Preparation of Biosurfactant

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Abstract— Biosurfactants are amphiphilic compounds produced on living surfaces, mostly on microbial cell surfaces, or excreted extracellularly and contain hydrophobic and hydrophilic moieties that confer the ability to accumulate between fluid phases, thus reducing surface and interfacial tension at the surface and interface respectively. They are a structurally diverse group of surface-active molecules synthesized by microorganisms. Biosurfactant was produced using *Pseudomonas aeruginosa* 10636 and 4673, with two different carbon sources i.e. glucose and glycerol at different concentration.optimisation of media, bacterial concentration and various physical parameters was carried out when ever required. It was found that both can utilize glucose and glycerol but *P. aeruginosa* 4673 can give better results with 5% glucose if compared.

Key words: Biosurfactants

I. INTRODUCTION

Biosurfactants are amphiphilic compounds produced on living surfaces, mostly on microbial cell surfaces, or excreted extracellularly and contain hydrophobic and hydrophilic moieties that confer the ability to accumulate between fluid phases, thus reducing surface and interfacial tension at the surface and interface respectively.[1] They are a structurally diverse group of surface-active molecules synthesized by microorganisms.

Rhamnolipids from *Pseudomonas aeruginosa*, surfactin from *Bacillus subtilis*, emulsan from *Acinetobacter calcoaceticus* and sophorolipids from *Candida bombicola* are some examples of microbial-derived surfactants.

Originally, biosurfactants attracted attention as hydrocarbon dissolution agents in the late 1960s, and their applications have been greatly extended in the past five decades as an improved alternative to chemical surfactants (carboxylates, sulphonates and sulphate acid esters), especially in food, pharmaceutical and oil industry[4]. Cunha CD, Do Rosario M, Rosado AS, Leite SGF (2004) The reason for their popularity as high-value microbial products is primarily because of their specific action, low toxicity, higher biodegradability, effectiveness at extremes of temperature, pH, salinity and widespread applicability, and their unique structures which provide new properties that classical surfactants may lack.

Biosurfactants possess the characteristic property of reducing the surface and interfacial tension using the same mechanisms as chemical surfactants. Unlike chemical surfactants, which are mostly derived from petroleum feedstock, these molecules can be produced by microbial fermentation processes using cheaper agro-based substrates and waste materials. During the past few years, biosurfactant production by various microorganisms has been studied extensively [17]. Yin H, Qiang Y, Jia Y, Ye J, Peng H, et al. (2009)

A. Classification and Chemical Nature of Biosurfactants

Chemically-synthesized surfactants are classified according to the nature of the polar groups, they carry. Microbially-derived biosurfactants are grouped depending on their chemical composition and the microbial source. Usually, the typical structure of biosurfactant includes a hydrophilic domain comprising amino acids or peptides, mono-, di-, or polysaccharides; and a hydrophobic domain consisting of unsaturated, saturated, or fatty acids[32]. Kosaric N (2001) The low-mass biosurfactants include glycolipids, lipopeptides and lipoproteins, phospholipids and fatty acids. High-mass biosurfactants include polymeric and particulate surfactants.

B. Applications of Biosurfactants

Due to the broad-range functional properties of biosurfactants and the diverse synthetic capabilities of microbes, a lot of research has been happening towards the large-scale production of biosurfactants[15]. Since they are readily biodegradable with lower toxicity than synthetic surfactants, biosurfactants have a high level of environmental acceptability. In the oil industry, the required purity specification is minimum, so that whole-cell broth could be used. When compared against chemical surfactants, biosurfactants are very selective in their action and are required in small quantities. They are effective under a broad range of oil and reservoir conditions. Also, they are ecofriendly in protecting coastal areas from damage due to synthetic chemicals[7]. *Bacillus licheniformis* secretes biosurfactants that are potentially useful for in situ microbially enhanced oil recovery operations. It is found in oilfield injection water and in addition to producing biosurfactants, has other properties like being anaerobic, halotolerant, and thermotolerant,. When Boscan Venezuelan heavy crude oil was treated with emulsan, viscosity was reduced from 200,000 to 100 cP (Hayes et al., 1986). The use of a biosurfactant for desludging of a crude oil storage tank was demonstrated by Banat et al., 1991, who achieved 90% recovery of the oil trapped in the sludge by using a biosurfactant-producing strain.

