Biosurfactant production by a newly isolated soft coral-associated marine *Bacillus* sp.E34: Statistical optimization and characterization

Mona E. M. Mabrouk[1*], Eman M. Youssif[2], Soraya A. Sabry[2]

[1.] Botany Department, Faculty of Science, Damanhour University, Damanhour, Egypt
[2.] Botany and Microbiology Department, Faculty of Science, Alexandria University, Alexandria, Egypt
[*] Corresponding Author: Mona E.M. Mabrouk, E-mail: mona_mabrouk_eg@hotmail.com

Abstract Marine biosurfactant-producing bacteria were isolated from the soft coral *Sarchophyton glaucum* collected from Red sea. The main criteria used for screening of biosurfactant producers were haemolytic activity, drop-collapse, oil displacement and emulsification index. Based on phenotypic characterization and analysis of 16S rDNA sequencing the most potent isolate was identified as *Bacillus* sp.E34. To the best of our knowledge, this work is one of the early attempts to isolate and characterize biosurfactant-producing bacteria from soft coarls. Molasses enhanced biosurfactant production and maximum production occurred at 96 h incubation. The Plackett–Burman design was implemented to screen the medium components that significantly influence the production. The biosurfactant was stable over a wide range of pHs, temperatures and salinity, and had a good degree of emulsification with different hydrophobic substrates. Based on FT-IR analysis it was identified as glycolipopeptide. The biosurfactant is a potential candidate for bioremediation of hydrocarbon-contaminated sites as well as in other industrial applications.

[Mona E. M. Mabrouk, Eman M. Youssif, Soraya A. Sabry. **Biosurfactant production by a newly isolated soft coral-associated marine *Bacillus* sp.E34: Statistical optimization and characterization.** *Life Sci. J* 2014; 11(10):756-768] (ISSN: 1097-8135). http://www.lifesciencesite.com. 123

Keywords: *Sarchophyton glaucum*; biosurfactant; *Bacillus* sp.E34; optimization; Plackett–Burman; characterization

1. Introduction

Biosurfactants are a unique class and structurally diverse group of surface-active amphipathic natural products produced by microorganisms such as bacteria, yeast and fungi. Microbial surfactants have potential applications in agriculture, cosmetic, food, pharmaceutical and environmental industries (Muthusamy et al., 2008) and has signifcantly increased recently not only due to their effectiveness at extreme conditions of temperature, pH and salinity but also because of their lower toxicity, better biodegradable nature, high foaming capability, better environmental compatibility in contrast to chemically synthesized surfactants (Onwosi and Odibo, 2013), and can be produced from renewable and cheaper substrates (Oliveira et al.,2013; Noparat et al.,2014).

One of the important points in the biotechnological process is to obtain maximum metabolite production with a low-cost substrate. This leads to the greater possibility for economical production and reduced pollution caused by those wastes (Saimmai et al., 2011). The promising future of biosurfactants appears to be specifically dependent upon the reduction of production costs by using abundant and low cost raw materials and the optimization of the operational cultivation conditions in order to achieve high yields (Dubey et al., 2012). Application of factorial design is a common practice in biotechnology for the optimization of media components and culture conditions (Mabrouk et al., 2012, 2013). The use of statistical optimization strategy has been successfully applied in the optimization of a medium for biosurfactant production (Onwosi and Odibo, 2013; Abbasi et al., 2013).

Marine microorganisms are a rich source of natural products with potential applications in drug discovery, environmental remediation, and the development of new resources for industrial processes (Debnath et al., 2007; Liu et al., 2010). Most of the novel marine natural compounds were isolated from indigenous or symbiotic microorganisms (Blunt et al., 2010). Although several publications (Kiran et al., 2009, 2010 a, b,c) reported the production of biosurfactant from marine sponges, up to our best knowledge no previous publications dealt with isolation and characterization of biosurfactant from coral-associated marine bacteria. Therefore, in the present study a coral-associated marine *Bacillus* sp. E34 was isolated and screened as a potent biosurfactant producer. Moreover, evaluation of low cost carbon sources and optimization of nutrient parameters for large-scale production were studied, in addition to characterization of the produced biosurfactant.

2. Materials and methods

Sample collection and isolation of coral –associated bacteria

The soft coral *Sarchophyton glaucum*was collected from Red Sea, Egypt by SCUBA diving at 10–15 m depth. For the isolation of associated bacteria, coral tissue (1 cm^3) was excised using a

sterile scissors. The excised portion was thoroughly washed three times with sterile sea water then the tissue was homogenized with saline. The resultant homogenate was serially diluted with sterile sea water and preincubated at 40°C for 1 h for the activation of dormant cells. An aliquot of 100µl was plated on Luria Bertani medium (LB) (Bertani, 2004) for isolation of marine bacteria. The inoculated plates were incubated at 30°C for 3 days. Colonies representing different morphological characteristics were picked, purified, maintained on LB agar slants and screened for biosurfactant production.

Screening for a potential biosurfactant producing strain

Different biosurfactant screening methods were carried to select the most potent isolate. The methods adopted were (a) Haemolytic activity on blood agar plates containing 5% (v/v) human blood (Walter et al., 2010), each strain was punctuated onto blood agar plates using sterile toothpicks and plates were visually inspected for zones of haemolysis around the colonies, after 48 h incubation at 37°C, colonies surrounded by a clear zone were presumed to produce biosurfactants (Ferhat *et al*., 2011), (b) Drop collapse test by adding mineral oil in 96-well microtitre plates (Khopade et al., 2012), the positive result was shown as drop collapse,(c) Oil spreading technique using crude oil (Kiran et al., 2010 b), and (d) Emulsification activity (Luna et al., 2013). The emulsification index (EI_{24} %) provides a rapid and reliable measure of the quantity of biosurfactant (Pal et al., 2009). Kerosene was added to cell free supernatant (CFS) in a ratio of 1:1 and vortexed vigorously for 2 min. After 24 h of incubation at room temperature, the height of the emulsified layer was measured. The emulsification index (EI_{24}) was calculated by using the following equation:

$$EI_{24}\ \% = \frac{\text{The height of emulsion layer}}{\text{The height of total solution}} \times 100$$

All the assays were performed in triplicate. A control sample was prepared by using 1 ml of culture medium before inoculation instead of the cell-free supernatant(CFS).

Identifcation of the efficient biosurfactant producer

Strain E34 was identified morphologically and physiologically according to Bergey´s Manual of Determinative Bacteriology (Holt et al. 1994). Identification was confirmed with 16S rDNA sequence analysis. 16S rDNA gene was amplified by polymerase chain reaction (PCR) using primers F(5'AGAGTTTGATCMTGGCTCAG3') and R (5'TACGGYTACCTTGTTACGACTT3'). DNA sequence was obtained using an ABI PRISM 377 automated DNA Sequencer (Perkin Elmer).The partial 16S rDNA gene sequence was uploaded to the National Center for Biotechnology Information (NCBI) database using BLASTN (http://www.ncbi.nlm.nih.gov/blast/; version 2.0) to search for 16S rDNA gene sequence homology, followed by the sequences alignment using Clustal W program (http://www.ebi.ac.uk/clustalw). A phylogenetic tree was constructed with PHYLIP package, version 3.6 (phylogeny inference package).

Culture condition

Biosurfactant production was performed in 250 ml Erlenmeyer flasks each containing 50 ml of sterilized medium containing (g/l): glucose, 20; $(NH_4)_2SO_4$, 3; tryptone, 10; K_2HPO_4, 2; KH_2PO_4, 0.2; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.1; yeast extract, 5; NaCl, 25. pH was adjusted to 7±0.2 with 1N NaOH or 1N HCl. Each flask was inoculated with 2% (v/v) of seed culture prepared in LB media of $OD_{550} \approx 1$. Fermentation flasks were incubated in a rotary shaker (160 rpm) at 37°C. Samples were collected aseptically at different time intervals for determination of bacterial growth (measuring O.D. at 550nm) and biosurfactant production. The measuring of emulsification index was used as the criterion of biosurfactant production (Luna et al., 2013).

