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Development of Low Cost Medium for the Production of Biosurfactants

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ABSTRACT

In the present investigation, the sugar industry byproduct, molasses has been tested for its suitability for biosurfactant production using *Pseudomonas aeruginosa* strain. Attempt has also been made to replace the costly nitrogen sources with agro-industrial byproducts to formulate low cost medium for biosurfactant production and other process parameters were standardized. *Pseudomonas aeruginosa* MTCC 2297 displayed maximum emulsification activity on molasses medium after 120 h of incubation period under optimized conditions. The molasses medium supplemented with soya-okra can be suitable medium for biosurfactant production using the above culture which can improve the economics of the process.

Key words: Biosurfactants, molasses, soya-okra, emulsification activity, *Pseudomonas aeruginosa,* optimization

INTRODUCTION

Biosurfactants are amphiphilic compounds which are produced mainly by hydrocarbon degrading microorganisms. Biosurfactants have shown wide range of applications in environmental protection and management, crude oil recovery, as antimicrobial agents in health care and food processing industries. These compounds are capable of reducing the surface tension of the culture broth and emulsification of insoluble carbon sources in the culture medium (Ron and Rosenberg, 2001; Rismani *et al*., 2006). Such surface properties made them good candidates for Enhanced Oil Recovery (EOR) (Banat *et al*., 2002). Some biosurfactants are known to have therapeutic applications as antibiotics and antifungal or antiviral compounds (Moussa *et al*., 2006). These can also be used in bioremediation of soil or sand or in the cleanup of hydrocarbon contamination in groundwater (Ron and Rosenberg, 2001; Thavasi *et al*., 2006).

The advantages of biosurfactants over synthetic (chemically derived) counterparts include lower toxicity, biodegradability, selectivity, specific activity at extreme temperatures, pH, salinity, the possibility of their production through fermentation (Abdel-Mawgoud *et al*., 2010). These advantages clearly put the biosurfactants ahead of the synthetic counterparts and have, as a result, elevated their commercial potential (Banat *et al*., 2000). Despite possessing many commercially attractive properties and clear advantages compared with their synthetic counterparts, the production of microbial surfactants on a commercial scale has not been realized because of their high production costs.

Successful commercialization of every product depends largely on its bioprocess economics. The cost can be reduced by selection of efficient strains, optimizing medium composition or by using alternative inexpensive substrates. The choice of inexpensive raw materials is important to the overall economy of the process as they account for major portion of the final production cost. Synthetic media commonly employed for the production of biosurfactants are not economically attractive and there is need to explore the inexpensive raw materials. Expensive traditional carbon sources for surfactant production could be replaced with cheaply available natural raw materials. The selection of waste substrates involves the difficulty of finding a residue with the right balance of nutrients to support optimal growth and production. Agro-industrial wastes with high content of carbohydrates or lipids meet the requirements for use as promising substrates for biosurfactant production (Panesar *et al*., 2010). Molasses is a by-product of sugar production, both from sugar cane as well as from sugar beet industry in India. It has many advantages because of its low price compared to other sources of sugar and the presence of several other compounds such as minerals, organic compounds and vitamins besides sucrose which are valuable for the fermentation process (Makkar and Cameotra, 2002).

The type and proportion of the biosurfactants produced depends on the bacterial strain, the carbon source used and the culture conditions (Soberon-Chavez *et al*., 2005). The production of different surface active compounds by microorganisms has been reported by many workers (Abdel-Mawgoud *et al*., 2010; Gharaei-Fathabad, 2011). Most of the work is based on glucose, sucrose, glycerol, or ethanol as the raw materials. The biosurfactant-producing microbes are distributed among a wide variety of genera. The nature of biosurfactant varies from strain to strain; therefore, it is essential to evaluate different available strains for their biosurfactant potential and their characterization. Moreover, the production economy is the major bottleneck in biosurfactant production, as is the case with most biotechnological processes. Thus, to reduce this cost it is desirable to use low-cost raw materials. In view of the above, the present work was carried out to formulate low cost medium for biosurfactant production and standardization of process parameters. The optimal addition of media components and selection of the optimal culture conditions will induce the maximum or the optimum productivity.

MATERIALS AND METHODS

All the materials used for the present investigation were procured in year 2009 and the study was conducted between June, 2009 and March, 2010.

