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Optimization of Surfactin Production by *Bacillus subtilis* ATCC 6633 in a Miniaturized Bioreactor

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ABSTRACT: In recent years, biosurfactants due to wide applications in chemical, petroleum, food and pharmaceutical industries, have been widely considered by researchers. Biosurfactants are produced by a series of microorganisms, so it is important to screen culture medium and operating conditions in miniaturized bioreactors prior to scaling up to large bioreactors.In this study, using a kind of miniaturized bioreactor called ventilation flask, optimal production conditions, including filling volume and shaking frequency to produce a surfactin-type biosurfactant by Bacillus subtilis ATCC 6633, were examined. Moreover, the effect of oxygen transfer rate (OTR) on the surfactin production was investigated according to Amoabediny and Büchs model. The results indicated that the maximum biomass and biosurfactant yield which obtained under optimal conditions (filling volume of 15 mL and shaking frequency of 300 rpm) were evaluated 0.3 g/L/h and 0.0485 g/L/h, respectively. Also, at the same conditions, the amount of surface tension decreased from 60.5 mN/m to 31.7 mN/m and the maximum oxygen transfer rate (OTR_{max}) obtained as 0.01 mol/L/h.

Key words: Biosurfactant, Surfactin,Optimization,Filling volume,Ventilation Flask

INTRODUCTION

Surface-active molecules or biosurfactants due to their unique properties such as low toxicity, high biodegradability, and high surface activity have been widely utilized in different industries like cosmetics, chemicals, food processing, pharmaceutical, agriculture, enhanced oil recovery and environmental bioremediation, during the past decade (Desai and Banat, 1997; Banat *et al.*, 2000; Moliterni *et al.*, 2012; Jokari *et al.*, 2012; Soltani *et al.*, 2012; Chen *et al.*, 2011; Mazaheri Assadi and Tabatabaee, 2010; Cherian and Jayachandran, 2009). These suitable features led to increase in demand for these bioproducts, and consequently the recognition of biosurfactants as alternatives to chemically synthesized surfactants in variety of fields. However, production of these molecules in large-scales has not been considered extensively because of their low production yields and high recovery and purification costs. To overcome these obstacles and to have cost-effective biosurfactants, it is proposed to use recombinant strain improvement, enhance the production yield, optimize the medium and bioreactor operation, and use inexpensive and renewable substrates such as sugars, oils, and wastes (Davis *et al.*, 2001; Wei *et al.*, 2004). Oxygen is the major substrate in aerobic biosurfactant production which affects the growth of the microorganisms, maintenance, productivity and bioreactor operation (Henzler and Schedel, 1991; Hilton, 1999). In Amoabediny and Büchs model, cotton in the sterile closure of ventilation flasks plays an important role in prevention of oxygen limitation or carbon dioxide inhibition and providing more aeration. Factors such as physical properties of gas and liquid,

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operational conditions, geometrical parameters of the bioreactor and also the presence of biomass, all are affecting parameters in oxygen consumption (Amoabediny and Büchs, 2007; Amoabediny *et al.*, 2009). The gas exchange capacity of shaking flasks is strongly dependent on two factors, the oxygen transfer through the gas-liquid interface and also the oxygen transfer through the sterile closure (Mrotzek *et al.*, 2001). Several researches have been carried out to optimize some of the variables such as filling volume, agitation and aeration rates and other operational conditions to enhance the production yield of biosurfactants (Mukherjee and Das, 2005; Chen *et al.*, 2006; Yeh *et al.*, 2006; Guez *et al.*, 2008). Current research using Amoabediny and Büchs model, considers the influence of filling volume (V_L) and shaking frequency (n) on surfactin production along with studying the cell growth in ventilation flasks.