Effect of carbon source

To economize biosurfactant production, glucose was replaced with 2% (w/v) of various hydrophilic carbon sources (including lactose, sucrose, fructose, glycerol and maltose), or 2%(v/v) of various hydrophobic carbon sources (including corn oil, sunflower oil, motor oil, olive oil soy bean oil and kerosene). Also, different low-cost substrates were tested at concentration of 2% (w/v) such as oilcakes of groundnut, sunflower, olive, soybean, cotton seed, in addition to rice straw, wheat bran, sugarcane bagasse, sugarcane molasses, potato peel and cheese whey.

Effect of pH

To study the effect of pH, the initial pH of the production media was adjusted at (5-10) with 1 N HCl or 1 N NaOH. The cultures were incubated under the same conditions described previously.

Experimental design and optimization of biosurfactant production

Optimum conditions for biosurfactant production by *Bacillus* sp. E34 were determined by Plackett-Burman design (Plackett and Burman ,1946). In this experiment, ten independent variables were screened in twelve combination organized according to the Plackett-Burman design matrix. Each variable was represented at two levels, high (+) and low (−), in 8 trials. The ranges of variables investigated in this study are shown in **Table 2**.Three centerpoint (0) replications were performed for the design atthe mid-level of each variable.Each row represents a trial, while each column represents independent variable. All emulsification indexes (EI_{24}%) were performed in

triplicate and treated as the response. Plackett–Burman experimental design based on the first-order model:

$$Y = \beta_0 + \sum \beta_i X_i$$

Where Y is the response (EI_{24} %), β_0 is the model intercept and β_i is the linear coefficient, and X_i is the independent variable. The main effect of each variable can be calculated using the following standard equation:

$$\text{Main effect} = [\Sigma R(H) - \Sigma R(L)]/N$$

Where R(H) and R(L) are observations of trials where the independent variable was present in high and low concentrations, respectively, and N is the number of the trials divided by 2. A main effect figure with a positive sign indicates that the high concentration of this variable is near to optimum and a negative sign indicates that the low concentration of this variable is near to optimum. Statistica"software (version 6.0, StatSoft, USA) was used for the experiment design and all statistical analyses. The variables with confidence levels above 95% were considered significantly influencing biosurfactant production.

Extraction and recovery of biosurfactant

The culture broth was centrifuged (12,000 rpm, 4°C, 15 min) to remove the cells and then sterilized with millipore membrane filter (Sartorius; Goettingen, Germany). The clear sterile supernatant served as the source of crude biosurfactant. The biosurfactant was recovered by cold acetone precipitation as described by Abouseoud et al.(2008).Three volumes of chilled acetone were added to CFS and allowed to stand for 12 h at 4°C. The precipitate was collected by centrifugation and evaporated to dryness to remove residual acetone. The yield of biosurfactant was gravimetrically estimated and expressed as g/L (Ismail et al., 2013).

Functional characterization of biosurfactant

Substrate specificity

The ability of the biosurfactant to emulsify liquid hydrocarbons was examined. Different hydrophobic phases were used instead of kerosene such as vegetable oils (soybean, olive, corn, sesame, flaxseed, mustard, sunflower and frying oil) and hydrocarbons (paraffin oil, motor oil, mineral oil, benzene, crude oil, xylene and toluene). Surface tension (ST) was measured using a digital surface tensiometer (TD1 LAUDA, Germany) working on the principle of the Du Nuoy ring method (Ismail et al., 2013). Foaming ability was determined (Abouseoud et al., 2008).

Stability

For thermal stability, the cell free broth was maintained at constant temperatures in the range 30 - 121 ºC for 30 min and then cooled to room temperature, before measuring the emulsification activity. For pH stability, the cell free supernatant was adjusted to various pHs from 4 to 11 with 1 N HCl or 1 N NaOH (Lotfabad et al., 2009). The emulsifying indexes were measured after fifteen minutes. Different concentrations of NaCl , (1–25%,w/v) were added to CFS and EI_{24} was measured after 30 min at 30°C±1(Aparna et al., 2012). Experiments were performed in triplicate.

Structural characterization

Chemical tests

For this purpose, the following reactions were carried out; cetyl trimethyl ammonium bromide (CTAB)/ methylene-blue agar test (Pradhan et al., 2014), Biuret test (Jamal et al., 2012), Ninhydrin reaction (Zhang et al., 2012) and phosphate test (Okpokwasili and Ibiene, 2006).

Biochemical analysis

Carbohydrate content was determined by the phenol–sulfuric acid method (Dubois et al., 1956) using D-glucose as a standard. Protein content was measured (Lowry et al.1951) standardized with bovine serum albumin and lipid content was determined according to method described by Aparna et al.(2012).

Fourier transform infrared spectroscopy (FTIR) analysis

Fourier transform infrared spectroscopy (FTIR) is most useful for identifying types of functional groups. FTIR spectrum of the dried biosurfactant was analyzed using a Bruker Tensor 37 FTIR, (Germany) spectrometer, equipped with a mercury–cadmium–telluride (MCT) detector cooled with liquid N_2 in wave number range of 4000–400 cm^{-1}. The analysis of IR spectra was carried out by using OPUS 3.1 (Bruker Optics) software, according to the method described by Donio et al. (2013).

Application of biosurfactant in removal of hydrocarbon from sand

The potential application of the biosurfactant in MEOR was evaluated using the 'sand pack column' [Abu-Ruwaida et al. (1991). Glass columns were packed with 100 g of acid-washed dry sand. Each column was saturated with 50 ml hydrocarbon (kerosene) and allowed to age for 24 h. The potential of the isolated surfactant for hydrocarbon recovery was estimated by adding 50 ml of the cell free supernatant in the column. The columns were incubated at 30°C for 24 h. Afterwards, the kerosene released was recovered from the bottom and its volume was measured (Kiran et al., 2009). Control assays were performed using distilled water at same conditions. All experiments were carried out in triplicate.

3. Results and discussion

Screening of biosurfactant producing marine bacteria

Marine microorganisms are good candidates for environmental and industrial purposes (Liu et al.,

2010). In this report, thirty-five isolates were isolated from the soft coarl *Sarchophyton glaucum* and screened for biosurfactant production. Among the different screening tests, isolate E34 showed highest emulsification index and highest oil displacement in addition to positive reactions towards the different methods (Table 1, Figure 1). On the basis of these observations, the present report focused on E34 as a potent biosurfactant producer.

Table 1. Biosurfactant screening by selected strains isolated from the soft coral *Sarchophyton glaucum*

Strains	Screening method			
	Haemolytic activity [a]	Drop collapse test	Oil displacement area (cm^2)	Emulsification index EI_{24} %
E1	+	-	30.2	30
E9	++++	+	49.7	45
E13	++++	-	43	50
E17	+	+	16	35
E18	++	-	28.4	40
E22	+++	+	45.6	45
E23	++	+	22.4	40
E34	++++	+	53.3	55

[a] Diameter of clear zone of haemolysis test; '+':0.3-0.7 cm, '++': 0.8-1.4 cm, '+++': 1.5-1.9 cm, '++++': 2-2.5 cm.

