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ORIGINAL ARTICLE

Statistical optimization of antifungal iturin A production from *Bacillus amyloliquefaciens* RHNK22 using agro-industrial wastes

P. Narendra Kumar, T.H. Swapna, Mohamed Yahya Khan, Gopal Reddy, Bee Hameeda *

Dept of Microbiology, University College of Science, Osmania University, Hyderabad 500 007, India

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Abstract Biosurfactants are secondary metabolites with surface active properties and have wide application in agriculture, industrial and therapeutic products. The present study was aimed to screen bacteria for the production of biosurfactant, its characterization and development of a cost effective media formulation for iturin A production. A total of 100 bacterial isolates were isolated from different rhizosphere soil samples by enrichment culture method and screened for biosurfactant activity. Twenty isolates were selected for further studies based on their biosurfactant activity [emulsification index (EI%), emulsification assay (EA), surface tension (ST) reduction] and antagonistic activity. Among them one potential isolate *Bacillus* sp. RHNK22 showed good EI% and EA with different hydrocarbons tested in this study. Using biochemical methods and 16S rRNA gene sequence, it was identified as *Bacillus amyloliquefaciens*. Presence of iturin A in RHNK22 was identified by gene specific primers and confirmed as iturin A by FTIR and HPLC. *B. amyloliquefaciens* RHNK22 exhibited good surface active properties and antifungal activity against *Sclerotium rolfsii* and *Macrophomina phaseolina*. For cost-effective production of iturin A, 16 different agro-industrial wastes were screened as substrates, and Sunflower oil cake (SOC) was favouring high iturin A production. Further, using response surface methodology (RSM) model, there was a 3-fold increase in iturin A production (using SOC 4%, inoculum size 1%, at pH 6.0 and 37 °C temperature in 48 h). This is the first report on using SOC as a substrate for iturin A production.

* Corresponding author. Tel.: +91 040 20052737; fax: +91 040 27090661.
E-mail address: drhami2009@gmail.com (B. Hameeda).
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1. Introduction

Microbial surfactants are a structurally diverse group of surface active molecules produced by a wide variety of microorganisms, including bacteria, fungi and yeasts. These are amphiphilic molecules with both hydrophilic and hydrophobic moieties that confer the ability to accumulate

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between fluid phases, thus reducing surface tension at surface and interface respectively (Ongena and Jacques, 2008; Mukherjee et al., 2006). Biosurfactants have advantages over their synthetic counter parts due to their low toxicity, higher biodegradation, better environmental compatibility at extreme temperature, pH, salinity and their ability to be synthesized from renewable feedstock (Romero et al., 2007). Biosurfactants have potential to be applied in pharmaceutical, cosmetics, petroleum, food industries and agriculture sector.

Global demand for microbial biosurfactant is valued at USD 12.7 million in 2012 and is expected to reach USD 17.1 million by 2020, expanding at a Compound annual growth rate (CAGR) of 4% from 2014 to 2020. Of the different biosurfactants, lipopeptides have projected peak annual US revenue of > US $1 billion and are approved in more than 70 countries (Meena and Kanwar, 2015; www.transparencymarketresearch.com). Members of the *Bacillus* genus are considered as efficient microbial factories for large scale production of lipopeptides such as iturin, surfactin and fengycin, inhibiting various fungal pathogens and protecting the crop plants (Singh et al., 2014; Jin et al., 2014). However, a significant obstacle to meet the large scale industrial application of biosurfactants is the high production cost (Makkar and Cameotra, 2002). Hence, optimization of medium composition is most important for the production of microbial metabolites at industrial scale because around 30–40% of production cost is estimated to be the cost of growth medium (Dhanya et al., 2008; Radhika et al., 2014). Agro-industrial wastes contain high amount of carbohydrate, proteins, lipids and are generally used as cattle feed or composted and disposed into land fill. Instead they can be used as substrates for cost effective production of microbial metabolites such as biosurfactants (Yarchoan and Arnold, 2014).

