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Production of biosurfactant at mesophilic and thermophilic conditions by a strain of *Bacillus subtilis*

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The ability of a *Bacillus subtilis* strain to grow and produce biosurfactant on different carbon and nitrogen sources under thermophilic conditions (45°C) was studied. The strain was able to reduce surface tension to 34 dynes cm^{-1} on 2% sucrose, and 32 dynes cm^{-1} on starch after 96 h of growth. The biosurfactant was stable at 100°C and within a wide pH range (3.0–11.0). Biosurfactant formation at mesophilic conditions (30°C) was also studied. The organism was able to produce the maximum amount of biosurfactant when nitrate ions were supplied as the nitrogen source. The potential application of the biosurfactant in oil recovery from desert oil fields, acidic and alkaline environments is demonstrated. The biosurfactant was identical to surfactin as confirmed by TLC and IR analysis.

Keywords: biosurfactant; thermophilic; *Bacillus subtilis*

Introduction

Biosurfactants are amphiphilic compounds produced by microorganisms which either adhere to cell surfaces or are excreted extracellularly in the growth medium [8,27]. Many microorganisms produce biosurfactants during growth on a wide variety of substrates. In recent years interest in biosurfactants has been generated due to their possible applications in environmental protection, crude oil drilling, and in the pharmaceutical and food processing industries [11,13]. Since the earliest reports on bacterial surfactants [28], a variety of biosurfactants have been described. These include glycolipids, phospholipids, lipopeptides, neutral lipids, fatty acids and lipopolysaccharides [5]. Several reports are available on the production of these biosurfactants on water-immiscible substrates, especially hydrocarbons [3,12]. Reports are also available on biosurfactant production on water-soluble substrates [10]. Some species of *Bacillus* and of the yeast *Rhodotorula* are efficient biosurfactant producers on these water-soluble compounds [10,24]. Surfactin, one of the most effective biosurfactants, was isolated from the cell-free culture medium after growth of *Bacillus subtilis* on glucose [4]. *Pseudomonas rubescus*, *Agrobacterium tumefaciens* and *Glucobacter cerinus* synthesize amino acid-containing lipids during growth on water-soluble compounds [23].

Synthesis of biosurfactants by microorganisms has been studied mainly in mesophilic environments using mesophilic organisms. There are very few reports on biosurfactant production by thermophiles. Thermophiles are of interest because of their potential for biotechnological applications in oil recovery in desert oil fields. Banat [2] isolated a thermophilic *Bacillus* strain on a hydrocarbon-containing medium. Yakimov *et al* [26] reported isolation of a strain of *Bacillus lichenformis* from a petroleum reservoir which was able to produce surfactant optimally at 5% NaCl concentration and temperatures between 35 and 45°C. Trebbau de Acevado and McInnerney [25] reported emulsifying activity from thermophilic and extremely thermophilic microorganisms. The bioemulsifier was effective over a wide range of pH and NaCl concentration up to 200 g L^{-1} and at temperatures up to 80°C.

In this communication, we report optimization of media and growth conditions for *Bacillus subtilis* for biosurfactant production under thermophilic conditions.

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Materials and methods

Organism

Bacillus subtilis MTCC 1427 was used in the present study and maintained on nutrient agar (Hi Media, Mumbai, India) plates.

Media and cultivation conditions

Nutrient broth was used for preparation of the inoculum. The composition of the nutrient broth used was as follows: beef extract 1.0 g, yeast extract 2.0 g, peptone 5.0 g, NaCl 5.0 g in a litre of distilled water. To make nutrient agar 15.0 g of agar was added to the nutrient broth. The cultures were grown in this broth for 6–8 h at 30°C ($OD_{600\ nm}$ 0.8–0.9). This was used as inoculum at the 2% (v/v) level. For biosurfactant synthesis a mineral salt medium with the following composition was utilized: 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 (g L^{-1}): 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 respective carbohydrate (glucose, starch, sucrose, sodium pyruvate, sodium acetate) was added to make the final concentration 2% (v/v). The hydrocarbon substrates (dodecane, hexadecane, and pristane) were also added separately at 2% (v/v) concentration. The concentration of ammonium nitrate, urea, ammonium sulphate and sodium nitrate was 0.3%.