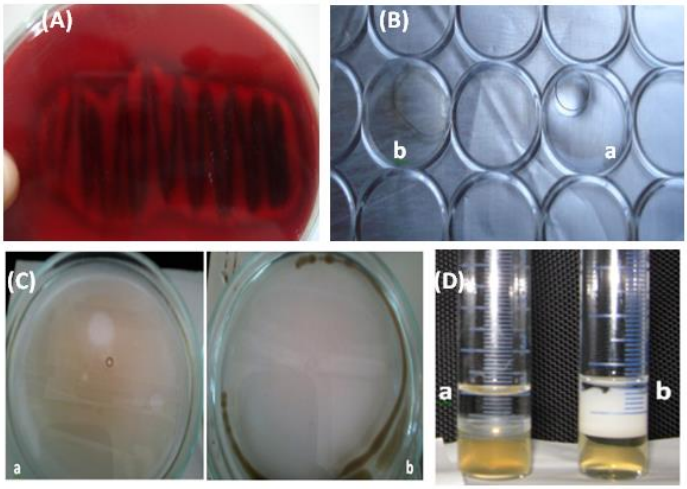

Figure 1. (A) Haemolytic activity of E34 strain on blood agar within 24 h of incubation at room temperature. (B) Drop collapse test: (a) Unculture media (control), not collapsed. (b) E34 supernatant on the oily surface, collapsed. (C) Oil displacment test: (a) Culture media alone (control) (b) the spreading of E34 biosurfactant on oil surface layer. (D) Emulsification index test: (a) Uncultured LB medium (b) Emulsion formation of E34 biosurfactant.

Identification of bacterial isolate

Cells of E34 were endospore forming, thin straight rods occurring singly. Colonies were mucoid, round and creamy on LB plates. Positive with oxidase, catalase and nitrate reduction. Hydrolyzed casein, starch and lipid and utilized citrate.

Partial sequencing of the 1008bp of 16S rDNA of the bacterial strain was analyzed with previously recorded sequences using BLASTN analysis; the 16S rDNA sequence showed 99% sequence homology to several species of the genus *Bacillus* (Figure 2). Hence, the strain was identified as *Bacillus* sp. E34.The 16S rDNA sequence was submitted in the GenBank database under accession number KF555377.

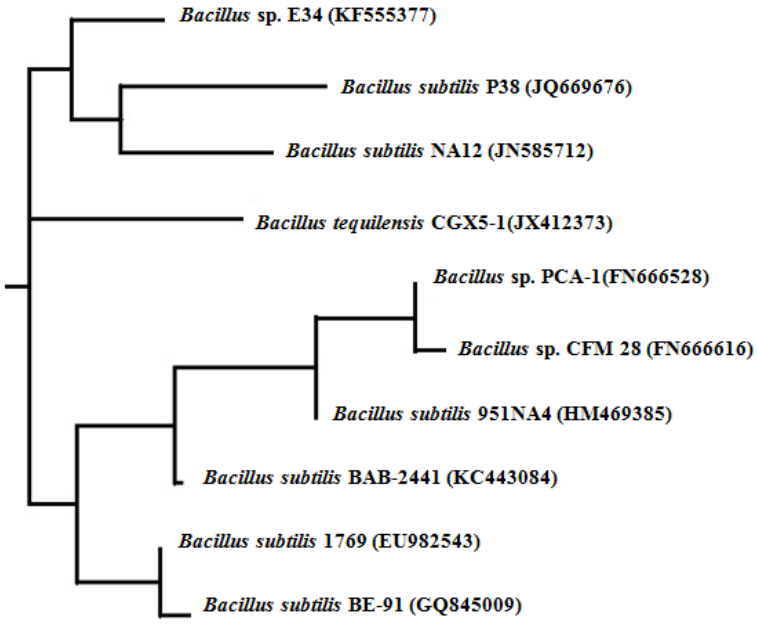

Figure 2. Phylogenetic tree based on 16S rDNA gene sequencing, and reference sequences extracted from the GenBank Database, showing the phylogenetic relationship of E34 within representative species of the genus *Bacillus.* The GenBank accession numbers for the 16S rDNA sequences are given in parentheses after the strain.

Kinetics of biosurfactant production

Growth curve (Figure 3) indicates a parallel relationship between biosurfactant production expressed as EI_{24}% and bacterial growth (OD550nm), suggesting that the production is growth associated. Most extracellularly secreted biosurfactants are growth associated (Zheng et al., 2012; Ismail et al., 2013). In parallel, biosurfactant production commenced at about 24 h (EI_{24} 10%), i.e., during the exponential phase, indicating its accumulation during growth phase and progressively increased. Production and accumulation of biosurfactant during the period between the logarithm and stationary phases have already been reported as commencement of biosurfactant production by *B. subtilis* BS5 (Abdel-Mawgoud et al., 2008); *B. subtilis* PTCC 1696 (Ghojavand et al., 2008); *B.* sp. I-15 (Ismail et al., 2013) and *B.mycoides* (Najafi et al., 2010).

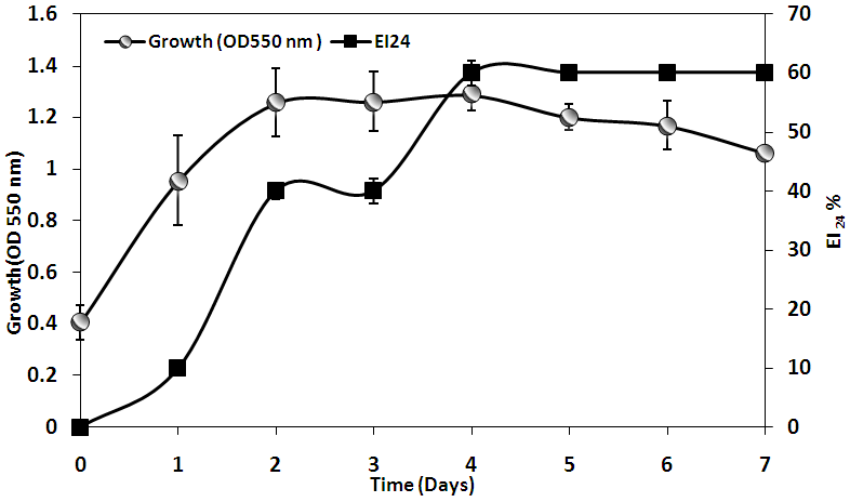

Figure 3. Growth and emulsification index of *Bacillus* sp. E34, grown at 37°C with shaking at 160 rpm with glucose as a carbon source.

Effect of different carbon sources

The carbon source has been reported in literature as a vital limiting factor in biosurfactant production process (Pal et al., 2009). Therefore, for selecting the optimal carbon source, the production medium was supplemented with 2% (w/v or v/v) of different carbon sources. Data in Figure 4 depict that sugar cane molasses supported the production of a biosurfactant with emulsification index (60%) comparable to simple sugars. Whereas, vegetable oils supported lower EI_{24}% compared to simple sugars. Moreover, no emulsification indeces were observed in supernatants of the bacterium grown on olive oil, corn oil, motor oil or kerosene. Molasses has already been reported for biosurfactant production by *Bacillus* spp. (Joshi et al., 2008; Saimmai et al., 2011; Al-Bahry et al., 2013). The principal reasons for the wide spread use of molasses as substrate is its low price compared to other conventional sugar sources like sucrose or glucose. Indeed, it is also due to its high total sugar contents mainly sucrose and its content of minerals, organic compounds, and vitamins, which are valuable for the fermentation process as it is a byproduct from processing of sugar-rich crops (Saimmai et al., 2011).

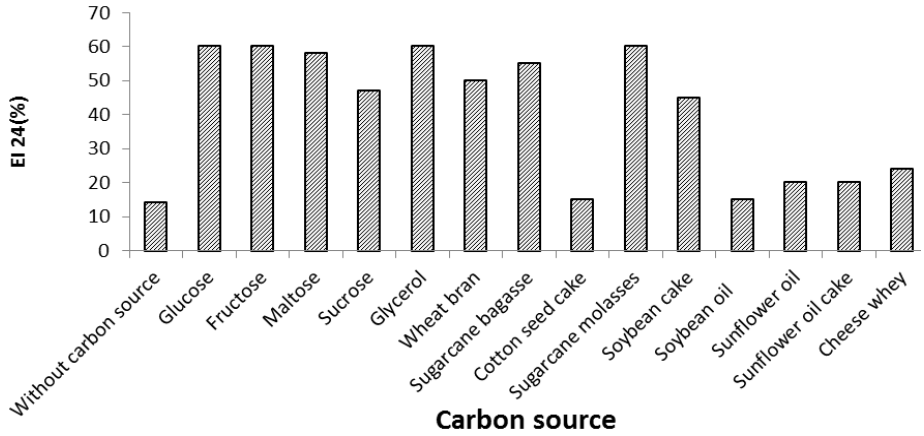

Figure 4. Effect of different carbon sources on emulsification activity of culture filtrate of *Bacillus* sp. E34 after 96 h incubation at 37°C.

