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Response surface modeling and optimization of major medium variables for glycolipopeptide production

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ABSTRACT

An unusual strain of *Pseudomonas aeruginosa* readily produced a glycolipopeptide compound which reduced surface tension of fermentation broth from 71.31 dynes/cm to 24.62 dynes/cm at a critical micelle concentration of 20.80 mg/L. A response surface methodology (RSM) involving a 2^4 full-factorial central composite rotatable design was used to optimize major medium parameters to improve yield. Critical input variables in the design included waste-frying oil (**X1**-carbon source), urea (**X2**-nitrogen source), Na_2HPO_4/KH_2PO_4 (**X3**-phosphates) and inoculum volume (**X4**), with biomass (**Y1**) and biosurfactant (**Y2**) concentrations as response variables. Maximum biosurfactant concentration of 23.86 g/L was obtained under optimized conditions set at (X_1, X_2, X_3, X_4) = (2, −1.5556, 2, 2) corresponding to 5% (v/v) waste frying oil, 1.19 g/L urea, 4.5 g/L phosphates and 10% (v/v-10^8 cfu/mL) inoculum volume. Production yield ($\mathbf{Y_{p/x}}$) increased from 1.91 in un-optimized medium to 3.76 under optimized conditions. Second-order quadratic models for both responses were significant ($P < 0.05$; adjusted R^2 = 99.72% and 99.95% for biomass and biosurfactant concentrations respectively) when data-fitting was attempted. The non-significant lack-of-fit for biomass ($F = 2.83$; $P = 0.105 > 0.05$) and biosurfactant ($F = 0.72$; $P = 0.697 > 0.05$) models suggest the adequacy of both models to explain data in the region of experimentation.

1. Introduction

Surface-active agents (surfactants) are amphiphilic compounds derived chemically or biologically and which partition themselves at interfaces thus lowering the free surface enthalpy per unit area of mixtures (Desai and Banat, 1997). World demand for surfactants has been on the rise by reason of the many applications which they have found in industries and the environment. However, industrial and environmental decontamination applications have been moving in the direction of green chemistry since environmental scientists chronicled the hazardous nature of most of the chemically-derived surface active compounds leaving these processes at the mercy of the biologically-derived alternative (Lima et al., 2011).

Green surfactants or biosurfactants come in a variety of chemical forms including glycolipids, lipopeptides and lipoproteins, phospholipids and polymeric types including glycolipopeptides (Zhang and Dequan, 2013). Glycolipopeptides are high-molecular-weight polymeric surface-active compounds produced by a variety of bacteria mostly of the genera *Corynebacterium* (Thavasi et al., 2007), *Pseudomonas* (Koronelli et al., 1983; Desai et al., 1988; Ilori and Amund, 2001; Ekpenyong et al., 2016) and *Bacillus* (Mabrouk et al., 2014). They act as excellent solubilizers and emulsifiers of hydrophobic substances thus facilitating their uptake and subsequent metabolism. The ability of hydrophobic organic compounds to be solubilized and transported to the immediate vicinity of microorganisms capable of metabolizing them is potentially the rate-limiting step in biodegradation (Cohen, 2002) and by extension bioremediation. High-molecular weight biosurfactants fulfill these tasks in microorganisms that elaborate them; however, their supplies for industrial and environmental applications are yet to meet up with their huge demand by reason of high production costs which has been the bane of all biotechnologically-derived products (Desai and Banat, 1997). Attempts at reducing production costs have been made through isolation of over-producing strains, genetic improvements of producing strains (Sekhon et al., 2011), medium and process development (Rodrigues et al., 2006a, 2006b) and use of low-cost substrates (Nitschke et al., 2004; Ekpenyong et al., 2016), with the ultimate goal of improving yield.

