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Production of biosurfactant by *Pseudomonas aeruginosa* PB3A using agro-industrial wastes as a carbon source

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Aims: To evaluate *Pseudomonas aeruginosa* PB3A strain for the production of biosurfactant using agro-industrial waste as a carbon source.
Methodology and results: *P. aeruginosa* PB3A strain was isolated from oil contaminated soil and was found to be a potential biosurfactant producing microorganism based on the following screening methods; hemolytic activity, drop collapse test, emulsification activity and surface tension measurement. The identification of the isolate was confirmed by 16S rRNA sequencing. The isolate exploited for the application of agro industrial wastes such as used castor oil, coconut oil, corn oil, motor oil, olive oil, olein, rapeseed oil, sunflower oil, barley bran, cassava flour waste, peanut cake, potato waste, rice bran and wheat bran for biosurfactant production by replacing the existing carbon source. Among the cheap substrates, both the used corn oil and cassava waste flour showed maximum productivity of 0.62 mg/mL and 0.60 mg/mL respectively when cultivated independently in the MSM medium.
Conclusion, significance and impact study: Present study was aimed at the application of agro-industrial wastes for biosurfactant production. The study indicates that agro-industrial wastes can be used as inexpensive substrates by replacing synthetic media for the production of biosurfactant.

Keywords: Toxicity, agro-industrial wastes, biosurfactant, drop collapse, hemolytic assay, surface tension.

INTRODUCTION

Surfactant belongs to amphiphatic molecules, consisting of a hydrophobic and a hydrophilic group which helps in reducing the surface and interfacial tension in water/hydrocarbon systems (Prantera *et al.*, 2002). The application of synthetic surfactants for the treatment of hydrocarbon-contaminated soil improves the restoration; however, they get accumulated in the ecological system leading to severe environmental damage. The biosurfactants or the surface active component synthesized by the microorganisms appears to be an excellent alternative approach for the synthetic surfactants. Besides possessing surface and emulsifying activities (Van Hamme *et al*., 2006; Singh *et al*., 2007) these bio surfactants exhibit antimicrobial and anti adhesive properties (Singh and Cameotra, 2004; Rodrigues *et al*., 2006). Microbial surfactants composed of diverse group of surface-active components are classified by their chemical composition and microbial origin which include lipopeptides, glycolipids, lipopolysaccharides, polysaccharide-protein complexes, protein-like substances, fatty acids, phospholipids, and neutral lipids (Van Hamme *et al*., 2006).

Distinct properties and physiological functions have been observed for different groups of biosurfactants produced by different microorganisms which include enhanced solubility of hydrophobic compounds, heavy metal binding, cell adhesion and aggregation, biofilm formation and quorum sensing (Ron and Rosenberg, 2001; Singh and Cameotra, 2004). Among the various microorganisms, bacteria are the important group of surfactant-producers although few yeasts and fungi are also reported (Desai and Banat, 1997). The most common biosurfactants are glycolipids and lipopeptides which include rhamnolipids synthesized by *Pseudomonas aeruginosa* (Nitschke *et al*., 2005), sophorolipids released by *Candida* species (Daverey and Pakshirajan, 2009), and surfactin and iturin synthesized by *Bacillus subtilis* strains (Ahimou *et al*., 2000).

In the present scenario, the major drawback that prevents the widespread use of microbial biosurfactants is the economic viability of the production costs. Currently various strategies have been evaluated in order to reduce the fermentation process economics and make it competitive with chemically synthesized surfactants (Makkar and Cameotra, 2002). Future research on biosurfactant has to be more focused on the economics of the fermentation processes, mainly through the usage of alternative low-cost production media (Makkar and Cameotra, 2002; Rodrigues and Teixeira, 2008).

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MATERIALS AND METHODS

Materials

All the chemicals and reagents used in the present study were of analytical grade and purchased from SRL Chemicals and Merck Chemicals, India. Used residual oil wastes were collected from various shops and household; agro-wastes were collected from local market and industries from Chennai and nearby area.

