Appl Biochem Biotechnol (2010) 162:510–523
DOI 10.1007/s12010-009-8889-0

Scale up and Application of Biosurfactant from *Bacillus subtilis* in Enhanced Oil Recovery

Hossein Amani · Mohammad Reza Mehrnia · Mohammad Hossein Sarrafzadeh · Manouchehr Haghighi · Mohammad Reza Soudi

Received: 15 June 2009 / Accepted: 14 December 2009 /
Published online: 19 January 2010

Abstract There is a lack of fundamental knowledge about the scale up of biosurfactant production. In order to develop suitable technology of commercialization, carrying out tests in shake flasks and bioreactors was essential. A reactor with integrated foam collector was designed for biosurfactant production using *Bacillus subtilis* isolated from agricultural soil. The yield of biosurfactant on biomass ($Y_{p/x}$), biosurfactant on sucrose ($Y_{p/s}$), and the volumetric production rate (Y) for shake flask were obtained about 0.45 gg^{-1}, 0.18 gg^{-1}, and 0.03 $gl^{-1}h^{-1}$, respectively. The best condition for bioreactor was 300 rpm and 1.5 vvm, giving $Y_{x/s}$, $Y_{p/x}$, $Y_{p/s}$, and Y of 0.42 gg^{-1}, 0.595 gg^{-1}, 0.25 gg^{-1}, and 0.057 $gl^{-1}h^{-1}$, respectively. The biosurfactant maximum production, 2.5 gl^{-1}, was reached in 44 h of growth, which was 28% better than the shake flask. The obtained volumetric oxygen transfer coefficient (K_La) values at optimum conditions in the shake flask and the bioreactor were found to be around 0.01 and 0.0117 s^{-1}, respectively. Comparison of K_La values at optimum conditions shows that biosurfactant production scaling up from shake flask to bioreactor can be done with K_La as scale up criterion very accurately. Nearly 8% of original oil in place was recovered using this biosurfactant after water flooding in the sand pack.

Keywords *Bacillus subtilis* · Bioreactor · Biosurfactant · Enhanced oil recovery · Foam · Scale up

Introduction

Biosurfactants are surface-active agents which are produced by microorganisms during their growth [1–3]. Biosurfactants are very important because of their biodegradability, low

H. Amani (✉) · M. R. Mehrnia · M. H. Sarrafzadeh
Oil and Gas Center of Excellence, School of Chemical Engineering, College of Engineering, University of Tehran, P.O. Box 11155-4563, Tehran, Iran
e-mail: hosn1_amani@yahoo.com

M. Haghighi
Australian School of Petroleum, University of Adelaide, Adelaide, 5000, Australia

M. R. Soudi
Department of Microbiology, Faculty of Sciences, Alzahra University, Vanak, Tehran, Iran

toxicity, ability to be produced from cheap raw materials, and effectiveness at extreme conditions of temperature, pH, and salinity [4–8]. One of the most important properties of biosurfactants is reduction of surface tension. These materials show low critical micelle concentration (CMC) values, surface tension reduction of the fermentation broth to less than 30 mN/m, and interfacial tension below 1 mN/m [2, 6, 9–18]. These properties are very important in microbial-enhanced oil recovery (MEOR) because the interfacial tension reduction causes the pressure required to release the oil trapped in the rock pores by capillary forces to drop, which displaces oil from the pores into the mobile liquid phase [19]. During the biosurfactant production in the presence of aeration and agitation, excessive foam is produced in the bioreactor because of lowering of the surface tension by the biosurfactant [1–3]. Using a simple foam fractionation column to recover biosurfactant from a bioreactor, Davis et al. [1] succeeded to concentrate biosurfactant from *Bacillus subtilis* ATCC 21332 in culture broth. The method was used in our investigation about the process of biosurfactant production.

One of the major obstacles in the biosurfactant commercial production is high production costs. The cost can be reduced by strain improvement, medium composition optimization by statistical methods, using alternative inexpensive substrates, or through the bioreactor operation and design [2, 3, 8, 20]. Recently, the use of shake flask in reduction of the process cost and time, operational facilitation, and determination of optimum condition in cultivation, and then scaling up of bioreactor has been taken into consideration [21, 22]. Several parameters including volumetric oxygen transfer coefficient, power input, agitation rate, superficial gas velocity, impeller tip speed, and Reynolds (Re) number are investigated as scale up criteria. To ensure successful scaling up of fermentation, it is essential to determine these criteria. There is a lack of fundamental knowledge about biosurfactant production scaling up. In order to develop suitable technology for possible commercialization, it is essential to carry out tests in shake flasks and bioreactors. The most utilized criterion in scaling up of aerobic fermentations is maintaining similarity between the volumetric oxygen transfer coefficient (K_La) at shake flask and bioreactor [21, 23]. In this study, K_La value measured in the shake flask was compared with K_La value obtained in the integrated bioreactor both under the optimum condition. In other words, the objective of this study was to scale up a biosurfactant process at equal K_La from 500 ml flask to 2.5 l bioreactor with a novel isolated strain, *B. subtilis*, from agricultural soil of Iran. Finally, the performance of this integrated bioreactor was compared to that of the same culture in shake flask. Also, this paper evaluates the potential of *B. subtilis* biosurfactant to be used in ex situ MEOR processes using the sand pack technique model.

Materials and Methods

Microorganisms

The strain used in this study was selected among 102 microorganisms isolated from agricultural soil of Iran. The isolates that showed blood hydrolysis and/or oil spreading were selected and used for further experiments [18]. Finally, one microorganism was selected due to its potential to cause the highest surface tension reduction to lower than 30 mN/m. Primary specification and biochemical characterization were performed and, thereafter, the strain was identified as *B. subtilis* according to its 16S rRNA sequences by Pasteur Institute (Paris, France).