Conventional medium optimization method involves one-factor-at-a-time variation approach while holding all other factors constant. Documented limitations of this one-dimensional approach have been its

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tedion, time-consumption and inability to address imminent factorial interactions, resulting in the generation of unreliable results and drawing of wrong conclusions (Abalos et al., 2002; Myers and Montgomery, 2002; Lenth, 2009). An alternative approach has been the use of response surface methodology (RSM) which handles multi-factorial experiments, incorporating designs and models with the objective of optimizing processes and product yields. It is a much exploited method for fermentation media optimization (Rodrigues et al., 2006a, b; Luna et al., 2011; Mnif et al., 2012) and involves a collection of statistical techniques for designing experiments (Lenth, 2009), building models (Myers and Montgomery, 2002; Hanrahan et al., 2007), evaluating the effects of factors and searching for process optimum where several factors are varied simultaneously (Kalil et al., 2000).

The aim of the present study was to optimize major medium variables for the production of glycolipopeptide by *Pseudomonas aeruginosa* Strain IKW1 using RSM. The input variables were carbon source, nitrogen source, phosphorus source and inoculum volume while biomass and biosurfactant concentrations were the response variables.

2. Materials and methods

2.1. The producing bacterium

The bacterium, *Pseudomonas aeruginosa* Strain IKW1, was earlier isolated by these authors (Ekpenyong et al., 2016) from a sub-surface water sample collected from Ikang River in Bakassi Local Government Area of Cross River State, Niger Delta region of Nigeria along coordinates N 04° 50.127′; E 08° 32.976′. The organism was identified by morphological and biochemical tests and its identity confirmed by molecular characterization using 16 S rRNA sequencing, as a strain of *Pseudomonas aeruginosa* with 100% sequence homology with a phthalic-acid degrading *Pseudomonas aeruginosa* Strain HNYM41 (GenBank accession no. **JN999891A**) earlier isolated from garbage-contaminated soil in India (Raorane et al., 2012).

A mineral oil-overlaid stock of the bacterium was retrieved from the University of Calabar Collection of Microorganisms (UCCM) and reactivated in Tryptic Soy agar by the quadrant-streak plate technique for 36 h. The bacterium was passed a second time through the reactivation process in the same medium for 24 h after which a loop-full of culture was used to inoculate freshly prepared Luria broth in 50 mL Erlenmeyer flask containing 10 mL of medium. Flasks were incubated on a rotary shaker at 150 rpm for 18 h at room temperature (28 ± 2 °C).

2.2. The optimization experiments

2.2.1. Selection of major medium variables

2.2.1.1. Selection of appropriate carbon source. The substrates glucose, cassava processing effluent (CPE), glycerol, crude oil, waste frying oil (WFO), olive oil and palm oil were included in the screen test. The minimal medium contained (g/L) KH_2PO_4 0.5; K_2HPO_4 1.0; $MgSO_4 \cdot 7H_2O$ 0.2; NaCl 0.5; NH_4Cl 1.0; $FeSO_4 \cdot 7H_2O$ 0.01 with pH adjusted to 7.0 using 1 M HCl/IM NaOH. Carbon sources were added at 1% (w/v or v/v) to the minimal medium in 100 mL Erlenmeyer flasks and flasks sterilized by autoclaving at 121 °C for 15 min. Flasks were incubated on orbital shaker agitating at 150 rpm at room temperature for 72 h after inoculating with 1 mL of an 18-h old Luria broth culture of the glycolipopeptide-producing bacterium.

Post-fermentation, cell-free fermentation broth of the respective carbon sources was obtained by centrifugation at 8000 × *g* for 10 min followed by membrane filtration with 0.45 μM and later 0.2 μM (Millipore). Biosurfactant quantification was performed as described by Ekpenyong et al. (2016). A one-way analysis of variance (ANOVA) was used to evaluate the effects of carbon sources on biosurfactant production at 95% confidence level. Significant means were compared to ascertain the location of the significance using Excel 2007 *t*-test for two samples assuming unequal variances.

