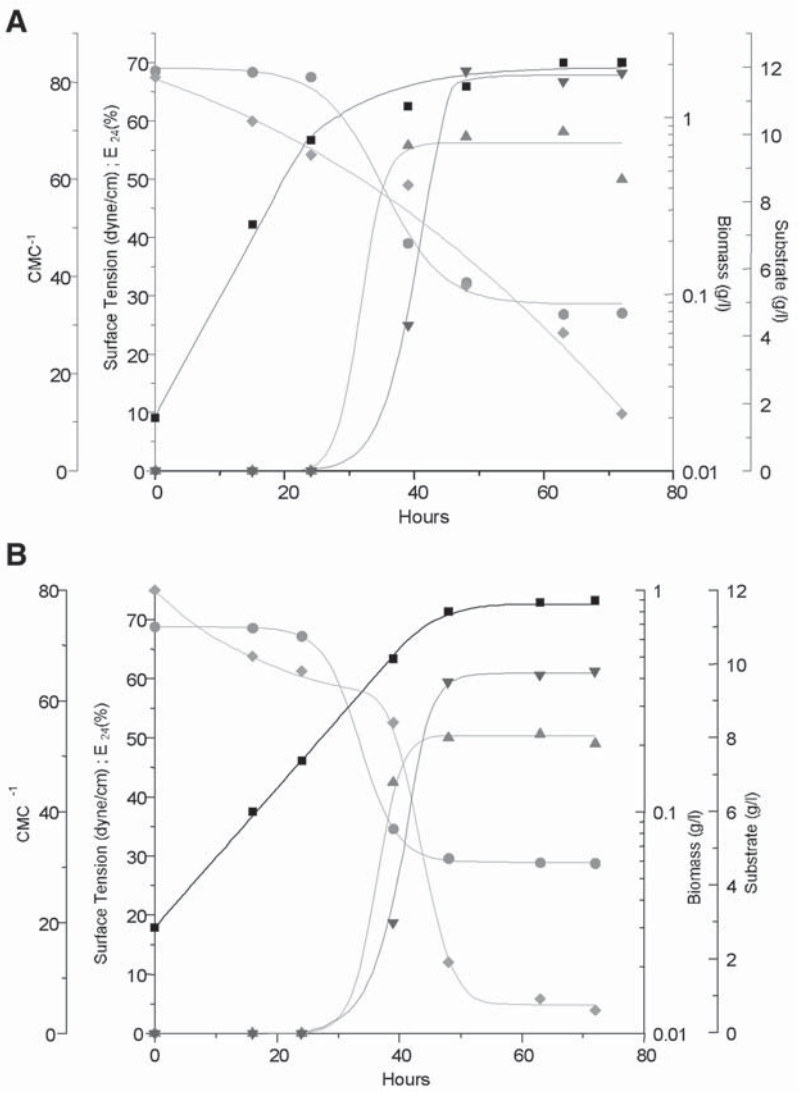

Fig. 6. Kinetics of growth and biosurfactant accumulation in batch culture of *B. subtilis* ATCC 6633 **(A)** with and **(B)** without supplementation of microsalts and EDTA: (●) surface tension; (▲) E24; (■) biomass; (◆) substrate; (▼) CMC^{-1}).

metabolite, which is in agreement with previous works *(20,35)*. This is also in agreement with the findings of previous reports that the onset of surfactin production occurred in the mid- to late-exponential phase *(20,24)*.

Thompson et al.'s *(24)* description of the kinetics of *B. subtilis* ATCC 21332 cultured in potato process waste streams medium presents the same correlation between biomass and surface tension. The minimum surface tension occurred near the onset of stationary phase. This behavior, as stated by these investigators, indicates a production of growth-associated surfactin.

As shown in Fig. 6B, a two-phase pattern occurred for the substrate uptake. It can be observed that during the exponential growth phase, sucrose assimilation by the bacteria was small, corresponding to about 20% of the initial amount introduced into the medium. However, after a 40-h process corresponding to the end of the growth phase, there was a rise in the substrate uptake, suggesting that the carbon source was directed to biosurfactant production, for the conditions tested. It should be emphasized that the fermentative process, when the medium was supplemented with microsalts and EDTA (Fig. 6A), generated a different substrate kinetics in comparison with that obtained for the nonsupplemented medium (Fig. 6B).

The addition of microsalts and EDTA resulted in a biomass yield on sucrose ($Y_{x/s}$) of 0.24 g/g and CMC^{-1}/dry cell weight of 54.5, quite different from that determined for the nonsupplemented medium, 0.08 g/g and 79.2, respectively. In a way, these results suggest that the presence of oligoelements stimulates cell growth rather than bioproduct synthesis.

Thompson et al. *(24)* also verified that the addition of trace minerals to potato process effluents has little effect on surfactin production by *B. subtilis* ATCC 21332. In fact, the addition of corn steep liquor had a detrimental effect on biosurfactant production, whereas the addition of trace minerals had no effect on surface tensions. On the other hand, growth rates were marginally higher with added nutrients.

Conclusion

Among the carbon sources tested, commercial sugar showed the best result for biosurfactant synthesis by *B. subtilis* ATCC 6633, reducing surface tension to 29.6 dyn/cm.

The maximum biosurfactant production was verified at pH 7.0 and 8.0. The addition of EDTA and microsalts favored microbial synthesis of surface-active compounds. On the other hand, the addition of yeast extract stimulated cell growth to the detriment of biosurfactant production. The most suitable concentration of commercial sucrose for biosurfactant synthesis was 10 g/L. Biosurfactant production occurred in the late-exponential phase, achieving its maximum value at the early stationary phase of growth. The values of surface tension that we obtained compare favorably with those obtained with commercial synthetic surfactants.

Acknowledgments

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