Media and Culture Conditions

All cultures were grown aerobically in liquid medium (medium E; pH 6.9) that contained (g l^{-1}): KH_2PO_4, 2.7; K_2HPO_4, 13.9; sucrose, 10; NaCl, 50; yeast extract, 0.5; and $NaNO_3$, 1. This solution was autoclaved at 121°C for 20 min and after cooling, 10 ml of solutions A, B, and C were added to 1 l of the above medium. Solution A contained 25 gl^{-1} of $MgSO_4$; solution B contained 100 gl^{-1} of $(NH_4)_2SO_4$; and solution C contained (g l^{-1}): EDTA, 0.5; $MnSO_4 \cdot H_2O$, 3; NaCl, 1; $CaCl_2 \cdot 2H_2O$, 0.1; $ZnSO_4 \cdot 7H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.1; $CuSO_4 \cdot 5H_2O$, 0.01; $AlK(SO_4)_2$, 0.01; $Na_2MoO_4 \cdot 2H_2O$, 0.01; boric acid, 0.01; Na_2SeO_4, 0.005; and $NiCl_2 \cdot 6H_2O$, 0.003. Solutions A and B were separately autoclaved, while solution C was filter sterilized [18]. Chemicals used in the growth media were with analytical grade and purchased from Merck. A loop of strain grown on a nutrient agar plate was added to 100 ml nutrient broth medium in a 500-ml Erlenmeyer flask. The flask was then incubated for 24 h at 150 rpm and 37°C in an orbital shaker (Climo-shaker ISF1-X Kuhner) and used as pre-culture.

Shake Flask Experiment

Ten milliliters of the pre-culture was used to inoculate a 500-ml flask containing 100 ml sterilized E medium. The flask was then incubated in the shaking incubator at 250 rpm and 37°C for 136 h. Fermentation was carried out in three 500-ml Erlenmeyer flasks containing 100 ml of E medium simultaneously (the experiment was repeated three times). Samples of the medium were taken for analysis at irregular time intervals. The cultures were centrifuged at 8,000×*g* for 30 min. After separation of biomass, further clarification was carried out by filtration of supernatant through 0.45-μm membrane.

Bioreactor Experiment

Fermentation was carried out in an integrated stirred tank bioreactor with accessories and automatic systems for dissolved oxygen (DO), pH, impeller speed, aeration rate, and temperature (Infors AG, Headofice, Switzerland). The reactor used was a 2.5-l batch stirred bioreactor with three baffle plates provided to reduce vertex effects and enhance the mixing. Two rushton impellers attached to the same shaft were used for agitation. Air was sparged through a pipe sparger placed below the bottom impeller. A foam collector was connected to the top of the bioreactor to recover the biosurfactant by withdrawing the foam produced during the process. The gas flow rates used were varied from 0.5 to 2 vvm. Impeller speed was varied from 200 to 350 rpm. The temperature was maintained at 37°C. The E medium was added to the bioreactor, which was autoclaved at 121°C for 20 min. After sterilization, the integrated bioreactor was cooled to 25°C and then, following the sterilization,10 ml of solutions A, B, and C were added to 1 l of E medium. The bioreactor was then inoculated (10% *v*/*v*) with pre-culture. Samples of the medium were taken for analysis at irregular time intervals.

Analysis of Sucrose

The concentration of sucrose was determined indirectly by the use of glucose assay kit (Parsazemun, Iran). This kit uses a spectrophotometric method, and its application is according to the manufacture instructions. Sucrose was firstly converted to glucose by adding HCl to supernatant.

Biosurfactant Analysis

After the separation of biomass and further clarification, the pH of supernatant was adjusted to 2 by 3 M HCl to allow the precipitation of biosurfactant. The precipitates were collected by centrifugation to obtain a crude biosurfactant. For further purification, the crude biosurfactant was dissolved in double-distilled water and was then extracted three times with an equal volume of dichloromethane. The solvent layer was harvested and evaporated. It was considered as semi-pure biosurfactant [24]. In order to evaluate the presence of impurity at this biosurfactant, we performed the thin-layer chromatography (with 25% 60 F254 plates and visualized under UV (254 nm)) [25].

Fourier Transform Infrared (FTIR) Spectroscopy and H Nuclear Magnetic Resonance (NMR) Analysis of Biosurfactant

To further confirm the results and gain more detailed structural information of the fermentation product of the strain, Fourier transform infrared (FTIR) were obtained with a Perkin-Elmer grating 1430 IR (Norwalk, CT, USA) in dry atmosphere. Absorption spectra were plotted using a built-in plotter. IR spectra were collected from 400 to 4,000 wave numbers (cm^{-1}) with resolution of two wave number per wave number. Sample was prepared by dispersing the solid (biosurfactant) uniformly in a matrix of KBr, compressed to form an almost transparent disc. H nuclear magnetic resonance (NMR) spectrum was recorded at 298 K on an AMX 300 NMR spectrometer (Bruker, 300 MHz) equipped with an Aspect 3000 computer (Bruker) locked to the deuterium resonance of solvent, CDL_3, without spinning. Data were recorded at 32 K (the number of data points per ppm of the plot).

Biomass Analysis

The conventional method of cell dry weight measurement was performed for biomass analysis. After the centrifugation of sample and separation of supernatant from the biomass, the biomass was washed twice in demineralized water and transferred into a pre-tared vial. The bacterial dry weight was determined after drying at 105°C for 24 h.

Surface Tension Measurement

The surface tension was measured at 25°C by a digital tensiometer (Kruss, K10ST, Germany) using the ring method.