MATERIALS & METHODS

Oxygen is an important substrate in bioproducts production (Henzler and Schedel, 1991) and shortage of oxygen highly affects the performance of the process (Calik *et al.*, 2004). Hence, for the accurate prediction and estimation of productivity and growth of microorganisms in biosurfactant production, oxygen transfer rate (OTR) under different operational conditions has to be considered carefully (Liu *et al.*, 2006). The bioprocesses are usually carried out under previously optimized conditions such as temperature, pH, pressure, mixing, and concentrations of nutrients with a previously chosen operating mode, like batch, fed-batch, continuous or resting cell (Garcia-Ochoa and Gomez, 2005). In these processes, when maximum oxygen transfer rate (OTR_{max}) (maximum amount of oxygen delivered by the gas-liquid mass transfer) is less than maximum oxygen uptake rate (OUR_{max}) (maximum oxygen consumption by microorganisms); oxygen limiting conditions will happen. The maximum oxygen uptake rate of an aerobic culture depends on the maximum growth rate (μ_{max}), the biomass oxygen yield ($Y_{X/O2}$) and the maximum biomass concentration (X_{max}) which can be calculated according to the following equation (Seletzky *et al.*, 2007):

$$OUR_{max} = \frac{1}{Y_{X/O_2}} . \mu_{max} . X_{max} \quad (1)$$

Where μ_{max} and X_{max} can be obtained experimentally and $Y_{X/O2}$ is found using Amoabediny and Büchs model (Amoabediny and Büchs, 2007).

The growth of *Bacillus subtilis* ATCC 6633 is explained by the following equation where K_S, S, $O_{2,L}$, and ko_2 are respectively the Monod constant (0.0045 g/L), the substrate concentration, the amount of dissolved oxygen, and the half saturation constant of oxygen (10^{-6} mol/L), reported by Amoabediny et al. (2009):

$$\mu = \mu_{max} . \frac{S}{S + k_S} . \frac{O_{2,L}}{O_{2,L} + ko_2} \quad (2)$$

Substrate consumption can be accordingly obtained by the following equation which describes biomass yield according to laboratory results (Amoabediny *et al.*, 2009):

$$\frac{\partial S}{\partial t} = - \frac{1}{Y_{X/S}} . \mu . X \quad (3)$$

In shake flasks, maximum oxygen transfer rate (OTR_{max}) strongly depends on to the surface area of gas-liquid interface, and the velocity of rotating liquid. The operational conditions, such as shaking rate, shaking diameter, flask size, flask shape, and liquid culture volume, all affects the maximum oxygen transfer rate (OTR_{max}). Amount of maximum oxygen transfer rate according to volumetric mass transfer coefficient ($k_L a$) and the partial pressure of oxygen in the gas phase is calculated (when $pO_{2,L}$ is zero) by the following equation (Amoabediny *et al.*, 2009):

$$OTR_{max} = k_L a . Lo_2 . po_2 \quad (4)$$

Where Lo_2 is the oxygen solubility in the solution (0.00019 mol/L/bar) which is obtained by the method presented by Schumpe (Schumpe *et al.*, 1982).

In the present study, the volumetric mass transfer coefficient ($k_L a$), oxygen consumption and the oxygen partial pressure in headspace of the shake flasks are calculated using Model Maker software developed by Amoabediny and Büchs (Amoabediny and Büchs, 2007; Amoabediny *et al.*, 2009). Assuming the unsteady-state gas transfer conditions in shake flasks, this model can be employed to predict suitable operational conditions and can prevent oxygen limitation and aeration problems.

In order to validate the unsteady state gas transfer model in a biological system, the fermentation of a strain of *Bacillus subtilis* ATCC 6633, as the model organism, was carried out in ventilation flasks (type f_1) equipped with sterile closure with a certain diameter and height (Amoabediny and Büchs, 2007) (Fig. 1).

The strain was grown on medium E consisting of KH_2PO_4 (2.7 g/L), K_2HPO_4 (13.9 g/L), sucrose (10 g/L), NaCl (50 g/L), yeast extract (0.5 g/L) and $NaNO_3$ (1 g/L) in pH=6.95. The medium was autoclaved at 121°C for 20 minutes. Then 10 mL of the following solution containing: $MgSO_4$ (25 g/L), $(NH_4)_2SO_4$ (100 g/L), was

