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Enhancing biosurfactant production from an indigenous strain of *Bacillus mycoides* by optimizing the growth conditions using a response surface methodology

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ABSTRACT

In this study, we have investigated the potential of a native bacterial strain isolated from an Iranian oil field for the production of biosurfactant. The bacterium was identified to be *Bacillus mycoides* by biochemical tests and 16S ribotyping. The biosurfactant, which was produced by this bacterium, was able to reduce the surface tension of media to 34 mN/m. Compositional analysis of the produced biosurfactant has been carried out by thin layer chromatography (TLC) and FT-IR. The biosurfactant produced by the isolate was characterized as lipopeptide derivative. Biosurfactant production was optimized by the combination of central composite design (CCD) and response surface methodology (RSM). The factor selected for optimization of growth conditions were pH, temperature, glucose and salinity concentrations. The empirical model developed through RSM in terms of effective operational factors mentioned above was found to be adequate to describe the biosurfactant production. Through the analysis, glucose and temperature were found to be the most significant factors, whereas pH and salinity had less effect within the ranges investigated. A maximum reduction in surface tension was obtained under the optimal conditions of 16.55 g/l glucose concentration, 39.03 °C, 55.05 g/l total salt concentration and medium pH 7.37.

1. Introduction

Biosurfactants are surface-active compounds produced by wide range of microorganisms. Microbial surfactants are composed of lipid, phospholipids, polysaccharide, protein and other biological macromolecules and contain various functional groups including carboxyl, amino and phosphate groups [1]. Biosurfactants are categorized by their chemical composition and microbial origin. They include glycolipids, lipopeptides, polysaccharide–protein complexes, protein-like substances, lipopolysaccharides, phospholipids, fatty acids and neutral lipids [2]. The most commonly isolated biosurfactants are glycolipids and lipopeptides. They include rhamnolipids released by *Pseudomonas aeruginosa* [3], sophorolipids from *Candida* species [4], as well as surfactin and iturin produced by *Bacillus subtilis* strains [5]. Lipopeptides are effective biosurfactants which are usually produced by *Bacillus* strains [6].

Surfactants display properties, for example, detergency, emulsification, foaming, and dispersion [7,8]. According to many literature, microorganisms have the ability of producing kind of surfactants, which these biosurfactants are low molecular weight polymers that efficiently reduce surface and interfacial tensions and high molecular weight molecules which are highly efficient emulsifiers [9,10]. Biosurfactants are stable even under extreme conditions, e.g. wide range of pH, high temperature, and salinity [11]. In recent years, much attention has been directed towards biosurfactants. These surface-active compounds have different advantages including low toxicity, high biodegradability, low irritancy, and compatibility with human skin [9,12]. Because of these superior properties, biosurfactants have a potential use in food, pharmaceutical, and cosmetic industries [1]. The unique properties of biosurfactants allow their use and possible replacement of chemically synthesized surfactants in a great number of industrial operations [1,13].

The identification and optimisation of the cultivation conditions that affect the surfactant production represent key points for the development of a cost-competitive process [14]. There are a number of operating parameters controlling biosurfactant production, which are required to be maintained within a certain range in operating conditions whereby the activity of bacteria with the resultant of maximum production of biosurfactant can be optimized. In this regard, temperature, pH of the medium, medium composition and salinity are of prime importance for control and optimization of biosurfactant production. The amount of biosurfactant synthesis

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depends greatly on the availability of carbon sources and on the balance between carbon and other limiting nutrients [15]. Factors affecting surfactant biosynthesis have been studied extensively, especially for *Pseudomonas*, *Candida* and *Rhodococcus* [16,17]. But there is dirt of information about optimal conditions for biosurfactant production from *Bacillus* species especially *Bacillus mycoides*.

Biosurfactant producers can only be effective if they are maintained at their optimal ambient conditions required for growth and activity. In this regard one of the best methodologies for designing the optimization experiments is response surface methodology (RSM). In statistical-based approaches, RSM has been extensively used in fermentation media optimization [18–20]. RSM is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions [21]. It is a statistically designed experimental protocol in which several factors are simultaneously varied [18]. In fact, the relationship between the response and the independent variables is usually unknown in a process; therefore the first step in RSM is to approximate the function (response) through analyzing factors (independent variables). In this study also the ambient growth conditions have been optimized by using this methodology.