Determination of Oxygen Volumetric Mass Transfer Coefficient (K_La)

The shake flask and the bioreactor for biosurfactant production were studied by using the static gassing out method for volumetric mass transfer coefficient (K_La) evaluation. In the case of shake flask and bioreactor, K_La was calculated by measuring the rate of oxygen transfer in nitrogen-purged medium. Before the estimation of the DO, the medium was purged with nitrogen to bring down the DO to the minimum level. This point was treated as zero time [3, 26]. Typical response curves for oxygen concentration against time for shake flask (on shaker at 250 rpm and 37°C) and bioreactor (300 rpm, 1.5 vvm, 37°C) for medium were drawn. According to two-film theory, the transfer rate of oxygen

from the gas phase to the liquid phase may be represented by the following general equation:

$$\mathrm{O.T.R} = \mathrm{dC/dt} = \mathrm{K_L a.}(\mathrm{C^*} - \mathrm{C_L}) \quad (1)$$

Where:

OTR: oxygen transfer rate (kg $m^{-3} s^{-1}$), C^*: oxygen concentration in the gas-liquid interface (kg m^{-3}), C_L: oxygen concentration in the liquid phase (kg m^{-3}), K_L: mass transfer coefficient (m s^{-1}), and a: specific surface of the interface (m^{-1}). The product $K_L a$ is often reported as a lumped parameter, because the two components are not easily separable experimentally. After integration from Eq. 1 and applying the boundary conditions $C_L=0$ when $t=0$, it is possible to calculate $K_L a$ from Eq. 2:

$$-\mathrm{Ln}(1 - C_L/C^*) = K_L \,\mathrm{a.t} \quad (2)$$

Logarithmic graphic of $-\mathrm{Ln}\,(1-C_L/C^*)$ versus time shows a linear tendency whose positive slope is the value of $K_L a$ [3, 26]. In our work C_L is a value of DO (%), C^* is the equilibrium DO concentration (%), and t is operation time (s). The DO values (C_L) was recorded as function of time until saturation was reached (i.e., DO=C^*).

Core Flooding Test

The petroleum reservoir rocks are a sandstone sample that appear to be solid but are not so solid. Therefore, even though a reservoir rock looks solid to the naked eye, a microscopic examination reveals the existence of tiny openings in the rock. These spaces or pores are the ones in which petroleum reservoir fluids are percents, much like a sponge holds water. The application of biosurfactant in MEOR was evaluated using the sand pack technique. This system consisted of a cylinder, which had a jacket for temperature control. Two fluid accumulators were connected to a variable rate injection pump to feed water, biosurfactant solution, and crude oil (19° API). Figure 1 shows the schematic diagram of core flooding apparatus. Sandstone core model was obtained from outcrops at Aghajari Mountains using a core cutter. In order to conduct the flooding experiments, the core was cleaned with toluene and then dried at 110°C and 50 mbar for 24 h. The core porosity, permeability, and pore volume were determined using PVT experiments as 17.37 (%), 14.45 (mD), and 65 (ml), respectively. Dried sand was placed in the core holder and was evacuated via a vacuum pump and finally saturated with 5% (by weight) NaCl solution. Then, the crude oil displaced the brine until no more water came up (to irreducible water saturation). After 24 h, the brine was injected with about 10 ml h^{-1} to the pack until no oil came out. At this point, the core was assumed ready for biosurfactant solution flooding. The injected flow rate was about 10 ml h^{-1} for 48 h at CMC concentration (50 mg l^{-1}). The amount of oil released was measured.

Results and Discussion

Growth and Biosurfactant Production in Shake Flask

With respect to our previously work [27], the best condition of biosurfactant in shake flask was obtained as 37°C and 250 rpm. The profiles of biomass concentration, biosurfactant

