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Production and characterization of lipopeptide from *Bacillus cereus* SNAU01 under solid state fermentation and its potential application as anti-biofilm agent

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ABSTRACT

This work was aimed to utilize peanut oil cake as a novel substrate to produce lipopeptide biosurfactant by *Bacillus cereus* SNAU01 under solid state fermentation (SSF). Central Composite Design (CCD) was performed to optimize the variables for biosurfactant production.

The Response Surface Methodology (RSM) results revealed, the optimal conditions for maximal production of biosurfactant were 8.18 g peanut oil cake as substrate, 2.5 ml inoculum level with pH 7 at 30 °C. The biosurfactant was characterized by FT-IR, TLC and GC–MS confirmed the presence of lipopeptide from *B. cereus* SNAU01. The Confocal Laser Scanning Microscopy (CLSM) proved the effective removal of biofilm by SNAU01 lipopeptide on glass surface. The biofilm disruption of SNAU01 lipopeptide was more efficient at 250 μg/ml against the selected pathogenic strains. The present findings indicated that peanut oil cake as suitable substrate for the production of lipopeptide under SSF by *B. cereus* SNAU01 and its potential application as anti-biofilm agent.

1. Introduction

Biosurfactants are amphiphilic surface active biological compounds produced by various microorganisms that can reduce the interfacial tension between different fluid phases (Nawawi et al., 2010; Ramani et al., 2012). The biosurfactant are surface active agents, which includes lipopeptide, glycolipids, phospholipids and peptides. Since the last decade, increasing attention has been paid to the isolation of biosurfactant producing organisms (Nalini and Parthasarathi, 2013). The biosurfactants have several advantages over chemical surfactants including high ionic strength tolerance, high temperature tolerance, higher biodegradability, lower toxicity, lower Critical Micelle Concentration (CMC), and higher surface activity (Gudina et al., 2011).

Several types of low molecular weight lipopeptide biosurfactant are produced by members of *Bacillus* species and their extensive application includes agriculture, cosmetic, food petroleum and pharmaceutical industries (Das et al., 2010). Only limited reports have described the biosurfactant production from *Bacillus cereus* strains. Sriram et al. (2011) reported lipopeptide biosurfactant produced by heavy metal tolerant strain *B. cereus* NK1 and exhibited significant reduction in biofilm formation by pathogens. Owing to their high surface activities and antimicrobial potency, lipopeptide biosurfactant produced by *Bacillus* sp. have garnered commercial attention (Wang et al., 2014). One possible strategy for reducing costs is the utilization of alternative substrates such as agro-industrial wastes (Mercade and Manresa, 1994).

Biosurfactant can be produced on various substrates and has potentiality of replacing the chemical surfactants which cause environmental hazards. However, the price of raw materials usage significantly increases the cost for production of biosurfactant. Most of the literature has reported the biosurfactant production using submerged fermentation (SmF) (George and Jayachandran, 2009; Nalini and Parthasarathi, 2013). The submerged fermentation pose problem with foam formation during the production (Lee and Kim, 2004; Yeh et al., 2006), hence solid-state fermentation (SSF) could be an alternate method for the biosurfactant production. Solid-state fermentation (SSF) is a process in which microorganisms grow on or within solid substrates or supports in the absence of free water (Pandey et al., 2000). There are very few reports on the production of biosurfactant by SSF (Kiran et al., 2010; Nalini and Parthasarathi, 2014). However, the fermentation process is significantly influenced by various physical and chemical parameters such as initial moisture content, incubation temperature, fermentation period, substrates, and additional nutrients such as mineral salts, etc. (Sharma and Arora, 2010).

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Response Surface Methodology (RSM) is an efficient statistical experimental strategy by which the optimal conditions of a multivariable system may be determined (Oskouie et al., 2008). Recently, Response Surface Methodology (RSM) a well know statistical tool was applied for optimization of culture parameters and its interaction on biosurfactant production under SSF using agro-industrial wastes (Nalini and Parthasarathi, 2014). RSM helps to identify the effective factors, study interactions, find optimum conditions, predict the optimum values of the variables and ensure the maximum production in limited number of experiments (Mohana et al., 2008). Therefore, there is a necessity to adapt RSM for an efficient biosurfactant production using a cost effective substrate bioprocessing.

Epstein et al. (2011) have demonstrated that under certain testing conditions, biosurfactants can be more effective than many traditional biofilm inhibition and or disruption strategies. Lipopeptides are one of the largest groups of biosurfactant that can effectively disperse microbial biofilms. Many of the current lipopeptides reported to inhibit or disperse biofilms originate from *Bacillus* or *Paenibacillus* (Kim et al., 2009; Quinn et al., 2012). Furthermore, new sights into advance studies on biofilm inhibition, disruption have now enabled researchers to design more for potentially effective biosurfactant against microbial biofilms.

Oil cakes/oil meals are by-products obtained after oil extraction from the seeds. Oil cakes are of two types, edible and non-edible. Edible oil cakes have a high nutritional value; especially have protein content ranging from 15% to 50% (www.seaoWndia.com) (Ramachandran et al., 2007). Depending upon the extraction methods the chemical composition of oil cake varies. These oilcakes are fairly rich in protein and are traditionally used as feed ingredients for farm animals. They are also used as aquaculture feeds (Singh et al., 2003). Peanut oil cake, being rich sources of carbon, nitrogen and also essential amino acid, servers as a suitable substrate for the growth of microorganisms (Roopesh et al., 2006). In the light of above, present study aims to explore the feasibility of peanut oil cake as potential substrate for the production of biosurfactant by *B. cereus* SNAU01 employing SSF. Furthermore, the potential application of biosurfactant on biofilm disruption against pathogenic bacterial strains were also investigated.