The aims of this work were to investigate the cultural factors affecting the production of the extracellular biosurfactants by a new strain of *B. mycoides*, isolated from oil-fields in southern tropical zone of Iran and to find the optimal composition of the growth medium for the production in flask-scale by using RSM. An insight into the preliminary characteristics of extracted biosurfactant has been provided as well.

2. Materials and methods

2.1. Preparation of culture medium

Mineral salts medium (MSM) used was medium E [22–24] that contained (g/l): 1 $(NH_4)_2SO_4$, 0.25 $MgSO_4$, 10 sucrose, 50 NaCl, 13.9 K_2HPO_4, 2.7 KH_2PO_4, 10 trace salt solution. The composition of the solution of trace elements was (g/l): 1 EDTA, 3 $MnSO_4{\cdot}H_2O$, 0.1 $FeSO_4{\cdot}7H_2O$, 0.1 $CaCl_2{\cdot}2H_2O$, 0.1 $CoCl_2{\cdot}6H_2O$ 0.1 $ZnSO_4{\cdot}7H_2O$, 0.01 $CuSO_4{\cdot}5H_2O$, 0.01 $AlK(SO_4)_2{\cdot}12H_2O$, 0.01 H_3BO_3, 0.01 $Na_2MoO_4{\cdot}2H_2O$. The pH of the medium was adjusted to 7 using 1N NaOH.

2.2. Isolation and identification of biosurfactant-producing bacteria

10 ml of oil sample was transferred to the 500 ml flask containing 100 ml of nutrient broth medium and incubated at two diverse temperatures (30 and 40 °C), with 170 rpm for 6 days. To isolate bacterial strains, samples (0.1 ml) of the enrichment culture were periodically taken and spread onto nutrient agar plates. Plates were incubated at 37 °C and colonies were observed after 24 h of incubation. 15–17 types of colonies were observed on plates and pure cultures of each morphologically distinct colonies were obtained by repetitive streaking onto nutrient agar.

To evaluate the production of biosurfactant and screening the isolates, we measured surface tension of culture samples. The strain which could reduce the surface tension of water to a lowest value was then selected as the highest biosurfactant-producing strain. Another method for screening the isolates was area of clear zone method [25] and ability of hydrolyzing blood on Blood Agar. For this latest method each strain was streaked onto blood agar plates and incubated for 48 h at 37 °C. The plates were visually inspected for zones of clearing around the colonies, indicative of biosurfactant production. The diameter of the clear zone depends on the concentration of the biosurfactant [26]. On the base of the results of these methods the best biosurfactant producer was selected and named SH2, and was maintained on nutrient agar slants at 4 °C.

Putative identification of the isolated organism was carried out by Pasture Institute, Tehran, Iran by performing gram staining, motility, oxidase, catalase and biochemical tests. The strain SH2 was then identified by matching the results with Bergey's Manual of Determinative Bacteriology [27].

2.3. DNA isolation for 16S rRNA analysis

The isolated bacterial strain was identified and characterized with conventional and molecular level technique. DNA of the isolate was extracted for 16S rRNA analysis. The late-exponential phase culture were harvested and washed with same basal media, cells were suspended in 576 μL of 10 mM EDTA–150 mM NaCl (pH 8.0) and followed by lyses of cells by sodium dodecyl sulfate and deproteination by chloroform-isoamylalcohol (24:1, v/v) and phenol–chloroform–isoamylalcohol (25:24:1, v/v/v), followed by precipitation by ethanol [28].

2.4. 16S rRNA sequencing

For identification of the bacterium, 16S ribotyping was carried out. The sequence analysis was done at Ribosomal Database Project (RDP) I & II and the Basic Local Alignment Search Tool (BLAST-n) analysis at National Center for Biotechnology Information (NCBI) server. The alignment of the sequence was done using CLUSTALW program at European Bioinformatics site. These sequence data have been submitted to the GenBank databases under accession number HM037178.