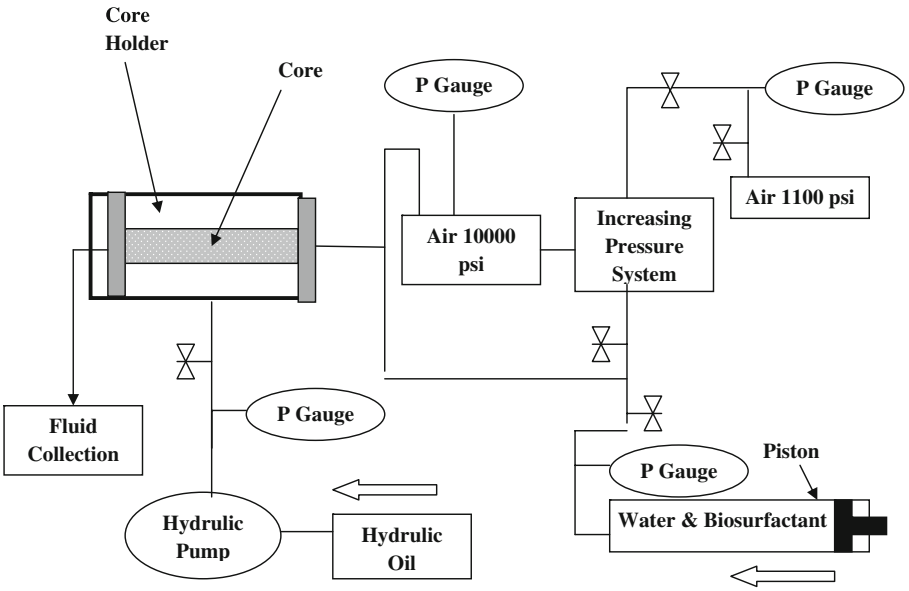

Fig. 1 Schematic diagram of core flooding apparatus

production, and substrate concentration versus time in the mentioned conditions are presented in Fig. 2. Figure 2 shows that the maximum biomass was reached in the 60 h of growth about 4 gl^{-1}, giving a yield of biomass on sucrose ($Y_{x/s}$) of 0.4 gg^{-1}. The growth continued until 60 h where the sucrose completely consumed. Specific growth rate was 0.023 h^{-1}, ascertained from the slope of the plot of Ln (biomass) versus time (from 10 to 60 h). Figure 2 also indicates the relations between sucrose consumption and cell growth. This figure also shows that the biomass concentration has been increased with time, and sucrose concentration has been decreased with cell growth. For this microorganism, the production of biosurfactant was proportional to the cell growth representing biosurfactant as a growth-associated product. In addition, at an initial sucrose concentration of 10 gl^{-1}, the concentration of biosurfcatant was reached to its maximum (1.8 gl^{-1}) at about 60 h of fermentation where the maximal cell concentration was observed. These results are in agreement with other research works [3, 8, 25]. We calculated the yield of biosurfactant on biomass ($Y_{p/x}$), the production yield ($Y_{p/s}$), and the volumetric production rate (Y) as 0.45 g g^{-1}, 0.18 gg^{-1}, and 0.03 $gl^{-1}h^{-1}$, respectively.

Growth and Biosurfactant Production in the Integrated Bioreactor

The effect of agitation rate (rpm) and aeration rate (vvm) on the production of boisurfactant was investigated. The experimental design and results are shown in Table 1. We obtained the optimum conditions for biosurfactant production in the integrated bioreactor.

According to Table 1, the best condition of biosurfactant production was obtained at 300 rpm and 1.5 vvm. Figure 3 shows a typical time course profile for our integrated bioreactor with agitation rate of 300 rpm and 1.5 vvm. The figure reveals that the maximum biomass was reached in the 44-h of growth about 4.2 gl^{-1}, giving a yield of biomass on sucrose ($Y_{x/s}$) of 0.42 gg^{-1}. Again, we calculated the $Y_{p/x}$, $Y_{p/s}$, and Y to have 0.595 gg^{-1}, 0.25 gg^{-1}, and 0.057 $gl^{-1}h^{-1}$, respectively. Aeration and agitation in this bioreactor have

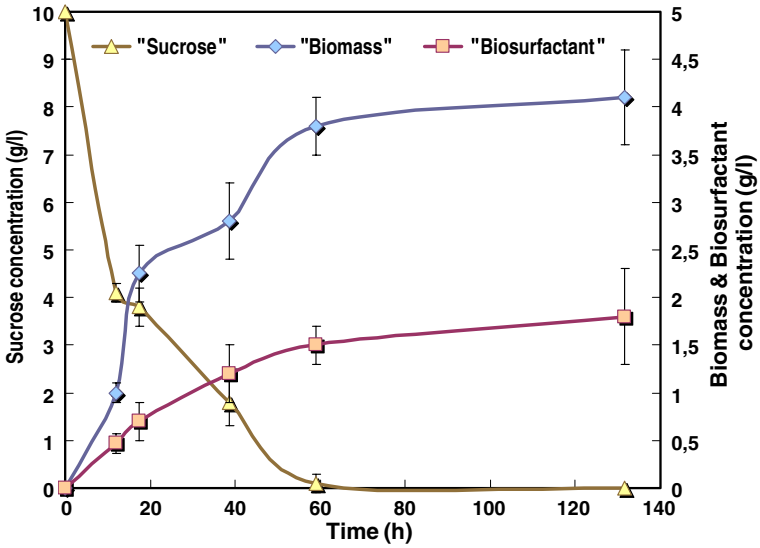

Fig. 2 Time course profiles of *Bacillus subtilis* cell growth, sucrose concentration, and biosurfactant concentration at 250 rpm and 37°C in the 500-ml flask containing 100 ml E medium

Table 1 Experimental design in batch fermentation of *Bacillus subtilis* under different condition of agitation and aeration.

Agitation rate (rpm)	Aeration rate (vvm)	Maximum production rate (mg $l^{-1}h^{-1}$)	Overall production rate (mg $l^{-1}h^{-1}$)	Maximum biosurfactant concentration (mg l^{-1})	Biosurfactant yield mg (g sucrose)$^{-1}$
200	2	88	25	2,310	170
250	2	114	30	2,415	200
300	2	120	38	2,200	230
350	2	102	29	1,800	118
200	1.5	99	28	2,200	160
250	1.5	122	33	2,340	210
300	1.5	142	41	2,500	250
350	1.5	120	33	1,800	100
200	1	83	25	1,950	120
250	1	118	29	2,100	135
300	1	124	34	2,200	200
350	1	100	31	1,800	110
200	0.5	55	17	1,830	97
250	0.5	78	21	1,920	116
300	0.5	83	28	2,000	133
350	0.5	50	25	1,400	68