Procurement and maintenance of bacterial cultures: *Pseudomonas aeruginosa* MTCC 2297 used in the present work was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The bacterial cultures were maintained on growth media containing: beef extract (1 g L^{-1}), yeast (2 g L^{-1}), peptone (5 g L^{-1}), sodium chloride (5 g L^{-1}). 250 ml. The cultures were maintained by subculturing, aseptically at fortnight intervals and stored at 4°C, until further use.

Preparation of starter culture: The bacterial culture was grown in 50 mL of media in 250 mL capacity Erlenmeyer flask, having the same composition as described above. After sterilization, the flasks were inoculated with a loopful of culture from capsule and incubated at 37°C for 24 h.

Media preparation: The composition of fermentation medium was (g L^{-1}): $NaNO_3$ (1.28), K_2HPO_4 (0.87), $MgSO_4.7H_20$ (0.1), NaCl (0.1), KCl (0.2), Tris (hydroxymethyl) aminomethane (6.5), glucose

(20); mineral salt solution (5 mL). The mineral salt solution contained the following ingredients (g L^{-1}): $FeSO_4$ $(NH_4)_2SO_4.6H_2O$, H_3BO_3, $COCl_2.6H_2O$, $CuSO_4.5H_2O$, $MnSO_4.H_2O$, $(NH_4)_6Mo_7O_{24}.4H_2O$, $ZnSO_4.7H_2O$. The pH of medium was initially adjusted to 6.7 by 1.0 M HCl. In molasses media, glucose in the above medium was replaced by molasses (2%). All the media prepared were autoclaved under standard conditions and used in the experimentation.

Production of biosurfactant: Experiments were carried out in batch system using Erlenmeyer flasks (500 mL capacity). For fermentation, a loopful of bacterial cells was taken from the seed culture and was added into a flask (5% inoculum) containing 100 mL of growth media. The flasks were incubated at 37°C for growth for 7 days. During the course of batch fermentation, samples were taken every 24 h from the liquid culture to monitor emulsification activity. The effect of different nitrogen sources (pulse flours and agricultural/food wastes such as pea pods, soya bean meal etc.) on emulsification activity was investigated by supplementing individually in the fermentation medium. These nitrogen sources were supplemented individually at the concentration equivalent to 0.12% N to test their effect on emulsification activity.

Effect of molasses concentration: To study the effect of molasses concentration, its concentration in the fermentation medium was varied from 2-7% and the media was inoculated with the screened bacterial culture and incubated at 37°C for 120 h to check its effect on emulsification activity.

Effect of nitrogen sources: Different agro-industrial/food industry byproducts which are potential nitrogen sources including pea pods, soy-okra, taro leaves and pulse flours like black gram, soya meal, green gram, masoor dal, moth and lentils etc., were supplemented individually in the fermentation medium to test their effect on emulsification activity.

Standardization of process parameters: Different process parameters such as pH, temperature and incubation time was monitored by varying the respective parameters.

Analytical techniques

Estimation of biomass: Biomass was estimated by the method explained by Guerra-Santos *et al.* (1984). Flasks incubated were withdrawn at different time intervals, followed by centrifugation at 8000 rpm for 15 min and extracted with water to remove the adhering hydrocarbon. This was followed by centrifugation and drying at 105°C overnight to obtain dry biomass.

Estimation of emulsification activity: The estimation of the emulsification activity was carried out using the method of Pruthi and Cameotra (1995).

Estimation of rhamnolipid: The estimation of rhamnolipid was carried by phenol sulphuric acid test (Dubois *et al.*, 1956).

RESULTS AND DISCUSSION

The effect of the following media components and process parameters was monitored for optimal biosurfactant production during the course of the present investigation.

Effect of molasses concentration: Different molasses concentrations (2.0-7.0%) were supplemented in the fermentation media to find out the optimal concentration of molasses for maximum emulsification activity. *P. aeruginosa* MTCC 2297 was inoculated in the fermentation medium and the results are presented in Fig. 1. It can be depicted that the emulsification activity was increased with the increase in molasses concentration up to 4% (w/v) and its higher concentration did not show any further improvement in the emulsification activity. The maximum emulsification activity at 4% molasses concentration was 64.96%. This concentration may be sufficient to induce as well produce the maximum level of biosurfactant in the bacterial cells. The results of Sarin and Sarin (2008) have indicated maximum emulsification activity at 2% molasses supplemented with glutamic acid. General trend of biosurfactant production initially increases with increasing carbon substrate concentration, until it reached a maximum value and then leveled off or decreased (Wei *et al.*, 2005). The decrease in emulsification activity with further increase in molasses concentration may also be due to high concentration of salts present in sugar cane molasses that may have raised the osmotic pressure above acceptable levels, reducing the cell viability and suppressing the biosurfactant production (Doelle and Doelle, 1990).