Growth studies were done in 1-liter flasks containing 200 ml medium at 30°C and 45°C with shaking at 200 rpm. For growth studies and biosurfactant production at different pH values, the pH of the medium was adjusted accordingly. Growth studies were done as mentioned above using 2% sucrose as the carbon source.

Experiments were done in duplicate and results reported are the average from 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 Scientific Co, Albany, 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 and purification

Bacterial cells were removed from surfactant-containing medium by centrifugation ($12\,352 \times g$, 20 min). The supernatant was subjected to acid precipitation by adding 6 N HCl to achieve a final pH of 2.0 and allowing a precipitate to form at 4°C. 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. The dried surfactant was extracted with solvent (chloroform : methanol; 65 : 15). The extract was dried with the aid of a rotary evaporator under vacuum. The material thus obtained was used for further analysis.

Biochemical analysis

The protein content of cell-free broth was estimated according to the method of Lowry *et al* [14] using BSA as standard. Lipid content was determined gravimetrically by weighing the pooled di-ethyl ether extracts of cell-free broth. Residual substrate concentration (total sugars) was estimated by the anthrone reaction [19].

Emulsification index (E_{24}) and sand pack test

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. The potential application of the product in MEOR was evaluated using the sand pack technique described by Abu Ruwaida *et al* [1]. A glass column (40.0×2.5 cm) was packed with 100 g of acid-washed sand. The column was then saturated with 100 ml of kerosene oil. The potential of the isolated surfactant for oil recovery was estimated by pouring 100 ml of aqueous solution of biosurfactant (1 mg ml^{-1}) into the column. The amount of oil released was measured.

Stability studies

Stability studies were done using the cell-free broth 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 at each point were measured. To study the pH stability of the cell-free broth, the pH of the cell-free broth was adjusted to different pH values and the surface tension was measured.

Results

Growth characteristics and biosurfactant production on different carbon sources

Bacillus subtilis MTCC 1427 was grown on different carbon sources (Table 1). Biosurfactant production was monitored by measuring the reduction in surface tension of the cell-free broth. The surface tension at day 0 was 68 and 62 dynes cm^{-1} for sucrose and starch, respectively. Surface tension reduction was greater with glucose, sucrose, starch and sodium pyruvate as carbon sources in comparison to other carbon sources viz sodium acetate and hydrocarbons. The strain was not able to grow or produce biosurfactant when sodium acetate and hydrocarbons were supplied as carbon sources.

Sucrose and starch were used as carbon sources to study the growth and biosurfactant production by the strain. A 2% concentration of these two substrates was found to be optimum for biosurfactant formation. Figures 1 and 2 illustrate biosurfactant production and growth characteristics of the *Bacillus* culture on 2% sucrose and 2% starch at 30°C and 45°C. Biosurfactant yield was maximum at 96 h of fermentation at 30°C when 2% sucrose was used as a carbon source. At this point biomass was maximum and surface tension, CMD^{-1}, CMD^{-2} were minimum. At 45°C maximum biosurfactant yield, though less in comparison to 30°C, was achieved in 72 h of fermentation and remained almost stationary until 96 h and decreased thereafter. Growth characteristics and biosurfactant production were similar at 30°C and 45°C. When starch was used as carbon source at the optimal concentration of 2%, at 30°C, the maximum reduction in surface tension was achieved within 24 h of fermentation (30 dynes cm^{-1}). However, the CMD^{-1} and CMD^{-2} value reduction was achieved only after 48 h

Table 1 Effect of carbon source in the mineral salt medium on surface tension of the cell-free broth measured after 48 h of growth

Carbon source	Surface tension (dynes cm^{-1}) at 30°C	Surface tension (dynes cm^{-1}) at 45°C
Control	68	68
Glucose	28	43
Sucrose	28	42
Starch[a]	29	31
Sodium pyruvate[b]	32	32
Sodium acetate	56	65
Pristane	52	51
Hexadecane	50	53
Dodecane	47	53

[a]Surface tension of uninoculated medium was 62 dynes cm^{-1}.
[b]Surface tension of the uninoculated medium was 56 dynes cm^{-1}.