2.5. Biosurfactant production

The biosurfactant producing bacterium was transferred to 5 ml Nutrient rich (NR) broth medium containing 1% yeast extract, 1.5% nutrient broth (Merck, Germany) and 1% ammonium sulfate and incubated at 37 °C, 180 rpm for 12 h as seed culture (optical density was 0.8 at 600 nm). Then after, 5 ml bacterial suspensions were transferred to a 2000 ml Erlenmeyer flask containing 500 ml of Minimal salt medium (MSM) and incubated on a rotary shaker incubator (180 rpm) at 37 °C. At different time intervals the samples were collected and monitored for biosurfactant production by measuring of surface tension (ST). The surface tension of cell-free samples was measured by Du Nuoy ring method using Krüss-K6 tensiometer. Area of clear zone by cell-free sample was measured according to Morikawa et al. [25].

2.6. Isolation of biosurfactant

The bacterial cells were removed from the culture broth by centrifugation (10,000 × *g* at 4 °C for 10 min). The collected supernatant was acidified with 6N hydrochloric acid solution to pH 2.0. A precipitated biosurfactant was allowed to settle at 4 °C overnight [29]. The precipitated biosurfactant was collected by centrifugation at 15,000 × *g* for 20 min. Following centrifugation, the precipitate was resolved in distilled water and adjusted to pH 7.0 using 1N NaOH, then after recentrifuged at 12,000 × *g* for 10 min. The solution was lyophilized and weighted [22–24].

2.7. Chemical characterization of biosurfactant

2.7.1. Thin layer chromatography (TLC)

Preliminary characterization of the biosurfactant was done by thin layer chromatography (TLC). A portion of the crude biosurfactant was separated on a silica gel 60 plate (Merck) using $CHCl_3$:CH_3OH:H_2O (85:15:2, v/v/v) as developing solvent system.

Table 1
Range of the parameters used for modeling the biosurfactant production and the specified codes for each parameter.

Variables	Coded values and the corresponding values of parameters				
	$-\alpha$	−1	0	1	$+\alpha$
A: Temperature	27.57	32	38.50	45	49.43
B: pH	4.31	5.5	7.25	9	10.19
C: Salinity	26.48	35	47.50	60	68.52
D: Glucose	3	6.45	11.50	16.55	20

The separated spots are visualized with ultraviolet light or by placing the plate in iodine vapor.

2.7.2. FT-IR spectra of the dried biosurfactants

FT-IR spectra of the dried biosurfactants were recorded on a Bruker 113 V FT-IR spectrometer equipped with a mercury–cadmium–telluride (MCT) detector cooled with liquid N_2. About 2 mg of dried biomaterial was milled with 200 mg of KBr to form a very fine powder. This powder was then compressed into a thin pellet which could be analyzed by FT-IR spectra measurement 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.

2.8. Optimization of culture conditions by RSM

RSM is a combination of mathematical and statistical techniques that is useful for analyzing the effects of several independent variables on the system response without the need of a predetermined relationship between the objective function and the variables [30–32]. In this regard to achieve the best results for growth and activity of bacterium, the composition of the media and growth conditions was optimized by using four different culture conditions with the help of RSM. To develop the model, four input parameters were considered as temperature, pH, salinity and glucose amount. The specified codes for each parameter and the range of the parameters used for modeling the biosurfactant production are given in Table 1.

As a result 21 experiments (Table 2) were performed. The culture samples were collected at time intervals. The surface tension of the sample was measured by using Du Nuoy ring method using Krüss-K6 tensiometer.

This is followed by analyzing the experimental results using the analysis of variance (ANOVA) technique to find out which factors had the most effective interactions and also which ones were most effectives for reduction of the surface tension.

3. Results and discussion

3.1. Isolation and identification of selected biosurfactant-producing bacterial strain

In the present study we have isolated Seventeen morphologically distinct microbial colonies as positive strains, ultimately one of the isolates, initially named SH2 which had highest biosurfactant production and activity was selected for the further studies. The primary abilities of the selected bacterial strain were including hemolytic activity (6 mm), oil displacement (11 mm) and reduction in ST values below 40 mN/m in MSM medium. The bacterial isolate was examined based on its morphological and biochemical characteristics. Morphological observations revealed that the colony was white and rhizoid form. It was characterized as motile, gram positive, spore-forming and MR positive with oxidase, catalase and nitrate reduction positive. The isolate hydrolysed starch, casein, gelatin and cellulose. The strain utilized glucose and not utilized mannitol. The isolate could utilize the glucose, fructose, maltose and sucrose but not xylose, lactose, melobiose and mannitol. Based on these results, the strain SH2 was preliminarily identified as *B. mycoides* and 16S rRNA ribotyping has confirmed that the isolated bacterium is belonged to *B. mycoides* (accession number HM037178).

