SHORT COMMUNICATION

Utilization of Molasses for Biosurfactant Production by Two *Bacillus* Strains at Thermophilic Conditions

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ABSTRACT: Traditionally, biosurfactants have been produced from hydrocarbons. Some possible substitutes for microbial growth and biosurfactant production include urban wastes, peat hydrolysate, and agro-industrial by-products. Molasses, a non-conventional substrate (agro-industrial by-product) can also be used for biosurfactant production. It has been utilized by two strains of *Bacillus subtilis* (MTCC 2423 and MTCC1427) for biosurfactant production and growth at 45°C. As a result of biosurfactant accumulation, the surface tension of the medium was lowered to 29 and 31 dynes/cm by the two strains, respectively. This is the first report of biosurfactant production by strains of *B. subtilis* at 45°C. Potential application of the biosurfactant in microbial enhanced oil recovery is also presented.
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KEY WORDS: *Bacillus*, biosurfactant, MEOR, molasses, thermophilic.

In recent years, biosurfactant synthesis has been studied extensively (1,2). These biosurfactants are amphiphilic in nature and reduce the surface tension of the medium in which they are being produced. These surface-active compounds have applications in industry, agriculture, mining, oil recovery, with functional properties as wetting agents, foaming agents and as emulsifiers in pharmaceutical and cosmetic products. These microbial surfactants are interesting because of their biodegradable nature and effectiveness at extreme temperatures, pH, and salinity (3,4).

However, from an economic standpoint, biosurfactants are not yet competitive with synthetics. Different ways should be explored to reduce production costs through high yields and product accumulation, economical engineering processes, and use of cost-free or cost-credit feedstocks for microorganism growth and surfactant production.

Traditionally, hydrocarbons have been the substrates of choice to produce biosurfactants and bioemulsifiers (5,6). It is assumed that surfactant production is induced to render hydrophobic substrates accessible to the cell (7). Water-soluble substrates also have been used (8,9). The latter are cheaper than hydrocarbons and are preferred substrates because single-phase fermentation is simpler than biphasic fermentation. Moreover, hydrocarbon substrates are unacceptable for many applications, such as in food, cosmetics, and pharmaceutical products.

A great variety of raw materials is currently available as nutrients for industrial fermentations. The spectrum, apart from traditional carbon and nitrogenous substrates, includes various agricultural and industrial by-products and waste materials. These agricultural feedstocks have advantages in being available in surplus and in being produced in regions with temperate-to-tropical climates.

Only a few attempts of using wastes for biosurfactant production of a few types of biosurfactants have been reported (10). Kosaric *et al*. (11) reported processes for production of sophorose lipids from urban wastes, which involved multiorganism strategies. Carbohydrate-rich waste is converted into triglycerides by oleaginous organisms, and a second microorganism, *Torulopsis bombicola*, converts these lipids to sophorolipids. Mulligan and Cooper (12) used water collected during drying of fuel-grade peat. This waste contains a significant amount of carbohydrates (glucose, galactose, and xylose) and some amino acids as substrates for microbial growth and surfactant production by *Bacillus subtilis*. They measured the critical micelle concentration (CMC) but provided no report of conversion yields. Koch *et al*. (13) used a genetically constructed lactose-utilizing strain of *Pseudomonas aeruginosa* for biosurfactant production with lactic whey from the dairy industry as a substrate for rhamnolipid production. Lactic whey is a rich source of carbohydrates (75% lactose and 12–14% protein). In addition, some organic acids, minerals and vitamins are also present. Sheppard and Mulligan (14) used peat hydrolyzate for biosurfactant production. Mercede *et al*. (15) reported use of olive oil mill effluent for rhamnolipid production by *Pseudomonas* sp. For enhanced rhamnolipid production, lipoidal substrates are the best. For this reason, Manersa *et al*. (16) have utilized olive oil as the sole source of carbon for production of rhamnolipids by *P. aeruginosa.* Ohno *et al*. (17–19) have reported production of iturin and surfactin by a strain of *B. subtilis* NB 22 on wheat bran and okara (soybean curd residue). Ghurye *et al*. (20) put forward a practical approach to biosurfactant production by non-aseptic fermentation of mixed cultures on molasses.