Effect of pH

The important characteristics of most organisms are their strong dependence on the pH for cell growth and production of metabolites. In our study, biosurfactant production maintained nearly constant values over pH 6-9 (Figure 5). Similar observation was reported by Najafi et al. (2010). Thus, it can be inferred that *Bacillus* sp. E34 excreted biosurfactant more effectively under neutral and alkalescent conditions.

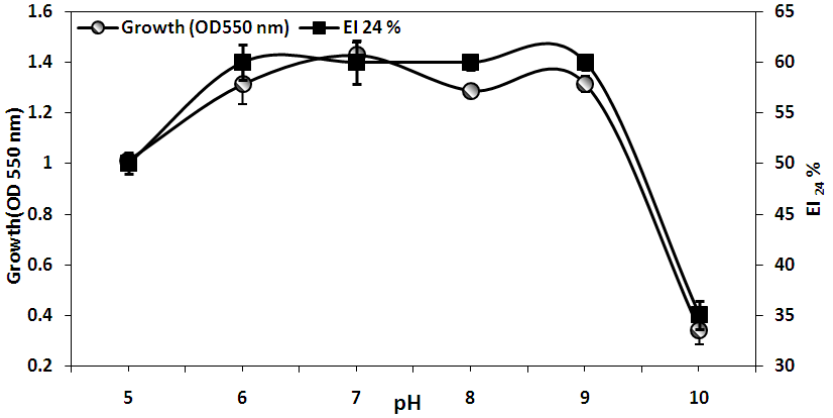

Figure 5. Effect of different pHs of the culture media on emulsification activity of culture filtrate of *Bacillus* sp. E34 incubated at 37°C for 96 h.

Optimization of biosurfactant production using statistical design

Plackett–Burman design is one of the so-called "screening designs". Such designs are traditionally used for identifying important factors among many potential factors. Therefore, in order to reach overproduction of biosurfactant by marine *Bacillus* sp. E34, a Plackett-Burman experimental design was conducted. The variables and

their levels were selected from literature data. The ten independent variables, their code and their respective high and low values are given in Table 2.The Plackett–Burman experimental design matrix for 12 trials at two levels of concentration for each variable with three center points along with the respective experimental response (EI_{24}%) are given in Table 2.

Table 2. Placket-Burman experimental design matrix with coded and levels of independent variables affecting biosurfactant production by marine *Bacillus* sp. E34 with EI_{24} % as response.

Trials	Levels	Variables (g/L)										Response
		$(NH_4)_2SO_4$	KH_2PO_4	$MgSO_4.7H_2O$	NaCl	Yeast extract	Tryptone	Molasses	K_2HPO_4	$CaCl_2$	Inoculum size[a]	
		N	K	Mg	Na	Y	T	M	K2	Ca	I	EI_{24} %
	-	2	0.1	0.1	15	3	5	10	1	0.05	1	
	+	4	0.3	0.3	35	7	15	60	3	0.15	3	
1		+1	-1	+1	-1	-1	-1	+1	+1	+1	-1	52
2		+1	+1	-1	+1	-1	-1	-1	+1	+1	+1	54
3		-1	+1	+1	-1	+1	-1	-1	-1	+1	+1	55
4		+1	-1	+1	+1	-1	+1	-1	-1	-1	+1	49
5		+1	+1	-1	+1	+1	-1	+1	-1	-1	-1	68
6		+1	+1	+1	-1	+1	+1	-1	+1	-1	-1	61
7		-1	+1	+1	+1	-1	+1	+1	-1	+1	-1	79
8		-1	-1	+1	+1	+1	-1	+1	+1	-1	+1	63
9		-1	-1	-1	+1	+1	+1	-1	+1	+1	-1	66
10		+1	-1	-1	-1	+1	+1	+1	-1	+1	+1	70
11		-1	+1	-1	-1	-1	+1	+1	+1	-1	+1	71
12		-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	32
13		0	0	0	0	0	0	0	0	0	0	60
14		0	0	0	0	0	0	0	0	0	0	60
15		0	0	0	0	0	0	0	0	0	0	59

[a]concentration % (v/v)

As clearly shown in Fig.6, production of biosurfactant was positively affected by molasses, tryptone, KH_2PO_4, yeast extract, NaCl,$CaCl_2$,K_2HPO_4 and inoculum size, while negatively affected by $(NH_4)_2SO_4$ and $MgSO_4.7H_2O$ within the tested range. Positive effect explains that if a higher concentration was used, a better response was achieved, while a negative effect means lower concentrations are favoured for better results.

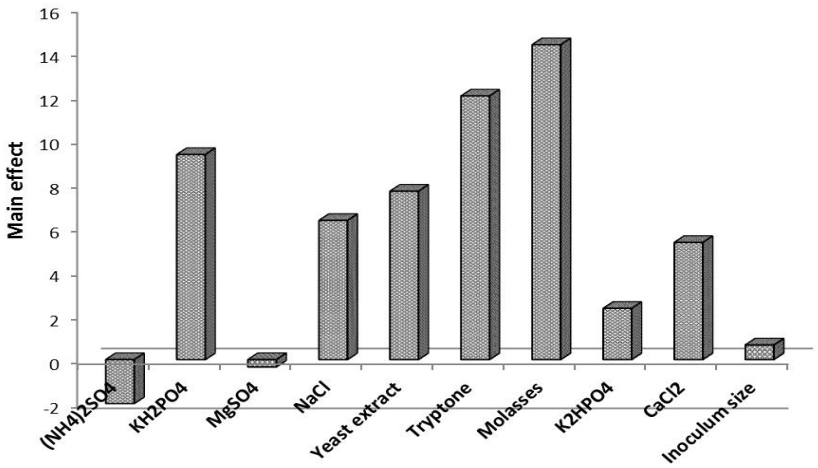

Figure 6. Main effect of the fermentation medium constituents on biosurfactant production by *Bacillus* sp. E34 according to the Placket-Burman experimental results.

Figure 7, illustrates the Pareto-Chart, with 95% confidence level, for estimated effects, in absolute values. The Pareto chart was used for identifying which estimated effects are most important and is a convenient way to view the results of a Plackett–Burman design (Strobel and Sullivan, 1999).

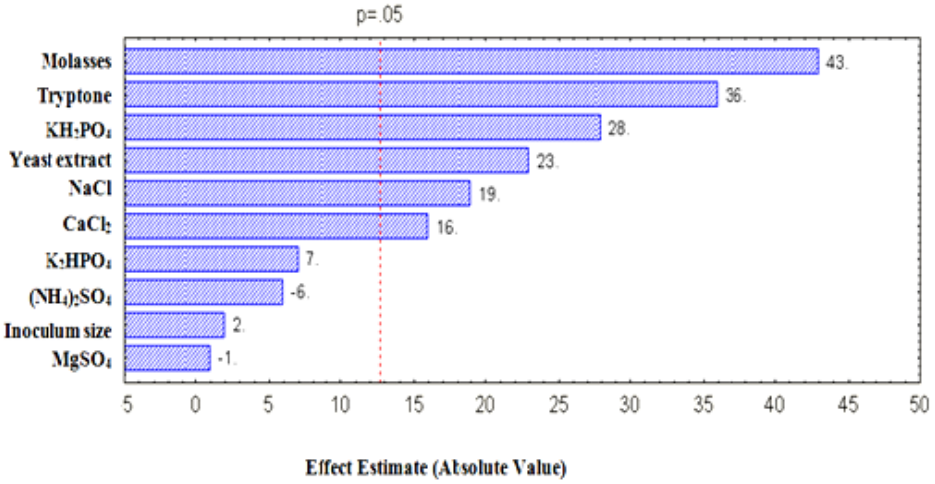

Figure 7. Pareto chart of standardized effects for emulsification index for the Placket-Burman design. The point at which the effect estimates were statistically significant (at $p \leq 0.05$) is indicated by the broken vertical line.