3.2. Biosurfactant production

Biosurfactant production of the new isolate was preliminary screened by hemolytic activity, the drop collapsing test, oil displacement method and measuring the surface tension of the solution. In drop collapsing test a flat drop was observed and in oil displacement method, a clear diameter of 11 mm was observed and the area was calculated as 94.985 mm^2. The surface tension of the free cell culture, decreased from 62 to 34 mN/m, and its properties, such as the surface tensions, remain invariable even after 72 h. The lowest surface tension was 34 mN/m at the middle of logarithmic phase (14 h) (Fig. 1). A reduction in the surface tension

Table 2
Experimental central composite design (CCD) runs in Design-Expert 7.1 and corresponding results (the response).

Run	Factor 1 Temperature	Factor 2 pH	Factor 3 Salinity	Factor 4 Glucose	Response Surface tension
1	38.50	7.25	47.50	11.50	37
2	38.50	10.19	47.50	11.50	61
3	27.57	7.25	47.50	11.50	55
4	38.50	7.25	47.50	20.00	33
5	38.50	7.25	26.48	11.50	40
6	32.00	5.50	35.00	6.45	50
7	38.50	7.25	47.50	11.50	37
8	45.00	9.00	60.00	6.45	48
9	38.50	7.25	47.50	11.50	34
10	38.50	7.25	47.50	3.00	40
11	38.50	4.31	47.50	11.50	60
12	32.00	9.00	35.00	16.55	54
13	38.50	7.25	68.52	11.50	41
14	32.00	5.50	60.00	6.45	52
15	32.00	9.00	60.00	16.55	49
16	49.43	7.25	47.50	11.50	48
17	38.50	7.25	47.50	11.50	34
18	38.50	7.25	47.50	11.50	35
19	45.00	9.00	35.00	6.45	46
20	45.00	5.50	35.00	16.55	53
21	45.00	5.50	60.00	16.55	50

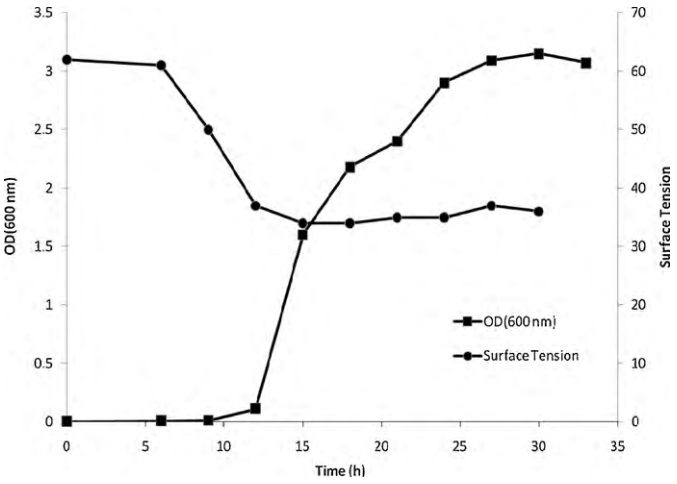

Fig. 1. Growth rate and biosurfactant production (surface tension reduction) versus time by a member of *Bacillus mycoides*.

of media as a result of biosurfactant production and accumulation during the period between the logarithm phase and stationary phases has already been reported for several other microorganisms [11,33,34]. This is one of the advantages of this isolate that can rich to the highest amount of produced biosurfactant and ultimately the lowest surface tension in about 14 h which comparable with other reports for bacterial strains like *B. subtilis* [11], *B. licheniformis* [35] and *Rhodococcus* [36] where they have reported between 28 and 72 h incubation for maximum production of biosurfactant. In case of reduction in surface tension to 34 mN/m point in several other studies by different strains the similar amount of reduction in surface tension with same or longer period of incubation have been reported [8,34,36]. In contrast, works carried out with *lactobacillus* strains the amount of reduction in surface tension was lower as compare to our work [37,38].