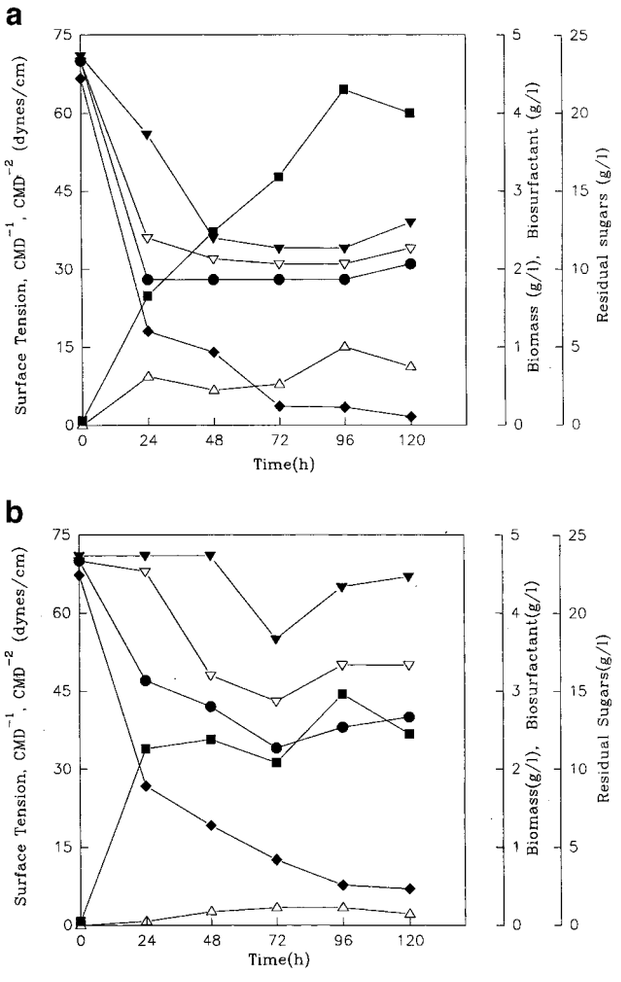

Figure 1 Growth, biosurfactant production and surface activity profiles of *Bacillus subtilis* MTCC 1427 grown in mineral salt medium with 2% sucrose as carbon source at (a) 30°C and (b) 45°C. The samples were taken every 24 h, centrifuged and cell-free broth was used for determination of surface tension (●), CMD values (CMD^{-1}, ▽; CMD^{-2}, ▼), biomass (■), biosurfactant estimation (△) and residual sugar analysis (◆).