C. Limitations in Biosurfactant Production

The main limiting factor is the economics of large-scale production of biosurfactants. As per Desai and Banat, 1997, the main limitations include:

1. Need for sterilization
2. Large capital investment
3. Poor yields from raw substrate materials
4. Problems in product recovery and purification
5. Problems in the control of the process, like, foaming

Difficulties in analyzing the finished products chemically due to their complex nature. Reactions are carried

out in dilute solution, so, poor volume efficiency for the plant[17].

Biosurfactants are generally microbial metabolites with the typical amphiphilic structure of a surfactant. This study investigated potential biosurfactants production of *Pseudomonas aeruginosa* 10636 using glucose and *glycerol* as substrates separately and compared it with the production in conventional medium. Krishnaswamy M, Subbuchettiar G, Ravi TK, Panchaksharam S (2008) *Pseudomonas aeruginosa* growing in BHMS (Bushnell hass mineral salt) medium with glucose as substrate decreased the surface tension from 72 of distilled water to 32 mN/m, this strain had higher reduction than *Bacillus subtilis* among all the substrates tested[21]. The selection of *Pseudomonas aeruginosa* for the separation of biosurfactant was determined.

II. Hydrocarbon Assimilation and Biosurfactant Production in Pseudomomas Aeruginosa Mutants

We isolated transposon Tn5-GM-induced mutants of Pseudomonas *aeruginosa* PG201 that were unable to grow in minimal media containing hexadecane as a carbon source. Some of these mutants lacked extracellular rhamnolipids, as shown by measuring the surface and interfacial tensions of the cell culture supernatants[11]. Furthermore, the concentrated culture media of the mutant strains were tested for the presence of rhamnolipids by thin-layer chromatography and for rhamnolipid activities, including hemolysis and growth inhibition of Bacillus subtilis. Mutant 65E12 was unable to produce extracellular rhamnolipids under any of the conditions tested, lacked the capacity to take up 14C-labeled hexadecane, and did not grow in media containing individual alkanes with chain lengths ranging from C12 to C19. However, growth on these alkanes and uptake of [14C]hexadecane were restored when small amounts of purified rhamnolipids were added to the cultures[21]. Mutant 59C7 was unable to grow in media containing hexadecane, nor was it able to take up [14C]hexadecane. Zinjarde SS, Pant A (2002) The addition of small amounts of rhamnolipids restored growth on alkanes and [14C]hexadecane uptake. In glucose-containing media, however, mutant 59C7 produced rhamnolipids at levels about twice as high as those of the wild-type strain. These results show that rhamnolipids play a major role in hexadecane uptake and utilization by P. aeruginosa.

III. Biosurfactant Production by a Soil Pseudomonas Strain Growing on Polycyclic Aromatic Hydrocarbon

The capaity of polycyclic aromatic hydrocarbon (PAH)-utilizing bacteria to produce biosurfactants was investigated [16]. Twenty-three bacteria isolated from a soil contaminated with petroleum wastes were able to form clearing zones on mineral salt agar plates sprayed with solutions of PAHs. Naphthalene and phenanthrene were utilized as sole substrates. Rahman PKSM, Gakpe E (2008) Biosurfactant production was detected by surface tension lowering and emulsifying activities from 10 of these strains grown in an iron-limited salt medium supplemented with high concentrations of dextrose or mannitol, as well as with naphthalene or phenanthrene[29]. Glycolipid determinations showed that in cultures of *Pseudomonas aeruginosa* 19SJ on naphthalene, the maximal productivity of biosurfactants was delayed compared with that in cultures grown on mannitol. However, when small amounts of biosurfactants and naphthalene degradation intermediates were present at the onset of the cultivation, the delay was markedly shortened. Production of biosurfactants was accompanied by an increase in the aqueous concentration of naphthalene, indicating that the microorganism was promoting the solubility of its substrate. Detectable amounts of glycolipids were also produced on phenanthrene. This is the first report of biosurfactant production resulting from PAH metabolism[25].

