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Biosurfactant production by a thermophilic *Bacillus subtilis* strain

RS Makkar and SS Cameotra

Institute of Microbial Technology, Sector 39-A, Chandigarh-160036, India

A strain of *Bacillus subtilis* was able to grow and produce a biosurfactant on 2% sucrose at 45°C. As a result of biosurfactant synthesis the surface tension of the medium was reduced from 68 dynes cm^{-1} to 28 dynes cm^{-1}. The strain had the capacity to produce the biosurfactant at high NaCl concentrations (4%) and a wide range of pH (4.5–10.5). The biosurfactant retained its surface-active properties after heating at 100°C for 2 h and at different pH values (4.5–10.5). A maximum amount of biosurfactant was produced when urea or nitrate ions were supplied as nitrogen source. The use of the biosurfactant at high temperatures, acidic, alkaline and saline environments is discussed. As a result of its action, 62% of oil in a sand pack column could be recovered, indicating its potential application in microbiologically enhanced oil recovery.

Keywords: biosurfactant; thermophilic; *Bacillus subtilis*; MEOR

Introduction

Increased interest in biosurfactants in recent years has stimulated attempts to enlarge the present range of microbial surfactants. Most of these biosurfactants are biodegradable and less toxic than their counterparts which are synthesized chemically [9]. These biosurfactants have potential for use in the oil industry, such as cleaning oil sludge from storage tanks and tankers, enhancing oil recovery processes, mobilizing heavy crude oil, transporting petroleum in pipelines and managing marine oil spills [4,9,10,14–16,24]. Other uses of biosurfactants are as food additives and emulsifiers for application in agricultural systems and in cosmetics [27].

A wide variety of microorganisms produce different types of surface-active agents. Many reviews are available which describe the structure and function of these biological agents [9,11–14]. *Bacillus* strains produce different classes of bioactive peptides showing a variety of antimicrobial and surface-active properties [17–20]. Surfactin, a lipopeptide produced by *Bacillus subtilis*, is one of the most effective biosurfactants known so far [2]. This compound inhibits fibrin clot formation and lyses erythrocytes, several bacterial spheroplasts and protoplasts. It lowers the surface tension of water from 72 dynes cm^{-1} to 27 dynes cm^{-1}. This is significantly lower than any other lipopeptide biosurfactant reported [6–8]. Most studies on biosurfactant production have been conducted on microorganisms under mesophilic conditions. Some field applications of these biosurfactants require high tolerance for temperatures, pH and salts. Banat [3] reported isolation of a thermophilic *Bacillus* strain on hydrocarbon-containing medium. Recently, Yakimov *et al* [28] reported isolation of a strain of *Bacillus lichenformis* from a petroleum reservoir which could produce surfactant optimally at 5% NaCl concentration and temperatures between 35 and 45°C.

In this paper we report biosurfactant production by a strain of *Bacillus subtilis* under thermophilic conditions. Optimization of media and growth conditions are also discussed. This is the first report describing biosurfactant production by a *Bacillus subtilis* at 45°C. Stability and the potential application of the product in oil recovery are presented.

Materials and methods

Organism

Bacillus subtilis MTCC 2423 was obtained from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The culture was maintained on nutrient agar plates.