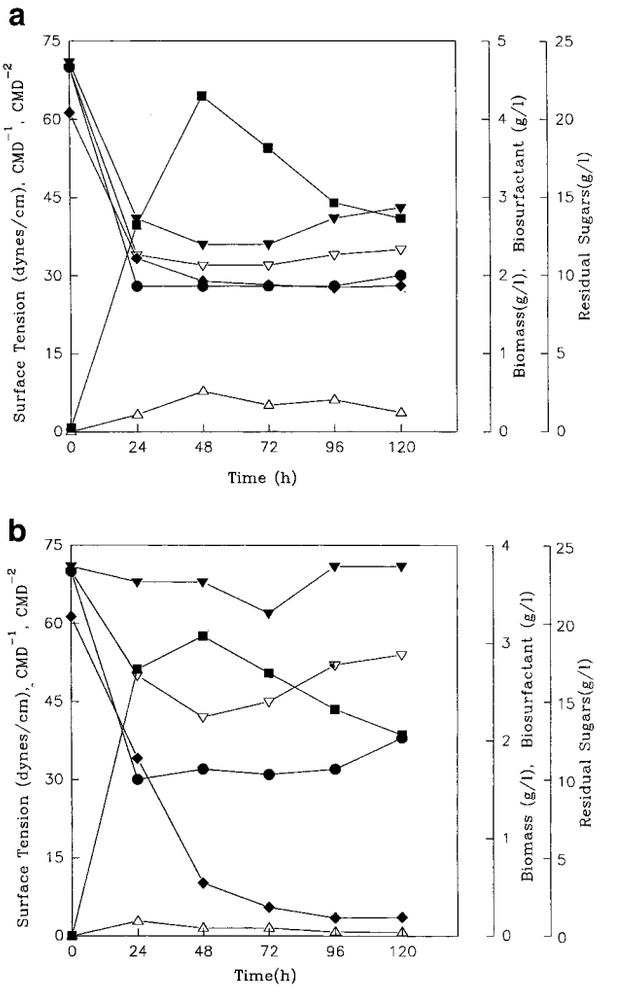

Figure 2 Growth, biosurfactant production and surface activity profiles of *Bacillus subtilis* MTCC 1427 grown in mineral salt medium with 2% starch as carbon source at (a) 30°C and (b) 45°C. The samples were taken every 24 h, centrifuged and cell-free broth was used for determination of surface tension (●), CMD values (CMD^{-1}, ▽; CMD^{-2}, ▼), biomass (■), biosurfactant estimation (△) and residual sugar analysis (◆).

of fermentation. At 45°C, the pattern of biosurfactant production was similar to that obtained at 30°C, but with lower yield. As evident from Figures 1 and 2, a major amount of substrate was utilized by the strain within 24 h of growth. The strain was able to grow and produce biosurfactant at a wide range of pH (pH 4.5–9.0) as shown in Table 2.

Effect of nitrogen source on biosurfactant production

Choice of nitrogen source affects the biosurfactant production as depicted in Figure 3. In nitrogen-depleted medium the least reduction in surface tension was achieved, while sodium nitrate and potassium nitrate were the two best sources of nitrogen of the five sources tested. Ammonium salts in the form of ammonium nitrate and ammonium sulphate were not utilized for biosurfactant production.

Studies on the properties of the biosurfactant

The cell-free broth was both pH and thermally stable as shown in Table 3. The biosurfactant activity was retained at a pH range of 3.0–11.0 with a minimum deviation in surface tension and CMD values. At pH 3.0 a negligible loss in activity (4 dynes cm^{-1}) was observed.

When the cell-free broth was heated in a boiling water bath for different time intervals it retained its surface activity (27–28 dynes cm^{-1}) even after heating for 60 min. The biosurfactant isolated by acid treatment had a lipid content of 30% and protein content of 1%. No significant change in lipid and protein content of the broth was observed after heat and pH treatments. The critical micelle concentration (CMC) of the biosurfactant was found to be 40 mg L^{-1}.

The Emulsification Index (E_{24}) value of the biosurfactant was found to be 33.33 with mobile oil demonstrating its capacity to emulsify oil. The biosurfactant solution was able to recover 56% of the oil adsorbed to the sand in the column. This indicates its potential use in MEOR (Microbial Enhanced Oil Recovery). The biosurfactant iso-