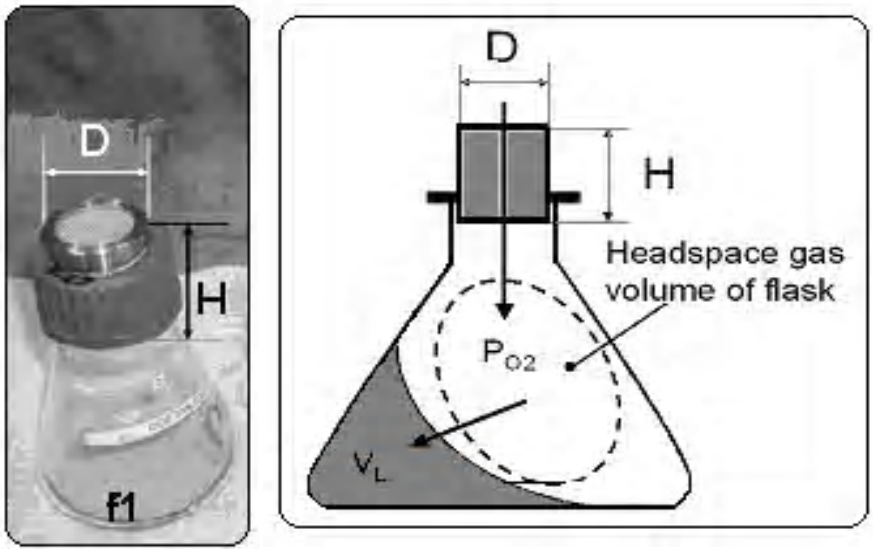

Fig. 1. The schematic of a miniaturized bioreactor (type f1) called ventilation flask (left) and the schematic representation of gas transfer inside the flask (right)

added to the medium after cooling and sterilizing. Also Wolin's trace metals solution containing the following compounds, was added after filter sterilized through a 0.22-µm membrane filter (Millipore, type GS): EDTA (0.5 g/L), $MnSO_4.H_2O$ (3 g/L), NaCl (1 g/L), $CaCl_2.2H_2O$ (0.1 g/L), $ZnSO_4.7H_2O$ (0.1 g/L), $FeSO_4.7H_2O$ (0.1 g/L), $CuSO_4.5H_2O$ (0.01 g/L), $AlK(SO_4)_2$ (0.01 g/L), $Na_2MoO_4.2H_2O$ (0.01 g/L), Boric acid (0.01 g/L), Na_2SeO_4 (0.005 g/L) and $Nicl_2.6H_2O$ (0.003 g/L) (Youssef *et al.,* 2004). One loop of *Bacillus subtilis*, was grown on a nutrient agar plate and then was added to nutrient broth medium (sterilized at 121°C for 20 minutes) as a pre-culture to make 100 mL of solution in 500 mL flask. This was incubated for 24 hours on an orbital shaker (X, climo-shaker ISF1-X Kuhner) at 150 rpm and 37°C in order to obtain the desired growth (OD_{600nm}=1). Then, 5% v/v of pre-culture was added to 5, 15 and 25 mL of E medium which has been poured in 250 mL ventilation flasks (type f_1). Then, ventilation flasks were placed in shaker incubator at 37°C for 30 hours at 150 and 300 rpm. All experiments were carried out in triplicates. After incubation, the culture was centrifuged at 6000 rpm for 20 minutes (Hettich Centrifuge, Universal 320, Germany) and the supernatant was separated from the biomass. The Biomass obtained after filtration on a 0.22 µm membrane (Whatman), washing with distillated water and then drying in oven at 100°C for 10 minutes. Then the filter was weighed to determine biomass weight by digital scale (Foss Tecator, Höganäs, Sweden) (Carrillo *et al.,* 1996). The pH of the supernatant was also measured by a digital pH meter (METTLER TOLEDO, Seven Easy, Germany). For determination of sucrose consumption, the modified phenol-sulfuric acid method was used. A volume of 2 mL of cell supernatant was mixed with 1mL of 5% phenol solution and 5mL of sulfuric acid, until vapor was formed. Then tubes were placed for 10 minutes in immovable state and after stirring them, the tubes were cooled in water at temperature between 20-30°C. Thereafter, the absorbance amount of the samples containing supernatant was read by spectrophotometer apparatus (Sigma Polemic) in wavelength of 480 nm (Dubois *et al.,* 1956). After removing the biomass by centrifugation, the pH of the filtrate was adjusted at 2, accomplished by addition of 3M HCI and allowing the biosurfactant to precipitate. The precipitate was collected and dried by filtration. Further purification was carried out by dissolving the precipitate in deionized water. Then this crude surfactin was extracted three times with dichloromethane at the same volumes to obtain brown solid. It was dissolved in deionized water and then centrifuged. At the last step, biosurfactant was filtered through Whatman filter paper and then was weighed (Kameda *et al.,* 1974). Moreover, the surface tension (ST) of the biosurfactant was determined by a KRUSS Tensiometer (KT100, KRUSS, Japan), using Du Nouy ring method (Cooper and Goldenberg, 1987). And finally to measure emulsification activity, 0.5 mL of crude oil was added to 2 mL of supernatant in test tube and then it was mixed by the vortex apparatus at high speed for 1 minute followed by maintaining for 24 hours. Then the emulsification index was calculated by dividing the measured height of the emulsion layer by the total height of mixture and then multiplying by 100 (Francy *et al.,* 1991).

