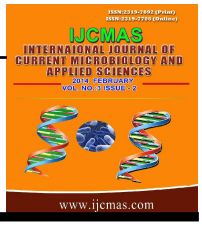
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 2 (2014) pp. 66-80**
http://www.ijcmas.com



Original Research Article

Enhanced production and characterization of biosurfactant produced by a newly isolated *Bacillus amyloliquefaciens* USTBb using response surface methodology

Radhika Chandankere[1], Jun Yao [1,2*], Kanaji Masakorala[1], A.K.Jain [3] and Ranjan Kumar[4]

[1]School of Civil & Environmental Engineering and National "International Cooperation Based on Environment and Energy", and Key Laboratory of "Metal and Mine Efficiently Exploiting and Safety" Ministry of Education, University of Science and Technology, Beijing, 100083 Beijing, PR China
[2]Key Laboratory of Biogeology and Environmental Geology of Chinese Ministry of Education, and Sino-Hungarian Joint Laboratory of Environmental Science and Health, University of Geoscience, Wuhan 430074, PR China
[3]Domain Expert, Petroleum Microbiology and Unconventional Oil/Gas, Oil and Natural Gas Corporation Ltd., Dehradun, India
[4]Department of Civil Engineering, Indian Institute of Technology Roorkee, Uttarakhand 247667, India
***Corresponding author e-mail:** yaojun@ustb.edu.cn*

A B S T R A C T

Bacillus species can serve as a major workhorse to produce biosurfactant for diverse biotechnological applications. In this study, an indigenous bacterial strain isolated from petroleum reservoir was found to be a persuasive biosurfactant-producer. Based on biochemical characteristics, gyrA and 16S ribotyping of the isolate, USTBb, was identified as *Bacillus amyloliquefaciens* with 100% identity. Strain USTBb was screened based on oil-displacement test, emulsification method and ability to reduce surface tension. USTBb biosurfactant had the ability to decrease the surface tension of water from 72 to 28 mN /m, with the critical micelle concentration (CMC) of 35 mg/l. To characterize the biosurfactant, fourier transforms infrared spectroscopy (FT-IR) and energy dispersive X-ray spectroscopy (EDS) method has been carried out to determine the functional groups and compositional analysis, respectively. Response surface methodology (RSM) with four variable Central composite design (CCD), based on a three level, was employed to optimize the biosurfactant production. The effect of four important growth parameters (temperature, pH, salinity and crude oil content as carbon course) was studied for enhanced biosurfactant production. With the optimized method, the relative biosurfactant production expressed as surface tension reduction was three-fold higher than that obtained in the un-optimized culture condition.

K e y w o r d s

Microbial Growth; Optimization; Biosurfactant; *Bacillus amylo-iquefaciens*; Characterization

Introduction

Biosurfactants referred as green chemicals are microbial - derived surface active molecules produced by variety of microorganisms, which either adhere to

cell surface or are excreted extracellular in the growth culture medium (Mulligan, 2005). Currently, almost all surfactants are being are derived from petroleum source. However, these synthetic surfactants are toxic and hardly degraded by microorganisms (Karanth *et al*., 1999). Therefore, in the recent years much attention have been attributed towards biosurfactant over chemically synthesized surfactants due to their ecological acceptance, low toxicity and biodegradable nature (Singh et al., 2013). Moreover biosurfactant are ease of synthesis, specific and effectiveness at extreme process conditions (Kebria *et al*., 2009). Moreover biosurfactant's ease of synthesis, specificity and effectiveness at extreme conditions (Kebria *et al*., 2009) makes them a preferred choice.

In the present day, there is enormous market demands for biosurfactants, but these compounds do not compete economically with synthetic surfactants owing to their higher production cost of biosurfactants. In this context, different methods could be used to increase the bio-product yield for developing an effective economical industrial process for the growth of microorganism (Vaz *et al*., 2012). One of the methods of accomplishing the above purpose is the screening of optimum culture conditions for maximum production of biosurfactant.

In this view, concentration of carbon source, pH of the medium, salinity and temperature are of key importance for optimization of biosurfactant productivity (Saikia *et al*., 2012). Previously, the factors affecting biosurfactant production have been extensively studied by various authors, especially for *Streptomyces* sp. and *Pseudomonas* sp. (Henkela *et al*., 2012; Lakshmipathy *et al*., 2010). But, is not much information about optimal parameters for biosurfactant production from *Bacillus* species particularly *Bacillus amyloliquefaciens*.

A few research on biosurfactant production from Bacillus sp. has been reported in the literature (Javaheri et al., 1985). However, to our best knowledge, this is the first report concerning the optimal biosurfactant production from crude oil. Present communication reports the optimization of growth parameters for biosurfactant production by a novel strain of *B. amyloliquefaciens*: an isolate from petroleum reservoir by using central composite design (CCD) of response surface methodology (RSM). Further, partial characterization of the extracted biosurfactant including critical micelle concentration, FT-IT and EDS have been provided as well.

Materials and Methods

Sampling and Isolation of the biosurfactant-producer strain

Crude oil sample was collected from oil producing well of petroleum reservoir located in DaGang oilfield, Tianjin, China. A persuasive biosurfactant-producer strain was isolated using minimal salt medium (MSM) by applying enrichment culture technique as mentioned in our previous studies (Chandankere *et al*., 2013). The isolated strain was maintained as master stock culture in 25 % (v/v) glycerol at -80 ^{o}C.

Screening and identification of biosurfactant-producer

Initially, the screeing of biosurfactant production by the isolated strain was carried out by oil-displacement test according to the method described

elsewhere (Ebrahimi *et al*., 2012). Further, emulsification activity of the cell-free culture supernatant against paraffin oil was carried out as mentioned by Paraszkiewicz *et al*., 2002. To confirm the biosurfactant production from the above mentioned tests, surface tension of the culture samples were measured by DuNouy Tensiometer.

The isolated strain was identified based on morphological and biochemical characterization (Buchanan, 1948) Further 16S rDNA and gyrA nucleotide sequencing for species identification. For this, the total genomic DNA was extracted, amplified and sequenced as described in the literature (De Clerck *et al*., 2004). The resulted 16S rDNA and gyrA gene sequences of the isolate are submitted to NCBI and the BLAST-n was used to analyze similarities (www.ncbi.nlm.nih.gov/BLAST). The phylogenetic tree was generated by using MEGA (Version 4.0) software (Chandankere *et al*., 2013).