Isolation and screening of biosurfactant producers

Bacterial strains used in this study were isolated from oil contaminated soils at Chennai as described by Saravanan and Vijayakumar (2012). The samples were serially diluted and preserved in nutrient agar slant for further screening of biosurfactant production. Hemolytic activity was performed as described by Carrillo *et al.*, (1996) with slight modifications. Isolated bacterial strains were screened for their hemolytic activity on blood agar plates with 5 vol. % of blood and incubated at 37 °C for 24 h. The hemolytic activity was distinguished based on the presence of a clear zone around the colonies. Using a 96 micro-well plate the drop collapse test was carried out as described by Bodour and Maier (1998). Two microliter of crude oil was added to the well and left to equilibrate for 24 h, followed by 5 µL of 48 h cell free supernatant of bacterial strain and the drop size was observed after 1 min with the aid of a magnifying glass. Positive result shows a flat drop and rounded drops were scored as negative which indicate a negative result for biosurfactant production. The emulsifying activity of biosurfactant was carried out according to Cooper and Goldenberg (1987). After 48 h, 2 mL of cell free supernatant and hydrocarbon (oil) was taken in a test tube and vortexed at high speed for 1 min and left to stand for 24 h. Emulsifying (E24) activity was expressed as the percentage of the total height occupied by the emulsion (Bodour *et al.*, 2004). Surface tension (ST) was determined using a Kruss processor tensiometer by the plate method (Płaza *et al.,* 2011). The bacterial cultures were cen¬trifuged at 10,000 rpm for 20 min and the cell free supernatant was analyzed for the determination of surface activity. All the assays were performed in triplicate with appropriate positive and negative controls.

Identification of bacterial strain

The selected isolate PB3A was identified by morphological and biochemical analysis based Bergey's Manual of Systemic Bacteriology (Holt *et al.*, 1994). The identity of bacterial strain PB3A was further confirmed by 16S rRNA gene sequence analysis. The total genomic DNA was extracted from an overnight culture in Luria Bertani broth using CTAB (Cetyltrimethylammonium Bromide) method (Ausubel *et al.*, 1994). The 16S rRNA gene amplification was done by universal 16S rDNA primers 27f (5'- GAGTTTGATCCTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') followed by purification using QIAquick Gel Extraction kits from Qiagen. DNA sequencing was performed using DTS cycle sequencing kit and Applied Biosystems automated DNA sequencer (Marchesi *et al.*, 1998). Nucleotide sequences were determined for both strands. Sequence obtained was aligned and used for similarity comparisons using the BLAST program (Altschul *et al.*, 1990).

Production of biosurfactant using agro-industrial wastes

In the present study, various solid and liquid wastes were used as cheap substrates for biosurfactant production. Different waste substrates such as used castor oil, coconut oil, corn oil, motor oil, olive oil, olein, rapeseed oil, sunflower oil, barley bran, cassava flour waste, peanut cake, potato waste, rice bran and wheat bran were amended in MSM medium by replacing glucose at a concentration of 1 % in the final composition according to Tahzibi *et al*. (2004) (g/L of distilled water: $NaNO_3$, 15; NaCl, 1.1; KCl, 1.1; KH_2PO_4, 3.4; K_2HPO_4, 4.4; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.00028; yeast extract, 0.5; glucose 1%). The selected strain PB3A was then inoculated into the medium amended with different wastes substrates and kept in shaker incubator (100 rpm) at 37 °C. After 48 h of incubation, cell-free supernatant was obtained by centrifuging the culture broth at 10,000 rpm for 20 min. The biosurfactant concentration in the cell-free culture broth was estimated using orcinol assay method (Tuleva *et al.*, 2002). One hundred microliter of each sample was mixed with 900 µL of a 0.19% orcinol solution (in 53% H_2SO_4) and heated at 80 °C for 30 min. The samples were then cooled to room temperature and absorbance was measured at 421 nm and compared with the standard curve prepared with L-rhamnose and expressed as rhamnose equivalents (RE) (mg/mL).

