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RESEARCH ARTICLE

STATISTICAL OPTIMIZATION OF BIOSURFACTANT PRODUCTION IN *BACILLUS AMYLOLIQUEFACIEN S*BS36, A BIOCONTROL AGENT FOR RICE MOTH

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ABSTRACT

Bacillus amyloliquefaciens BS36 isolated from soil was tested for its biosurfactant production potential by utilizing different natural waste carbon substrates and its insecticidal activitywas evaluated. Among several natural substrates used in the present study, orange peel was found to be best substrate for biosurfactant production yield with surface tension of 80.04 mN/m and emulsification activity of 65%. Plackett-Burman experimental design was used to maximize biosurfactant production. Biosurfactant production increased 1.5 fold (28.17 mN/m) within 24 hrs compared to its production under the unoptimized conditions (41.88 nM/m). Qualitative analysis revealed that the isolated biosurfactant is of lipopeptide nature. Biosurfactant produced by *B. amyloliquefaciens* BS36 has an insecticidal activity against rice moth (*Corcyra cephalonica*).

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INTRODUCTION

Our daily basic activities are mostly dependent on the use of some kind of surfactants (toothpaste, detergents and cosmetic products, pharmaceutical by products, household cleaning products, herbicides or pesticides (Geys *et al.,* 2014). Huge demand for most of the surfactants is currently met by synthetically derived biosurfactant from petroleum feedstock. Environmental regulations and the increased awareness towards environmental friendly technologies have enhanced the search for biodegradable compounds of natural origin (Banat *et al.,* 2010).In recent years, owing to high biodegradability, increased foaming, activity at extreme temperature, pH and salinity, and lower toxicity of microbial derived surfactants allow possible replacement of chemically derived surfactants. Nevertheless, the wide-scale production of biosurfactants has not been commercialized due to the expensive production cost, low production yield and high recuperation charge (Behary *et al.,* 2012; Slivinski *et al.,* 2012). Some of the strategies which can improve biosurfactant production is by usage of low cost renewable substrates, optimization of growth media, screening for overproducing strain to maximize productivity (Jamal *et al.,* 2012).

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Formulation of a production medium involves selection of the right nutrientsat their correct levels to provide an ideal microenvironment which supports growth and metabolite production (Mukherjee *et al.,* 2008). Success of biosurfactant production depends on the development of cheaper processes and the use of low cost raw materials, which account for 10 - 30% of the overall cost (Cameotra and Makkar, 1998). Million tons of waste is generated each year throughout the world. Use of waste containing right balance of carbohydrates and lipids as feedstock to support optimum bacterial growth, biosurfactant production will help in better management of waste. With the increasing expansion of agro-industrial activities, large quantities of lignocellulosic residues are generated annually worldwide (Sanchez, 2009).

Although utilization of several agro-industrial wastes has been reported for biosurfactant production, the full potential of the whole range is yet to be investigated. In this context, this study was aimed at selecting significant media components by Plackett-Burman statistical approach. Plackett-Burman design was selected based on its ability to screen and evaluate the relevant medium components that affects biosurfactant production so as to generate manageable set of components as well as indicating how each component affects the overall response (Plackett and Burman, 1946; Ruchi *et al.,* 2008).

MATERIALS AND METHODS

Organism and culture conditions: The biosurfactant producing isolate was isolated in our laboratory from an agricultural soil, in Bangalore, India. It was identified as *B. amyloliquefaciens* BS36 by 16S rDNA gene sequencing. Comparison of the partial 16S rDNA gene sequence (1329 bp) of BS36 isolate with other bacterial sequences from the NCBI gene bank database showed highest degree of identity (<99%) with *B. amyloliquefaciens.* It was grown in nutrient broth and maintained in 40% glycerol and stored at 4°C.