Production and extraction of biosurfactant

The biosurfactant production was carried out in mineral salt medium (MSM) with the same composition as used in our previous study (Chandankere *et al*., 2013). Briefly, the biosurfactant-producer strain was transferred to 4 ml Luria-Bertani broth medium containing (g/l): peptone 10, NaCl 5 and yeast extract 10 and incubated at 180 rpm, 37 ºC for 12 h. Subsequently, 4 ml bacterial suspensions were transferred to 1000 ml Erlenmeyer flask containing 500 ml of MSM and incubated at above mentioned conditions. To determine the biosurfactant production, culture samples were collected at specified time intervals to assay the surface tension (ST). The biosurfactant was extracted by removing the bacterial cells through centrifugation at 10,000 rpm at 4 ºC for 20 min and precipitation of biosurfactant from the supernatant 1 N HCl to pH 2 and the precipitated product was collected by centrifugation with the conditions mentioned above. Finally, the precipitate was dissolved in sterilized distilled water and adjusted to pH 7 using 1N NaOH and the solution was lyophilized and weighed.

Experimental design for biosurfactant production

Central composite design (CCD) was used for designing the experimental runs because of its efficiency and inherent advantages over other designs of RSM. In the present study temperature (X_1), pH (X_2), salinity (X_3) and crude oil concentration (X_4) were chosen as four independent variables and the response variable was the surface tension (ST) reduction. The codes and ranges of each variable used for optimizing biosurfactant production are given in Table 1. A second order polynomial equation was used for regression analysis to estimate the response function as below:

$$Y = \alpha_0 + \sum_{n=1}^{4} \alpha_i X_i + \sum_{n=1}^{4} \alpha_{ii} X_i^2 + \sum \sum_{n=1}^{4} \alpha_{ij} X_i X_j \quad (1)$$

where Y is the response (surface tension, ST), α_0 is the constant coefficient α_i, α_{ij} and α_{ij} are the coefficients estimated by the regression; they represent the linear, quadratic and cross-product effects of X_1, X_2, X_3 and X_4 respectively on the response. All the designed experiments were carried out in triplicates.The statistical analysis, experimental design and building of the mathematical model were performed by

the statistical program package "MINITAB RELEASE 16" followed by analysis of variance (ANOVA).

Characterization of biosurfactant

Determination of critical micelle concentration (CMC)

Different concentrations of the biosurfactant in the range of 10-80 mg/l were prepared from the stock solution of biosurfactant (0.1 g/l) in water. CMC was determined by measuring the surface tension of the dilutions at room temperature. The CMC was defined by plotting the surface tension as a function of biosurfactant concentration. These experiments were conducted in three independent experiments

Fourier transforms infrared spectroscopy (FT-IR) Energy Dispersive X-ray Spectroscopy (EDS) analyses

The FT-IR analysis was carried out on a GX-FTIR system (Perkin Elmer, USA) as mentioned in our previous study (Chandankere *et al*., 2013). The dried biosurfactant sample mounted over the copper grid was used for quantitative elemental analysis using SEM-EDX (Oxford Instruments, UK).

Results and Discussion

Biosurfactant-producer strain identification

A novel biosurfactant producing strain USTBb formed creamy white color, round and convex colonies, not glossy and 1-2 mm diameter. Furthermore, the strain USTBb was Gram-positive and rod shaped (Fig. 1). The strain USTBb was identified as *Bacillus* based on the biochemical and morphological characteristics (Joo *et al*., 2007). The strain showed catalase activity, methyl red test, starch hydrolysis, gas production, and glucose/lactose fermentation. Tests for urease, Voges–Proskauer, indole and citrate utilization were negative. The 16S rDNA sequence of USTBb and using GenBank BLAST tool, the isolate USTBb showed high similarities (> 99 %) (data not shown) to *B. velezensis*, *B. amyloliquefacien*, *B. methylotrophicus*, *B. atrophaeus* and *B. substilis.*

Previously, it was questioned by other authors about the discrimination of these species on the basis of 16S rDNA gene analysis (De Clerck *et al*., 2004). Therefore, gyrA sequence could accurately classify the related taxa of *Bacillus subtilis* group. GyrA sequence of strain USTBb showed 100 % similarity with *B. amyloliquefaciens* and according to phylogenetic tree analysis; it was closely associated with *B. amyloliquefaciens* (Figure. 2). The16S rDNA and gyrA sequences of the isolated strain USTBb are available in the GenBank under the accession number of **JX202779** and **KF496216**, respectively.

Production of biosurfactant

Biosurfactant production by newly isolate was firstly screened by oil-displacement test, emulsification assay and measuring the reduction in surface tension of the culture solution. Initially, the results of oil spreading test carried out for cell-free supernatant exhibited biosurfactant production by producing a large clearing or oil displacement zones (~ 3 in diameters) (Figure. 3a-b). Emulsifying capacity of the same test sample against parafilm oil additionally supported the biosurfactant production potential (Figure.

3c). Figure. 4 shows the growth kinetics and biosurfactant production by *Bacillus* sp. USTBb. The surface tension of the cell-free supernantant was reduced from 62 to 28 mN/m, which remained unchanged even after 48 h. The minimum surface tension was 28 mN/m after incubation, during the middle of exponential phase (24 h), after which there was insignificant change in the surface tension of the cell-free supernantant. Similar results was observed by other authors (Vaz *et al*., 2012) and recommended that the biosurfactant concentration in the culture broth probably exceeded/reached its CMC at this time of cultivation.

After 24 h of incubation, the emulsification capacity of the cell-free culture broth was not maximum and continued to rise with further biosurfactant production. This discloses that the concentration of biosurfactant was adequate for micelle creation after 24 h of incubation, ahead of which invariable surface tension is observed. Earlier, same growth pattern by *Rhodococcus* sp. strain was observed by other researchers (Shavandi *et al*., 2011).