Statistical analysis

The data represent the arithmetical averages of at least three replicates. The results were represented as mean value ± standard deviation (SD). The statistical analysis was performed using MS office Excel 2007 for calculating mean, standard deviation and standard error.

RESULTS AND DISCUSSIONS

Various bacterial isolates obtained from oil contaminated soil were screened for the presence of biosurfactant activities using blood hemolytic test, drop collapse test, emulsification index (E24) and Surface tension according to Satpute *et al.* (2008) who described the significance of more than one screening method for the primary screening of potential biosurfactant producers. Among the different bacterial strain, PB3A showed hemolytic colonies (Figure 1) on blood agar which was similar to the report done by Mulligan *et al.* (1984). The strain also showed a flat drop appearance (Figure 2) in the drop collapse, a sensitive method to detect biosurfactant production as suggested by Jain *et al.* (1991).

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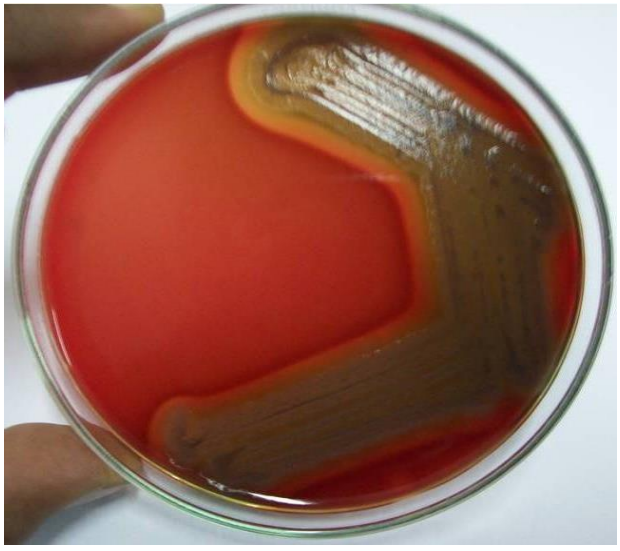

Figure 1: PB3A showing hemolytic colonies on Blood agar.

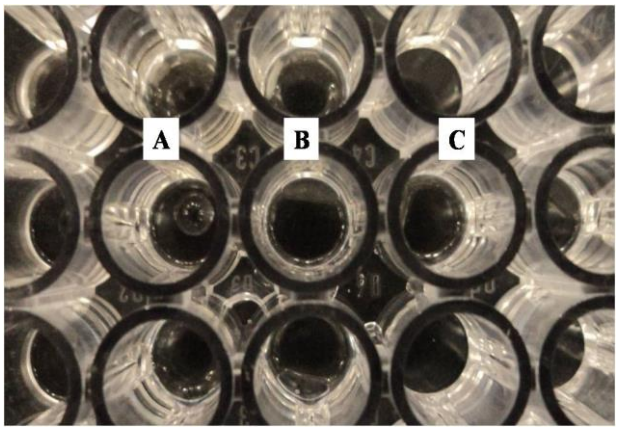

Figure 2: PB3A showing flat drop in (C) in drop collapse test. A, Negative control; B, positive control.

Although the use hemolytic activity and drop collapse test constitute an easy method to screen biosurfactant production, determination of surface tension and emulsification activity (E24) was performed to confirm the biosurfactant producers (Plaza *et al.*, 2011). Among the isolates, the strain PB3A reduced the surface tension of the culture medium up to 43 mN/m and also showed the highest emulsification index of about 65.5% against crude oil.

The efficient isolate PB3A was selected based on the results of screening tests and identified using partial sequence obtained from their 16S rRNA gene. The 16S rRNA sequences were aligned and showed that the isolate was *Pseudomonas aeruginosa*. The sequence PB3A showed high similarity of more than 97% with *P. aeruginosa* when compared with the other sequences existing in the GenBank using BLAST search. The 16S rRNA sequence was then deposited in the GenBank database under the accession number of KF029593.1. The results of morphological and biochemical tests of the selected isolate PB3A is summarised in the Table 1. The isolate PB3A was motile, Gram negative bacilli and showed positive result for oxidase, citrate, urease and gelatine liquefaction and negative for other tests. The isolate was able to ferment mannitol, xylose and galactose. The results of the morphological and biochemical tests confirmed that the isolate PB3A was *P. aeruginosa* (Holt *et al.*, 1994).