2. Materials and methods

2.1. Sampling site and isolation of biosurfactant producing microorganism

For isolation of biosurfactant producing bacteria, the samples of hydrocarbon-contaminated soil were collected from Cuddalore district, Tamil Nadu, India. One gram of each soil sample was inoculated into 100 ml of Mineral Salt Medium (MSM) with diesel oil (2%v/v) as carbon source and enriched flask was incubated at 30 °C for 4 days on a rotary shaker at 180 rpm. The composition of Mineral Salt Medium ($g\,l^{-1}$) used in this study was: $NaNO_3$ (2.5); K_2HPO_4 (1.0); KH_2PO_4 (1.0); KCl (0.1); $MgSO_4 \cdot 7H_2O$ (0.5); $CaCl_2$ (0.01); $FeSO_4 \cdot 7H_2O$ (0.01) and yeast extract (0.1). The pH was adjusted to 6.8 ± 0.2 and the medium was autoclaved. After 4 days, 1.0 ml of the culture was transferred to fresh media containing diesel oil (2%v/v) and re-incubated for another 4 days. After three cycles of enrichment, 0.1 ml of cultures from the last enriched flask were plated on to MSM Agar plates and three successive subcultures were performed from the enriched culture flask. The isolates were maintained on Nutrient agar (HiMedia) at 4 °C and stored as frozen stock culture at − 70 °C in 25% glycerol for future use. The selected isolates were screened for the production of biosurfactant.

2.2. Screening of biosurfactant producer

In order to analyse the capability of the strain to produce biosurfactant, the isolates were subjected to screening methods. Biosurfactant production was preliminary assessed by drop collapsing test (Youssef et al., 2004) and oil displacement test (Morikawa et al., 1993). The haemolytic activity was performed on blood agar plates containing 5% (v/v) human blood. For this assay, the culture (50 μl) was spot inoculated at the centre of blood agar plates and incubated at 37 °C for 48 h. The plates were observed for the zone of clearance around the colony. The diameter of the clear zone is a qualitative method used as an indicator of biosurfactant production (Mulligan et al., 1984). Lipase activity was performed according to Sriram et al. (2011). All the experiments were done in triplicates. The best strain was selected for further studies.

2.3. Identification of biosurfactant producing microorganism

The morphological and biochemical characterization of the selected biosurfactant producing strain was performed according to Bergey's manual of determinative bacteriology, 9th edition (Holt et al., 1994). Molecular identification was carried out by 16S rRNA gene sequencing. DNA from the selected bacterium was extracted using microbial DNA extraction kit (Insta-GeneTM Matrix, Bio-Rad). The PCR was carried out using 27F/1492R primers (27F: 5′-AGA GTT TGA TCM TGG CTC AG-3′, 1492R-5′ TAC GGY TAC CTT GTT ACG ACT T-3′) for amplification and then 35 amplification cycles at 94 °C for 45 s, 55 °C for 60 s, and 72 °C for 60 s. Unincorporated PCR primers and dNTPs were removed from PCR products by using Montage PCR Clean up kit (Millipore). The PCR products of approximately 1400 bp were sequenced by 518F/800R primers (518 F:5′- CCA GCA GCC GCG GTA ATA CG-3′, 800R-5′ TAC CAG GGT ATC TAA TCC-3′). The sequencing products were resolved on an Applied Biosystems model 3730 XL automated DNA sequencing system (Applied BioSystems, USA). The 16S rRNA gene sequence was compared with Gen Bank nucleotide database (NCBI) using BLAST and BLASTX algorithms. The sequence alignments and the phylogenetic tree construction was conducted in MEGA software version 5.2 (Tamura et al., 2011). The phylogenetic tree was constructed using a neighbour joining method and assessed with 1000 bootstrap replications.

2.4. Substrate

In the present study, Agro-industrial by-products such as coconut oil cake, castor oil cake, gingelly oil cake, peanut oil cake, palm oil cake, and sunflower oil cake were used as substrate. All the aforementioned agro-industrial by-products were obtained from the local market in Chidambaram, India. All the substrates were dried at 60 °C for 48 h, blended to fine powder using a mixer-grinder, sieved using 1.0 mm sieve, packed in air-tight polythene bags and stored in moisture-free container at room temperature. All these substrates were directly used in the fermentation media without any pre-treatment.

2.5. Solid-state fermentation (SSF)

In the current study, Solid state fermentation (SSF) was developed for the optimization of biosurfactant production. The production of biosurfactant was performed in 250 ml Erlenmeyer flask containing 5.0 g substrate and to this salt solution was added to obtain final moisture content of 65%. The composition of the salt solution was as follows ($g\,l^{-1}$): NH_4NO_3 (5); NaCl (9); $MgSO_4 \cdot 7H_2O$ (1); pH 7.0. Substrates were sterilized at 121 °C for 15 min, cooled and inoculated with 2.0 ml inoculum of *B. cereus* SNAU01 and incubated at 30 °C for seven days. The optical density