Table 3 shows the statistical parameters obtained after the Design-Expert analysis. Analysis of variance (ANOVA) was performed in order to find the effect and contribution of each variable. The *p*-values were used as a tool to determine the significance of each of the coefficients. The smaller the magnitude of *p*, the more significant is the corresponding coefficient. Values of *p* less than ≤ 0.05 indicate model terms that are significant. The coefficient and the corresponding *p* values suggest that, among the input variables, molasses, tryptone, KH_2PO_4, yeast extract, NaCl and $CaCl_2$ are significant model terms.

Table 3. Analysis of variance (estimated effect, regression coefficient, and corresponding t, p-values and confidence level of each variable) described for biosurfactant production in Plackett-Burman design experiments.

Factors	Coefficient	Effect	Standered Error	Sum of squares	Contribution % (PC)	*t*-value	*p*-value	Significance level
Intercept	60	-		1722.00	-	-	-	-
$(NH_4)_2SO_4$	-1	-2.00000	0.333333	12.000	0.696864111	-6	0.1051	89.4863
KH_2PO_4	4.666667	9.33333	0.333333	261.333	15.1761324	28	0.0227	97.7273
$MgSO_4$	-0.16667	-0.33333	0.333333	0.333	0.019337979	-1	0.5000	50
NaCl	3.166667	6.33333	0.333333	120.333	6.987979094	19	0.0335	96.6525
Yeast extract	3.833333	7.66667	0.333333	176.333	10.24001161	23	0.0277	97.2338
Tryptone	6	12.00000	0.333333	432.000	25.08710801	36	0.01768	98.2321
Molasses	7.166667	14.33333	0.333333	616.333	35.7916957	43	0.0148	98.5198
K_2HPO_4	1.166667	2.33333	0.333333	16.333	0.948490128	7	0.0903	90.9666
$CaCl_2$	2.666667	5.33333	0.333333	85.333	4.955458769	16	0.0397	96.0263
Inoculum size	0.333333	0.66667	0.333333	1.333	0.077409988	2	0.2952	70.4833

R^2 =0.9998; Adj. R^2 =0.9978
***p*≤ 0.05 were considered to be significant.**

In our study, molasses, the carbon source in the medium, demonstrates the highest level of significance in positive term. A *t*-value of 43 and a very low *p*-value 0.0148 demonstrate its significance and large effect on the biosurfactant. This implies that besides supporting growth and normal metabolism, a large part of carbon is diverted towards the biosurfactant production pathway. The carbon source has been reported in literature as a vital limiting factor in production process (Abouseoud et al., 2008; Mukherjee et al., 2008; Nawawi et al., 2010).

In the present work, yeast extract and tryptone, a source of organic nitrogen, vitamins, amino acids and trace elements, were found to possess a positive significant effect. Besides growth, nitrogen is an important constituent of the peptide part of the lipopeptide biosurfactants. According to previous reports (Jacques et al., 1999; Abbasi et al., 2013), yeast extract significantly influenced biosurfactant production by *Rhodococcus* sp.MTCC2574,*Pseudomonas aeruginosa* MA01and *B. subtilis* S499.

KH_2PO_4, a source of K^+ and PO^{-3}_4, had positive significant effect on production process. It has a buffering action in the media. A positive significant effect of KH_2PO_4 on biosurfactant production by *B. subtilis* S499 was previously reported (Jacques et al., 1999). In contrast to the obtained results, low concentration of two-potassium phosphate salts has been reported to increase biosurfactant production by marine *Bacillus* sp. (Mukherjee et al., 2008).

NaCl, a source of Na^+ and Cl^- ions in the medium, another critical component possessed a positive effect that signifies its effectiveness at higher concentrations in experimental design. These findings are in accordance with *Bacillus mycoides* which showed high tolerance to salts concentrations and maximum production in presence of 5.5% NaCl (Najafi et al., 2010) . In contrast to our results, NaCl had negative effect on biosurfactant production by marine *Bacillus* sp. (Mukherjee et al., 2008).

$CaCl_2$, the source of calcium in the medium, was found to be significant in terms of *p*-value (0.039).A positive effect of $CaCl_2$ predicts an increase in emulsification activity upon increasing its concentration in the medium. Previous reports described that $CaCl_2$ is statistically insignificant for biosurfactant production (Nawawi et al., 2010 ; Mukherjee et al., 2008).

Higher cell density increased biosurfactant yields, appropriate inoculum size is critical for biological compound produced by microorganisms (Saimmai et al., 2011).

The adequacy of the model was checked using analysis of variance (ANOVA), the R^2 value (multiple correlation coefficient) should be in the range of 0–1.0, closer to 1 denotes better correlation between the observed and predicted values. In this case the value of R^2 (0.9998) indicates that the variability in the response could be explained by the model and thus the model is capable of explaining 99.98% of the variation in response.

In order to validate the obtained data and to evaluate the accuracy of the applied Plackett-Burman statistical design, a verification experiment was carried out in triplicates. The optimized medium was thus composed of (g/L) molasses, 60; tryptone, 15; yeast extract, 7; $(NH_4)_2SO_4$, 2 ; KH_2PO_4, 0.3 ; $MgSO_4$, 0.1; NaCl, 35; K_2HPO_4, 3; $CaCl_2$, 0.15 and inoculated with 3% inoculum size. The emulsification indexes before and after optimization were 60% and 77%, respectively. Thus, a significant increase (1.28 -fold) in the emulsification index was achieved by Plackett-Burman optimization.

Characterization of *Bacillus* sp. E34 biosurfactant

Functional characterization

Under optimized cultivation conditions, the crude biosurfactant recovered by cold acetone precipitation was a brown colored material with a yield 6 g/L. Furthermore, the surface tension of *Bacillus* sp. E34 cell free broth was reduced from 44.13±0.38 mN/m to 26.10±0.25mN/m. Our values are in agreement with values obtained by other researchers (Saimmai et al., 2011). Aqueous solutions of recovered product showed good foaming ability more than 50% and stable for more than 5 h, which shows its potential application in coal and mineral flotation (Abbasi et al., 2012).

Emulsification property

Bacillus sp. E34 biosurfactant efficiently emulsified various hydrocarbons and oils (Figure 8). Generally less efficient to emulsify vegetable oils compared to hydrocarbons. It could emulsify only toluene, soybean, sunflower, mustard and olive oils to less than 50%. No emulsions were observed with corn oil, frying oil, sesame oil and flaxseed oil (data not shown). Parafffin oil, kerosene and mineral oil were the best hydrocarbons showing EI_{24}% value of 77. The highest emulsifying activity (84.5 %) was recorded with crude oil. Our data refer to the good potentiality of the product for applications in microbial-enhanced oil recovery and remediation of different types of hydrocarbon pollution as a means of their direct removal or as a promoter of biodegradation (Jadhav et al., 2011).