IV. Biosurfactant Production by Pseudomomas Aeruginosa GS3 From Molasses

Pseudomonas aeruginosa GS3 produces rhamnolipid biosurfactants during growth on molasses and cornsteep liquor as the primary carbon and nitrogen sources, respectively [19]. After 96 h of growth the culture supernatant fluid had a rhamnose concentration of 0·24 g l−1 and reduced the interfacial tension against crude oil from 21 mN m−1 to 0·47 mNm−1. The rhamnolipids were able to form stable emulsions with n-alkanes, aromatics, crude oil and olive oil. These studies indicate that renewable, relatively inexpensive and easily available resources can be used for important biotechnological processes. Adamczak M, Bednarski W (2000)

V. Factors Affecting Biosurfactant Production Using Pseudomonas Aeruginosa CFTR-6 Under Submerged Condition

Studies on the biosurfactants produced by *Pseudomonas aeruginosa* CFTR-6 revealed that they consisted of glycolipids R-1 and R-2. A time course study of fermentation indicated that the appearance of glycolipids in the fermentation broth coincided with the exhaustion of nitrogen and the commencement of the stationary phase with respect to biomass[26]. The effect of variation of the media components such as carbon, nitrogen, phosphate and metal ions has been investigated. The following values were found to be optimum for biosurfactant production: glucose, 20 g dm^{-3}; carbon to nitrogen ratio, 38; phosphate, 30 mmol dm^{-3}; $MgSO_4.7H_2O$, 100 mg dm^{-3}. Addition of iron to the medium significantly diminished the glycolipid yield [14].

VI. Correlation of Nitrogen Metabolism With Biosurfactant Production by Pseudomonas Aeruginosa

A direct relationship between increased glutamine synthetase activity and enhanced biosurfactant production was found in *Pseudomonas aeruginosa* grown in nitrate and Proteose Peptone media. A chloramphenicol-tolerant strain showed a twofold increase in biosurfactant production and glutamine synthetase activity. Increased ammonium and glutamine concentrations repressed both phenomena [22].

VII. OIL WASTES AS UNCONVENTIONAL SUBSTRATESFOR RHAMNOLIPID BIOSURFACTANT PRODUCTION BY PSEUDOMONAS AERUGINOSA LBI

Oil wastes were evaluated as alternative low-cost substrates for the production of rhamnolipids by *Pseudomonas aeruginosa* LBI strain. Wastes obtained from soybean, cottonseed, babassu, palm, and corn oil refinery were tested. The soybean soapstock waste was the best substrate, generating 11.7 g/L of rhamnolipids with a surface tension of 26.9 mN/m, a critical micelle concentration of 51.5 mg/L, and a production yield of 75%. The monorhamnolipid $RhaC_{10}C_{10}$ predominates when *P. aeruginosa* LBI was cultivated on hydrophobic substrates, whereas hydrophilic carbon sources form the dirhamnolipid $Rha_2C_{10}C_{10}$ predominantly[18].

VIII. KINETICS OF BIOSURFACTANT PRODUCTION BY PSEUDOMONAS AERUGINOSA STRAIN BS2 FROM INDUSTRIAL WASTES

Batch kinetic studies were carried out on rhamnolipid biosurfactant production from synthetic medium, industrial wastes viz. distillery and whey waste as substrates. The results indicated that the specific growth rates (μ_{max}) and specific product formation rates (V_{max}) from both the wastes are comparatively better than the synthetic medium, revealing that both the industrial wastes (distillery and whey) can be successfully utilized as substrates for biosurfactant production.

Pseudomonas species is well known for its capability to produce rhamnolipid biosurfactant with potential surface active properties when grown on different carbon substrates (Parra et al., 1989; Koch et al., 1988; Mercade et al., 1993). Reports have been published on production of rhamnolipid biosurfactant from pure substrate (Santos er al., 1984; Mercade et ul., 1988; Santos et al., 1986), but kinetic studies on biosurfactant production from wastes are rarely published[7]. Techno-economically feasible production of biosurfactants which may compete with chemical surfactants, is of industrial intere.st. In this context, studies were undertaken for the production of biosurfactant through biotechnological routes employing inexpensive substrates (Kosaric (!I 01.) 1984; Koch PI al., 1988; Mercade er al., 1993). Authors have reported previously, that the industrial wastes could successfully be utilized as substrates for production of biosurfactants (Juwarkar et al., 1994). In the present work: the Michaelis-Menten and Monod type of kinetics of the biosurfactant production by *Pseudomonus aeruginosuna* strain RS2 from industrial wastes and pure substrate has been reported, which is useful in developing a continuous process for a large scale production of biosurfactant from industrial wastes[4].