RESULTS & DISCUSSION

Bacillus subtilis ATCC 6633 was cultivated in different filling volumes (V_L) of medium to determine the effect of filling volume on cell growth and biosurfactant production. Filling volumes of 5, 15 and 25 mL were utilized at shaking frequency of 300 rpm to cultivate the organism. The duration of each experiment was determined 30 hours. Effect of different filling volumes on pH and biomass concentration can be observed in Fig. 2 which shows that after 14 hours, the pH of the culture medium decreases from 6.95 to 6.18, 6.11, and 6.29 in filling volumes of 5, 15, and 25 mL, respectively. While at the same time, biomass concentration increases during the exponential growth phase to 3.9, 4.21 and 3.33 g/L in filling volumes of 5, 15 and 25mL, respectively. Also, Fig. 1 depicts that the biomass concentration and pH in all the cultures gradually start to decrease after 14 hours as the microorganisms' growth rate diminishes.

Effect of different filling volumes on carbon source consumption and also biosurfactant concentration can be observed in Fig. 3 which indicates that residual sucrose concentration in the culture in filling volume of 25 mL approximately equals 1.17 g/L after 14 hours and it can be concluded that the culture does not consume the entire carbon source under these conditions. Whereas, this value in filling volumes of 5 and 15 mL reaches approximately zero after 14 hours. On the other hand, the maximum biosurfactant concentration in filling volume of 15 mL is obtained 0.68 g/L, while at the same conditions, the maximum biosurfactant concentration infilling volumes of 5 and 25 mL, is 0.623 and 0.527 g/L, respectively and it shows that after elapsed time of 14 hours, as indicated in the curve of biomass concentration, the biosurfactant concentration gradually decreases.

Effect of different filling volumes on surface tension and emulsification activity can be observed in Fig. 4 which clearly indicates that the surface tension decreases from 60.5 mN/m to 32.98 mN/m, 31.7 mN/m and 34.85 mN/m in filling volumes of 5, 15 and 25 mL after 14 hours, respectively. Also, emulsification activity gradually increases to reach its maximum value, equals with 90.64%, 95.25%, and 78.76%, respectively in filling volumes of 5, 15 and 25 mL. Consequently, in filling volume of 15 mL, the decrease in surface tension value is approximately 10 % more than reduction in filling volume of 25 mL. By Considering the enhancement of surface tension in filling volume of 25 mL, it can be concluded that, in this volume oxygen limitation occurs significantly, which can be predicted by Amoabediny and Büchs's model, too.

In the next step, *Bacillus subtilis* ATCC 6633 was cultivated in different shaking frequencies (n) to determine the effect of agitation rate on cell growth and biosurfactant production. Shaking frequencies of 150 and 300 rpm were employed in filling volume of 15 mL to cultivate the organism. Effect of agitation rate on pH and biomass concentration is depicted in Fig.5. As can be seen in the figure, pH of the bacterial culture medium decreases from 6.95 to 6.11 at 300 rpm and to 6.24 at 150 rpm during the exponential growth phase after, while biomass concentration increases during this phase after the same time passes. So that maximum biomass concentration is measured 4.21g/L and 3.22 g/L at 300 and 150 rpm, respectively. Also, Figure 4 depicts that increase in pH and decrease in biomass concentration, begins after 14 hours because of diminishing of the microorganisms' growth rate.

The results of investigation through the effect of agitation rate on carbon source consumption and also biosurfactant concentration are indicated in Fig. 6. It can be observed that at shaking rate of 300 rpm the residual sucrose is used up completely, while its concentration at shaking frequency of 150 rpm approximately reaches to 0.9 g/L at the end of the experiment, and it reveals that the culture does not consume all the sucrose. On the other hand, maximum biosurfactant concentration at shaking frequencies of 300 and 150 rpm, after 14 hours, equals 0.68 g/L and 0.52 g/L, respectively, and after elapsed time of 14 hours, as indicated in the curve of biomass concentration, residual sucrose concentration gradually decreases.