Model fitting, and Experimental results of biosurfactant production by strain USTBb

The statistical analysis using RSM is used to model the biosurfactant production in terms of surface tension reduction and to determine the most significant process parameters and their interactions. The proposed design matrix of 30 trial experiments is given in Table 2. The final response equation for biosurfactant production in terms of surface tension reduction is as given below

$$ST = 381.835+8.76\ X_1 + 0.67\ X_2 - 3.216\ X_3 - 4.03\ X_4 + 2.818\ X_1^2 - 1.085\ X_2^2 - 0.026\ X_3^2 - 1.0216\ X_4^2 + 0.442\ X_1 \times X_2 + 1.87\ X_1 \times X_3 - 0.297\ X_1 \times X_4 + 0.56\ X_2 \times X_3 - 0.33\ X_2 \times X_4 - 0.067\ X_3 \times X_4 \quad (2)$$

where ST denotes surface tension (mN/m); X_1, X_2, X_3 and X_4 denotes the coded values with pH, salinity (%) and crude oil content (%) respectively. The value of regression coefficient of R^2 = 99.85% indicated the suitability of the quadratic equation to predict the biosurfactant production in terms of surface tension reduction (Table 3). The significant lack of fit *F*-value, of 1.92 indicates that lack of fit is not significant relative to pure error. The model predicted R^2 of 99.05% is as close to the adjusted R^2 of 99.49%, indicating the model to be the best fit for optimization. The predicted values and the experimental values were found to be in close agreement as shown in Figure. 5.

Table 3 imply that the linear positive effects of temperture ($P < 0.01$), pH of the solid medium ($P < 0.01$), salinity concentration ($P < 0.01$), and crude oil content ($P < 0.01$) predominate over the quadratic and interactive effects. Futher, these terms had a very significant influence on the surface tension reduction as $P < 0.01$ at 99% confidence limit. Experimental runs designed according to the CCD of RSM at arbitrarily selected different levels shows that temperature (X_1) has the greatest linear positive ($P < 0.01$) effect on ST, followed by the positive linear positive effect of pH of the solid medium (X_2), effect of salinity (X_3), and crude oil content (X_4) ($P < 0.01$). Considering the different interactions, the positive effect of temperature × pH of solid medium, pH of solid medium×

salinity concentration, and temperature × salinity have the most effect on surface tension reduction ($P < 0.01$). These significant interactions mean that the temperature and pH of the medium predominantly effects the ST.

Optimization of experimental model for biosurfactant production

In order to interpret the significance of the four variables and to determine the interaction of each variables for maximum response, the experimental results were represented in the form of three-dimensional response surface curves (Figure. 6a-e). The maximum surface tension reduction is indicated by the surface confined to the smallest curve of the plot. It also reveals the optimum values of the process conditions for all parameters, and are: temperature, 35-40 ºC; pH of the solid medium, 6.8-8.5; salinity concentration; 48–55%; and crude oil content 1.5-2.5%.

The optimum values of the test variables were: pH of the medium 6.98; temperature 35.86 ºC; salinity concentration 49.57 %; and crude oil content 2.10 %. Further validation of the proposed model was performed by biosurfactant production experiments with the above mentioned optimal values. The predicted surface tension reduction was 27.80 mN/m, while the experimental result was 28.13 mN/m.

This provided the validation of model proposed in this study. From all the results obtained after optimization process, strain USTBb increased the biosurfactant production more than 3-folds (2.2 to 6.85 g/l) when compared with un-optimized conditions. Recently, it was reported about theenhanced biosurfactant production of 3.3 g/l from an indigenous strain of *Bacillus* mycoides under optimized growth conditions (Najafi et al., 2010).

Characterization of biosurfactant

The CMC is an important characteristic of a biosurfactant which is defined as the biosurfactant concentration requisite to form micelle (Macdonald *et al*., 1981). Figure. 7 represents the measurement of ST as a function of biosurfactant concentration. The CMC of the biosurfactant isolated from *Bacillus* sp. USTBb was 35 mg/l and corresponding surface tension was 28 mN/m. The result showed the high effectiveness of the biosurfactants as they could reduce the surface tension of distilled water from 72 to 28 mN/m. Nitschke and Pastore (19) observed a close results to that obtained in our present study. Moreover, the ability to reduce ST below 35 dyne/cm is one of the criteria used to select biosurfactant-producing microorganisms. Furthermore, there was an insignificant change in the ST up to the end of the cultivation as discussed in section 3.3. The behavior might be due to the attainment of CMC by the biosurfactant, beyond which the ST remained constant (Macdonald *et al*., 1981).

Figure. 8a depicts the FT-IR spectrum of the dried biosurfactant which showed broad stretching peaks at 3620 cm^{-1}, 1650 cm^{-1} and 1482 cm^{-1} representing the stretching modes for N-H, C=0 and C-N bond respectively and disclosed strong adsorption bands of peptides. This indicates that the biosurfactant produced by *Bacillus* sp. USTBb is a lipopeptide. The stretching peaks around 2781 cm^{-1}, 1250 cm^{-1} and 658 cm^{-1} attribute to the presence of hydrocarbon chain (C-H) position and sulphate group (S-O and C-O-S), respectively. These results were comparable with biosurfactant produced from other strains of *Bacillus* sp. (Thaniyavaran *et al*., 2006).

Table.1 Experimental range of four variables with specified codes at three levels in Central composite design (CCD) for biosurfactant production

		Level of variables in design		
Independent variable	Codes	-1	0	+1
Temperature (ºC)	X_1	25	35	45
pH	X_2	5	7	9
Salinity (%)	X_3	30	50	70
Crude oil content (%)	X_4	0.5	2	3.5

Table.2 Results of experimental design matrix for optimization biosurfactant production with respect to surface tension (mN m^{-1}) reduction by using Central composite design (CCD) in thirty trials.