Molasses is a by-product of the sugar cane industry in India. It is the major raw material for production of baker's

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yeast, citric acid, feed yeasts, acetone/butanol, organic acids, and amino acids. The principal reasons for widespread use of molasses as substrate are its low price, compared to other sources of sugar, and the presence of several other compounds besides sucrose. These include minerals, organic compounds and vitamins, which are valuable for the fermentation process. Composition of the molasses at 75% dry matter is total sugars—48–56%; nonsugar organic matter, 9–12%; protein (N × 6.25), 2–4%; potassium, 1.5–5.0%; calcium, 0.4–0.8%; magnesium, 0.06%; phosphorus, 0.6–2.0%; biotin, 1.0–3.0 mg/kg; pantothenic acid, 15–55 mg/kg; inositol, 2500–6000 mg/kg; thiamine, 1.8 mg/kg.

In this communication, we report the utilization of molasses, which is a nonconventional substrate for biosurfactant production, by two strains of *B. subtilis* at thermophilic conditions (45°C). This is the first report of biosurfactant production by *B. subtilis* strains at 45°C on molasses. Application of the biosurfactant in microbial enhanced oil recovery (MEOR) is also presented.

MATERIALS AND METHODS

Cultivation of organism. Bacillus subtilis MTCC 1427 and *B. subtilis* MTCC 2423 were grown on minimal medium of the following composition: KNO_3, 3.0 g; Na_2HPO_4, 2.2 g; KH_2PO_4, 1.4 g; NaCl, 0.1 g; $MgSO_4$, 0.6 g; $CaCl_2$, 0.04 g; $FeSO_4$, 0.02 g per liter of water at pH 6.8. Molasses was added at a concentration of 2% total sugars. The flasks containing 250 mL minimal medium were inoculated with 2% (vol/vol) of an 8–10 h grown nutrient broth (Hi-Media, Mumbai, India). The cultures were incubated at 45°C on a shaker incubator (200 rpm). Samples were withdrawn every 24 h for analysis of surface activity, biomass, and biosurfactant production.

Isolation of biosurfactant and biomass. Cells were separated from the broth by centrifugation at 10,000 rpm for 25 min. The supernatant (cell-free broth) collected was acidified with 6.0 N HCl to pH 2.0 and kept overnight at 4°C. The precipitated biosurfactant thus obtained was dried and weighed. Biomass was measured by washing the pelleted cells with normal saline and recentrifugation at 10,000 rpm. The cells were dried overnight at 65°C and biomass was estimated gravimetrically.

Surface activity measurement. Surface tension and critical micelle dilution (CMD^{-1} and CMD^{-2}) were determined with a duNouy Tensiometer (CSC No 70535, CSC, Utech Products, Inc., Albany, NY). All measurements were made on cell-free broth. CMD^{-1} and CMD^{-2} measurements were done by measuring the surface tension of 10-times and 100-times diluted cell-free broth.

Emulsification and sand pack test. Application of the product in MEOR was evaluated by the sand-pack technique. A glass column (40.0 × 2.5 cm) was packed with 100.0 g of acid-washed sand. The column was then saturated with 100.0 mL of kerosene oil. The ability of the isolated surfactant to recover oil was estimated by pouring 100·0 mL of aqueous solution of biosurfactant (1.0 mg·mL^{-1}) into 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.

RESULTS AND DISCUSSION

Time course of biosurfactant production and cell growth. Figure 1 shows the time course of biosurfactant production and cell growth of *B. subtilis* MTCC 2423 on molasses. Biosurfactant production (as evident from surface tension lowering) starts from day 1 and continues till 96 h of fermentation. CMD^{-1} and CMD^{-2} values follow a similar pattern as surface tension lowering. Maximal biosurfactant production was achieved in 96 h of fermentation, and CMD values (measure of concentration) are minimal at this point. Cell growth (biomass) remains almost stationary from 24 to 96 h of fermentation. As evident from the figure, maximal biosurfactant production is achieved in the late stationary phase.

For *B. subtilis* MTCC 1427 (Fig. 2), production of biosurfactant starts after 24 h and continues till 96 h of growth. Maximal biosurfactant production is achieved around 72 h of fermentation. CMD^{-1} and CMD^{-2} values at this point are minimal. As depicted in the figure, growth of the organism was maximal in 48 h.