Effect of agitation rate on the surface tension is illustrated in fig. 7 which clearly indicates that surface tension decreases from 60.5 mN/m to 31.7 mN/m, at 300 rpm, and decrease to 34.88 mN/m, at 150 rpm, after 14 hours. Consequently, at shaking frequency of 300 rpm, the reduction in surface tension is approximately 10% more than decrease in surface tension at agitation rate of 150 rpm. Also, emulsification activity increases and researches to 95.25% and 83.15% at 300 and 150 rpm, respectively. Furthermore, as expected, surface tension gradually increases and emulsification activity decreases after 14 hours.

The observed differences in maximum biomass amount and biosurfactant productivity between different filling volumes are due to oxygen limitation which takes place in filling volume of 25 mL. The maximum biomass yield is 0.278, 0.3 and 0.237 g/L/h and the biosurfactant productivity is 0.045, 0.048 and 0.037 g/L/h in filling volumes of 5, 15 and 25 mL, respectively. Thus, according to these results together with using Amoabediny and Büchs's model, it can be concluded that the best filling volume in which oxygen limitation does not occur is 15 mL. Table 1 indicates the values of different parameters which are calculated for various filling volumes at shaking frequency of 300 rpm and inoculation percentage of 5% v/v.

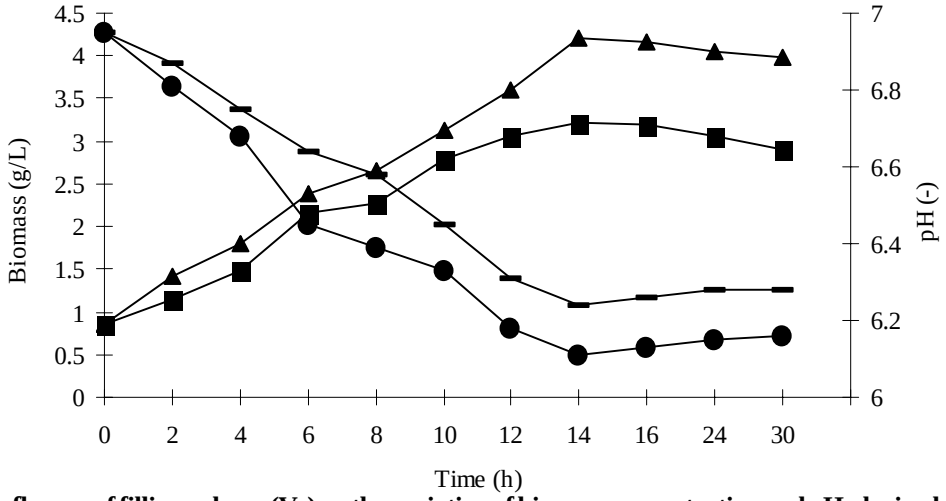

Fig. 2. Influence of filling volume (V_L) on the variation of biomass concentration and pH, during biosurfactant synthesis by *Bacillus subtilis* ATCC 6633 in shaking frequency of 300 rpm and inoculation percentage of 5% v/v: Biomass, 5 mL (-), Biomass, 15 mL (■), Biomass, 25 mL (●), pH, 5 mL (■), pH, 15 mL (▲), pH, 25 mL (◆)