Screening of organic nitrogen sources: In the present year, low cost organic nitrogen sources were tested for the effect on the emulsification activity. For this purpose, agriculture/food wastes such as pea pods, soy-okra, taro leaves and pulse flours like black gram, soya meal, green gram, masoor dal, moth and lentils etc. were supplemented individually in the fermentation medium to test their effect on emulsification activity. Among all the organic nitrogen sources tested, pea pods and soy-okra showed highest emulsification activity (Fig. 2). Soy okra and pea pods showed similar emulsification activity but pea pods showed lesser emulsion stability as compared to soy-okra after 24 h. Therefore, soy-okra having good emulsification activity, stability and clear formability was selected as nitrogen source in further studies for the optimization of the process.

The nitrogen source can be important key to regulation of biosurfactant biosynthesis. There is evidence that nitrogen plays an important role in the production of surface-active compounds by microorganisms. It has been reported that generally a preference is shown for nitrogen source with the assimilatory pathway that costs least in terms of energy required to assimilate the same quantity of inorganic nitrogen into amino acids (Flynn *et al.*, 1997; Cheng *et al.*, 1999). Although the data on the use of agriculture/ food wastes such as pea pods, soy-okra is scarcely available, however, reports on the use of inorganic nitrogen sources are available. It is important that an optimum C/N ratio is required by *P. aeruginosa* for maximum biosurfactant production

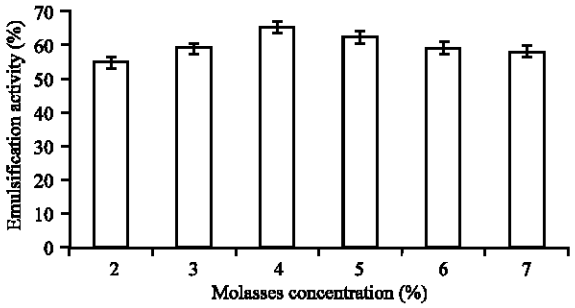

Fig. 1: Effect of molasses concentration on emulsification activity of *P. aeruginosa* MTCC 2297. Bars indicate the standard deviation from triplicate determinations

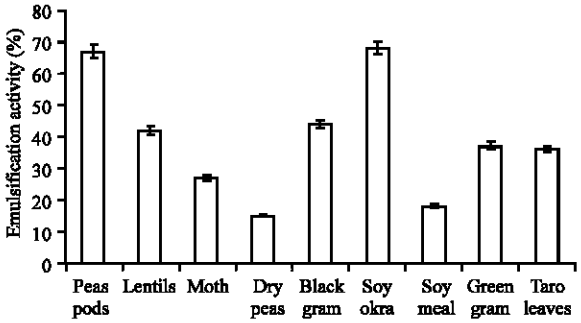

Fig. 2: Screening of nitrogen sources for the emulsification activity in batch culture of *P. aeruginosa* MTCC 2297. Bars indicate the standard deviation from triplicate determinations

(Guerra-Santos *et al.*, 1984). *Arthrobacter paraffineus* ATCC 19558 preferred ammonium to nitrate for biosurfactant production. Urea also resulted in good surfactant production (Duvnjak *et al.*, 1983). An investigation of rhamnolipid production by *Pseudomonas* 44 Ti on olive oil showed that sodium nitrate was the best nitrogen source (Robert *et al.*, 1989). Syldatk *et al.* (1985) showed that nitrogen limitation increased the production of some biosurfactants but also changed the composition of the biosurfactants. Results also suggest that nitrogen source is an important factor for biosurfactant production. Some reports mentioned that biosurfactant production is more efficient under nitrogen-limiting conditions (Benincasa *et al.*, 2002). It has also been reported that maximal biosurfactant synthesis was found under conditions of nitrogen limitation during the stationary phase of growth (Syldatk *et al.*, 1985; Mulligan and Gibbs, 1989). As evident from the results that soya-okra as nitrogen source with molasses based medium gave the maximum emulsification activity of 67%, therefore, it was selected in further experimentation.