Biosurfactant stability

Bacillus sp. E34 biosurfactant was found to exhibit quite stable emulsification activity from pH 7 to 11, showing higher stability at alkaline than acidic conditions (Figure 9A). No emulsification index was shown at pH lower than 6.The obtained results are in good agreement with previous observations (Gudina et

al., 2012; Al-Bahry et al., 2013) as the stability of biosurfactant at different pH values is mostly in the alkaline range. The biosurfactant was thermostable in a range from 30 ºC to 100 ºC (average of EI_{24}=77%)(data not shown). Similar behaviors were observed with *B.* spp., *B. licheniformis*TR7 and *B. subtilis* SA9 (Gudina et al., 2012; Saimmai et al., 2011). Emulsification activity (EI_{24}=50%) was found to be functionally stable at varying salinity 5 -13% NaCl ((Figure 9B). Although a previous report (Desai and Banat, 1997) showed that concentrations above 2% NaCl are enough to inactivate a synthetic surfactant, emulsifying activity observed in this study remained unchanged up to 4 % NaCl. This special ionic strength tolerance offers the biosurfactant more suitability for oil-related applications most of which are in highly saline conditions (Shavandi et al., 2011). Therefore, it can be concluded that stability of the E34 biosurfactant to the environmental stresses prevalent in the oil reservoirs such as high temperature, salinity and different pH strengths reveals its suitability for oil well injection, enhanced oil recovery and bioremediation of soil as well as spills in the marine environment (De Gusmão et al., 2010).

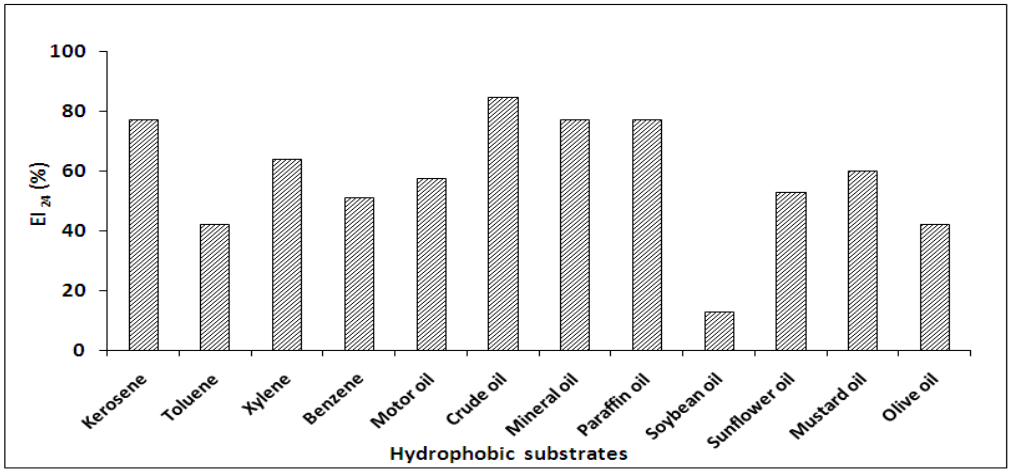

Figure 8. Emulsification index (EI_{24} %) of *Bacillus* sp. E34 biosurfactant produced in the optimized medium against some hydrophobic substrates.

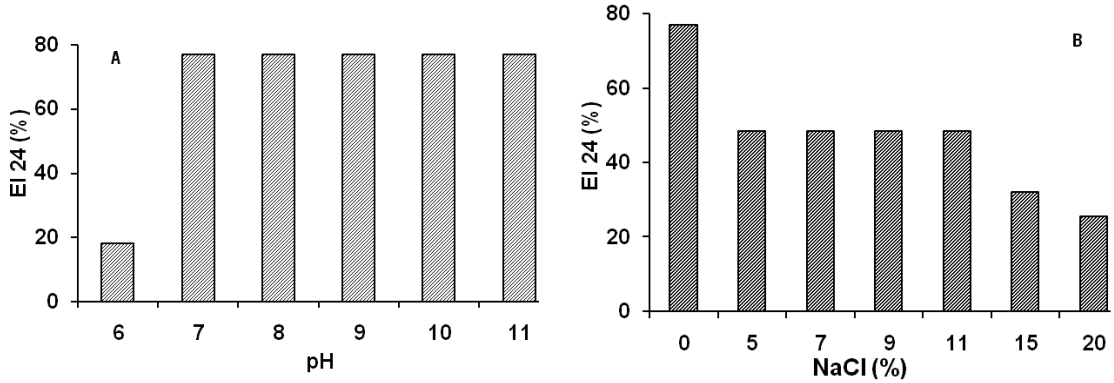

Figure 9. Effect of pH (A) and salinity (B) on the emulsifying activity.

Structural characterization

Biochemical reaction

The positive reaction with CTAB/methylene-blue agar; indicates that the biosurfactant produced is glycolipids or anionic surfactant in nature.Similar observations were reported for biosurfactant produced by *Klebsiella pneumonia* and *Pseudomonas aeruginosa* (Jamal et al., 2012; Samanta et al., 2012). The positive result with Biuret reagent, indicates the presence of peptide bond proteins. Ninhydrin reaction was negative, indicating that the peptide has a blocked N-terminal. These findings are in accordance with those obtained by *B. licheniformis* PTCC 1595 (Dehghan-noudeh et al., 2010). The result of preliminary identification revealed that the biosurfactant produced by *Bacillus* sp.E34 has a glycolipopeptide structure.

Biochemical analysis

Compositional analysis revealed that it was a mixture of carbohydrate, protein and lipid with a combination of 11 %, 20 %, 65 % (w/w) respectively. The quantification of compound gave a higher ratio in lipid and protein compared to carbohydrate.

Fourier Transform Infrared Spectroscopy (FTIR)

The molecular composition of *Bacillus* sp. E34 biosurfactant was evaluated by FTIR. The obtained peaks are consistent with those commonly found in the IR spectra of the biosurfactant produced by several *Bacillus* species (Das et al., 2008; Oliveira et al., 2013). FTIR spectroscopy (Figure 10) reveal a strong broad absorption band in the range of 3250-3700 cm^{-1} with a maximum at 3464.8cm^{-1} which represents -OH, -CH, and -NH stretching vibrations. This is characteristic of carbon-containing compounds with amino groups bonds of protein (Ismail et al., 2013). Another strong sharp peak observed at 1640.4 cm^{-1} signifies CO-NH stretching vibration, suggesting the presence of carbonyl functionality present in carboxylate or amide moieties of protein and peptide amines (Saimmai et al. 2011, Jain et al., 2012). The weak band at 1403.7 cm^{-1} is in the absorption range 1370-1470 cm^{-1} resulting from deformation and bending vibrations of–C-CH_2 and –C-CH_3 groups (alkyl groups) in aliphatic chains (Ismail et al., 2013). Also, the strong sharp band observed at 1097.2 cm^{-1}indicates the presence of polysaccharide or polysaccharide-like substances (Aparna et al., 2012). IR absorption found at 868 cm^{-1} was due to out of plane C–H bending, characteristic of aromatic compounds (Das et al. 2008).The adsorption peak of CH (858–934 cm^{-1}) suggests that the polysaccharides composed of sugar derivatives (Zheng et al. 2012). The absorption peak observed at 530.76 cm^{-1} known to be characteristics of sugar derivatives. Therefore, it can be concluded that the biosurfactant produced by *Bacillus* sp. E34 was glycolipopeptide in nature.

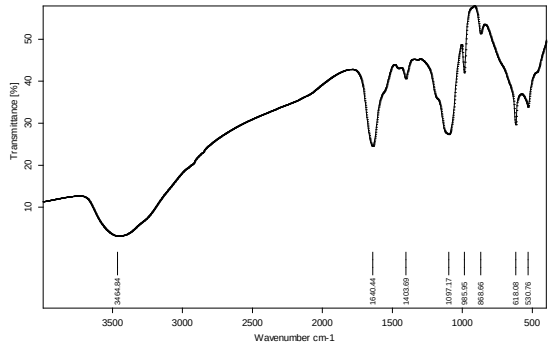

Figure10. Fourier Transform Infrared Spectrum (FTIR) of biosurfactant produced by *Bacillus* sp. E34.

