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Comparison of lipopeptide biosurfactants production by *Bacillus subtilis* strains in submerged and solid state fermentation systems using a cheap carbon source: Some industrial applications of biosurfactants

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Abstract

Biosurfactants, in general has the potential to aid in the recovery of subsurface organic contaminants (environmental remediation) or crude oils (oil recovery). However, high production and purification costs limit its use in these high-volume applications. In the present study, the efficiency of two *Bacillus subtilis* strains viz., DM-03 and DM-04 for the production of biosurfactants in two fermentation systems viz., solid state fermentation (SSF) and submerged fermentation (SmF) was compared. Both the *B. subtilis* strains produced appreciable and equal amount of crude lipopeptide biosurfactants (*B. subtilis* DM-03: 80.0 ± 9 mg/gds in SmF and 67.0 ± 6 mg/gds in SSF; *B. subtilis* DM-04: 23.0 ± 5.0 mg/gds in SmF and 20.0 ± 2.5 mg/gds in SSF) in the two different fermentation systems using potato peels as cheap carbon source. These thermostable lipopeptide biosurfactants produced by *B. subtilis* strains either in SSF or in SmF, exhibited strong emulsifying property and could release appreciable amount of oil from saturated sand pack column. Further, it was shown by biochemical analysis, RP-HPLC profile and IR spectra that there is no qualitative and qualitative differences in the composition of crude biosurfactants produced either in SmF or in SSF system.

Keywords: *Bacillus subtilis*; Emulsification index; Lipopeptide biosurfactant; Potato peel; Solid state fermentation; Submerged fermentation; Surface tension

1. Introduction

Surfactants are amphipathic molecules consisting both hydrophilic and hydrophobic moieties that partition preferentially at the interface between fluid phases having different degrees of polarity and hydrogen bonding; e.g., oil and water or air and water interfaces [1]. The surface and interfacial tension reducing properties of surfactants confer excellent detergency, emulsifying, foaming and dispersing traits; those make them some of the most versatile process chemicals. With increasing environmental awareness and emphasis on a sustainable society in harmony with the global environment, during the recent years, natural surfactants of microbial origin, commonly referred to as biosurfactants are getting much more attention compared to chemical surfactants owing to mild production condition, lower toxicity, higher biodegradability and environmental compatibility [2,3]. All the stated qualities of biosurfactants have prompted their tremendous applications in environmental protection as well as in food, cosmetic, biopesticide and pharmaceutical industries [4,5].

A survey of literature shows that biosurfactants are produced by a wide variety of microorganisms; however the chemical nature of biosurfactant is dependent on the producing species [6]. Among the biosurfactant producing potential microbes, *Bacillus subtilis* are known to produce cyclic lipopeptides (CLPs) including surfactins, iturins, fengycins, and lichenysins, as the major classes of biosurfactants [7–9]. Our previous studies have shown that lipopeptide biosurfactants secreted by thermophilic *B. subtilis* DM-03 and DM-04 strains possess mosquitocidal and antimicrobial activities [9,10].

A major obstacle on the way of wide-scale industrial application of biosurfactant is the high production cost coupled with less production rate as compared to commercially available synthetic surfactants. Therefore, if the production cost becomes competitive with the synthetic surfactants, and as the commercial availability of biosurfactant increases, the

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industrial use of biosurfactant can be expected to grow tremendously in the coming decade. To achieve this goal, during the recent years, efforts have been directed to explore the means to reduce the biosurfactant production costs through improving the yield, and the use of either cost-free or low-cost feed stocks or agricultural byproducts as substrate(s) for biosurfactant production. Many of the cheaper byproducts such as peat hydrolysate [11], olive-oil mill effluent [12], soapstock and waste-water from sunflower oil [13], de-proteinized whey [14], wheat bran and okara [15,16], molasses [17] and potato effluent [18] have been targeted as sole source of carbon for biosurfactant production by microbes in submerged fermentation (SmF).

Although there are certain apparent advantages of SmF in process control and easy downstream processing of products; however, over the past couple of years, solid state fermentation (SSF) involving growth of microbes on moist solid substrate(s) in the absence of free-flowing water, has gained a tremendous momentum owing to certain advantages over the conventional SmF, like low production cost, saving of water and energy, less waste effluent problem and stability of the product due to less dilution in the medium [19,20]. Therefore, SSF has found several potential applications in the industrial production of value added products, such as industrially important microbial enzymes, bioinsecticides, secondary metabolites and pharmaceuticals [20,21].

The objective of the present study was two fold. First to compare the efficiency of lipopeptide biosurfactant production by *B. subtilis* DM-03 and DM-04 strains in SmF and SSF systems using dried potato peels (a kitchen waste product) as a cheap carbon source, and to study the influence of various process parameters on the biosurfactant yield. To the best of our knowledge, this is the first report describing the biosurfactant production using potato peel as a substrate. The second objective was to characterize some of the biochemical properties of isolated biosurfactant and their possible industrial applications.

2. Materials and methods

2.1. Microorganisms

Isolation, characterization and taxonomic identification of biosurfactant producing microorganisms viz., *B. subtilis* DM-03 and DM-04 strains were done by following the standard biochemical and morphological tests, and by gas-chromatographic (GC) analysis of bacterial cellular fatty acid methyl esters [9,22]. These bacteria, isolated from a traditional fermented food and crude petroleum-oil contaminated soil sample from North-East India, respectively, were capable of secreting biosurfactant at thermophilic growth condition [9]. They were subcultured on nutrient agar plates before use as inoculums for biosurfactant production study.

