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Optimization of rhamnolipid biosurfactant production by mangrove sediment bacterium *Pseudomonas aeruginosa* KVD-HR42 using response surface methodology

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ABSTRACT

Rhamnolipid biosurfactant production by a novel *Pseudomonas aeruginosa* strain KVD-HR42 was optimized with statistical approaches. The produced biosurfactant showed surface active properties with stable emulsification activities. Based on the results of Plackett–Burman design, first-order polynomial model was developed and the following significant variables were determined viz., Karanja oil, sodium nitrate and pH. Response surface methodology experimental design was performed by Box–Behnken design to study the concentration of each component. The response plots resulted in the following optimized conditions; Karanja oil (23.85 g/L) sodium nitrate (9.17 g/L) and pH (7.8) which yielded an average biosurfactant production of 5.90 ± 2.1 g/L at 48 h, and 37 °C temperature. The statistical approach resulted in enhanced biosurfactant production. The biosurfactant showed excellent emulsion forming capabilities and could reduce the surface tension to 30.14 mN/m at a CMC value of 100 mg/L. The biosurfactant was found to be stable at extreme conditions of temperature, pH and NaCl concentrations. Additionally surface active nature of the crude biosurfactant was demonstrated using oil displacement assay with a clearance zone of 19.26 ± 0.23 cm^2. Our results signify that the biosurfactant has a great industrial potential as a cleansing agent at adverse environmental conditions and provide better alternative to synthetic surfactants.

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1. Introduction

Microbial surfactants have gained a great deal of attention in the past few decades due to their unique properties such as high biodegradability, lower toxicity and better stability (Mulligan, 2005; Mukherjee et al., 2006). These molecules are structurally diverse group of surface-active bio molecules produced by bacteria, fungi and yeasts which include glycolipids, lipoaminoacids, lipopeptides, lipoproteins, lipopolysaccharides, phospholipids, monoglycerides and diglycerides. Among these, the glycolipids produced by strains of Pseudomonads have received much attention due to their notable tensioactive and emulsifying properties (Maier and Soberon-Chavez, 2000; Mukherjee et al., 2006). Moreover chemical surfactants synthesized from petrochemical or oleo chemical sources (Desai and Banat, 1997) have been widely used in large scale industrial applications, particularly for cleansing purposes as detergents, surface cleaners and also in environmental remediation field. Due to the non-biodegradability, ability to accumulate and toxicity caused by excessive use of synthetic surfactants, application of ecofriendly technologies, biodegradable compounds of natural origin are gaining remarkable interest (Banat et al., 2010; Cameotra et al., 2010). Biosurfactants serve as natural choice for such processes as they possess potential advantages over synthetic surfactants for example lower toxicity, biodegradability and resistance to a wide range of pH and temperature values (Banat et al., 2010; Cameotra et al., 2010). Like synthetic surfactants, they exhibit physicochemical properties and characteristics such as detergency, emulsification, de-emulsification, foaming and wetting (Banat et al., 2000). Furthermore these molecules also have the abilities to reduce superficial and interfacial tension between solids and liquids.

However, biosurfactants have limited applications owing to their high production costs, which can be lowered by process optimization, downstream processing strategies, agro-industrial waste fermentation and use of hyper-producer strains (e.g., mutant and recombinant strains) (Wei et al., 2004; Perfumo et al., 2010). In order to enhance biosurfactant productivity, the selection

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Table 1
Physical and chemical characteristics of karanja oil (Bobade and Khyade, 2012; Meher et al., 2004).

Physical characteristics	Characteristic value	Chemical characteristics	Characteristic value
Color & Odor	Dark brown & Repulsive	Acid value (mg KOH/g)	5.06
Density at 15°C kg/m3	889	Saponification value (mg KOH/g)	187
Specific Gravity	0.925	Unsaponifiable matter (w/w per cent)	2.6
Flash point°C	116	Iodine value (g/100 g)	86.5
Cloud point°C	22	Carbon residue per cent (w/w)	0.08
Pour point°C	15.8	Ash content per cent (w/w)	0.003
Fire Point	230		
Boiling Point°C	316		

of suitable media components and optimal culture conditions are highly indispensable. The limitations of classical methods of media optimization can be overcome by statistical experimental designs using Plackett–Burman design (PBD) and response surface methodology (RSM) (Tanyildizi et al., 2005; Lotfy et al., 2007). PBD is one of the widely used statistical design technique for screening of medium components and the obtained variables were further optimized in a 2^3 factorial Box–Behnken design (BBD) (Plackett and Burman, 1944; Box, 1952).