Run	X_1	X_2	X_3	X_4	Surface tension
1	35	5	30	2	42 ± 1.24
2	45	7	30	2	40 ± 2.03
3	45	9	50	2	38 ± 1.91
4	35	9	50	3.5	44 ± 2.10
5	35	9	30	2	35 ± 1.65
6	35	7	50	2	28 ± 1.03
7	35	7	70	3.5	42 ± 1.36
8	35	7	50	2	28 ± 2.53
9	25	7	50	3.5	40 ± 2.44
10	35	7	30	3.5	33 ± 1.81
11	35	9	50	0.5	49 ± 1.52
12	25	5	50	2	51 ± 1.36
13	45	7	50	0.5	43 ± 1.78
14	25	7	30	2	45 ± 2.09
15	45	5	50	2	31 ± 1.60
16	35	5	50	0.5	38 ± 2.46
17	35	7	30	0.5	39 ± 2.13
18	25	9	50	2	32 ± 1.02
19	45	7	50	3.5	51 ± 1.24
20	35	9	70	2	48 ± 2.08
21	25	7	50	0.5	36 ± 1.69
22	35	5	50	3.5	34 ± 1.48
23	35	7	50	2	28 ± 2.31
24	45	7	70	2	52 ± 1.73
25	35	7	70	0.5	44 ± 1.41
26	35	5	70	2	46 ± 1.73
27	25	7	70	2	50 ± 2.64
28	45	9	50	0.5	39 ± 1.09
29	35	5	30	3.5	51 ± 2.23
30	25	7	50	0.5	36 ± 1.38

Table.3 Analysis of variance (ANOVA) and result of regression model of Central composite design (CCD) for biosurfactant production.

Source	SS	DF	Coefficient	*F*-value	*P*-value
Model	1625.92	14	-	175.2	0.0001
Constant	-	-	381.835	-	0.0001
Temperature (X_1)	8.019	3	8.76	20.17	0.0021
pH (X_2)	6.524	1	0.67	9.987	0.0045
Salinity (X_3)	63.098	1	-3.216	524.72	0.0032
Crude oil content (X_4)	12.791	1	-4.031	7.212	0.0162
Temperature (X_1) × Temperature (X_1)	10.12	1	2.818	27.345	0.0015
pH (X_2) × pH (X_2)	4.651	1	-1.085	15.065	0.0067
Salinity (X_3) × Salinity(X_3)	2.097	1	-0.026	6.982	0.0043
Crude oil content (X_4) × Crude oil content (X_4)	5.32	2	-1.026	1.309	0.0002
Temperature (X_1) × pH (X_2)	1.09	1	0.442	0.023	0.0022
Temperature (X_1)× Salinity(X_3)	3.876	1	1.87	0.164	0.0038
Temperature(X_1) × Crude oil content (X_4)	7.812	1	-0.297	1.734	0.009
pH (X_2)× Salinity (X_3)	11.871	2	0.561	0.345	0.0016
pH (X_2)× Crude oil content (X_4)	9.056	1	-0.331	3.321	0.0028
Salinity (X_3) × Crude oil content(X_4)	2.998	1	-0.066	0.961	0.0055
Residual	25.16				
Lack of fit	46.02	10		1.92	0.1083
Pure error	9.15	4	1.02		

Dev = 0.58; Adequate Precision = 30.7537; R^2 = 99.85%; R^2 (pred) = 99.05%; R^2 (adj) = 99.49%; SS, Sum of squares; DF, Degree of freedom; MS, Mean square.

Figure.1 Cells of *B. amyloliquefaciens* USTBb. (A) Gram staining of USTBb; (B) SEM photographs of USTBb

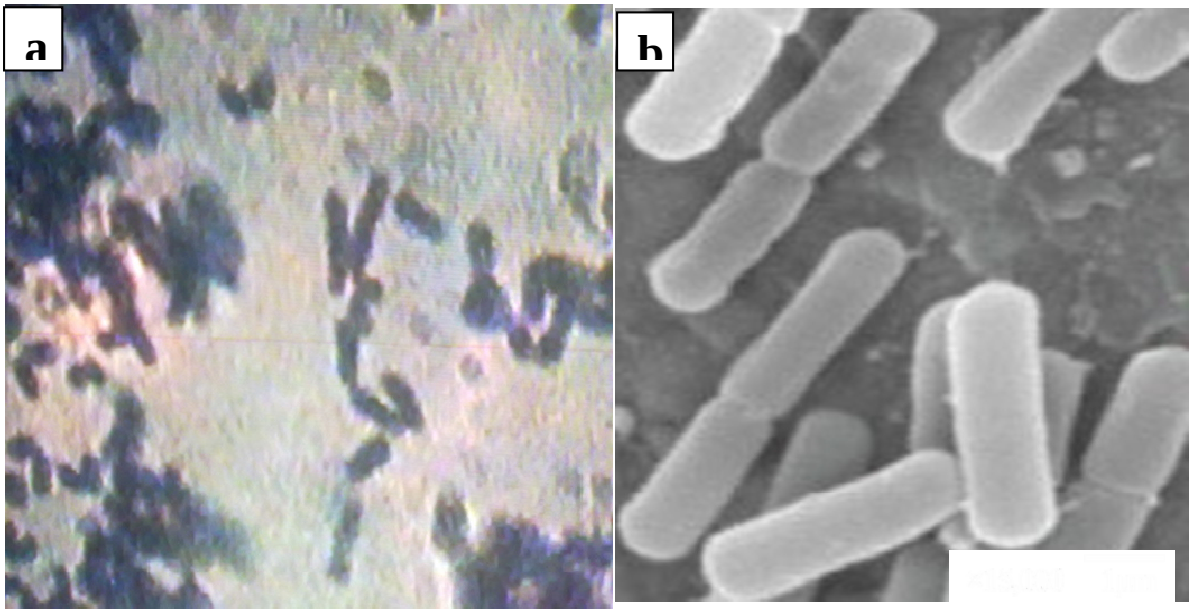


Figure.2 Phylogenetic neighbor-joining tree constructed by using partial *gyrA* sequences of Bootstrap values (expressed as percentages of 1,000 replications). The scale bar corresponds to 0.005-estimated nucleotide substitution per sequence position. Accession numbers are given in parentheses