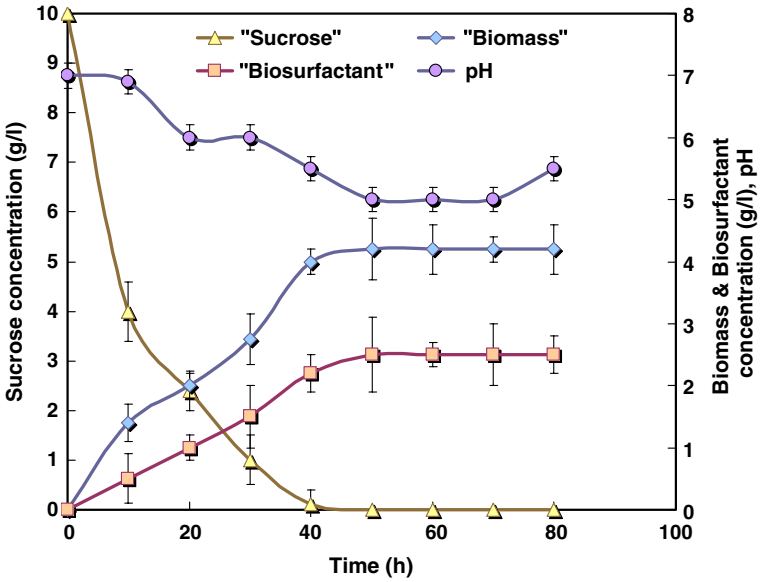

Fig. 3 Time course profiles of *Bacillus subtilis* cell growth, sucrose concentration, and biosurfactant concentration at 300 rpm, 1.5 vvm, and 37°C in the integrated bioreactor containing 1 l E medium

main roles in enhanced biosurfactant production because these parameters increase the oxygen required by better mixing medium, which transfer oxygen from the gas into the liquid phase. The growth continued until 44 h where the sucrose has been completely consumed. The pH drop during the biosurfactant production might be due to formation of acid by microorganism during fermentation [3]. There was a similar reduction of surface tension in both the samples collected from bioreactor and flask. This result might be due to the low CMC of biosurfactant. However, in the bioreactor and shake flask, the maximum crude biosurfactant production was 2.5 and 1.8 gl^{-1}, respectively, which showed 28% better bioreactor performance than the shake flask. In this bioreactor, significant foaming occurred between 1 and 6 h of culture time. At the end of fermentation, 100 ml foam was collected. Concentration of biosurfactant in the foam collector was around three times greater than

Table 2 Comparison of the results obtained for shake flask and integrated bioreactor with comparable studies.

Microorganism *Bacillus subtilis*	Maximum biomass production (g l^{-1})	Maximum biosurfactant concentration (g l^{-1})	Y g l^{-1} h^{-1}	$Y_{X/S}$ g g^{-1}	$Y_{P/X}$ g g^{-1}	$Y_{P/S}$g g^{-1}	References
Shake flask	4	1.8	0.03	0.4	0.45	0.18	This study
Intregrated bioreactor	4.2	2.5	0.057	0.42	0.595	0.25	This study
Shake flask	1.9	0.092	0.0038	0.645	0.071	0.046	[2]
Intregrated bioreactor	0.52	0.136	0.0097	0.26	0.262	0.068	[2]
Intregrated bioreactor		6.45	0.106			0.161	[3]

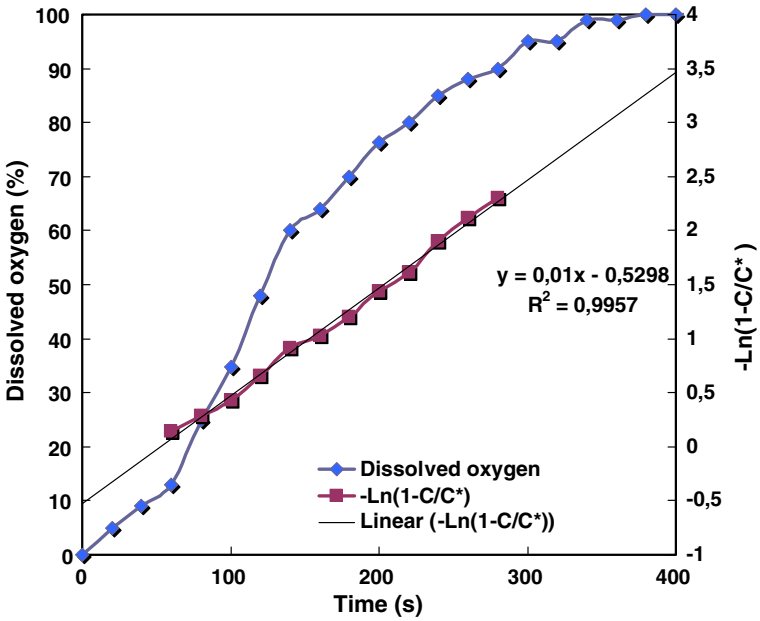

Fig. 4 Plot of dissolved oxygen level and −ln (1−*C*/*C**) versus time for shake flask at 250 rpm and 37°C

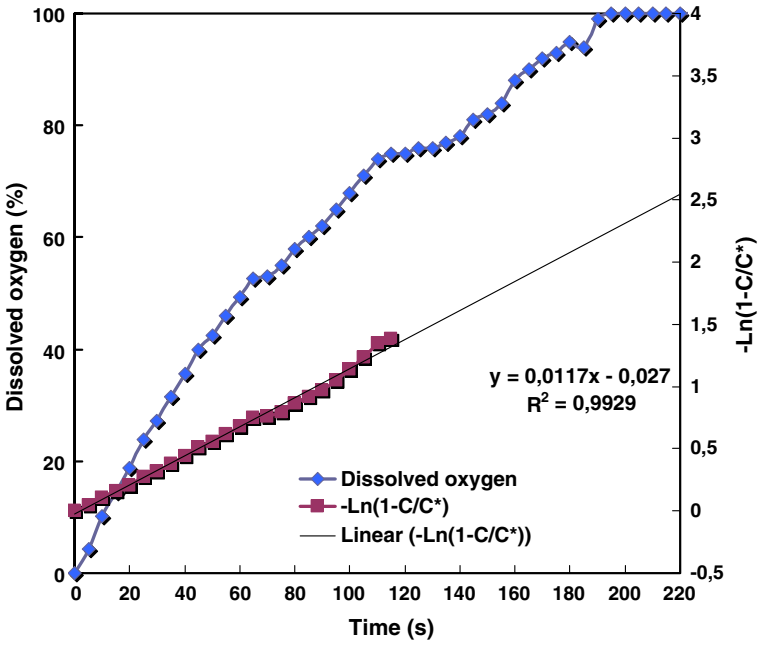

Fig. 5 Plot of dissolved oxygen level and −ln (1−*C*/*C**) versus time for bioreactor (300 rpm, 1.5 vvm, and 37°C)