Table 1: Morphological and biochemical characteristics of the strain PB3A.

Tests	Results
Gram stain	–
Motility	+
Oxidase	+
Catalase	–
Indole production	–
MR test	–
VP test	–
Citrate	+
H_2S	–
Gelatin liquefaction	+
Mannitol fermentation	+
Glucose fermentation	–
Xylose fermentation	+
Maltose fermentation	–
Galactose fermentation	+
Lactose fermentation	–
Sucrose fermentation	–
Nitrate reduction	–
Urea hydrolysis	+

The carbon source present in the production medium influences the bio surfactant production (Davis *et al.*, 1999; Adamczak and Bednarsk 2000). Patel and Desai (1997) used whey wastes from dairy industries for the microbial growth and biosurfactant production at commercial scale against synthetic medium. Youssef *et al.* (2004) also screened for various substrates such as brewery effluents, molasses and fruit and vegetable decoction for production of biosurfactants using *Bacillus* strains.

The biosurfactant production depends upon the type of carbon substrate present in the production medium (Davis *et al.*, 1999). In the present study, the various agro-industrial wastes were used as carbon sources for the biosurfactant production. Different waste substrates such as used castor oil, coconut oil, corn oil, motor oil, olive oil, olein, rapeseed oil, sunflower oil, barley bran, cassava flour waste, peanut cake, potato waste, rice bran and wheat bran were used as alternative source for carbon in the biosurfactant production medium. Among the cheap substrates used corn oil and cassava flour waste shows the maximum biosurfactant production of

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0.62 mg/mL and 0.60 mg/mL respectively (Figures 3 and 4).

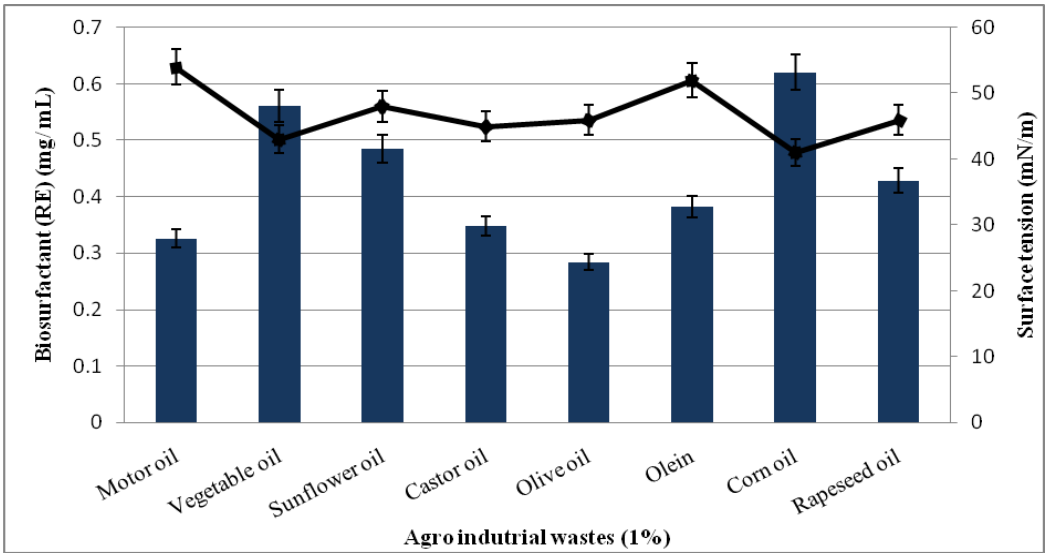

Figure 3: Effect of agro-industrial cheap substrates (used oils) on biosurfactant production.