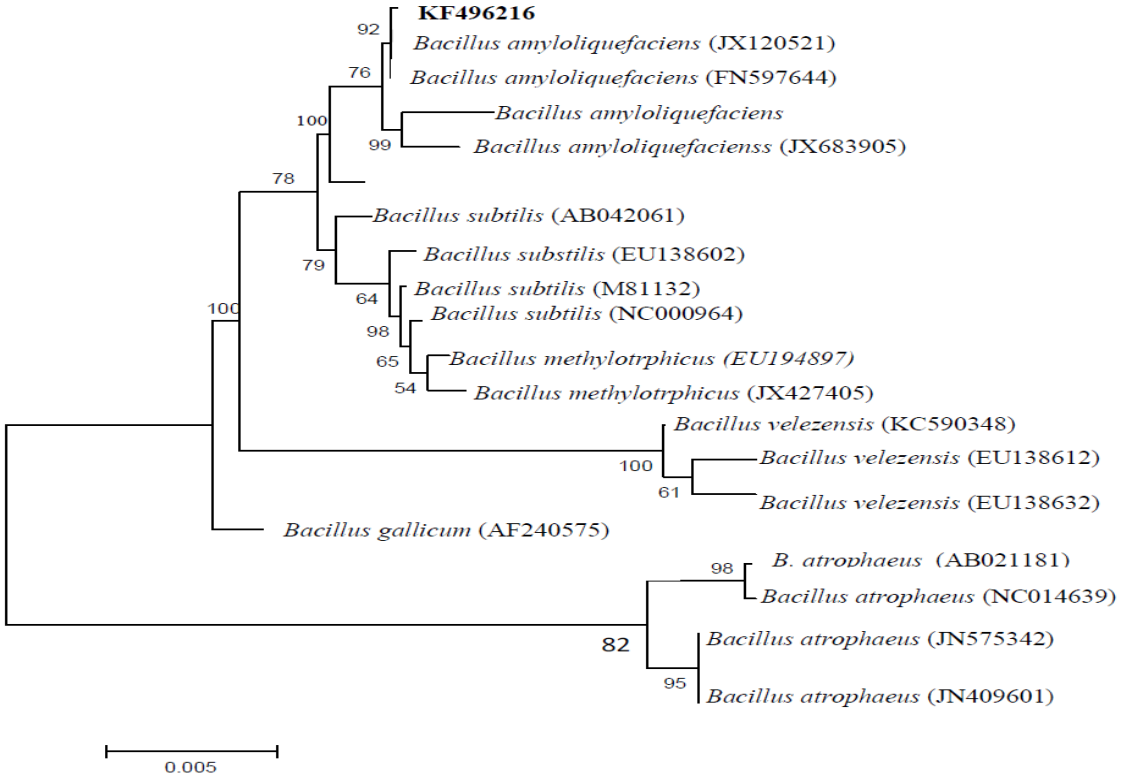


Figure.3 Oil-displacement test. (a) Negative control (Milli-Q water), and (b) zone of clear and (c) Emulsifying activity of cell free supernatant of *B. amyloliquefaciens* USTBb against paraffin oil exhibiting biosurfactant production displacement formed by cell-free supernatant of *B.amyloliquefaciens* USTBb

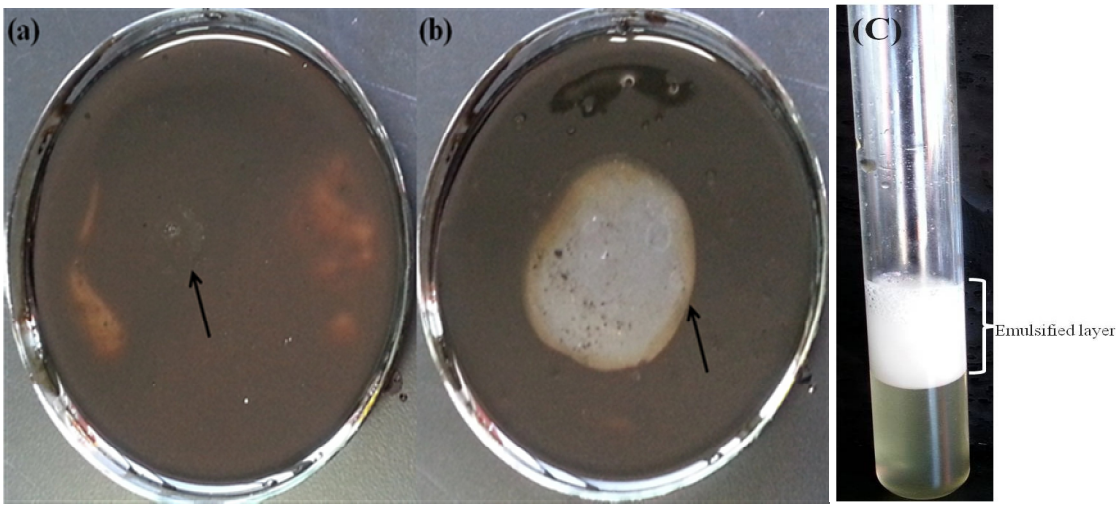


Figure.4 Growth kinetics (OD) and biosurfactant production (surface tension reduction/emulsification index) versus time by *B. amyloliquefaciens* USTBb. Error bars represent the standard deviation of three independent measurements.