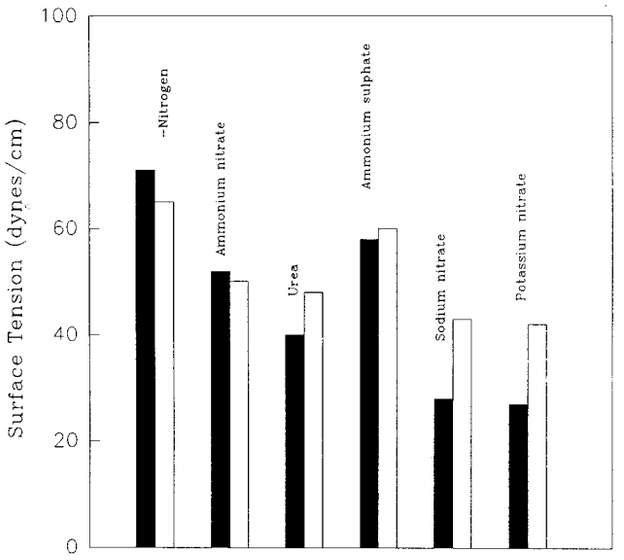

Figure 3 Effect of nitrogen source in mineral salt medium on surface tension measured after 48 h of growth. The organism was grown at 30°C (■) and 45°C (□) for 48 h, centrifuged and cell-free broth used for determination of surface tension.

Table 2 Growth and production of biosurfactant by *Bacillus subtilis* at extreme pH conditions. The organism was grown in minimal medium supplemented with 2% sucrose at 30°C

pH	Time (days)	Surface tension (dynes cm^{-1})	Biomass (g L^{-1})	Biosurfactant (g L^{-1})
4.5	1	29	2.78	0.316
	2	30	2.98	0.260
	3	30	3.82	0.272
	4	30	3.67	0.240
9.0	1	29	1.17	0.294
	2	29	2.60	0.808
	3	29	2.61	0.760
	4	29	2.20	0.688

lated from 30°C and 45°C growth gave similar results for both E_{24} value and sand pack oil recovery.

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). Developing the TLC gave white dry spots when sprayed with distilled water with R_f values similar to surfactin (Sigma, St Louis, MO, USA). The IR spectrum of the purified biosurfactant at mesophilic and thermophilic conditions was similar (overlapping) to the spectra obtained with pure surfactin, indicating that the surfactant obtained was surfactin.

Discussion

Most of the work on biosurfactant production by microorganisms has been in mesophilic environments, the organisms mainly belonging to the genera *Acinetobacter*, *Pseudomonas* and *Bacillus*. The majority of known biosurfactants are synthesized by microorganisms grown on water-immiscible hydrocarbons [9,10], but there are many reports of biosurfactant production on water-soluble substrates such as glucose, glycerol and ethanol [4,16]. In the present study conditions were standardized for the maximum biosurfactant production by *Bacillus subtilis* MTCC 1427 at mesophilic and thermophilic growth conditions. A number of studies have indicated that the type of medium and growth conditions can influence the type and yield of the biosurfactant. In this regard we studied the influence of both carbon and nitrogen sources. The carbon source, particularly the carbohydrate, has a major effect on the type of glycolipids formed. Glucose, fructose and sucrose lipids are formed by *Arthrobacter paraffineus* and several species of *Corynebacterium*, *Norcardia*, and *Brevibacterium* during growth on the corresponding sugar [21]. The *Bacillus* strain used in this study was able to utilize glucose, sucrose, starch and sodium pyruvate for biosurfactant production. The organism was not able to grow or produce biosurfactant when sodium acetate and hydrocarbons were presented as the carbon sources. Growth and biosurfactant production by *Bacillus subtilis* was studied in minimal medium supplemented with sucrose and starch as carbon sources at a concentration of 2%.

The nitrogen source in the medium influences the production of biosurfactant [5]. *Arthrobacter paraffineus* showed a preference for ammonium salts and urea as the nitrogen source [6]. Robert *et al* [18] while investigating rhamnolipid production by *Pseudomonas* 44Ti on olive oil reported that sodium nitrate was the best nitrogen source. Similar results have been noted for *Pseudomonas aeruginosa* [17] and *C. tropicalis* IIP-4 [20]. In the present study we found that both sodium nitrate and potassium nitrate are the preferred nitrogen sources (Figure 3). *Bacillus subtilis*