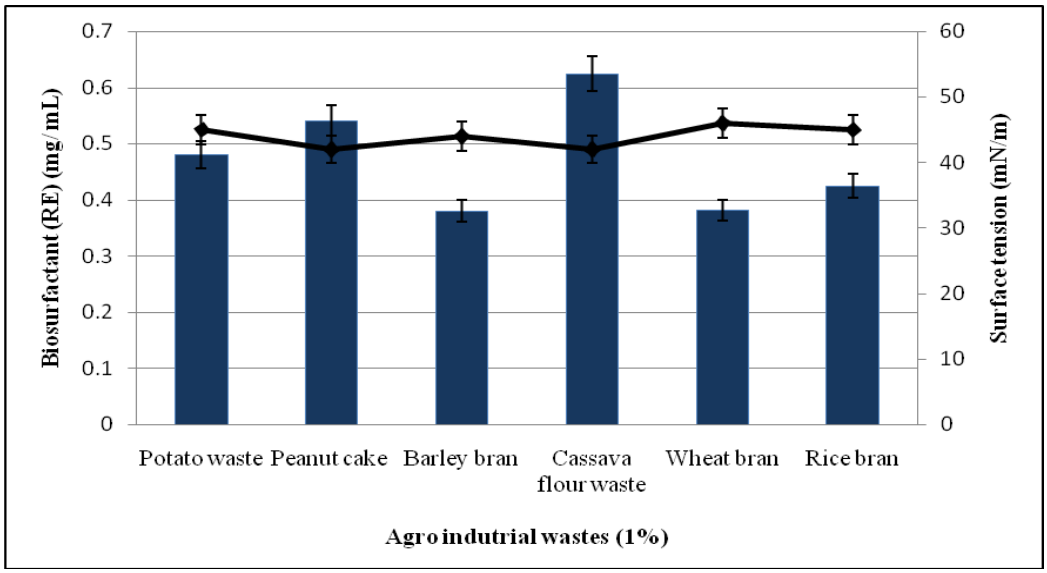

Figure 4: Effect of agro-industrial cheap substrates (solid wastes) on biosurfactant production.

Similarly surface tension values of the used corn oil and cassava flour waste amended medium after 48 h showed 41 mN/m and 42 mN/m respectively which were similar to the study conducted by Patel and Desai (1997) and Dubey and Juwarkar (2004). Among the cheap substrates, used Castor oil, motor oil, olive oil, olein, and wheat bran showed lower amount of biosurfactant production ranging from 0.28 mg/mL to 0.38 mg/mL compared to coconut oil, rapeseed oil, sunflower oil, barley bran, potato waste, peanut cake and rice bran which showed significant production ranging from 0.42 mg/ml to 0.56 mg/mL. Sheppard and Mulligan (1987) studied the utilization of peat hydrolyzate for the production of biosurfactant production and Mercadé *et al.,* (1993) along with his co-workers also reported application of olive oil mill effluent for production of rhamnolipid by *Pseudomonas* sp. Manersa *et al.* (1991) studied the production of rhamnolipids by *P. aeruginosa* using olive oil as the carbon source in the production medium. Similar studies were also reported by Rashedi *et al.* (2006) and Raza *et al.* (2007) who have utilized molasses as a cheap carbon substrate for the production of biosurfactant. The present work on the application of

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cheap substrate for the biosurfactant production was in agreement with the other studies reported in the literature.

CONCLUSION

The present study deals with application of industrial wastes as substrates for the biosurfactant production. Efforts have been made to use different cheap substrates such as used oil and solid wastes for bio surfactant production. This approach would be economic in developing new strategies to increase the volume of productivity. Among the waste cooking oils corn oil and in case of solid wastes, cassava waste flour were suitable substrates for biosurfactant production using *P. aeruginosa* PB3A, a strain isolated from oil contaminated soils. Further optimization and production strategy have to be studied for the culture medium to increase the productivity of biosurfactant. However, further research is needed to develop biotechnological processes for increase productivity using complex wastes with nutritional potential for biosurfactant production.

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