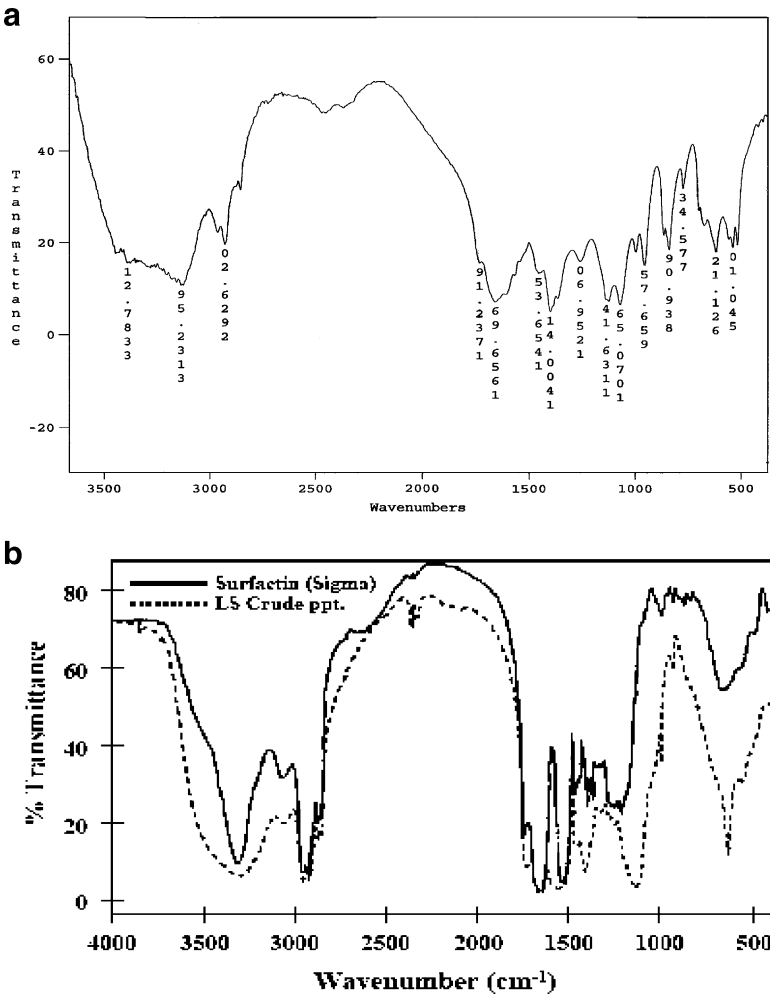

Fig. 6 Transmission Fourier transform infrared spectra of biosurfactant product, (**a**) from this study and from (**b**) purified commercial surfactin [6]

that in the culture medium (7.5 gl^{-1}). Specific growth rate of 0.0347 h^{-1} was obtained in the bioreactor.

For a better comparison between different researches, some parameters such as $Y_{x/s}$ $Y_{p/x}$, $Y_{p/s}$, and Y were calculated and presented in Table 2. We note that the maximum biosurfactant production (6.45 gl^{-1}) by Yeh et al. [3] was produced under recycle of biomass condition. As seen in Table 2, higher $Y_{p/s}$ and $Y_{p/x}$ were reached in our integrated bioreactor than in shake flask, which were comparable to other researches. However, Chen et al. [2] reported that maximum biomass concentration at bioreactor was less than that in the shaken culture, indicating a poorer growth of their microorganism in the bioreactor.

As seen in Table 2, the maximum biomass and biosurfactant concentration in the bioreactor are more than in the shake flask, which may be due to the foam separation process enhancing biosurfactant production or better agitation and aeration of medium in

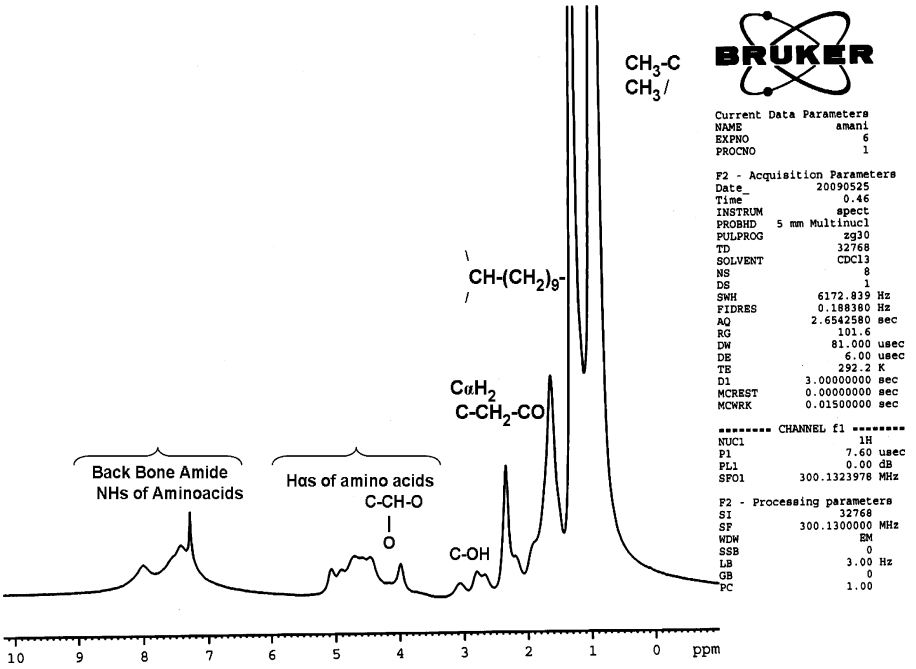

Fig. 7 H nuclear magnetic resonance spectrum of the biosurfactant produced by *Bacillus subtilis* isolated from agricultural soil of Iran

bioreactor [1–3]. These results show the potential effectiveness of the microorganism and the technique used in biosurfactant production.