Selection of suitable natural waste substrates for biosurfactant production: To evaluate natural waste substrates as carbon sources for biosurfactant production, different substrates such as banana peel, orange peel, lemon peel, potato peel, wheat bran and curd whey were used. Production of the biosurfactant was carried out in 250 ml Erlenmeyer flask containing 50 ml of the basal media composed of (g/l) K_2HPO_4 – 0.68; Na_2HPO_4 – 4.5; $MgSO_4.7H_2O$ – 0.1; $NaNO_3$ – 6.5; Glycerol – 30 and yeast extract 0.5. All waste were dried and powdered before use. To the basal media different substrates at a concentration of 1% (w/v) was added and sterilized at 121°C, 15 lbs pressure for 15 min. After sterilization flask was cooled to room temperature and 1% (v/v) of 24 h old bacterial broth culture was inoculated and incubated at 37°C for 72 h. For every 24 h broth was collected and centrifuged at 10,000 rpm at 4°C for 10 min. Supernatant was analyzed for biosurfactant production by measuring Emulsification Index and surface tension . The substrate yielding the maximum biosurfactant production was identified and selected for further study.

Surface tension measurement: The surface tension of the liquid was measured with a stalagmometer (Walter *et al.,* 2000). The surface tension was determined on the basis of the number of drops that fell per volume, the density of the sample and the surface tension of the reference liquid (water). The surface tension is calculated by

$$\sigma_L = \frac{\sigma_W \cdot N_W \cdot \rho_L}{N_L \cdot \rho_W}$$

Where, σ_L is the surface tension of the liquid under investigation, σ_W is the surface tension of water N_L is the number of drops of the liquid, N_W is the number of drops of water, ρ_L is the density of the liquid, ρ_w is the density of water

Emulsification test (E_{24}): The emulsifying capacity was evaluated by an emulsification index (E_{24}). Emulsification test was carried out using petrol as described by Cooper and Goldenberg (1987).To 1ml of cell free supernatant obtained after centrifugation (10,000 rpm for 10 min)2 ml of hydrocarbon (petrol) was added and vortexed for 2 minutes to ensure homogenous mixing of both the liquids. The emulsification index was observed after 24 h and it was calculated by using the formula

$$\text{Emulsification index} = \frac{\text{Height of the emulsion layer}}{\text{Total height}} \times 100$$

Selection of culture media conditions by plackett – burman experimental design: To find out the important medium components, Plackett–Burman design was applied and it is a design of fractional planused to identify the medium components which had significant effects on biosurfactant production. In this study 11 variables (variable *k* = 11, Table 1) were selected to evaluate their effect on biosurfactant production in 12 experiments and surface tension was used as a response. Each variable was tested at two levels, high (+1) and low (-1) levels. The effect of each variable was determined by the following equation:

$$E (X_i) = 2 (\Sigma M_i^+ - M_i^-) / N \quad \ldots\ldots (1)$$

Where, E (X_i) is the concentration effect of the tested variables. M_i^+ and M_i^- represent biosurfactant production from the trials, where the independent variable (X_i) measured was present at high and low concentrations, respectively. *N*, total number of the trials equals to 12. When the sign is positive, the influence of the variable upon biosurfactant production is greater at higher concentration and when negative, the influence of the variable is greater at low concentration. The standard error (SE) of concentration effect was the square root of the variance of an effect, and the significance level (*P* value) of each concentration effect was determined using the Student's *t*- test:

$$t (X_i) = E (X_i) / SE \quad \ldots\ldots (2)$$

Where, E (*X*i) is the effect of variable X_i. The statistical analysis of the results was performed with the aid of statistical software package Design Expert 10.0 (State-Ease, Minneapolis, MN, USA).Each trial was carried out in 100 ml Erlenmeyer flasks with 50 ml working volume. For inoculation, the flasks were allowed to cool down to room temperature before transferring 1% (v/v) inoculum into the production media. All experiments were carried out in triplicate.

Characterization of biosurfactant production: Cell free broth culture of BS36 was subjected to Phenol – Sulfuric acid method (Ellaiah *et al.,* 2002), Biuret test (Feigner *et al.,* 1995), Phosphate test (Okpokwasili and Ibiene, 2006) to characterize the nature of biosurfactant.