TLC analyses were performed in order to find out the purity and R_f value of the product. TLC of the ethyl acetate extract showed a spot (R_f; 0.59) on a TLC plate developed with solvent system (chloroform:methanol:water = 85:15:2). Similar result for other lipiopeptide type biosurfactants also reported [8].

Infrared spectrum of the isolated biosurfactant disclosed a broad stretching peak at 3161 cm^{-1}, characteristic of hydroxyl and amine groups (Fig. 2). Absorption around 2967 cm^{-1} is assigned to the symmetric stretch (γ C–H) of CH_2 and CH_3 groups of aliphatic chains. Also, an intense absorption band at 1691.65 cm^{-1} showed stretching mode of the CO–N bond and a weak symmetric stretching peak around 1455 cm^{-1} indicate the presence of ester carbonyl groups (C═O in COOH) in the biosurfactant. The ester carbonyl group was proved from the band at 1244 cm^{-1} which corresponds

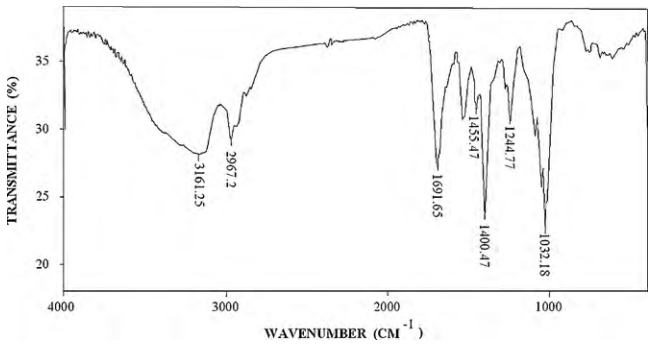

Fig. 2. IR spectrum profile of dried biosurfactant produced by *Bacillus mycoides* SH2 grown on MSM medium.

to C–O deformation vibrations. Also absorption around 1400 cm^{-1} is characterized as aromatic group. Cyclic lipopeptides produced by bacilli like surfactin (produced by *B. subtilis*) and lichenysin (produced by *B. licheniformis*) are, the most effective biosurfactant discovered so far [13].

3.3. Optimization of biosurfactant production

Medium composition such as carbon sources, nitrogen sources, salinity and other growth factors are strongly influenced cell growth and the accumulation of metabolic products, thus the optimization of these parameters can improve the bacterial efficiency. As mentioned, RSM can be an excellent approach to study a process response and to figure out the best correlation among the parameters of a process. This is done via developed models based on the statistical methods to investigate the relation between the inputs and outputs of any process. With the help of the RSM, we can execute the statistical models and to evaluate the effect of parameters of a particular process as well as to optimize the conditions for desirable responses. The RSM is utilized as a statistical design to model the reduction in surface tension (biosurfactant production) process and to determine the significance of growth parameters and their interactions.

The factors affecting the biosurfactant production have been extensively studied in recent years, but few of these studies used proper statistical tools for experimental design. The classical method of medium optimization consists in changing one variable at time and keeping the others at fixed level. However in this study we have optimized the growth conditions of the SH2 strain by the help of RSM for designing the experiments with aim to achieve highest rate of biosurfactant production. Due to the complex nature of biological processes, it is very difficult to predict distinctively the effects of all parameters, which may have multiple interactions. Therefore, RSM was applied to build up an empirical model for modeling biosurfactant production in terms of the operational parameters of medium temperature, pH, salinity and glucose concentration. Design-Expert 7.1 suggested a quadratic equation for decrease in surface tension, ST as

$$\begin{aligned} ST = {} & 35.79 - 2.08(A) + 0.3(B) - 0.17(C) - 2.08(D) - 3.33(A \times B) \\ & + 0.25(A \times C) + 1.30(A \times D) - 0.25(B \times C) - 1.08(B \times D) \\ & - 1.50(C \times D) + 5.26(A^2) + 8.45(B^2) + 1.38(C^2) - 0.039(D^2) \end{aligned}$$

where ST is surface tension (mN/m) (biosurfactant production), A, B, C and D are coded values pertaining to the temperature, pH, salinity and glucose amount, respectively.

ANOVA results of the quadratic model in Table 3 revealed that the model equation derived by RSM by Design-Expert 7.1 could adequately be used to describe the biosurfactant production under a wide range of operating conditions. For the model, there was no lack of fit and the quadratic R^2 was 0.9868. According to software and specified section of optimization, the statistical confidence of the model is 95%, which is an appropriate area.