Conditions of cultivation

Nutrient broth (Hi Media, India) was used for preparation of the inoculum. The cultures were grown in this broth for 6–8 h at 45°C ($OD_{600\ nm}$ 0.8–0.9). This was used as inoculum at 2% (v/v). For biosurfactant synthesis a mineral salts medium was utilized; it contained (w/v) KNO_3 (0.3%), Na_2HPO_4 (0.22%), KH_2PO_4 (0.014%), NaCl (0.001%), $MgSO_4$ (0.06%), $CaCl_2$ (0.004%), $FeSO_4$ (0.002%) and 0.1 ml of trace element solution containing 2.32 g $ZnSO_4 \cdot 7H_2O$, 1.78 g $MnSO_4 \cdot 4H_2O$, 0.56 g H_3BO_3, 1.0 g $CuSO_4 \cdot 5H_2O$, 0.39 g $Na_2MoO_4 \cdot 2H_2O$, 0.42 g $CoCl_2 \cdot 6H_2O$, 1.0 g EDTA, 0.004 g $NiCl_2 \cdot 6H_2O$ and 0.66 g KI. The carbohydrate (glucose, trisodium citrate, sucrose, sodium pyruvate, sodium acetate) and other carbon sources (yeast extract, beef extract) were added to make the final concentration 2% (w/v). The hydrocarbon substrates (dodecane, hexadecane, decane, kerosene and pristane) were added separately at 2% (v/v) concentration. The concentration of ammonium nitrate, urea, ammonium sulphate, sodium nitrate or other nitrogen sources was 0.3%.

Growth studies were done in 1-L flasks containing

Correspondence: SS Cameotra, IMTECH, Sector 39-A, Chandigarh-160036, India
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200 ml medium at 45°C with shaking at 200 rpm. For growth studies and biosurfactant production at different NaCl concentrations and pH, the NaCl concentration and pH of the medium were adjusted accordingly. Growth studies were done using 2% sucrose as the carbon source. Experiments were done in duplicate and the results reported are averages of three independent experiments.

Biomass determination

Fifty-millilitre samples at different time intervals of fermentation were centrifuged at $12\,352 \times g$ for 25 min. Biomass obtained was dried overnight at 105°C and weighed.

Surface activities

Surface tension and Critical Micelle Dilution (CMD^{-1} and CMD^{-2}) were determined using a Du-Nouy Tensiometer (CSC No. 70535, CSC, Fairfax, VA, USA). All measurements were made on cell-free broth obtained by centrifuging the cultures at $12\,352 \times g$ for 25 min. For CMD measurements the cell-free broth was diluted 10 times (CMD^{-1}) and 100 times (CMD^{-2}) respectively.

Biosurfactant isolation

Samples at different fermentation times were centrifuged at $12\,352 \times g$ for 25 min to get cell-free broth. Biosurfactant in the cell-free broth was precipitated by adjusting the pH of the broth to 2.0 using 6 N HCl and keeping it at 4°C overnight. The precipitate thus obtained was pelleted at $12\,352 \times g$ for 20 min, redissolved in distilled water, adjusted to pH 7.0, freeze dried and weighed. Further purification of the biosurfactant was done by extraction of the freeze-dried acid precipitate with chloroform : methanol (65 : 15). The extract was evaporated by rotatory evaporation under vacuum. The product obtained was used for chemical characterization of the surfactant.

Chemical characterization of biosurfactant

Preliminary characterization of the biosurfactant was done by thin layer chromatography (TLC). The components of the $CHCl_3 : CH_3OH$ extract were identified by TLC on silica gel 60 (Merck, Darmstadt, Germany) in $CHCl_3 : CH_3OH : H_2O$ (65 : 15 : 1). Components were detected by spraying with distilled water and heating the plates at 110°C for 5 min or by spraying with 50% sulphuric acid and charring plates at 120°C for 20 min. Free amino groups were detected by spraying the plates with 0.2% ninhydrin in acetone.

Biochemical analysis

The protein content of cell-free broth was estimated according to the method of Lowry *et al* [19] using BSA as standard. Lipid content was determined gravimatically by weighing the pooled diethyl ether extracts of cell-free broth. Residual substrate concentration (total sugars) was estimated by the anthrone reaction [23].

Sand pack test and emulsification index (E_{24})

The application of the product in microbiologically enhanced oil recovery (MEOR) was evaluated using the sand pack technique described by Abu Ruwaida *et al* [1]. A glass column (40 × 2.5 cm) was packed with 100 g of acid-washed sand. The column was then saturated with 100 ml of kerosene. The activity of the isolated surfactant in oil recovery was estimated by pouring 100 ml of aqueous solution of biosurfactant (1.0 mg ml^{-1}) onto the column. The amount of oil released was measured. For estimation of the emulsification index, 6 ml of motor oil was added to 4 ml of the culture broth in a graduated tube and vortexed at high speed for 2 min. The emulsion stability was determined after 24 h. The E_{24} was calculated by measuring the emulsion layer thus formed.