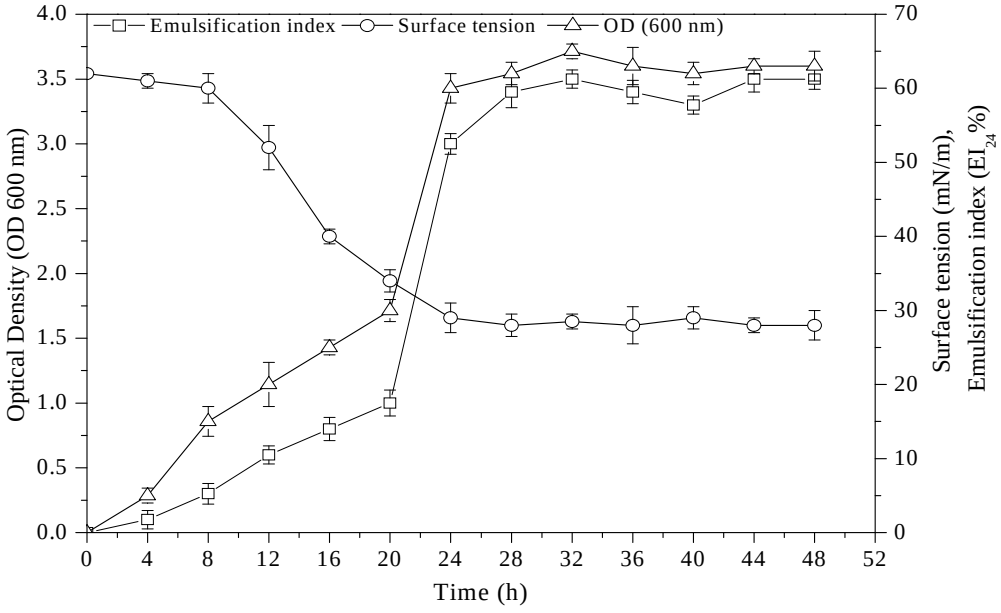


Figure.5 Plot of predicted verus actual biosurfactant concentration (surface tension reduction) for *B. amyloliquefaciens* USTBb

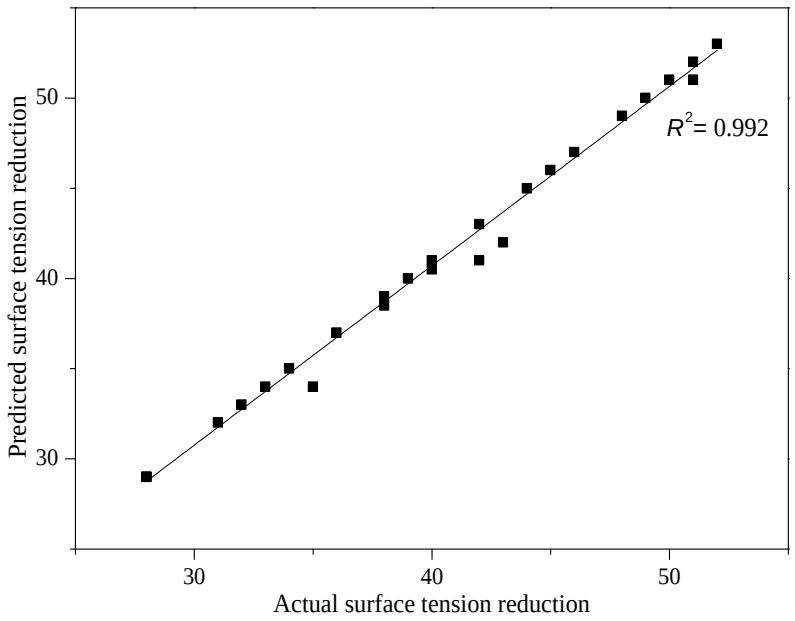


Figure.8b represents the EDX spectrum, which revealed the atomic and weight percentage of five elements (C, O, Na, S and Cl) present in the biosurfactant produced by *Bacillus* sp. USTBb. The distribution of cation (Na) element suggested its link with the negative charge of the sulphate group (Jain *et al*., 2012). Further, the anionic nature of the extracted biosurfactant was proved by the presence of carboxyl and acetyl functional groups in the biosurfactant.

In this work, *B. amyloliquefaciens* USTBb have been successfully isolated and identified. Interestingly, the isolate was found to be a potent biosurfactant-producer. The strain USTBb exhibited biosurfactant production at wide range of pH and temperature. Also, this strain showed high emulsification activity and a potential ability to reduce surface tension of the culture medium to 28 mN/m. The central-composite design of RSM provided a feasible and effective tool for optimizing the growth parameters for enhanced biosurfactant production. These favourable properties of USTBb biosurfactant, suggested its possible use as an efficient tool in bioremediation of hydrocarbon contamination and enhanced oil recovery (EOR). And consequently, merit for advance investigate.

Figure.6 Three-dimensional response plots for the minimum surface tension (maximum biosurfactant production). Surface tension reduction as a function of (a) pH and temperature; (b) pH and salinity; (c) pH and crude oil content; (d) temperature and salinity; and (e) temperature and crude oil content (carbon source).