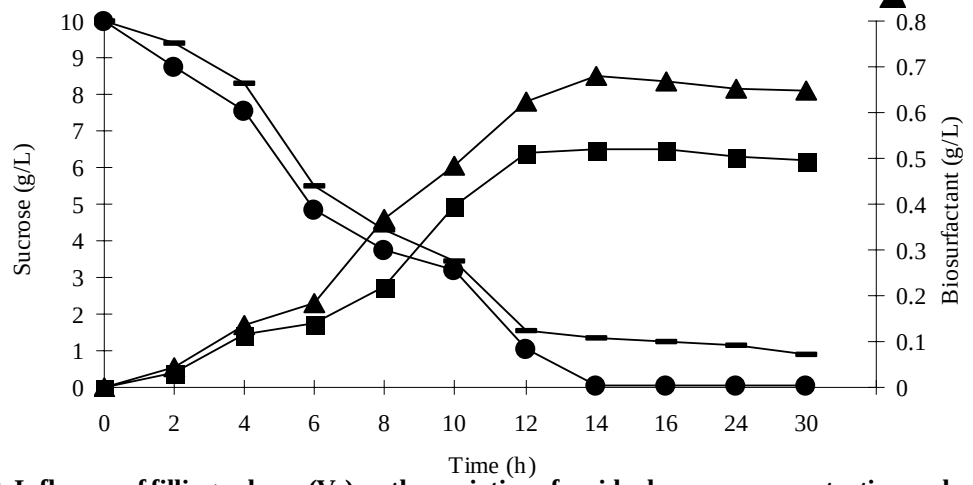

Fig. 3. Influence of filling volume (V_L) on the variation of residual sucrose concentration and surfactin concentration during biosurfactant synthesis by *Bacillus subtilis* ATCC 6633 in shaking frequency of 300 rpm and inoculation percentage of 5% v/v: Sucrose, 5 mL (▲), Sucrose, 15 mL (◆), Sucrose, 25 mL (●), Biosurfactant, 5 mL (■), Biosurfactant, 15 mL (▲), Biosurfactant, 25 mL (-)

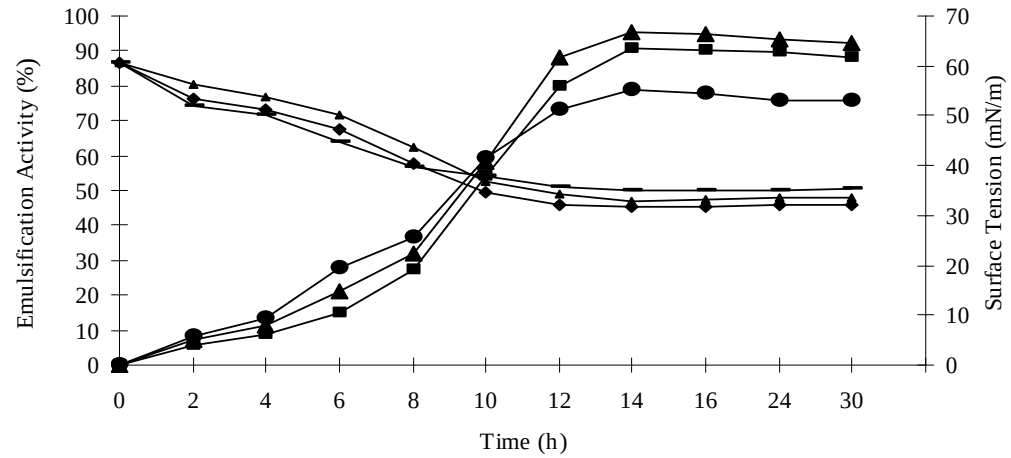

Fig. 4. Influence of filling volume (V_L) on the variation of emulsification activity (EA) and surface tension (ST) during biosurfactant synthesis by *Bacillus subtilis* ATCC 6633 in shaking frequency of 300 rpm and inoculation percentage of 5% v/v: EA, 5 mL (■), EA, 15 mL (▲), EA, 25 mL (●), ST, 5 mL (▲), ST, 15 mL (◆), ST, 25 mL (-)