2.2.1.2. Selection of appropriate nitrogen source. The nitrogen sources investigated included asparagine (amino nitrogen), NH_4Cl (ammonium nitrogen), KNO_3 (nitrate nitrogen), $NaNO_3$ (nitrate nitrogen), Peptone (complex organic nitrogen) and Urea (simple organic nitrogen). All nitrogen sources, except urea, were added to fermentation medium (composition as in Section 2.2.2) at 1% (w/v) concentration. Waste frying sunflower oil served as carbon source at 1% (v/v). Flasks were sterilized as described earlier. Upon cooling, the flask labeled for urea was supplemented with filter-sterilized urea (0.2 μM Millipore) at 1% (w/v). Further preparations involving inoculations, incubations, biosurfactant quantification and statistical analyses were as described in Section 2.2.1.1.

2.2.1.3. Selection of appropriate phosphorus source. The effect of various combinations of phosphate on biosurfactant production was evaluated. The combinations were prepared in the ratio of 2:1 because this ratio, in the trial experiments, brought the medium pH very close to neutral and so needed very minimal adjustments with extraneous chemicals. The combinations investigated included K_2HPO_4/KH_2PO_4, Na_2HPO_4/NaH_2PO_4, K_2HPO_4/NaH_2PO_4 and Na_2HPO_4/KH_2PO_4. The different phosphate combinations were added at 1% (w/v) to fermentation medium with composition as given in Section 2.2.1 supplemented with 1% (v/v) waste frying oil and 1% (w/v) urea. Further preparations involving inoculations, incubations, biosurfactant quantification and statistical analyses were as described in Section 2.2.1.1.

2.2.1.4. Selection of appropriate inoculum size. An 18-h old Luria broth culture of the test isolate was centrifuged at 8000 rpm for 15 min and the cells washed twice with sterile distilled water. Cells were then diluted to obtain concentrations of 10^{10}, 10^9, 10^8 10^7, 10^6 and 10^5 cfu/mL using McFarland standards. The various concentrations were added at 1% (v/v) to fermentation flasks with medium composition as given in Section 2.2.1, supplemented with 1% (v/v) waste frying oil, 1% (w/v) urea and 1% (w/v) Na_2HPO_4/KH_2PO_4. Further preparations involving inoculations, incubations, biosurfactant quantification and statistical analyses were as described in Section 2.2.1.1.

2.2.2. Response surface methodology

2.2.2.1. Fermentation medium for biosurfactant production. The minimal medium for glycolipopeptide production contained (g/L) $MgSO_4 \cdot 7H_2O$ 0.2; NaCl 0.5; $CaCl_2$ 0.5; KCl 0.5 and $FeSO_4 \cdot 7H_2O$ 0.05, supplemented with 1 mL of trace mineral solution containing (g/L): $ZnSO_4 \cdot 7H_2O$ 0.005; $MnSO_4 \cdot 4H_2O$ 0.005; H_3BO_3 0.005; $CuSO_4 \cdot 5H_2O$ 0.005; $CoCl_2 \cdot 6H_2O$ 0.005; $MoNa_2O_4 \cdot 2H_2O$ 0.005 and $NiCl_2 \cdot 6H_2O$ 0.005. The concentrations of waste frying oil (carbon source), urea (nitrogen source) and Na_2HPO_4/KH_2PO_4 (phosphates) were added according to experimental design before adjusting pH to 7.0. The waste frying oil was obtained from sunflower oil with composition (% w/w) stearic acid 2.21, palmitic acid 6.11, volatile fractions 16.23, oleic acid 22.34 and linoleic acid 50.76. The levels of the test factors are presented in Table 1 which illustrates the design codes and their actual values.

2.2.2.2. Experimental design and fermentation studies

2.2.2.2.1. The design. A 2^4 full-factorial central composite rotatable design (CCRD) for four factors (independent variables), each at five levels, was selected as the experimental design employed to fit the multiple regression models using MINITAB 17 (trial version). The test variables included carbon source-X_1, nitrogen source-X_2, phosphates-X_3 and inoculum volume-X_4. Actual level of each factor was calculated using the equation of Myers and Montgomery (2002);