Effect of soya-okra concentration: Different concentrations (0.10-0.30% w/v) of soy-okra were added to the fermentation media to find out its effect on the emulsification activity. It can be observed from Fig. 3 that an increase in concentration of soy-okra beyond 0.2% (w/v) resulted in decrease of emulsification activity. At high concentration (0.3%, w/v) of soya-okra, very low emulsification activity was observed which may be due to the fact that an optimum C/N ratio is required by *P. aeruginosa* for maximum biosurfactant production and an increase in the nitrogen concentration resulted in lower rhamnose concentration as well as higher interfacial tension values (Guerra-Santos *et al.*, 1984). It was also observed during the experimentation that the increase in the concentration of soy-okra greater than 0.25% (w/v), resulted high soy mass in the fermentation broth which in turn hinder the growth of organism and made emulsion turbid with low emulsification activity. Some reports mentioned that biosurfactant production is more efficient under nitrogen-limiting conditions (Benincasa *et al.*, 2002).

Effect of pH: In any fermentation process, pH is an important factor which can effect the product formation by a microorganism. Therefore, pH of the fermentation media with molasses concentration of 4% (v/v) and soy-okra concentration of 0.15% (w/v) was varied from 6.0 - 8.0 to study the effect of pH on emulsification activity. At very low and very high pH i.e. at 6.0 and 8.0, low emulsification activity was observed (Fig. 4). This may be due to the reason that the

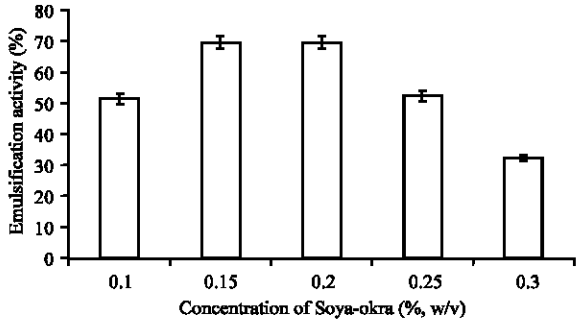

Fig. 3: Effect of soya-okra concentration on emulsification activity of *P. aeruginosa* MTCC 2297. Bars indicate the standard deviation from triplicate determinations

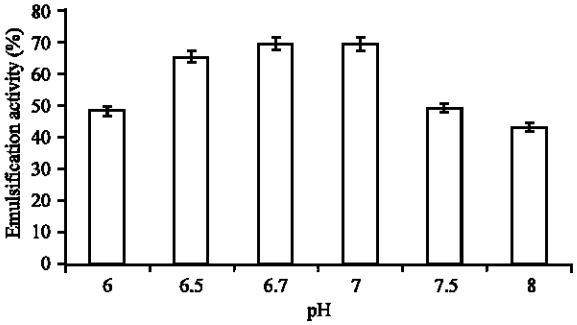

Fig. 4: Effect of pH of fermentation medium on emulsification activity of *P. aeruginosa* MTCC 2297. Bars indicate the standard deviation from triplicate determinations

P. aeruginosa MTCC 2297 strain grows only near neutral pH. Any change in media alkalinity or acidity hinders the growth of microorganism, thereby effects biosurfactant production. These results suggested that the optimal pH for emulsification activity with the strain was in the range of 6.7-7.0, neutral pH. Thus, pH of 6.7 for the fermentation medium was selected in the further experimentation. In previous reports, biosurfactant production in *Pseudomonas* spp. has been reported to be maximum at pH range from 6 to 6.5 and decreased sharply above pH 7 (Guerra-Santos *et al.*, 1986). An optimal pH of 5.8 has been suggested for biosurfactant production using *Serratia marcescens* (Bidlan *et al.*, 2007).

Effect of temperature: The effect of temperature on emulsification activity was studied by cultivating *P. aeruginosa* MTCC 2297 in media with soy-okra concentration at 0.15%, (w/v) and molasses concentration of 4%, (v/v) at temperature range of 25-45°C. Good emulsification activity was observed in the temperature range of 35-37°C (Fig. 5). Biosurfactant production increased with temperature until 37°C and then decreased sharply above 37°C. At very high temperature like 40°C and above, there was very low emulsification activity. Previous reports also indicated optimal growth and biosurfactant production from *Pseudomonas* at 37°C (Wei *et al.*, 2005; Gunther *et al.*, 2005). Thus maximum emulsification activity of *P. aeruginosa* MTCC 2297 was at 35-37°C. It was concluded that maximum emulsification activity in batch culture of *P. aeruginosa* MTCC 2297 was at 35-37°C, with molasses concentration of 4%, (v/v) and soy-okra concentration of 0.15 %, (w/v) at pH 6.7. In further experimentation, a temperature of 37°C was selected.