Determination of Insecticidal activity: Bioassay was carried out against larvae of rice moth (*Corcyra cephalonica*) under starvation for 24 hours. Crude biosurfactant was used to prepare different concentrations (1 - 5 %) in distilled water and larvae were transferred to individual glass tubes and incubated at room temperature for 24-48 h, followed by counting the time taken for the death of larvae and number of dead larvae in each tube. Distilled water served as control (Kim *et al.,* 2011).

RESULTS AND DISCUSSION

In the present study, biosurfactant production by *B. amyloliquefaciens* BS36 with different natural substrates as carbon sources was studied using a mineral salt medium for 72 h. In the case of orange peel, banana peel and wheat bran, the maximum biosurfactant production was observed on the first 24 h (day 1) (Figure 1) whereas in case of potato peel and lemon peel on the third day (72 h) by reduced surface activity. Among the substrates tested, surface tension reduction and high percentage of emulsification was observed with orange peel substrate (80.04 mN/m; 65%) followed by banana peel (95.89 mN/m), wheat bran (97.29 mN/m) and curd whey (98.56 mN/m). Accordingly, orange peel was used for further studies as a carbon source to enhance the biosurfactant production. In the present investigation, orange peel showed increased biosurfactant production compared to other natural substrates.

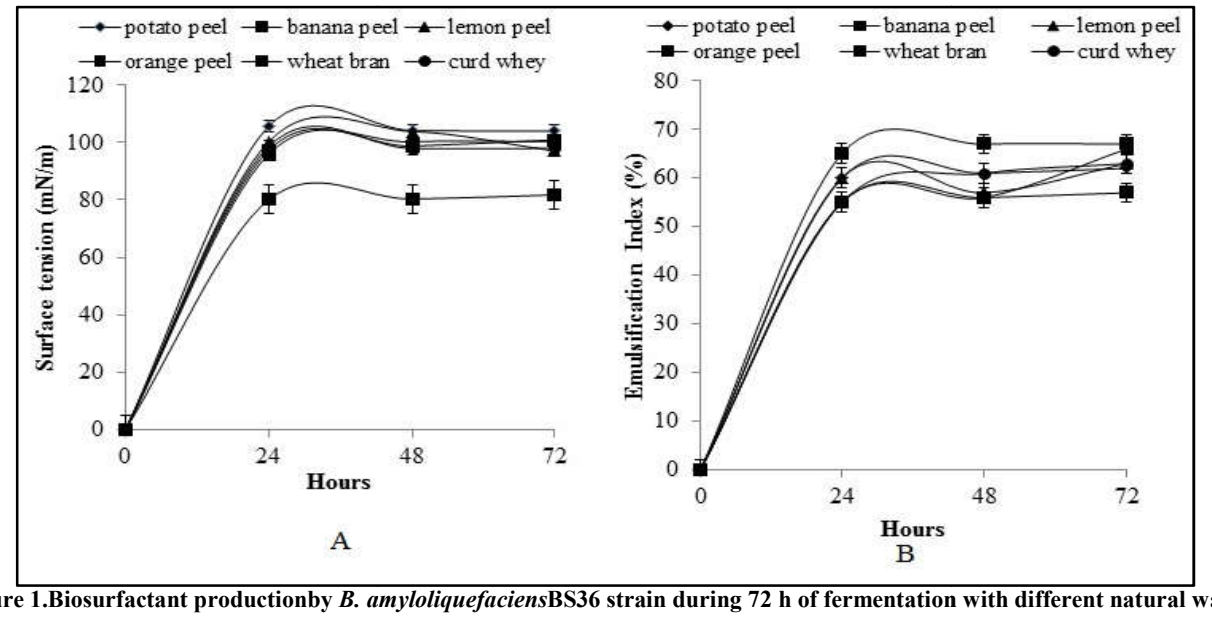

Figure 1.Biosurfactant productionby *B. amyloliquefaciens*BS36 strain during 72 h of fermentation with different natural waste substrates