Stability studies

Stability studies were done using cell-free broth from cells grown on sucrose for 72 h, obtained by centrifuging the cultures at $12\,352 \times g$ for 25 min. Fifty millilitres of broth were heated in a boiling water bath for different time intervals and cooled to room temperature. Surface tension and CMD values were then measured. For studying the pH stability of cell-free broth, pH was adjusted to different pH values and surface tension and CMD values were measured.

Results

Growth characteristics and biosurfactant production on different carbon sources

Bacillus subtilis MTCC 2423 was grown on each of 12 carbon sources (Table 1). Since biosurfactants reduce the surface tension of the medium in which they grow, biosurfactant production was monitored by measuring the reduction in surface tension of the cell-free broth [5,6]. The surface tension of the cell-free broth at day 0 is indicated in parentheses for different carbon sources. Surface tension reduction was greater when glucose, sucrose, tri-sodium citrate, sodium pyruvate, yeast extract and beef extract were used as carbon source. Sodium acetate inhibited growth and was not used by the strain for biosurfactant production. The organism was able to grow on *n*-hexadecane and pristane but did not produce biosurfactant on *n*-hexadecane or pristane. None of the other water-insoluble carbon sources (hydrocarbon) was used by the strain for biomass and biosurfactant production.

Table 1 Effect of carbon sources (2%) on biosurfactant production after growth of *Bacillus subtilis* for 72 h at 45°C

Carbon source	Surface tension (dynes cm^{-1})	Biomass (g L^{-1})	Biosurfactant (g L^{-1})
Sucrose	28 (68)	2.60	0.744
Glucose	29 (68)	1.85	0.652
Sodium pyruvate	32 (64)	1.77	0.376
Sodium acetate	45 (71)	0.035	0.112
Yeast extract	30 (42)	1.90	0.308
Beef extract	32 (46)	1.29	0.349
Tri-sodium citrate	31 (73)	0.543	0.597
Hexadecane	52 (68)	2.4	0.241
Dodecane	53 (68)	0.340	0.127
Decane	57 (68)	0.400	0.134
Kerosene	47 (68)	0.750	0.093
Pristane	55 (70)	1.74	0.145

Readings in parentheses indicate surface tension of the cell-free broth at day 0.

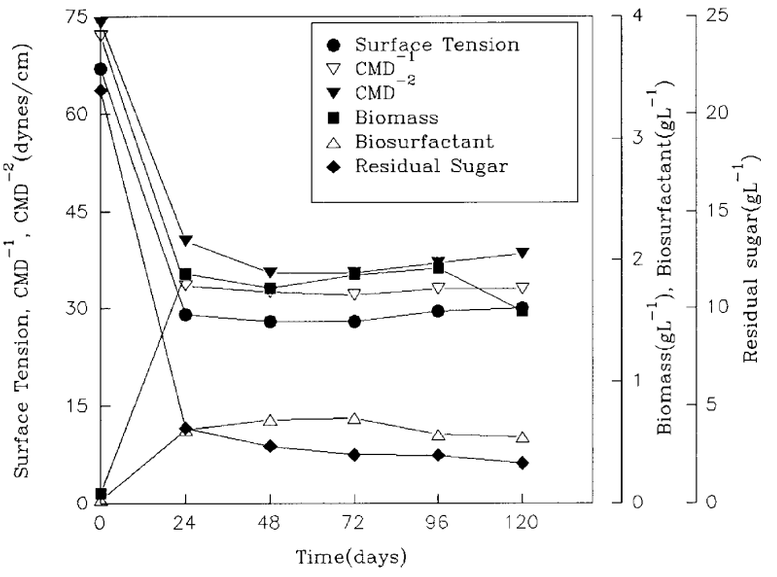

Figure 1 Growth, biosurfactant production and surface activity of *Bacillus subtilis* during growth on a mineral salts medium supplemented with 2% sucrose at 45°C.