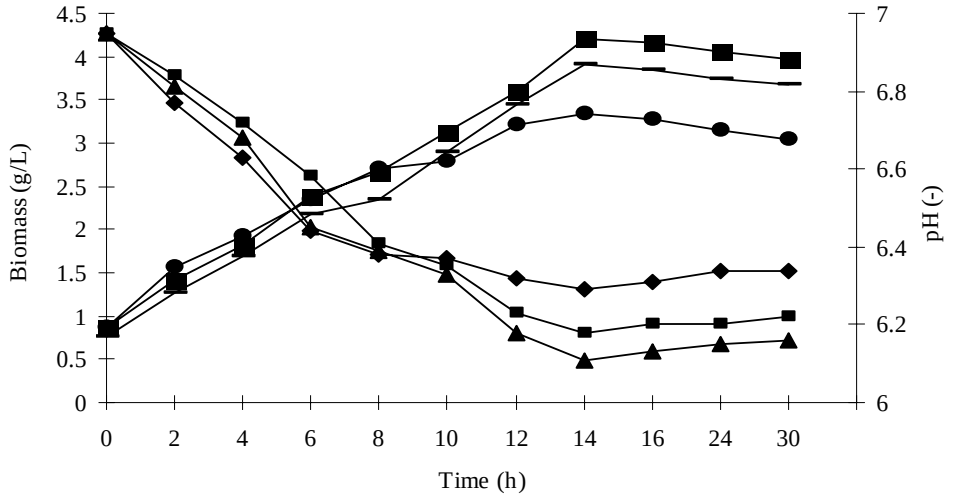

Fig. 5. Influence of agitation rate (rpm) on the variation of biomass concentration and pH during biosurfactant synthesis by *Bacillus subtilis* ATCC 6633 in filling volume of 15 mL and inoculation percentage of 5% v/v: Biomass, 150 rpm (■), Biomass, 300 rpm (▲), pH, 150 rpm (-), pH, 300 rpm (●)

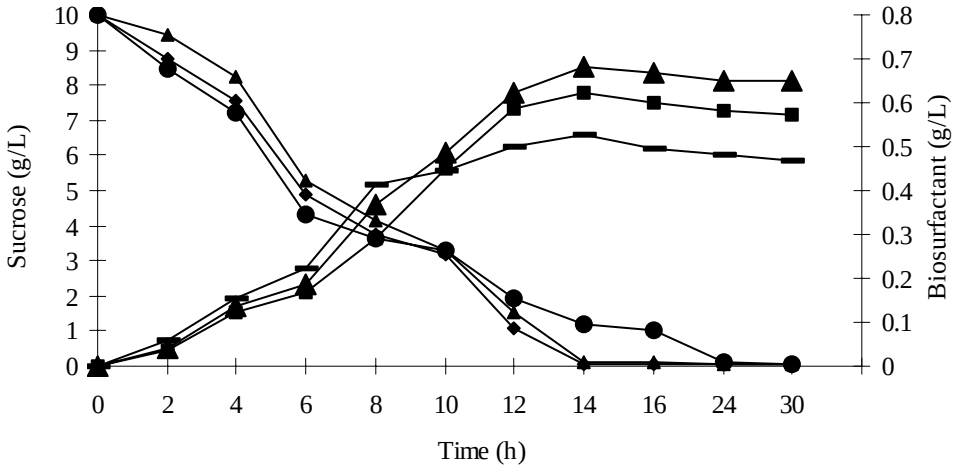

Fig. 6. Influence of agitation rate (rpm) on the variation of residual sucrose concentration and surfactin concentration during biosurfactant synthesis by *Bacillus subtilis* ATCC 6633 in filling volume of 15 mL and inoculation percentage of 5% v/v: Sucrose, 150 rpm (-), Sucrose, 300 rpm (●), Biosurfactant, 150 rpm (■), Biosurfactant, 300 rpm (▲)

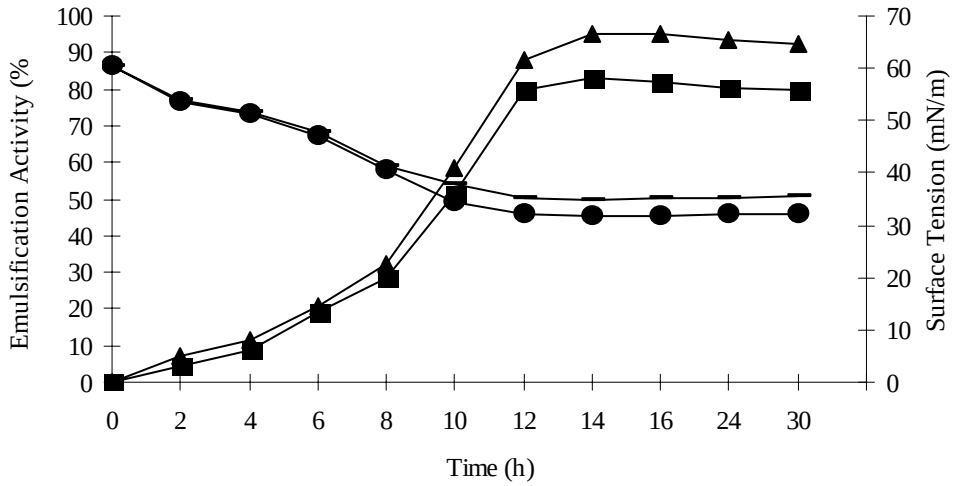

Fig. 7. Influence of agitation rate (rpm) on the variation of emulsification activity (EA) and surface tension (ST) during biosurfactant synthesis by *Bacillus subtilis* ATCC 6633 in filling volume of 15 mL and inoculation percentage of 5% v/v: EA, 150 rpm (■), EA, 300 rpm (▲), ST, 150 rpm (-), ST, 300 rpm (●)