The predicted versus experimental plot for ST showed that actual values were distributed near to the straight line (Fig. 3), which indicated that actual values were very close to the predicted ones (R^2 = 0.9868). Thus, it was a suitable model to predict the biosurfactant production efficiency using aforementioned experimental conditions.

Biosurfactant production is temperature and pH dependent with optimum production occurring in a particular range where the bacterial strain is most active, e.g. mesophiles at 35–40 °C and pH 7–7.5. Optimum temperature of each type of bacteria takes place in a relatively well-defined range of temperatures at which these microorganisms operate most efficiently. This indicates the

Table 3
The analysis of variance (ANOVA) for the response surface quadratic model.

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	Prob > *F*
Model	1415.54	4	353.89	105.09	<0.0001
Residual	9.20	4	2.30		
Lack of fit	11.00	2	5.50	2.39	0.2073
Pure error	9.20	4	2.30		
R^2	0.9868				

temperature dependent character of the biosurfactant production processes. Increasing temperature results not only in the usual augmentation in the chemical reaction rate but also, within limits, in faster microbial metabolism.

Bacillus mycoide is active in the pH range of 5–9. pH of the growth medium significantly affects the growth and activity of *Bacillus* species. The important characteristics of most organisms are their strong dependence on the pH for cell growth and production of secondary metabolites. Strain SH2 was able to produce biosurfactant in a pH range of 5.0–9.0, although the maximal yield of the biosurfactant was obtained at pH 7.37. (Fig. 4(A)). This ability to produce biosurfactant at wide range of pH with almost similar rate is one of the good properties of this isolate as compare to some other bacterial strains.

Fig. 4(A) presents temperature and pH effects on reduction of surface tension at fixed glucose and salinity concentration of 11.5 and 47.5 g/l, respectively. As can be seen from Fig. 4(A) the pH had a definite influence on the production of biosurfactant by the strain SH2. The production was drastically declined at higher pH when compare to the lower pH, where the production was less affected. Since the marine microorganisms are obviously grown well at pH 7.0, the decreased pH might have not much affected the production, when compare to the higher pH, where the production was drastically declined.

Temperature was one of the critical parameters that have been controlled in bioprocess. Strain SH2 is active in broad range of temperature (20–50 °C) and having maximum growth and activity at higher temperature which indicates it is moderate thermopile. The results in the present study revealed the production of biosurfactant by *B. mycoides* reached maximum at 39.03 °C (Fig. 4 (A)). However at lower and higher temperatures than 39.03 °C almost there were equal declines in biosurfactant production. Having this temperature as an optimum was expected because the isolated bacterium is moderate thermopile. Therefore, we can conclude that for a particular bacterial strain there is a specific optimum growth temperature and another specific temperature at which the bacterial activity is maximum. High pH values (7–8) at high temperatures (37–42 °C) may have a positive effect on biosurfactant production whereas at low pH values and high temperatures biosurfactant production was significantly decreased. Growth and biosurfactant production of the isolate at high temperature increases the potential application of this bacterium especially in oil industries as compare to other organisms like some strain of *B. subtilis* [11], *P. aeruginosa* [34] *Lactobillus* [37,38] *and Rhodococcus* [36].

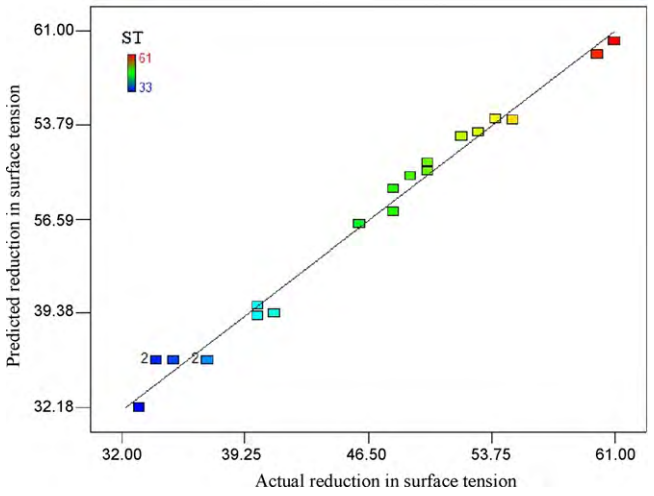

Fig. 3. Experimental reduction in surface tension vs. predicted reduction in surface tension.