Sucrose was used as the carbon source to study growth and biosurfactant production by the strain. Other carbon sources were not used as they are expensive substrates when compared to sucrose. A 2% concentration of sucrose was found to be optimum for biosurfactant formation. Figure 1 illustrates biosurfactant production and growth characteristics of the *Bacillus* culture on 2% sucrose at 45°C. Biosurfactant yield was maximum at 72 h of fermentation. At this point surface tension, CMD^{-1}, CMD^{-2} were minimum. Most of the substrate was utilized by the strain within 24 h of growth.

Effect of nitrogen source on biosurfactant production

The nitrogen source affects biosurfactant production as depicted in Table 2. In nitrogen-free medium, the least reduction in surface tension was achieved, whereas sodium nitrate, potassium nitrate and urea were the best sources of nitrogen of those tested. Ammonium sulphate was not utilized for growth and biosurfactant production but the organism was able to utilize ammonium nitrate.

Chemical characteristics of the biosurfactant

For detection of purified biosurfactant the most suitable solvent system was $CHCl_3 : CH_3OH : H_2O$ (65 : 15 : 1). When sprayed with distilled water TLC plates contained white dry spots with Rf values similar to Surfactin (Sigma, St Louis, MO, USA). Black spots were obtained upon charring with sulphuric acid. The IR spectrum of the purified biosurfactant was similar (overlapping) to the spectrum of pure surfactin indicating that surfactant obtained was similar to surfactin.

Properties of the biosurfactant

The cell-free broth was both pH and thermo-stable as shown in Figures 2 and 3. The biosurfactant activity was retained over a pH range of 4.5 to 10 with minimal deviation in surface tension and CMD values.

When the cell-free broth was heated in a boiling water bath, it retained its surface activity (28–29 dynes cm^{-1}) even after heating for 2 h (Figure 3). The biosurfactant recovered by acid precipitation was reconstituted in differ-

Table 2 Effect of nitrogen sources (0.3%) on biosurfactant production after growth of *Bacillus subtilis* for 72 h at 45°C

Nitrogen source	Surface tension (dynes cm^{-1})	Biomass (g L^{-1})	Residual sugar (g L^{-1})	Biosurfactant (g L^{-1})
Urea	29	1.41	1.30	1.012
Peptone	31.5	2.29	1.17	0.327
Yeast extract	30 (42)	2.09	1.73	0.458
Beef extract	29 (46)	1.9	0.495	0.493
Tryptone	29	2.02	1.8	0.238
Nitrogen-free medium	47 (69)	0.21	17.70	0.090
Potassium nitrate	29 (68)	2.27	1.41	0.731
Sodium nitrate	29.5 (68)	2.3	1.1	0.724
Ammonium nitrate	33 (70)	2.16	1.19	0.317
Ammonium sulphate	56 (71)	0.897	13.45	0.170

Readings in parentheses indicate surface tension of the cell-free broth at day 0.