Table 1. Calculated values for various filling volumes at shaking frequency of 300 rpm and inoculation percentage of 5% v/v

V_L (mL)	Y_{xmax} (g/L /h)	Y_{pmax} (g/L/h)	μ_{max} (1/h)	consumed O_2 (mol)	OTR_{max} (mol/L/h)	k_La (1/s)	q_{in} (vvm)	Oxygen limitation
5	0.278	0.045	0.2181	0.0018	0.0260	0.1830	5.65	-
15	0.300	0.048	0.2292	0.0020	0.0100	0.0721	1.88	-
25	0.238	0.037	0.1688	0.0023	0.0065	0.0467	1.13	+

Table 2. Calculated values for various shaking frequencies in filling volume of 15 mL and inoculation percentage of 5% v/v

N (rpm)	Y_{xmax} (g/L/h)	Y_{pmax} (g/L/h)	μ_{max} (1/h)	consumed O_2 (mol)	OTR_{max} (mol/L/h)	k_La (1/s)	q_{in} (vvm)	Oxygen limitation
150	0.23	0.037	0.1804	0.0009	0.0046	0.0325	1.88	+
300	0.30	0.048	0.2292	0.0020	0.0100	0.0721	1.88	-

The results indicate that, according to Amoabediny and Büchs's model, oxygen limitation significantly occurs at shaking frequency of 150 rpm, whereas, at shaking frequency of 300 rpm oxygen limitation does not take place. The maximum biomass yield is 0.23 and 0.3 g/L/h and the maximum biosurfactant productivity is 0.037 and 0.048 g/L/h at shaking frequencies of 150 and 300 rpm, respectively. Thus, it can be enlightened that the best shaking frequency without oxygen limitation and with maximum biosurfactant concentration is 300 rpm. At this shaking frequency, OTR_{max} and k_La obtained by the model is 0.01 mol/L/h and 0.0721 1/s, respectively.

Table 2 indicates the values of different parameters which are calculated under various conditions of shaking frequencies in filling volume of 15 mL and inoculation percentage of 5% v/v.

Using filling volumes of 10, 25, 42 and 60 mL in RAMOS flasks, Guez et al could produce surfactin in less than 24 hours, employing *Bacillus subtilis* ATCC 6633, in which due to absence of yeast extract, the OTR started to increase after latency period of 10 hours and OTR_{max} was evaluated 0.02, 0.014, 0.009 and 0.007 mol/L/h, respectively at each filling volume (Guez *et al.*, 2008). While in this study, using the same organism and yeast extract in culture medium, surfactin was produced after 14 hours in ventilation flasks and OTR_{max} was calculated 0.026, 0.01 and 0.0065 mol/L/h, in filling volumes of 5, 15, 25 mL, respectively, which are better results compared with results, obtained in the Guez's research. Yeh et al, using a special design of batch bioreactor and glucose as carbon source, could produce surfactin after 60 hours, at shaking frequency of 300 rpm. In that study, the k_La and q_{in} were evaluated 0.0132 1/s and 1.50 vvm, respectively (Yeh *et al.*, 2006). However, as mentioned before, in present study, using sucrose as carbon source, at the same shaking frequency and time interval of 14 hours better results were obtained (k_La and q_{in} were evaluated 0.0721 1/s and 1.88 vvm, respectively).

CONCLUSION

In this research, the acquired results indicate that the use of unsteady state model is so useful to determine the optimum conditions of biosurfactant production in a miniaturized bioreactor. By this model, it became possible to predict whether oxygen limitation occurred or not. It was also utilized to determine OTR_{max} and k_La under different conditions in ventilation flasks as a type of miniaturized bioreactors. As indicated, the best results in ventilation flasks under non oxygen limiting conditions were obtained at filling volume of 15 mL and shaking frequency of 300 rpm. Thus, this method of biosurfactant production can be taking into account by scholars and scientists in order to commercialize its applications.

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