In microbial fermentation, potentially influential variables are numerous and when desired to screen a large number of factors; experimental designs for first-order models, such as the factorial design or Plackett–Burman design, can be used. Plackett–Burman factorial designs are used for reliable short listing of medium components in fermentation for further optimization and allow one to obtain unbiased estimates of linear effects of all factors with maximum accuracy for a given number of observations. However, they do not give an optimum value for each variable and further optimization is needed (Ikram and Ali, 2005). Response surface methodology (RSM) has been widely used to evaluate and understand the interactions between different physiological and nutritional parameters (Laxman et al., 2005). RSM, which includes factorial design and regression analysis and can be used to evaluate the effective factor, build models, provide information about the interaction between variables and multiple responses at the same time (Dhouha et al., 2012). The objective of this work is to screen rhizosphere bacteria for biosurfactant production, characterize high biosurfactant producing bacterial isolate and develop a cost effective medium formulation for iturin A production.

2. Material and methods

2.1. Media used for growth of microorganisms

Nutrient broth (NB) and mineral salts medium (MSM) [$NaNO_3$ (0.5), $MgSO_4{\cdot}7H_2O$ (0.5), KCl (0.1), $FeSO_4$ (0.01) K_2HPO_4 (0.5), KH_2PO_4 (0.5) g/L], pH 7 and temperature 37 °C were used for bacterial growth. Potato dextrose agar (PDA), glucose casaminoacids yeast extract medium (GCY) and Kings B (KB) medium were used for fungal growth at 30 °C.

2.2. Isolation of biosurfactant producing bacteria by enrichment culturing

Rhizosphere soil samples of varied crop plants were collected from different areas of Telangana and Andhra Pradesh states of India. Enrichment culturing method was performed by the method described by Dubey and Juwarkar (2001), 10 g of soil sample was added to 90 mL of mineral salts medium (MSM) in 250 mL flasks, amended with kerosene (1 mL or 5 mL or 10 mL) for enrichment and incubated at 37 °C, 180 rpm for 72 h. The enriched soil samples were subjected to serial dilution and appropriate dilutions were spread on nutrient agar plates and incubated at 37 °C for 24–48 h. Colonies of pure cultures were isolated and further characterized by Gram's staining and spore staining. The cultures of selected *Bacillus* spp., were persevered as glycerol stocks (−70 °C) for further studies.

2.3. Preliminary screening for biosurfactant activity

From the above preliminary screening, hundred bacterial isolates were isolated and tested for biosurfactant activity using the following methods like microplate and penetration methods, oil-spread method, blue-agar plate method, blood haemolysis test, lipase assay, emulsification index (EI) and emulsification assays (EA) for preliminary qualitative screening of biosurfactant activity. All the bacterial isolates were inoculated in NB medium and incubated at 37 °C for 24 h. Based on the assay, overnight grown culture or cell free supernatant was used as required.

2.3.1. Microplate method

In this method the culture supernatant (100 μL) of each bacterial isolate was added separately into 96 well microplate placed on a graph paper. Then the plate was observed for curvature of lines on the graph sheet under each well. Curvature of graph lines under the well is a preliminary indication of biosurfactant activity (Vaux and Cottingham, 2001).

2.3.2. Penetration method

All the wells of 96 well microplates were filled with 150 μL hydrophobic paste consisting of oil and silica gel. Then the paste was overlaid with 10 μL of oil. To this, 90 μL of cell free supernatant and 10 μL of staining solution (safranin) were added and biosurfactant activity was identified. Based on the results obtained by microplate and penetration methods, twenty isolates were selected for further studies.

2.3.3. Oil spread method

Oil spread assay described by Plaza et al. (2006) was performed according to which 50 mL of distilled water was taken in a Petridish, and 20 μL of crude oil was overlaid uniformly on water surface. Then, 10 μL of cell free supernatant was added over