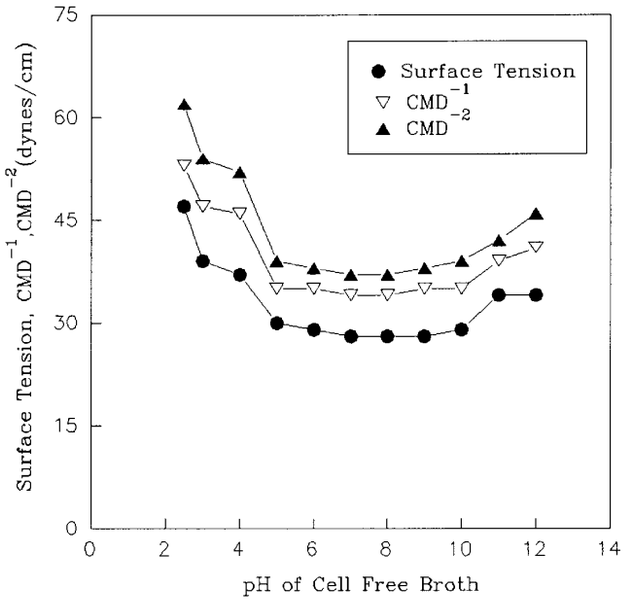

Figure 2 Influence of pH on surface activity of the cell-free broth obtained from growth of *Bacillus subtilis* on 2% sucrose for 72 h at 45°C.

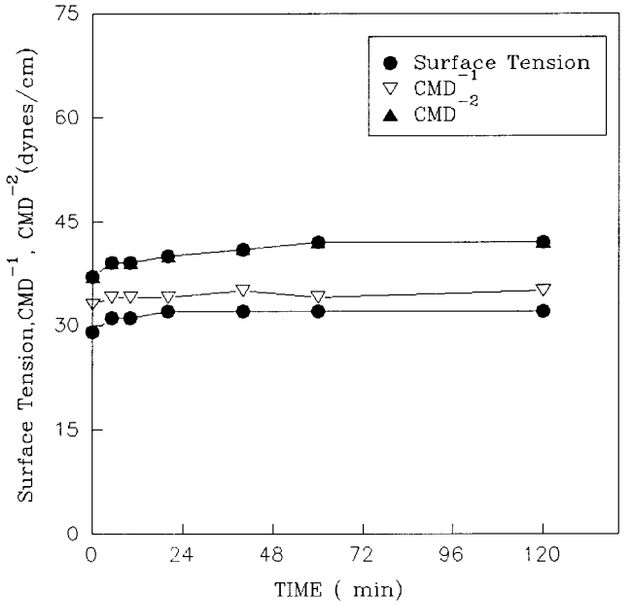

Figure 3 Effect of temperature (100°C) on surface activity of the cell-free broth obtained from growth of *Bacillus subtilis* on 2% sucrose for 72 h at 45°C.

ent concentrations as shown in Table 3. The biosurfactant exhibited reduction of surface tension.

The biosurfactant isolated by acid treatment had a lipid content of 17.05%, protein content of 13.2% and carbohydrate content of 3.152%. The remaining portion was inorganic salts. The critical micelle concentration (CMC) of the biosurfactant was 35 mg L^{-1}.

The Emulsification Index (E_{24}) value of the biosurfactant was 90 with motor oil, demonstrating its capacity to emulsify oil. When the column was washed with distilled water, only 28% of the oil was recovered. However, 62% of the oil was recovered from the sand pack column when surfactant solution was added, indicating the potential use of the biosurfactant in MEOR.

Table 3 Surface activity of the reconstituted biosurfactant obtained by acid precipitation

Sample No.	Concentration of biosurfactant (mg ml^{-1})	Surface tension (dynes cm^{-1})
1	100	30
2	50	33
3	20	36
4	2	39
5	1	39
6	0.05	42

Growth of the organism at different NaCl concentrations and pH values

As shown in Table 4 the organism grew and produced biosurfactant at different NaCl concentrations (0.01–4%) and pH values (4.5–10.5). High NaCl concentration in the medium did not affect the capability of the organism to grow and to synthesize biosurfactant, but biosynthesis of surfactant was reduced at 4% NaCl. When the organisms were grown at pH values between 6.5 and 10.5, there was little difference in biomass and biosurfactant production, but both were markedly lower at a medium pH of 4.5.