Application of biosurfactant in removal of hydrocarbon from sand

The potential use of biosurfactant in microbial enhanced oil recovery (MEOR) was evaluated using the sand pack column technique. Residual hydrocarbon (kerosene) in columns was mobilized during passage of the biosurfactant containing broth and began to exude with the effluent. About 45% of residual oil was recovered using biosurfactant containing broth showing the potential of the product in oils spill accidents. Biosurfactants produced by *B.licheniformis* TR7 and *B. subtilis* SA9 removed around 45–49% of the spiked oil, respectively (Saimmai et al.,2011). Moreover, biosurfactants produced by different strains of *B. subtilis* recovered between 19% and 22% of oil (Pereira et al., 2013). From this result, it can be concluded that the biourfactant obtained from marine *Bacillus* sp. E34-can be used as an alternative to chemical surfactants for enhanced oil recovery, cleaning of oil reservoirs,and bioremediation of spilled oils in soil or marine environments (Kiran et al., 2009). As a result it may perhaps leads to the reduction of mortality rate of the marine creatures and increased the dissolved oxygen level as well as the light penetration (Kiran et al., 2009).

Conclusion

Considering the need of new strains for the production of novel surface active molecules, the present study brings out a new insight on the exploration of marine environment for biosurfactant producers and process optimization for industrial applications. By increasing the biosurfactant yield via experimental design approach, the production cost of biosurfactant would markedly be reduced, enhancing feasibility of commercial application a powerful biosurfactant. The ability to form stable emulsions with different hydrocarbons as well as exhibiting a high level of pH, salinity and thermal stability, shows clear perspectives for its use in extreme environmental conditions in bioremediation and other industrial fields.

Corresponding Author:
Dr. Mona E.M. Mabrouk
Botany Department, Faculty of Science, Damanhour University, Damanhour, Egypt
E-mail: mona_mabrouk_eg@hotmail.com

Auhtors:
Mona E.M. Mabrouk
Affiliation: Botany Department, Faculty of Science, Damanhour University, Damanhour, Egypt
E-mail: mona_mabrouk_eg@hotmail.com

Eman M. Youssif
Affiliation**:** Botany and Microbiology Department, Faculty of Science, Alexandria University, Alexandria, Egypt
E-mail: Emanyoussif87@yahoo.com
Soraya A. Sabry
Affiliation**:** Botany and Microbiology Department, Faculty of Science, Alexandria University, Alexandria, Egypt
E-mail: sabrysoraya@yahoo.com

References

1. Muthusamy K, Gopalakrishnan S, Ravi Tk, Sivachidambaram P. Biosurfactants: properties, commercial production and application. Curr Sci 2008; 94:736-746.
2. Onwosi CO, Odibo FJC. Use of response surface design in the optimization of starter cultures for enhanced rhamnolipid production by *Pseudomonas nitroreducens*. African J Biotechnol 2013; 12:2611-2617.
3. Oliveira DW, Franca ÍW, Félix AK, Martins JJ, Giro ME, Melo VM, Gonçalves LR. Kinetic study of biosurfactant production by *Bacillus subtilis* AM005 grown in clarified cashew apple juice. Colloids Surf B: Biointerfaces 2013; 101:34–43.
4. Noparat P, Maneerat S ,Saimmai A. Utilization of palm oil decanter cake as a novel substrate for biosurfactant production from a new and promising strain of *Ochrobactrum anthropi* 2/3. World J Microbiol Biotechnol 2014; DOI 10.1007/s11274-013-1493-z.
5. Saimmai A, Sobhon V, Maneerat S. Molasses as a whole medium for biosurfactants production by *Bacillus* strains and their application. Appl Biochem Biotechnol 2011; 165:315-335.
6. Dubey KV, Charde PN, Meshram SU, Yadav SK, Singh S, Juwarkar AA. Potential of new microbial isolates for biosurfactant production using combinations of distillery Waste with other Industrial Wastes. J Petrol Environ Biotechnol 2012; 12:1-11
7. Mabrouk M, Abou-Zeid D-M, Sabra W. Application of Plackett–Burman experimental design to evaluate nutritional requirements for poly (γ-glutamic acid) production in batch fermentation by *Bacillus licheniformis* A13. African J Appl Microbiol Res 2012; 1:6-18.
8. Mabrouk MEM, ElAhwany AMD, Beliah, MMB, Sabry SA. Xanthan production by a novel mutant strain of *Xanthomonas campestris*: Application of statistical design for optimization of process parameters. Life Sci J 2013; 10:1660-1667.
9. Abbasi H, Hamed MM, Lotfabad TB, Zahiri HS, Sharafi H, Masoomi F, Moosavi-Movahedi AA, Ortiz A, Amanlou M, Noghabi KA. Biosurfactant-producing bacterium, *Pseudomonas aeruginosa* MA01 isolated from spoiled apples: physicochemical and structural characteristics of isolated biosurfactant. J Biosci Bioengin 2012; 113:211–219.
10. Debnath M, Paul AK, Bisen PS. Natural bioactive compounds and biotechnological potential of marine bacteria. Curr Pharm Biotechnol 2007; 8:253–260.
11. Liu X, Ren B,Chen M, Wang H,Kokare CR,Zhou X,Wang J, Dai H, Song F, Liu M, Wang J, Wang S, Zhang L. Production and characterization of a group of bioemulsifiers from the marine *Bacillus velezensis* strain H3 Appl Microbiol Biotechnol 2010; 87:1881–1893.
12. Blunt JW, Copp BR, Munro MH, Northcote PT, Prinsep MR. Marine natural products. Nat Prod Rep 2010; 27:165–237.
13. Kiran GS, Hema TA, Gandhimathi R, Selvin J, Thomas TA, Ravji TR, Natarajaseenivasan K. Optimization and production of a biosurfactant from the sponge-associated marine fungus *Aspergillus ustus* MSF3. Biointerfaces 2009; 73:250-256.
14. Kiran GS,Sabu A,Selvin J..Synthesis of silver nanoparticles by glycolipid biosurfactant produced from marine *Brevibacterium casei* MSA19. J. Biotechnol. 2010 a*;* 148, 221-225
15. Kiran GS, Thomas TA, Selvin J.Production of a new glycolipid biosurfactant from marine *Nocardiopsis lucentensis* MSA04 in solid-state cultivation. Colloids Surf B: Biointerfaces. 2010 b; 78:8–16.
16. Kiran GS, Thomas TA, Selvin J, Sabarathnam B, Lipton AP. Optimization and characterization of a new lipopeptide biosurfactant produced by marine *Brevibacterium aureum* MSA13 in solid state culture. Bioresour. Technol 2010c;101: 2389–2396.
17. Bertani G. Lysogeny at mid-twentieth century: P1, P2, and other experimental systems. J Bacteriol. 2004; 186:595–600.
18. Walter V, Syldatk C, Haussmann R. In: Sen R (ed) Biosurfactants : landes bioscience and springer science. 2010; pp 1–13.
19. Ferhat S, Mnif S, Badis A, Eddouaouda K, Alouaoui R, Boucherit A, Mhiri N, Moulai-Mostefa N, Sayadi S. Screening and preliminary characterization of biosurfactants produced by *Ochobactrum* sp.1C and *Brevibacterium* sp. 7G isolated from hydrocarbon-contaminated soils. Int Biodeter Biodegrad . 2011; 65:1182-1188.
20. Khopade, A, Ren B, Liu XY, Mahadik K, Zhang L, Kokare C. Production and characterization of biosurfactant from marine *Streptomyces* species B3. J Colloid Interface Sci 2012; 367:311-318.
21. Luna JM, Rufino, RD, Sarubbo LA Campos-Takaki GM. Characterization, surface properties and biological activity of a biosurfactant produced from industrial waste by *Candida sphaerica* UCP0995 for application in the petroleum industry. Colloids Surf B: Biointerfaces 2013; 102:202– 209.
22. Pal MP, Vaidya BK, Desai KM, Joshi RM, Nene SN, Kulkarni BD. Media optimization for biosurfactant production by *Rhodococcus erythopolis* MTCC 2794: artificial intelligence versus a statistical approach. J. Ind. Microbiol. Biotechnol 2009; 36: 747–756.
23. Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams T. Bergey's Manual of Determinative Bacteriology (9thedn). Hensyl, W.R. (ed.) 1994; 40-169.United States, Baltimore, Williams & Wilkins.
24. Plackett RL, Burman, JP. The design of optimum multifactorial experiments. Biometrika 1946; 33:305-325.
25. Ismail W, Al-Rowaihi IS, Al-Humam AA, Hamza RY, El Nayal AM, Bououdina M. Characterization of a lipopeptide biosurfactant produced by a crude-oil- emulsifying *Bacillus* sp. I-15. Int Biodeter Biodegrad 2013; 84:168-178.
26. Abouseoud M, Maachi R, Amranec A, Boudergua S, Nabi A. Evaluation of different carbon and nitrogen sources in production of biosurfactant by *Pseudomonas fluorescens*. Desalination 2008; 223:143–151.