A culture medium for isolation and growth of bacteria is essentially a mixture of necessary chemical compounds to supply all the elements required for cell mass production and sufficient energy for biosynthesis and maintenance. Provision of nutrient salts is required to maintain the optimum growth and hence maximum biosurfactant production with the quantity of nutrients apparently being dependent on the availability of substrate, i.e. carbon source, nitrogen source and salts.

The salinity was found to be one of the critical parameter in the production of biosurfactant, in the absence of the salt production and growth was very low. Since the location that this bacterium was isolated from has high salinity (between 10 and 30%) therefore the isolate shows high tolerance to salts concentrations and it showed optimum activity in the salt supplemented medium (Fig. 4(B)).

Fig. 4(B) shows the effects of salt concentrations and temperature on biosurfactant production at fixed glucose concentration of 11.5 g/l and pH 7.25. Increasing the salt concentrations had no considerable effect on biosurfactant production. The strain showed maximum biosurfactant production with 5.5% NaCl supplementation. High salinity at high temperatures can enhance the efficiency of the biosurfactant production whereas low salinity and high temperatures may have negative effect on biosurfactant production. This refers to the nature of the bacterium as it is halotolerant and moderate thermopile.

The joint effects of salt concentration and pH are shown in Fig. 4(C) with glucose concentration fixed at 11.5 g/l and temperature 38.5 °C. Reduction in surface tension gradually increased with increasing pH and salt concentration reaching a final plateau value near 7.25 for pH and 47 g/l for salt concentration. However at high salinity concentrations and pH range 7–8 biosurfactant production and reduction in surface tension was highest.

Fig. 4(D) shows glucose concentration and medium pH effects on biosurfactant production at fixed temperature of 38.5 °C and salts concentration of 47.5 g/L. The biosurfactant production by SH2 strain was consistently increased by increasing the glucose concentration, 16.55 g/l glucose shown to be the best concentration for the maximum production of biosurfactant. The production was declined at higher concentrations of glucose. The possible reason for this reduction is that, for each kind bacterium there is limit for carbon source concentration in the medium and also more important the ratio of the carbon per nitrogen should not exit or reduce from some limit. The response surface of reduction in surface tension gradually increased with increasing glucose concentration from 6 g/l up to 16.55 g/l at fix pH value of 7. However at pH range from 6.5 to 8 at any glucose concentrations biosurfactant production and reduction in surface tension increased.