Currently, the use of agro-industrial byproducts for production of biosurfactants is one of the much adopted fermentation method as they serve as cheaper and economical substrates for higher yields and also helps in waste disposal. The use of low-cost substrates is an essential factor for the overall economic recovery as they account for 50% of the total production cost (Makkar and Cameotra, 1999; Rodrigues et al., 2006). Several studies reported the use of agro-industrial wastes such as molasses, whey milk, distillery waste, olive oil mill effluent, soap stock, cassava waste, potato substrates etc. (Makkar and Cameotra, 1999; Henkel et al., 2014; Gudiña et al., 2015) and vegetable oils as carbon substrates including palm seed oil, olive oil, sunflower oil, safflower oil, canola oil, soybean oil and corn oil (Rahman et al., 2002; Wu et al., 2008; Oliveira et al., 2009). In view of increasing food demand and price hikes of edible plant based oils (Ko-Sin et al., 2010) non-edible vegetable oils may serve as best alternate substrates for rhamnolipid production. Therefore, non-edible oils such as Karanja oil was preferred as substrate for rhamnolipid production.

Millettia pinnata commonly known as Karanja (Family: Fabaceae) is a fast-growing tree native to tropical and temperate Asia. It grows abundantly along the coasts, riverbanks and tidal forests in India and highly tolerant to salinity. The seeds contain an average of 28–34% oil with high percentage of polyunsaturated fatty acids (Sarma et al., 2005). Karanja oil also contains oleic acid (44.5–71.3%) as the major fatty acid followed by linoleic (10.8–18.3%), palmitic (3.7–7.9%) and stearic (2.4–8.9%) acids, eicosenoic acid (9-eicosenoic acid) in reasonable amounts (9.5–12.4%) (Karmee et al., 2005). It is popular due to its low cost, easy extraction of seed oil and ready availability. Till date there are no reports for the use of non-edible karanja oil for rhamnolipid production. The present work aims to optimize the factors directly responsible for rhamnolipid production by mangrove sediment bacteria using statistical designs and RSM for higher yield and also help in microbial enhanced oil recovery (MEOR).

2. Materials and methods

2.1. Microorganisms and Culture Conditions

A biosurfactant producing strain *Pseudomonas aeruginosa* KVD-HR42 (Deepika et al., 2015 unpublished data) was used in this study. The strain was isolated from oil contaminated mangrove wetlands of Krishna estuary, Andhra Pradesh, India, genotypically identified as *Pseudomonas aeruginosa* strain KVD-HR42 (GenBank Accession Number-KJ872835). The medium components for production of biosurfactant were followed as per Deepika et al. (2014), Karanja oil (20 g/L) was used as sole carbon source. The pH of the medium was adjusted to 7.5 and incubated at 35 °C at 150 rpm for 48 h. In order to qualitatively detect extracellular rhamnolipid biosurfactant production, blue agar plates containing cetyltrimethylammonium bromide (CTAB 0.2 g/L), methylene blue (5 mg/L) and agar (15 g/L) were used (Siegmund and Wagner, 1991). The appearance of dark blue halos around the bacterial colonies was an indication of rhamnolipid biosurfactant production. Additionally the surface tension reduction (STR) was also used as a criterion for primary screening of biosurfactant producing isolates using tensiometric analysis (K11 Tensiometer, KRUSS-Germany). *P. aeruginosa* ATCC type strain DS10-129 was used as positive control strain in the screening experiment.

2.2. Selection of optimal carbon and nitrogen sources

In order to use cheap industrial by-products and agricultural wastes as cost-effective alternative substrates for microbial growth and rhamnolipid production, karanja oil was used as sole source of carbon and energy. The physical and chemical characteristics of karanja oil are shown in Table1 as reported by Bobade and Khyade (2012) and Meher et al. (2004). $NaNO_3$ was used as nitrogen source. The culture conditions were same as described above.

2.3. Experimental design and statistical analysis using Plackett–Burman design

The PB design was used to evaluate the individual medium components that considerably influenced the rhamnolipid biosurfactant production. In this study, nine variables (including Karanja oil, $NaNO_3$, $K_2HPO_4.3H_2O$, KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2$, KCl, NaCl and pH) were selected to study the individual variable effect on biosurfactant production. Based on this, 9 independent variables were selected for the study, evaluated in 12 experimental trials (ST. 1). Each variable was used at two concentrations (high and low), designated as +/− levels (Table 2).The concentration levels were also selected by one factorial experiment. Plackett Burman Design is showed on the first order polynomial model,

$$Y = \beta_0 + \Sigma\beta_i X_i (i = 1, 2, 3 \ldots\ldots\ldots\ldots k) \quad (1)$$

where Y is the response (Biosurfactant yield), β_0 is the model intercept and β_i is the linear coefficient, and X_i is the level of the independent variable. The experimental design and statistical analysis of the data were done by Minitab statistical software package (v 17.0) (Minitab Inc., PA). In the present study the trials were run in duplicates and the analyzed biosurfactant was taken as the response. Based on regression analysis, variables with a significance level of 95% ($P < 0.05$) were considered as significant factors.

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