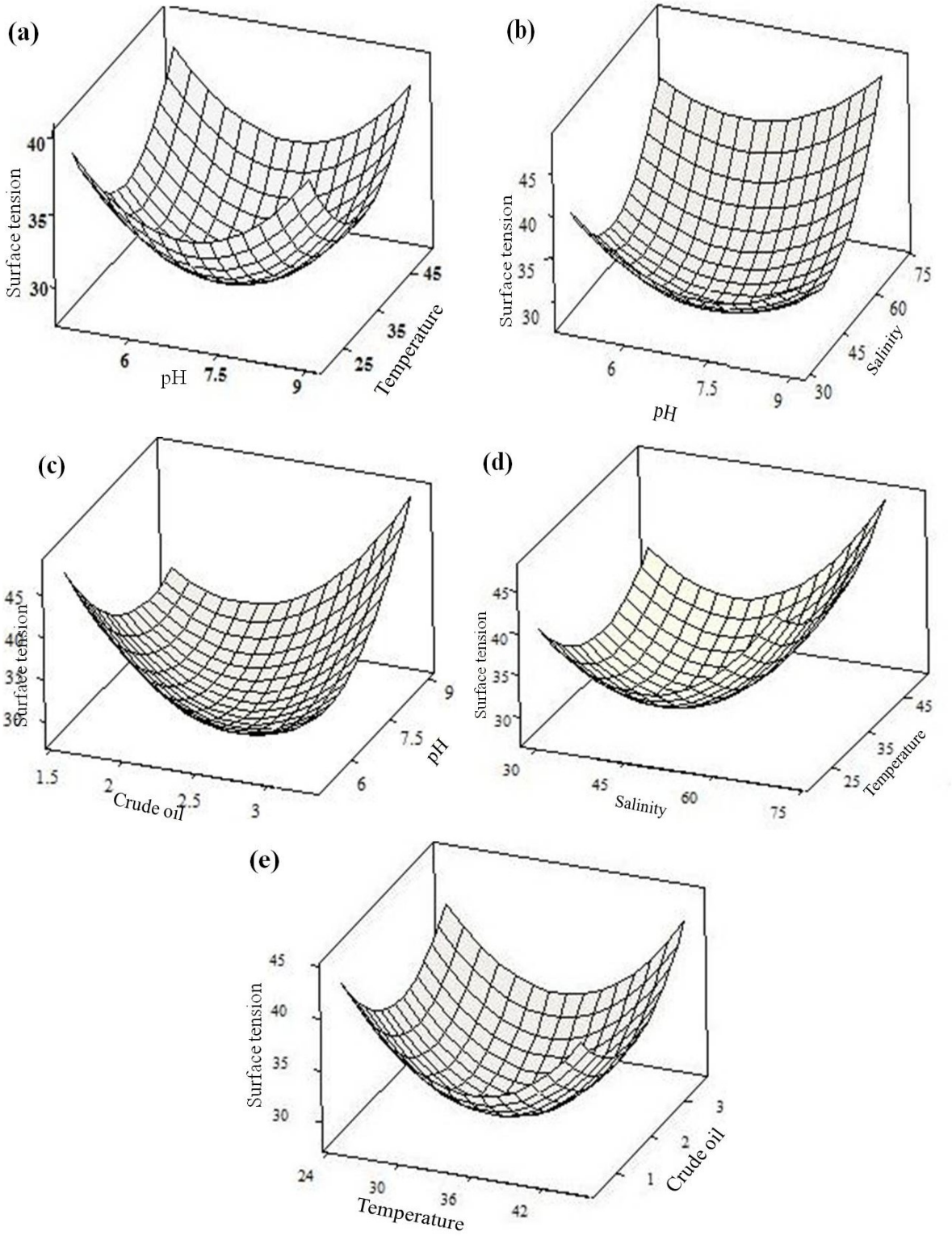


Figure.7 Effect of increasing the biosurfactant concentrations on surface tension. Error bars represent the standard deviation of three independent measurements

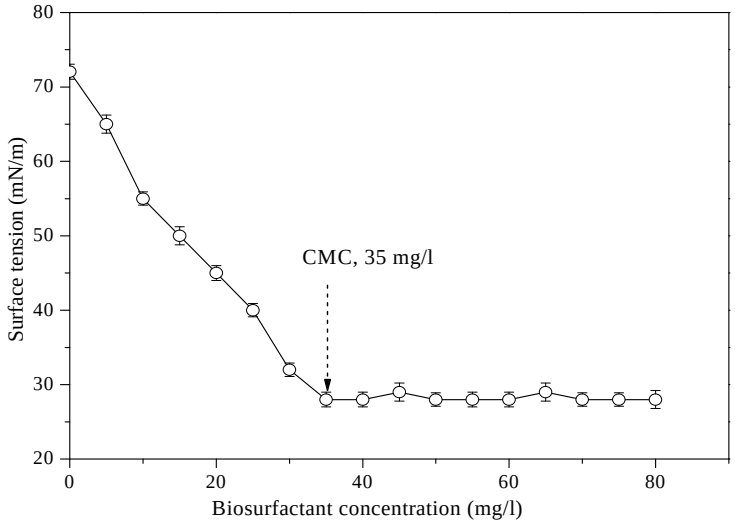
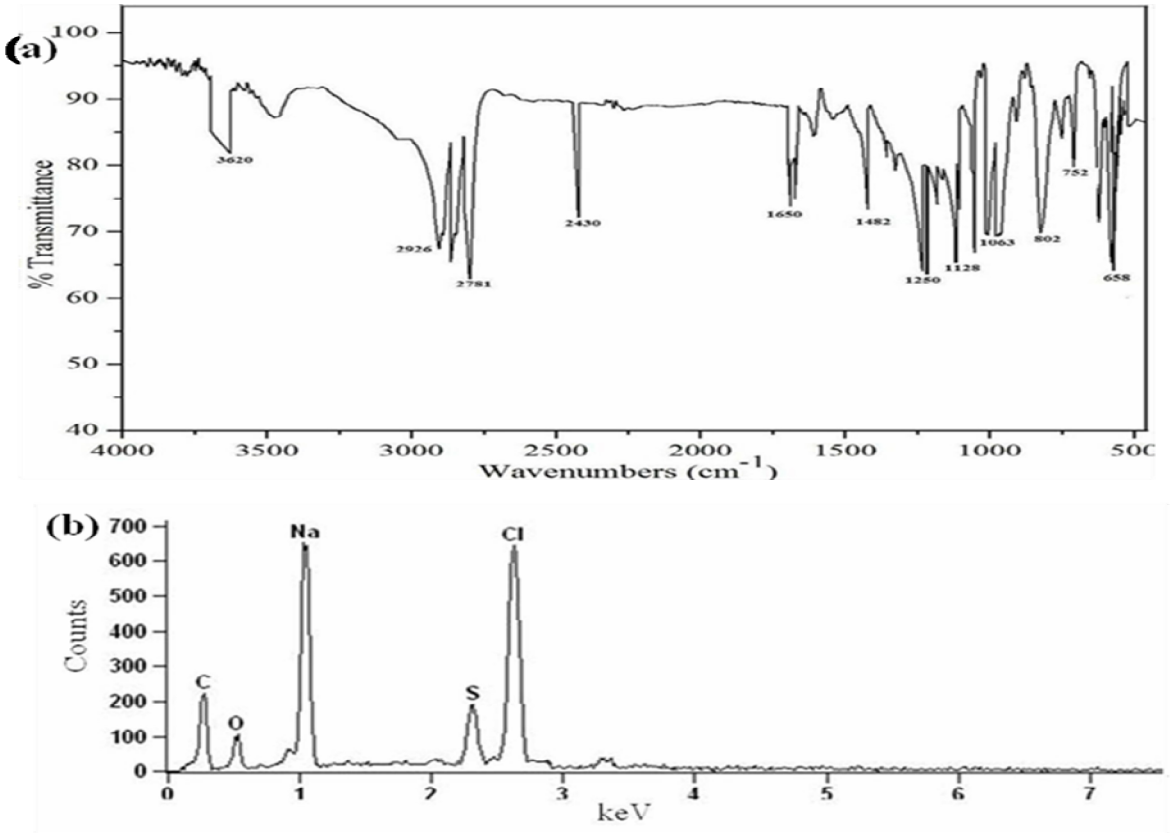


Figure.8 Characterization of the dried biosurfactant from *Bacillus* sp. USTBb grown on mineral salt medium (MSM). (a) FT-IR spectrum profile and (b) EDX spectrum



Acknowledgement

This work is supported in part by grants from the International Joint Key Project from the Chinese Ministry of Science and Technology (2010DFB23160), International Joint Key Project from the National Natural Science Foundation of China (40920134003), National Natural Science Foundation of China (41273092), and National Outstanding Youth Research Foundation of China (40925010), the Overseas, Hong Kong and Macau Young Scholars Collaborative Research Fund (41328005).