Scale Up from Shake Flask to Bioreactor

The values of $K_L a$ for shake flask and bioreactor obtained from the slope of the plot of $-\mathrm{Ln}(1-C_L/C^*)$ versus time. The results are shown in Figs. 4 and 5. The first linear part of each curve was taken into account, and the comparison between shaking flasks and bioreactor was made based on this part of both figures.

The obtained $K_L a$ value at optimum condition in the shake flask (37°C and 250 rpm) was around 0.01 (s^{-1}; Fig. 4). The $K_L a$ value can be manipulated in bioreactor by the rate of vvm and rpm. At optimal combination of agitation and aeration (Table 1), the best $K_L a$ in

Table 3 Effect of water flooding and biosurfactant flooding on oil recovery.

OOIP (ml)	Water flooding		OIP_{WF} (ml)	Biosurfactant flooding after water flooding		
	Recovery			Recovery		
	ml	%OOIP		ml	%OOIP	$\%_{WF+BF}$
65	40.17	61.8	24.83	5.3	8.15	69.9

OOIP original oil in place (the total hydrocarbon content of an oil reservoir), *OIP* oil in place after water flooding, *BF* biosurfactant, *WF* water.

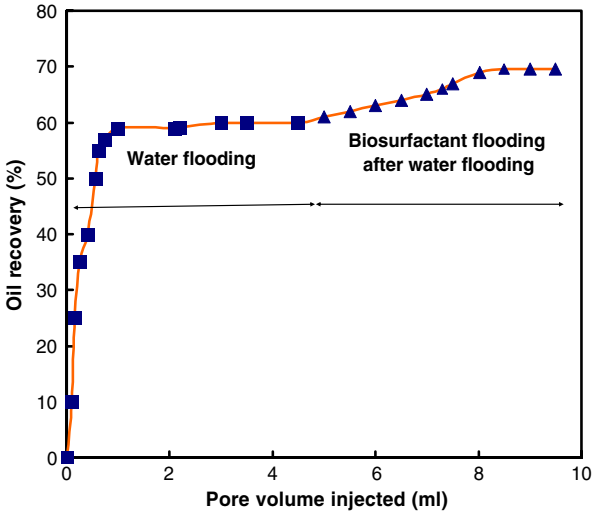

Fig. 8 Relationship between water (*squares*) and biosurfactant (*triangles*) flooding with pore volume in core flooding; the injected flow rates were about 10 ml h^{-1} for 48 h

bioreactor is 0.0117 (s^{-1}; Fig. 5). Comparison of K_La values at optimum conditions shows less than 17% error for these values so that the scale up of biosurfactant production from shake flask to bioreactor can be done with K_La as scale up criterion with high accuracy. In other words, among several parameters including volumetric oxygen transfer coefficient, power input, agitation rate, superficial gas velocity, impeller tip speed, and Re number, K_La can be successfully chosen as a scale up parameter for the optimum biosurfactant production. Therefore, in order to produce biosurfactant, first, the K_La must be estimated at optimum condition in a shake flask, and then using the same value of K_La in bioreactor, some parameters such as rpm will be estimated.

Chemical Characterization of the Biosurfactant

In order to assess the product purity, we first performed the thin-layer chromatography analysis. These results suggested a considerable purity grade of the fermentation product. To confirm the biosurfactant production, the FTIR of commercially obtained purified surfactin and biosurfactant of this study were compared. The results of FTIR analysis are shown in Fig. 6.

In this figure, the FTIR spectrum of biosurfactant from the strain showed strong absorption bands indicating the presence of a peptide component at 3,132–3,387 cm^{-1} resulting from N–H stretching mode. At 1,650 cm^{-1}, the stretching mode of CO–N bond was observed. The presence of an aliphatic chain was indicated by the C–H stretching modes at 2,929 and 1,070–1,456 cm^{-1}. The band at 1,732 cm^{-1} was due to lactone carbonyl absorption. These patterns were identical to those of surfactin.

For further confirming the results of this study, we carried out the H NMR analysis. The results are shown in Fig. 7. Results obtained with H NMR indicated that the molecule is a lipopeptide. Almost all of the back bone-amide-NH groups are in the region from 7.2 to 8 ppm downfield from tetramethylsilane. Alpha hydrogens of the amino acids come into resonance from 3.98 to 5 ppm. A doublet at δ=0.854 ppm for the $(CH_3)_2$–CH group,

indicating terminal branching in the fatty acid component. Remaining spectra clearly confirmed the presence of β-hydroxy fatty acid. Therefore, we think our strain has produced a lipopeptide, possibly surfactin.

Sand Pack Core Flooding Tests

A 50-mg/l solution of the biosurfactant (CMC concentration) in brine was prepared and used to enhance oil recovery in laboratory cores. The effect of water flooding followed by biosurfactant flooding in the core is presented in Table 3.