2.2. Preparation of substrate

Potato peels (PP) were collected from Tezpur University hostel canteens and washed first with tap water followed by distilled water to remove the adhered surface dust particles. Then blenching operation was carried out by immersing the peels in hot water (75–80 °C) for 20 min. Peels were then oven dried at 45 °C for nearly 36 h. The dried material was grinded in a mixer grinder (Remi) to form a paste. The paste was re-dried overnight at 45 °C and sterilized at 121 °C, 15 lbs pressure for 15 min and stored at 4 °C before further use.

2.3. Production of biosurfactant by solid state fermentation and optimization of process conditions

Five gram of substrate (PP) was taken in 500 ml Erlenmeyer flasks and to this, 2.0 ml of M9 medium (composition in g l^{-1}: Na_2HPO_4, 6.0; KH_2PO_4, 3.0; NH_4NO_3, 1.0; NaCl, 1.0; $CaCl_2$, 0.014; $MgSO_4{\cdot}7H_2O$, 0.245; thiamine–HCl solution, 1.0 ml and 1 ml of micronutrients solution) was added, mixed thoroughly and autoclaved at 121 °C, 15 lbs pressure for 15 min. Before autoclaving, the pH of the M9 medium was adjusted to 8.0 and 7.0 for *B. subtilis* DM-03 and DM-04 strains, respectively [9]. The flasks were cooled to room temperature and then inoculated with 2.0 ml of 24-h grown bacterial culture (OD at 600 nm between 0.79 and 0.81) under sterile conditions and incubated at 45 °C temperature (DM-03 strain) and 55 °C temperature (DM-04 strain) for various time period (24, 36, 48, 72, 96 h). To study the influence of other culture parameters on biosurfactant production, effects of initial moisture content of the substrate (25%, 50%, 75%, 100%, 150%, 200%), inoculum size (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 ml), co-carbon sources (glucose, fructose, maltose, starch, and glycerol at 0.5%, w/v or v/v) and co-nitrogen sources (NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, yeast extract, beef extract, tryptone and peptone at 0.1%, w/v) were investigated. Uninoculated flasks and flasks without substrates(s) served as controls. Biosurfactant production was expressed as mean and standard deviations based on the results obtained with triplicate flasks.

2.4. Submerged fermentation and optimization of process conditions

Initially, the biosurfactant production was carried out in 250 ml Erlenmeyer flask containing 50 ml of M9 media and 2.0% (w/v) PP as substrate. Batch fermentation was carried out in a 5 l Bioflow 110 Fermentor (New Brunchwick Scientific, USA) with a working volume of 3 l, operating with foam/anti-foam probe system and by using 2.0% (w/v) PP as a substrate. The agitation speed was 200 rpm, provided by a centrifuge propeller. O_2 and pH electrodes were used for the control of the conditions. Although these bacteria are capable of growing in a wide range of temperature (25–60 °C), but biosurfactant production study was performed at their optimum growth conditions. Accordingly, the incubation temperature and pH were adjusted to 55 °C and 7.0 (for DM-04) and 45 °C and 8.0 (for DM-03), respectively. The cells were harvested at 24, 48, 72 and 96 h and the cell-free clear supernatant was used for the surface activity assessment and biosurfactant separation.

For optimizing the other culture parameters on biosurfactant production, inoculum size (0.5 ml, 1.0 ml, 2.0 ml, 3.0 ml, 4.0 ml, 5.0 ml, and 6.0 ml into 50 ml of media in Erlenmeyer flask), co-carbon sources (glucose, fructose, maltose, starch glycerol 0.5%, w/v or v/v) and co-nitrogen sources (NH_4Cl, KNO_3, $NaNO_3$, yeast extract, beef extract and peptone at 0.1%, w/v) were studied.

2.5. Determination of bacterial growth, surface activity of culture supernatants and isolation of biosurfactant

For isolation of biosurfactant produced in SSF, a known quantity of fermented matter was mixed with distilled water (1:5, w/v) by stirring on a magnetic stirrer for 30 min at room temperature (~25 °C), followed by centrifuging the whole content at 10,000 × *g* for 10 min at 4 °C to remove the insoluble matters. Crude lipopeptide surfactant was isolated from the medium as described previously [9] and biosurfactant recovery was expressed as amount of crude biosurfactant obtained per g of dry substrate (gds).

For determination of biomass and biosurfactant yield in SmF, the culture broth was sieved through a muslin cloth to remove the residual potato peels. The muslin cloth was then washed three times with PBS (phosphate buffered saline) to remove the bacterial cells adhering to the muslin cloth. Biomass was determined gravimetrically as described by Makkar [23] and biosurfactant isolation was done as described above. Protein content of the culture supernatant was estimated by Lowry's method [24] using bovine serum albumin as protein standard. Surface tension, critical micelle concentration (CMC) of biosurfactant and critical micelle dilution (CMD^{-10}, CMD^{-100}) of cell-free culture supernatants were determined using a Du-Nouy Tensiometer (Kruss 9 KT Tensiometer, Kruss, Germany) at room temperature (~25 °C) using the ring correction mode of the instrument.