Table 3 pH and thermostability of cell-free broth of *Bacillus subtilis* MTCC 1427. The organism was grown in minimal medium supplemented with 2% sucrose at 30°C for 48 h

(a) pH studies

pH of cell-free broth	Surface tension (dynes cm^{-1})	CMD^{-1} (dynes cm^{-1})	CMD^{-2} (dynes cm^{-1})
3.0	32	36	40
5.0	28	32	35
6.0	28	31	35
7.0	28	31	34
8.0	29	31	35
10.0	30	32	35
11.0	31	35	38

(b) Thermostability studies

Time of heating at 100°C (min)	Surface tension (dynes cm^{-1})	CMD^{-1} (dynes cm^{-1})	CMD^{-2} (dynes cm^{-1})
0	27	30	34
5	27	30	34
10	27	30	34
20	27	31	34
40	28	31	35
60	28	31	35

MTCC 1427 was not able to utilize ammonium ions, but exhibited a preference for nitrate ions. Ammonium ions inhibited the utilization of nitrate ions for biosurfactant production as observed by less reduction in surface tension when ammonium nitrate was provided as nitrogen source. Nitrogen was required for the biosurfactant production as is evident from the observation that when there was no nitrogen in the medium, reduction in surface tension was not observed.

Of the several envisioned industrial applications of the biosurfactants the greatest potential use is by the oil industry. For applications in the oil industry the biosurfactant produced by *B. subtilis* MTCC 1427 has potential with E_{24} value of 33.33 against diesel oil and sand pack recovery of 56%. The high stability of the biosurfactant (at a wide pH range and temperature) and the ability of the organism to grow at extremes of pH and at thermophilic temperature (45°C) makes it very suitable for the extreme conditions encountered in the application field. Moreover, the ability of the organism to produce biosurfactant on molasses (a substrate added in oil wells as a rich source of carbohydrate) makes the organism itself a possible candidate for *in situ* oil recovery [15].

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. There are reports for *Arthrobacter paraffineus* [7] and *Pseudomonas* sp DSM-2874 [22] of alteration in the composition of biosurfactants due to temperature of growth. However, we did not observe any change in composition of the biosurfactant when the strain was grown at 45°C.

Biosurfactant production by the strain at 45°C and its stable nature suggest 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 could be of potential application in acidophilic and alkalophilic environments.