Comparison of biosurfactant production by the two strains. Comparing biosurfactant production by the two strains indicates that MTCC 2423 produces more biosurfactant than MTCC 1427. Maximal biosurfactant production in both strains was achieved in the late stationary phase. Biomass formation was higher in MTCC

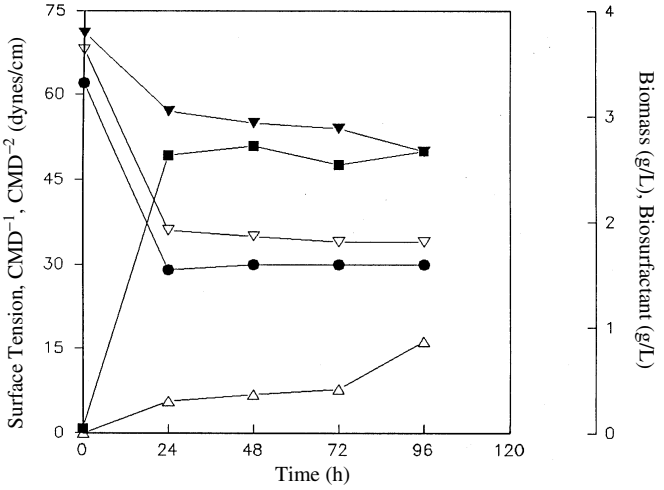

FIG. 1. Growth, biosurfactant production and surface activity profiles of *Bacillus subtilis* MTCC 2423, grown in mineral salt medium supplemented with molasses. Symbols: ■ biomass; △ biosurfactant; ● surface tension; ▽ CMD^{-1}, (critical micelle dilution)$^{-1}$; ▼ CMD^{-2}, (critical micelle dilution)$^{-2}$.

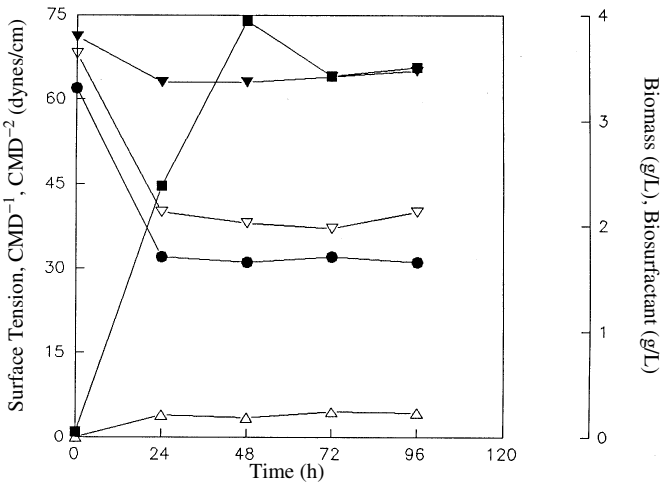

FIG. 2. Growth, biosurfactant production, and surface activity profiles of *B. subtilis* MTCC 1427, grown in mineral salt medium supplemented with molasses. Symbols: ■ biomass, △ biosurfactant, ● surface tension, ▽ CMD^{-1}, ▼ CMD^{-2}. See Figure 1 for abbreviations.

2423, suggesting higher utilization of the substrate for biosurfactant formation by the latter strain. The results indicate that biosurfactant production at 45°C on molasses as nonconventional substrate was achieved by both strains.

Emulsification index and sand pack test. Biosurfactant obtained by both strains showed E_{24} values of 80 and 95 for *B. subtilis* MTCC 1427 and MTCC 2423, respectively. Oil recovery was 34% for MTCC 1427 and 38.46% for 2423. These values indicate potential application of these biosurfactants in MEOR.

Updegraff (21) reported application of biosurfactants produced from molasses as substrates in MEOR. Molasses could be a preferred substrate because it can be injected together with bacterial cultures into the oil wells for enhanced oil recovery. The injected bacterial culture would then utilize the molasses as growth substrate and displace oil either by production of surfactants or gas production, resulting in reduction in the oil viscosity. Conditions prevailing in the oil well are not congenial for the growth of mesophilic organisms or production of biosurfactants. Under those conditions, the two *Bacillus* species used in this study could be model organisms because they can utilize molasses at 45°C and produce biosurfactants.

We conclude that biosurfactant production from molasses as growth substrate is a relatively inexpensive and economical process, which can be easily adapted to field conditions for treating contaminated soil with hydrophobic pollutants and for MEOR.

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