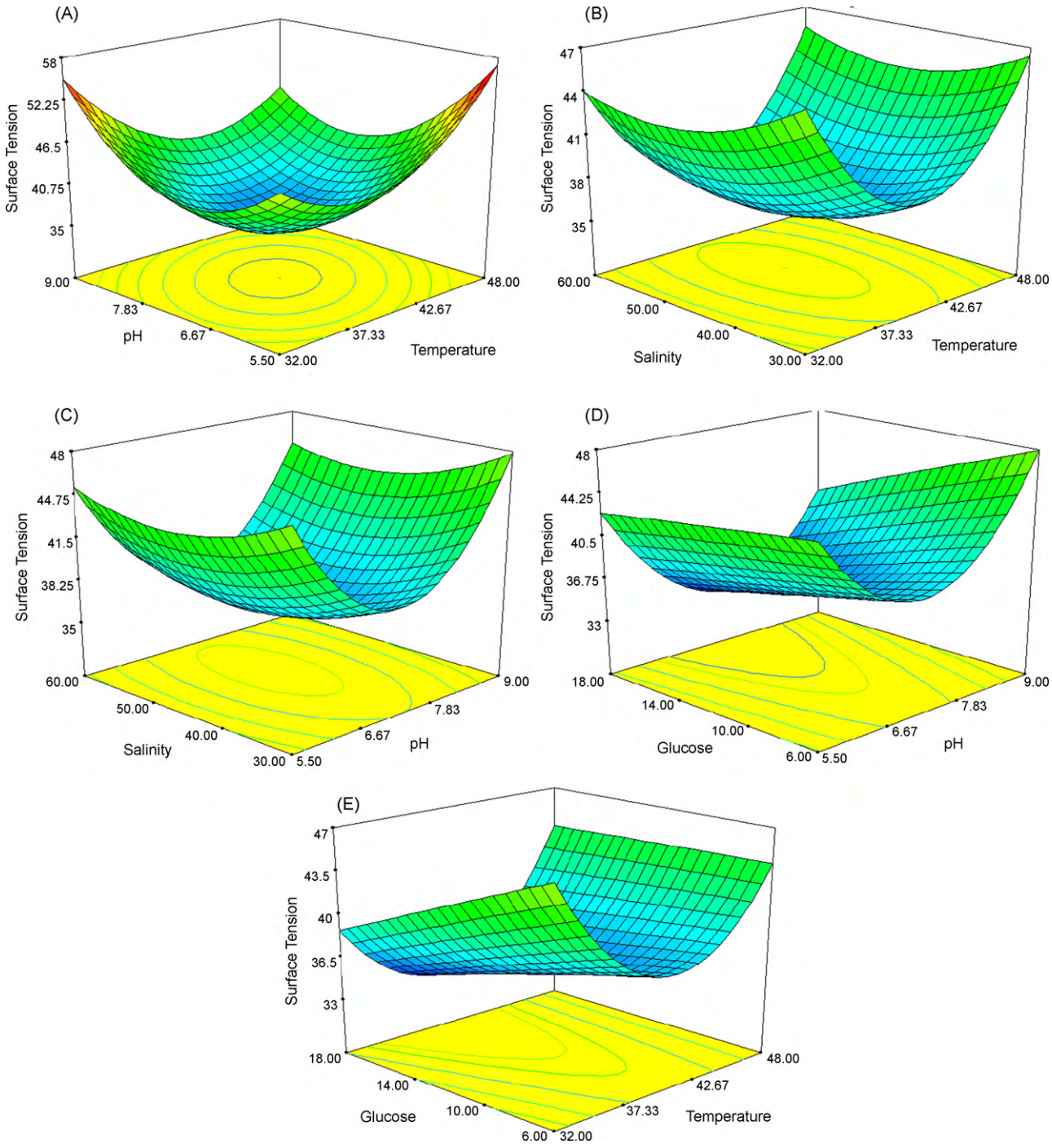

Fig. 4. Three dimensional plots for the minimum surface tension (maximum biosurfactant production). RSM plots were generated using the data shown in Table 2. Inputs were the 21 experimental runs carried out under the conditions established by the CCD design. (A) Reduction in surface tension as a function of temperature and pH. (B) Reduction in surface tension as a function of temperature and salinity. (C) Reduction in surface tension as a function of pH and salinity. (D) Reduction in surface tension as a function of pH and glucose concentration. (E) Reduction in surface tension as a function of temperature and glucose concentration.

Fig. 4(E) shows glucose concentration and temperature effects on biosurfactant production and ultimately reduction in surface tension at fixed pH of 7.25 and salts concentration of 47.5 g/l. In the figure, reduction in surface tension increases almost linearly with temperature in the range of 32–42 °C, while increasing the glucose concentration increased the reduction in surface tension. Like joint effect of glucose and pH here also reduce in surface tension, gradually increased with increasing glucose concentration from 6 up to 16 g/l at fix temperature value of 40 °C. This was expectable because 40 °C is the best temperature for growth and activity of the bacterium and whiles the glucose concentration increases in the medium more carbon source is accessible by the isolate which ultimately can increase the growth and the efficiency of the biosurfactant production.

In conclusion after optimization process the amount of biosurfactant production by SH2 strain increased around 2-fold as compare to basic conditions before optimization (from 1.7 to 3.3 g/l).

4. Conclusions

In the current investigation, we have successfully isolated the indigenous strain of *B. mycoides*. The advantages of this bacterium are the ability of biosurfactant production at wide range of pH (5–9) and temperature (20–50 °C) and tolerance to high salt concentration. Further this bacterium could reduce the surface tension of the medium to 34 mN/m by producing biosurfactant. In conclusion, the biosurfactant produced by the strain SH2 was a kind of preferable surface-active substance, having potential application in bioremediation of crude oil contamination. However to the best of our knowledge this is one of the first reports regarding production and optimization of biosurfactant by *B. mycoides* strain.

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