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References

1 Abu-Ruwaida A, IM Banat, S Hadithrto, A Salem and M Kadri. 1991. Isolation of biosurfactant-producing bacteria product characterization and evaluation. Acta Biotechnol 11: 315–324.
2 Banat IM. 1993. The isolation of a thermophilic biosurfactant-producing *Bacillus* sp. Biotechnol Lett 15: 591–594.
3 Cooper DG and JE Zajic. 1980. Surface active compounds from microorganisms. Adv Appl Microbiol 26: 229–253.
4 Cooper DG, CR MacDonald, SJB Duff and N Kosaric. 1981. Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions. Appl Environ Microbiol 42: 408–412.
5 Desai AJ, RM Patel and J Desai. 1994. Advances in the production of biosurfactants and their commercial applications. J Sci Ind Res 53: 619–629.
6 Duvnajale Z, DG Copper and N Kosaric. 1983. Effect of nitrogen source on surfactant production by *Arthrobacter paraffinus* ATCC 19558. In: Microbial Enhanced Oil Recovery (Zajic JE, DG Cooper, TR Jack and N Kosaric, eds), pp 66–67, Pennovell Books, Tulsa, Oklahoma.
7 Duvnajale Z, DG Copper and N Kosaric. 1982. Production of surfactant by *Arthrobacter paraffineus* ATCC 19558. Biotechnol Bioeng 24: 165–175.
8 Fiechter A. 1992. Biosurfactants: moving toward industrial application. Tibtech 10: 208–217.
9 Guerra-Santos L, O Kappeli and A Fiechter. 1984. *Pseudomonas aeruginosa* biosurfactant production in continuous culture with glucose as carbon source. Appl Environ Microbiol 48: 301–305.
10 Haferberg D, R Hommel, R Claus and HP Kleber. 1986. Extracellular microbial lipids as biosurfactants. Adv Biochem Eng 33:53–93.
11 Hurting-Muller R, R Blaszczyk, F Wagner and N Kosaric. 1993. Biosurfactants in environment control. In: Biosurfactants and Biotechnology. Surfactant Science Series, Vol 48 (Kosaric N, ed), pp 447–469, Marcel Dekker, New York.
12 Kosaric N, NCC Gray and WL Cairns. 1983. Microbial emulsifier and de-emulsifier. In: Biotechnology, Vol 3 (Rehm HJ and G Reed, eds), pp 575–592, Verlag Chemie, Weinheim.
13 Kosaric N, NCC Gray and WL Cairns. 1987. Microbial de-emulsifiers. In: Biosurfactants and Biotechnology. Surfactant Science Series, Vol 25 (Kosaric N, NCC Gray and WL Cairns, eds), pp 247–321, Marcel Dekker, New York.
14 Lowry OH, NJ Rosenberg, AL Farr and RJ Randall. 1951. Protein measurement with Folin phenol reagent. J Biol Chem 193: 265–275.
15 Makkar RS and SS Cameotra. 1997. Utilization of molasses for biosurfactant production by two *Bacillus* strains at thermophilic conditions. J Am Oil Chem Soc 74: 887–889.
16 Palejwala S and JD Desai. 1989. Production of an extracellular emulsifier by a gram negative bacterium. Biotechnol Lett 11: 115–118.
17 Ramana KV and NG Kranth. 1989. Production of biosurfactants by the resting cells of *Pseudomonas aeruginosa* CFTR-6. Biotechnol Lett 11: 437–442.
18 Robert M, ME Mercade, MP Bosch, JL Parra, MJ Espuny, MA Manresa and J Guinea. 1989. Effect of the carbon source on biosurfactant production by *Pseudomonas aeruginosa* 44Ti. Biotechnol Lett 11: 871–874.
19 Roe JH. 1955. Analysis of total hexoses found in glycoprotein. J Biol Chem 212: 335–338.
20 Singh M, V Saini, DK Adhikari, JD Desai and VR Sista. 1990. Production of bio-emulsifier by a SCP producing strain of *Candida tropicalis* during hydrocarbon fermentation. Biotechnol Lett 12: 743–746.
21 Suzuki T, H Tanaka and S Itoh. 1974. Sucrose lipids of *Arthrobacteria*, *Corynebacteria* and *Norcardia* grown on sucrose. Agr Biol Chem 38: 557–563.
22 Syldatk C, S Lang, U Matulovic and F Wagner. 1985. Chemical and physical characterization of four interfacial-active rhamnolipids from *Pseudomonas* sp DSM 2874 grown on n-alkanes. Z Naturforsch 40:51–60.
23 Tahara Y, M Kameda, Y Yamada and K Kondo. 1976. A new lipid: the ornithine and taurine containing cerilipin. Agr Biol Chem 40: 243–244.
24 Takahara Y, Y Hirose, N Yasuda and K Nitsugi. 1978. Effect of peptide lipids, produced by *Bacillus* on the enzymatic lysis of gram negative bacterial cells. Agr Biol Chem 40: 1901–1903.
25 Trebbau de Acevado G and MJ McInnerney. 1996. Emulsifying activity in thermophilic and extremely thermophilic microorganisms. J Ind Microbiol 16: 1–7.
26 Yakimov MM, N Kenneth, V Wray and HL Fredrickson. 1995. Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface *Bacillus licheniformis* BA550. Appl Environ Microbiol 61: 1706–1713.
27 Zajic JE and W Steffens. 1984. Biosurfactants. CRC Rev Biotechnol 1: 87–106.
28 Zobell CE. 1947. Bacterial release of oil from oil bearing materials. Oil Gas J 46:36–37.