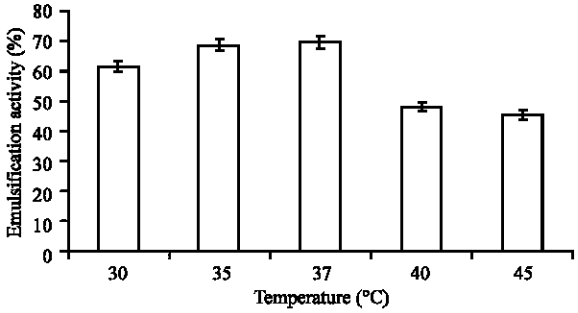

Fig. 5: Effect of temperature on emulsification activity in batch culture of *P. aeruginosa* MTCC 2297. Bars indicate the standard deviation from triplicate determinations

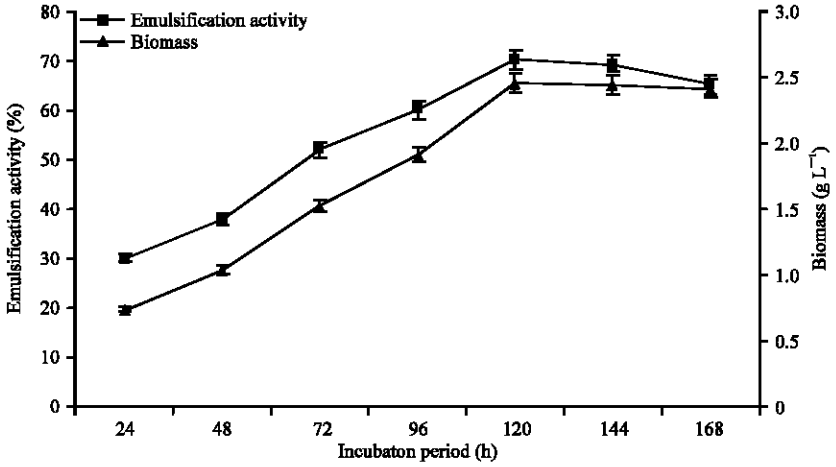

Fig. 6: Effect of incubation period on biomass production and emulsification activity in batch culture of *P. aeruginosa* MTCC 2297. Bars indicate the standard deviation from triplicate determinations

Effect of incubation period: The fermentation media prepared from the above set of parameters i.e., molasses concentration of 4 % (v/v), soya-okra concentration 0.15% (w/v), having pH 6.7 was incubated at 37°C and the samples were taken at regular intervals. From the results it can be observed that there was continuous increase in the emulsification activity of biosurfactants as a function of incubation period (Fig. 6). Emulsification activity increased up to 120 h and beyond this incubation period, it was slightly decreased which may be due to exhaustion of nutrients and metabolic changes in the medium. Rhamnolipid content was directly related to emulsification activity, with increase in time rhamnolipid content also increased with maximum of 256 mg mL^{-1} with emulsification activity of 70% at 120 h. Maximum biomass was reached at 3.24 g L^{-1} at 120-144 h of incubation period. The observations are consistent with work of previous findings (Maier and Soberon-Chavez, 2000; Wei *et al.*, 2005; Sifour *et al.*, 2007) that biosurfactant is a secondary metabolite secreted by bacterial cultures in stationary phase of growth. Recent studies have reported the maximum emulsification activity of 62% at 2% molasses medium after 120 h of incubation period (Sarin and Sarin 2008). An optimum incubation period of 168 h using *P. aeruginosa* MM 1011 has been reported (Tahzibi *et al.*, 2004) while the mutant strain displayed higher production after 120 h of incubation. In case of yeast cultures some workers have reported maximum emulsification activity after 96 h of cultivation using mineral medium (Sarubbo *et al.*, 2006).

CONCLUSION

From the above studies, it can be concluded that molasses medium supplemented with soya-okra can be suitable medium for biosurfactant production using *P. aeruginosa* MTCC 2297. The fermentation media having molasses (4%) and soya-okra (0.15%) with pH 6.7-7.0 incubated at 35-37°C gave maximum emulsification activity (70%) after 120 h of incubation period. It is well known phenomenon that the amount and type of a raw material contribute considerably to the production cost in most of biotechnological processes, so the above medium can serve as low-cost medium for biosurfactant production thus can make the process economical.

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