Figure 8 shows the relationship between water, biosurfactant flooding, with pore volume injected in core flooding; the injected flow rates were about 10 ml h^{-1} for 48 h. The recovery value of crude oil after water flooding was obtained at 61.8% of original oil in place, whereas nearly 8% of original oil in place was recovered using biosurfactant after water flooding (Table 3). The results indicated that biosurfactant mobilized oil in the sand and should have a significant role in enhanced oil recovery. Therefore, this microorganism produces biosurfactant that can lower oil–water interfacial tension thus causing emulsification. The drop in interfacial tension increases the capillary number. The increased capillary numbers are associated with reduction of residual oil saturation.

Conclusions

Due to the lack of fundamental knowledge about scale up of biosurfactant production, we investigated the use of shake flask to determine the optimum condition in cultivation and then scaling up to bioreactor. We designed an integrated reactor with foam collector and carried out tests in shake flasks and bioreactor in order to develop suitable technology for possible commercialization. Comparison of $K_L a$ values at optimum conditions shows that scale up of biosurfactant production from shake flask to bioreactor can be done with $K_L a$ as scale up criterion with high accuracy. The high values of $Y_{x/s}$ $Y_{p/x}$, $Y_{p/s}$, and Y indicate that a lipopeptide (possibly surfactin) was produced more efficiently by the strain in proposed integrated bioreactor than other researches. The maximum biosurfactant production in bioreactor was also reached to about 2.5 gl^{-1}, which was 28% better than the shake flask. This result might be due to the foam separation process enhancing biosurfactant production. Nearly 8% of original oil in place was recovered using this biosurfactant after water flooding in sand.

Acknowledgements The authors gratefully acknowledge the Petroleum Engineering and Development Company of Iran (PEDEC) for funding this project (contract No 553 MTN).

References

1. Davis, D. A., Lynch, H. C., & Varley, J. (2001). *Enzyme and Microbial Technology., 28*, 46–354.
2. Chen, C. Y., Baker, S. C., & Darton, R. C. (2006). *Chemical Technology and Biotechnology, 81*, 1923–1931.
3. Yeh, M. S., Wei, Y. H., & Chang, J. S. (2006). *Process Biochemistry, 41*, 1799–1805.
4. Banat, I. M. (1995). *Bioresource Technology, 51*, 1–12.
5. Desai, J. D., & Banat, I. M. (1997). *Microbiology Molecular Review, 61*, 47–64.

6. Fang, X., Wang, Q., Bai, B., Liu, X. C., Tang, Y., Shuler, P. J., et al. (2007). SPE No: 106048.
7. Mulligan, C. N. (2005). *Environmental Pollution, 133*, 183–198.
8. Joshi, S., Bharucha, C., Jha, S., Yadav, S., Nerurkar, A., & Desai, A. J. (2008). *Bioresource Technology, 99*, 195–199.
9. Kim, H. S., Yoon, B. D., Lee, C. H., Suh, H. H., Oh, H. M., & Katsuragy, T. (1997). *Fermentation and Bioengeering, 84*, 41–46.
10. Wei, Y. H., & Chu, I. M. (1998). *Enzyme Microbial Technology, 22*, 724–728.
11. Davis, D. A., Lynch, H. C., & Varley, J. (1999). *Journal of Enzyme Microbial Technology, 25*, 322–329.
12. Demin, W., Jiecheng, C., Qun, L., Lizhong, L., Changjiu, Z., Jichun, H. (1999). Malaysia Conference, SPE 57304.
13. Makkar, R. S., & Cameotra, S. C. (1999). *J Surfactants Detergents, 2*, 237–241.
14. Maier, R. M., & Soberon-Chavez, G. (2000). *Applied Microbiology and Biotechnology, 54*, 625–633.
15. Abalos, A., Pinaso, A., Infante, M. R., Casals, M., Garcia, F., & Manresa, A. (2001). *Langmiurir, 17*, 1367–1371.
16. Daoshan, L., Shouliang, L., Yi, L., & Demin, W. (2004). *Colloids and Surfaces, 244*, 53–60.
17. Wei, Y. H., Wang, L. F., & Chang, J. S. (2004). *Biotechnology Progress, 20*, 979–983.
18. Youssef, N. H., Duncan, K. E. D., Nagle, D. P., Savage, K. N., Knapp, R. M., & McInerney, M. J. (2004). *Journal of Microbiology Methods, 56*, 339–347.
19. Gabitto, G. F. (2006). SPE 102328 presented at the Annual Technical Conference and Exhibition held in San Antonio, Texas.
20. Rao, R. S., Kumar, C. G., Prakasham, R. S., & Hobbs, P. J. (2008). *Biotechnology journal, 3*(4), 510–523.
21. Shukla, V. B., Veera, U. P., Kulkarni, P. R., & Pandit, A. B. (2001). *Biochemical Engineering Journal, 8*, 19–29.
22. Yezza, A., Tyagi, R. D., Valero, J. R., Surampalli, R. Y., & Smith, J. (2004). *Journal Industrial Microbiology and Biotechnology, 31*(12), 545–552.
23. Garcia-Ochoa, F., & Gomez, E. (2009). *Bioresource Advance, 27*, 153–176.
24. Cooper, D. G., Macdonald, R. C., Duff, S. J. B., & Kosaric, N. (1981). *Applied and Environmental Microbiology, 42*, 408–412.
25. Yeh, M. S., Wei, Y. H., & Chang, J. S. (2005). *Biotechnology Progress, 21*, 1329–1334.
26. Ory, I., de Romero, L. E., & Cantero, D. (1999). *Journal of Bioprocess Engineering, 20*, 73–75.
27. Amani, H., Mehrnia, M. R., Haghighi, M., Sarrafzadeh, M. H. (2009). *Journal of Applied Biochemistry and Biotechnology.* Under review.