IX. BIOSURFACTANT PRODUCTION BY PSEUDOMONAS FLUORESCENS 378: GROWTH AND PRODUCT CHARACTERSTICS

An isolate of *Pseudomonas fluorescens*, strain 378 was shown to produce a novel surface active compound (code name AP-6). The compound is unique in being a high molecular weight compound but has, in some aspects, properties of a low molecular weight surfactant. The product is extracellular and its formation appeared to be partly growth-associated. Using a semisynthetic medium, fermentor cultivations were performed in the pH range 6.8–8.4[5]. The product yield was optimal at pH 8.0 and gave a final concentration of 210 times critical micelle dilution. At higher pH, specific growth rate, final biomass and product concentration decreased. It consists mainly of carbohydrates and protein, the molecular weight is 1×10^6 and the isoelectric point is pH 9.1.

The surface tension of an aqueous solution reached 27 mN/m which is a very low value even compared to other surfactants of considerably lower size and the critical micelle concentration was less than 10 mg/l in 0.9% (w/v) NaCl[9]. The kinetics of the adsorption process at the air-water interface was studied using the drop volume technique, and the reaction was found to be rapid, considering the size of the molecule. A concentration as low as 0.025 g/l reached a surface tension of 30 mN/m within 70 s.Biosurfactants and bioemulsifiers are most commonly, different types of glycolipids, although there are exceptions, such as surfactin, a lipopeptide produced by *Bacillus subtilis*, or emulsan, a 1 M dalton heteropolysaccharide produced by *Acinetobacter calcoaceticus*. Biosurfactants often have the advantage of being biodegradable and, to find applications, characteristics different from those of existing synthetic or microbiological surfactants are needed[17]. Furthermore, the use of carbohydrates instead of hydrocarbons as carbon and energy sources facilitates the fermentation process (Guerra-Santos et al. 1984).

X. CONCLUSION

A. Bio-process optimization

Type, quality and quantity of biosurfactant production is dependent on the cultural conditions i.e. pH, temperature, agitation, aeration, dilution rate, the concentration of metal ions, the nature of the carbon and nitrogen sources. There are lots of studies regarding biosurfactant production relating the optimization of their physicochemical properties [59]. Environmental factors are exceptionally significant in the yield and characteristics of the biosurfactant produced. In order to acquire large quantities of biosurfactant, it is essential to optimize the process conditions. An efficient and economic bioprocess is the bottleneck for any profit-making biotechnology industry. Several elements, media compositions and precursors affect the process of biosurfactant production. Different elements such as nitrogen, iron, and manganese affect the production of biosurfactants. Limitation of nitrogen enhances biosurfactant production in P. aeruginosa strain BS-2 and U. maydis[18] . Addition of iron and manganese to the culture medium increased the production of biosurfactant by B. subtilis. The relative proportions of different elements to carbon in the reaction mix, such as C: N, C: P, C: Fe or C: Mg affects biosurfactant production. The classical method of medium optimization involves changing one variable at a time, while keeping the others at fixed levels; however, this method is time consuming and does not guarantee the optimal metabolite production. A statistical optimization strategy response surface methodology (RSM) has been developed for

the optimization of process. Response surface methodology (RSM) explores the relationships between several explanatory variables and one or more response variables[43]. This method could be used to determine the optimum media, inoculum and environmental conditions for the enhanced production of surfactin by *B. subtilis* . RSM has also been applied to enhance biosurfactant production by *P. aeruginosa* AT10, the probiotic bacterial strains *Lactococcus lactis* and *Streptococcus thermophilus* and by *B. licheniformis* for the concomitant production of biosurfactants and protease RG1 using agro-products such as cornstarch and soy flour as carbon and nitrogen sources respectively. Such optimization methods would help the industry to design the best combination of cheaper substrates for media production and to use the most favorable environmental conditions for improved biosurfactant production[56]. Current developments in the area of optimization of fermentation conditions have resulted in a considerable enhancement in production yields, making them more commercially attractive. Using the methods like experimental factorial design and response surface analysis, it is possible to conclude optimal operating circumstances to obtain a higher cellular growth, thus a higher cell-bound biosurfactant production yield. Optimization through factorial design and response surface analysis is a general practice in industrial technique for the optimization of cultural conditions.

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