References

Buchanan, R.E. 1948. How bacteria are named and identified. In: Breed, Murray, Hitchens (eds) Bergey's Manual of Determinative Bacteriology, 6th edn.. The Williams & Wilkins Co., Baltimore, pp 39-38.

Chandankere, R., Jun, Y., Choi , M.M.F., Masakorala, K., and Chan, Y. 2013. An efficient biosurfactant-producing and crude-oil emulsifying bacterium *Bacillus methylotrophicus* USTBa isolated from petroleum reservoir. Biochem. Eng. J. 74: 46–53.

De Clerck, E., Vanhoutte, T., Hebb, T., Geerinck, J., Devos, J., and De, P. 2004. Isolation, Characterization, and Identification of Bacterial Contaminants in Semifinal Gelatin Extracts. Appl. Environ. Microbiol. 70: 3664–3672.

Ebrahimi, A., Tashi, N., Lotfalian, S. 2012. Isolation of biosurfactant producing bacteria from oily skin areas of small animals. Jundishapour J. Microbiol .5: 401-404.

Henkela, M., Müllera, M.M., Küglera, J.H., Lovagliob, R.B., Contierob, J., Syldatka, C., and Hausmannc, R. 2012. Rhamnolipids as biosurfactants from renewable resources: Concepts for next-generation rhamnolipid production. Process. Biochem 4: 1207-1219.

Jain, R.M., Mody, K., Mishra, A., and Jha, B. 2012. Physicochemical characterization of biosurfactant and its potential to remove oil from soil and cotton cloth. Carbohydr Polym. 89: 1110–1116.

Javaheri, M., Jenneman, G.E., McInerney, M.J., and Knapp, R.M. 1985. Anaerobic Production of a Biosurfactant by *Bacillus licheniformis* JF-2. Appl. Environ. Microbiol. 50: 698-700.

Joo, M.H., Hur, S.H., Hanand, Y.S., and Kim, J.Y. 2007. Isolation, Identification, and Characterization of *Bacillus* strains from the Traditional Korean Soybean-fermented Food, Chungkookjang. J. Appl. Biol. Chem .50: 202-210.

Karanth, N.G., Deo, P.G., and Veenanadig, N.K. 1999. Microbial production of biosurfactants and their importance. Curr. Sci. 77: 116-123.

Kebria, D.Y, Khodadadi, A., Ganjidoust, H., Badkoubi, A., and Amoozegar, M.A. 2009. Isolation and characterization of a novel native *Bacillus* strain capable of degrading diesel fuel. Int J Environ. Sci. 6: 435-442.

Lakshmipathy , T.D., Prasad, A.S., and Kannabiran, K. 2010. Production of Biosurfactant and Heavy Metal Resistance Activity of *Streptomyces Sp*. VITDDK3-a Novel Halo Tolerant *Actinomycetes* Isolated from Saltpan Soil. Adv. Biol. Res. 4: 108-115.

Macdonald, C.R., Cooper, D.G., Zajic, J.E. 1981. Surface-Active Lipids from *Nocardia erythropolis* Grown on Hydrocarbons. Appl. Environ. Microbiol. 41: 117-123.

Mulligan, C.N. 2005. Environmental applications for biosurfactants. Environ. Pollut .133: 183-98.

Najafi, A.R., Rahimpour, M.R., Jahanmiri, A.H., Roostaazad, R., Arabian, D., and Ghobadi, Z. 2010. Enhancing biosurfactant production from an indigenous strain of *Bacillus mycoides* by optimizing the growth conditions using a response surface methodology. Chem. Eng. J. 163: 188–194.

Nitschke, M., and Pastore, G.M. 2006. Production and properties of a surfactant obtained from Bacillus *subtilis* grown on cassava wastewater. Bioresour. Technol. 97: 336-341.

Paraszkiewicz, K., Kanwal, A., Długonski, J. 2002. Emulsifier production by steroid transforming filamentous fungus *Curvularia lunata*. Growth and product characterization. J. Biotechnol. 92: 287–294.

Saikia, R.R., Deka, S., Deka, M., and Sarma, H. 2012. Optimization of environmental factors for improved production of rhamnolipid biosurfactant by *Pseudomonas aeruginosa* RS29 on glycerol. J. Basic. Microbiol. 52: 446-457.

Shavandi, M., Mohebali, G., Haddadi, A., Shakarami, H., and Nuhi, A. 2011. Emulsification potential of a newly isolated biosurfactant-producing bacterium, *Rhodococcus* sp. strain TA6. Colloids Surf B: Biointerfaces. 82: 477–482.

Singh, S.P., Bharali, P., and Konwar, B.K. 2013. Optimization of Nutrient Requirements and Culture Conditions for the Production of Rhamnolipid from *Pseudomonas aeruginosa* (MTCC 7815) using *Mesua ferrea* Seed Oil. Indian. J. Micrbiol. 53: 467-476.

Thaniyavaran, J., Roongsawang N., Kameyama, T., Haruki, M., Imanaka, T., Morikawa, M., and Kanaya, S. 2006. Production and Characterization of Biosurfactant from *Bacillus licheniformis* F2.2. Biosci. Biotechnol. Biochem. 67: 1239-1244.

Vaz, D.A., Gudiña, E.J., Alameda, E.J., Teixeira, J.A., and Rodrigues, L.R. 2012. Performance of a biosurfactant produced by a Bacillus subtilis strain isolated from crude oil samples as compared to commercial chemical surfactants. Colloids Surf. B: Biointerface.s 89: 167-174.