27. Lotfabad TB, Shourian M, Roostaazad R, Najafabadi AR, Adelzadeh MR, Noghabi KA. An efficient biosurfactant-producing bacterium *Pseudomonas aeruginosa* MR01, isolated from oil excavation areas in south of Iran. Colloids Surf B: Biointerfaces 2009; 69:183–193.
28. Aparna A, Srinikethan G, Smitha H. Production and characterization of biosurfactant produced by a novel *Pseudomonas* sp. 2B. Colloids Surf B: Biointerfaces 2012; 95:23–29.
29. Pradhan K, Pradhan N, Sukla LB, Panda PK, Mishr BK. Inhibition of pathogenic bacterial biofilm by biosurfactant produced by *Lysinibacillus fusiformis* S9. Bioprocess Biosyst. Eng 2014; 37:139–149.
30. Jamal P, Nawawi WM, Alam MZ. Optimum medium components for biosurfactant production by *Klebsiella pneumoniae*. Australian J Basic Appl Sci 2012; 6:100-108.
31. Zhang X, Xu D, Yang G, Zhang H, Li J, Shim H. Isolation and characterization of rhamnolipid producing *Pseudomonas aeruginosa* strains from waste edible oils. African J. Microbiol. Res 2012; 6:1466-1471.
32. Okpokwasili GC, Ibiene AA. Enhancement of recovery of residual oil using a biosurfactant slug. African J Biotechnol 2006; 5:453-456.
33. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956; 28:350-356.
34. Lowry HO, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951; 193:265-275.
35. Donio MBS, Ronica FA, Viji VT, Velmurugan S, Jenifer JS C A, Michaelbabu M, Dhar P, Citarasu T. *Halomonas* sp. BS4, A biosurfactant producing halophilic bacterium isolated from solar salt works in India and their biomedical importance. Springer Plus 2013; 2:149-159.
36. Abu-Ruwaida A, Banat IM, Hadithto S, Salem A, Kadri M. Isolation of biosurfactant-producing bacteria product characterization, and evaluation. Acta Biotechnol 1991; 11:315–324.
37. Zheng C, Li Z, Su J, Zhang R, Liu C, Zhao M. Characterization and emulsifying property of a novel bioemulsifier by *Aeribacillus pallidus* YM-1. J Appl Microbiol 2012; 113:44–51.
38. Abdel-Mawgoud AM, Aboulwafa MM, Hassouna, NAH. Optimization of surfactin production by *Bacillus subtilis* isolate BS5. Appl Biochem Biotechnol 2008; 150:305-325.
39. Ghojavand H, Vahabzadeh F, Roayaei E, Shahakai A. Production and properties of a biosurfactant obtained from a member of the *Bacillus subtilis* group (PTCC 1696). J. Colloid Interface Sci 2008; 324:172–176.
40. Najafi AR, Rahimpour MR, Jahanmir AH, Roostaazad R, Arabian D, Ghobadi Z. Enhancing biosurfactant production from an indigenous strain of *Bacillus mycoides* by optimizing the growth conditions using a response surface methodology. Chem Eng J 2010; 163:188–194.
41. Joshi S, Bharucha C, Jha S, Yadav S, Nerurkar A, Desai AJ. Biosurfactant production using molasses and whey under thermophilic conditions. Bioresour Technol 2008; 99:195–199.
42. Al-Bahry SN, Al-Wahaibi YM, Elshafie AE, Al-Bemani AS, Joshi SJ, Al-Makhmari HS,Al-Sulaimani HS. Biosurfactant production by *Bacillus subtilis* B20 using date molasses and its possible application in enhanced oil recovery. Int Biodeter Biodegrad 2013; 81:141–146.
43. Strobel RJ, Sullivan GR. Experimental design for improvement of fermentations. In Manual of Industrial Microbiology and Biotechnology. (Demain, AL, Davies GE, eds.) 1999; 80-93 United Kingdom, ASM Press.
44. Mukherjee S, Das P, Sivapathasekaran C, Sen R. Enhanced production of biosurfactant by a marine bacterium on statistical screening of nutritional parameters. Biochem Eng J 2008; 42:254–260.
45. Nawawi WM, Jamal P, Alam MZ. Utilization of sludge palm oil as a novel substrate for biosurfactant production. Bioresour Technol 2010; 101:9241–9247.
46. Jacques P, Hbid C, Destain J, Razafindralambo H, Paquot M, De Pauw E,Thonart P. Optimization of biosurfactant lipopeptide production from *Bacillus subtilis* S499 by Plackett-Burman design. Appl Biochem Biotechnol 1999; 77:223–233.
47. Jadhav M, Kagalkar A, Jadhav S, Govindwar S. Isolation, characterization, and antifungal application of a biosurfactant produced by *Enterobacter* sp. MS16. European J Lipid Sci Technol 2011; 113:1347–1356.
48. Gudina EJ, Pereira JFB, Rodrigues LR, Coutinho JAP, Teixeira JA. Isolation and study of microorganisms from oil samples for application in microbial enhanced oil recovery. Int Biodeter Biodegrad 2012; 68:56–64.
49. Desai JD, Banat IM. Microbial production of surfactants and their commercial potential. Microbiol Mol Biol Rev 1997; 61:47-64.
50. Shavandi M, Mohebali G, Haddadi A, Shakarami H, Nuhi A. Emulsification potential of a newly isolated biosurfactant-producing bacterium, *Rhodococcus* sp. strain TA6. Colloids Surf B: Biointerfaces 2011; 82:477–482.
51. De Gusmão, CAB, Rufino RD, Sarubbo LA. Laboratory production and characterization of a new biosurfactant from *Candida glabrata* UCP1002 cultivated in vegetable fat waste applied to the removal of hydrophobic contaminant. World J Microbiol Biotechnol 2010; 26:1683–1692.
52. Samanta A, Pal P, Mandal A, Sinha C, Lalee A, Das M, Kaity S, Mitra D. Estimation of biosurfactant activity of an alkaline protease producing bacteria isolated from municipal solid waste. Cent European J Exp Biol 2012; 1:26-35.
53. Dehghan-noudeh G, Moshafi MH, Payam K, Farideh A Studies on bioemulsifier production by *Bacillus licheniformis* PTCC 1595. African J Biotechnol 2010; 9:352-356.
54. Jain RM, Mody k, Mishra A, Jha B. Isolation and structural characterization of biosurfactant produced by an alkaliphilic bacterium *Cronobacter sakazakii* isolated from oil contaminated wastewater. Carbohydr Polym 2012; 87: 2320–2326.
55. Das P, Mukherjee S, Sen S. Improved bioavailability and biodegradation of a model polyaromatic hydrocarbon by a biosurfactant producing bacterium of marine origin. Chemosphere 2008; 72: 1229–1234.
56. Pereira JFB, Gudina EJ, Costa R, Vitorino R, Coutinho JAP, Teixeira JA, Rodrigues LR. Optimization and characterization of biosurfactant production by *Bacillus subtilis* isolates towards microbial enhanced oil recovery applications. Fuel 2013; 111: 259–268.