Discussion

Studies on oil-degrading and biosurfactant-producing microorganisms deal almost exclusively with mesophiles, mainly belonging to the genera *Acinetobacter*, *Pseudomonas* and *Bacillus*. Haferberg *et al* [14] and Gurra Santos *et al* [13] reported that the majority of known biosurfactants are synthesized by microorganisms grown on water-immiscible hydrocarbons, but some have been produced on water-soluble substrates such as glucose, glycerol and ethanol [8,20]. In the present study conditions were standardized for maximum biosurfactant production by *Bacillus subtilis*

Table 4 Growth, biosurfactant production and surface tension values during growth of *Bacillus subtilis* at different NaCl concentration and pH on 2% sucrose

	Surface tension (dynes cm^{-1})	Biomass (g L^{-1})	Biosurfactant (g L^{-1})
Concentration of salt %			
0.01	28	1.87	0.936
0.05	28	2.25	0.628
1.0	29.5	2.43	0.694
2.0	30	2.02	0.612
4.0	32	2.77	0.408
pH of medium			
4.5	35	0.200	0.108
6.5	28	1.24	0.604
7.0	28	2.19	0.840
8.5	29	1.86	0.604
10.5	29	1.57	0.648

MTCC 2423 under thermophilic growth conditions. Since the type of medium and growth conditions can influence the type and yield of biosurfactant, we studied the influence of both carbon and nitrogen sources. The carbon source, particularly the carbohydrate, influences the type of glycolipids formed. Suzuki *et al* [25] observed that glucose-, fructose- and sucrose-lipids are formed by *Arthrobacter paraffineus* and several species of *Corynebacterium*, *Norcardia* and *Brevibacterium* during growth on the corresponding sugar. The *Bacillus* strain used in this study was able to utilize glucose, sucrose, and sodium pyruvate for biosurfactant production. Biosurfactant was not synthesized when sodium acetate was used as the carbon source. Growth and biosurfactant production by *Bacillus subtilis* were studied in minimal medium supplemented with sucrose as carbon source at a concentration of 2%. The strain was allowed to grow on the hydrocarbons (pristane, dodecane, decane or kerosene) for 10 days but no growth was observed. The strain was able to utilize *n*-hexadecane and pristane for biomass formation (growth), but did not form biosurfactant on either *n*-hexadecane or pristane.

Nitrogen (ammonium or nitrate ions) regulates the production of biosurfactant [9]. *Arthrobacter paraffineus* showed preference for ammonium salts and urea as a nitrogen source [10]. Investigations on rhamnolipid production by *Pseudomonas* 44Ti on olive oil showed that sodium nitrate was the best nitrogen source [22]. Similar results were obtained using a *Pseudomonas aeruginosa* culture [21]. In the present study, both sodium nitrate and potassium nitrate were preferred nitrogen sources (Table 2). *Bacillus subtilis* MTCC 2423 was not able to utilize ammonium sulphate, but exhibited preference for nitrate ions. Nitrogen was required for biosurfactant production; when there was no nitrogen in the medium, there was negligible reduction in surface tension and amount of biosurfactant produced.

Preliminary chemical characterization of the biosurfactant by TLC shows it to be similar to surfactin. The similarity of the surfactant with standard surfactin was confirmed by IR analysis.

Stability studies of the product in the culture broth indicate the surfactant to be thermostable and pH stable. Moreover, biosurfactant was produced by the organism in the pH range of 4.5 to 10.5. The reconstituted biosurfactant isolated by acid treatment exhibited surfactant activity.

Sand pack tests indicated a recovery of around 62% oil suggesting that the biosurfactant may be useful in oil recovery. Biosurfactant production by the strain at 45°C and its stable nature suggests its possible use in desert oil fields. In addition, the ability of the organism to grow and produce biosurfactant over a wide range of pH (4.5–10.5) and at NaCl concentrations up to 4% could be of potential application in acidophilic, alkalophilic and saline environments.

The potential use of the stable biosurfactant produced by the strain in the oil industry for cleaning sludge in storage tanks, oil mobilization and MEOR is apparent. The use of such a thermophilic strain in large scale production in any future commercial application would be of advantage compared to a mesophilic strain due to reduction in cooling cost during production and less chance of contamination.

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