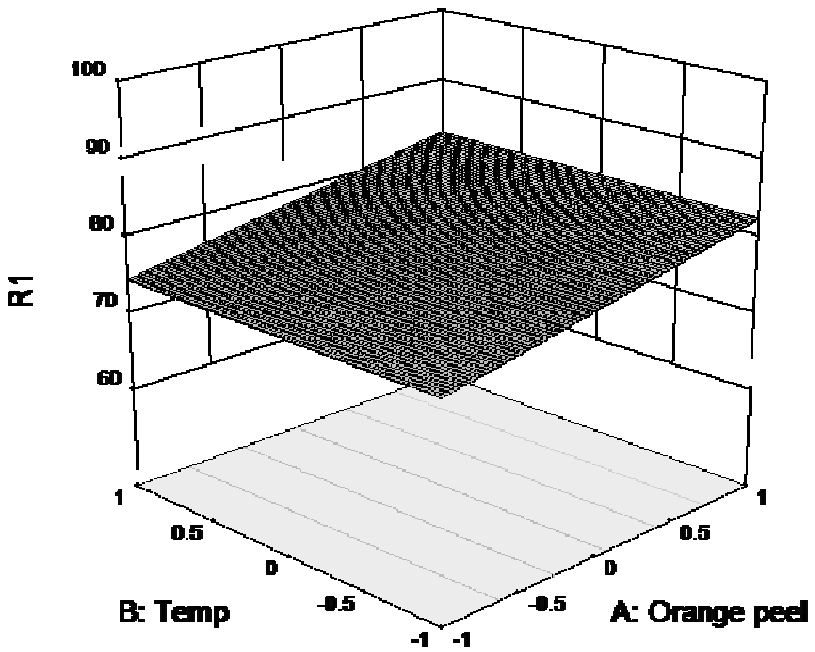

Figure 2. Interaction between orange peel and temperature

Kumar *et al.* (2016) who reported biosurfactant yield of 1.796 g/L and emulsification activity of 75.17 % with orange peel as substrate. George and Jayachandran (2009) reported orange peel to be best substrate generating 9.18 g/l of rhamnolipid biosurfacatant with a surface tension reduction upto 31.3mN/m. On the other hand Sharma *et al.* (2015)in his studies used potato peels as a potential useful substrate for biosurfactant production by *B. pumilus* DSVP18 with an ability to reduce surface tension from 72 to 28.7 mN/m. Potato substrates were evaluated as a carbon source for surfactant production by *B. subtilis* ATCC 21332 (2000).Thus, the ability of microorganisms to degrade a substrate varies greatly with the physio-chemical characteristics of the substrate and the crystallinity degree of substrate. Makkar and Cameotra (2002) have reported high contents of carbohydrates and lipids in Agro waste which might would be the cause of increased biosurfactant production.

Selection of culture media conditions by plackett – burman experimental design: Production economy is the major interest in secondary metabolites production, as in the case with most biotechnological processes. Often, the amount and type of fermentative media components can contribute considerably to the production cost. One possibility explored extensively is the application of experimental planning methodology to enhance biosurfactant production through optimization of nutritional requirements. Plackett-Burman design is one of the screening designs used for identifying relevant factors among many potential factors for further optimization. Methodology of Plackett-Burman makes it possible to consider a large number of variables and avoids the loss of information, which might be essential in the optimization process. Statistical optimization methodology helps in formulating suitable media with an advantage of being rapid and reliable in short listing of nutrients at varying concentrations leading to significant reduction in the total number of experiments.

Table 1.Variables representing medium components used in Plackett – Burman design

Variables	Medium components (factors)	Low level (-)	High level (+)
X_1	Orange peel (g/l)	0.25	2.5
X_2	Temperature (°C)	37	45
X_3	pH	4	9
X_4	Aeration (rev min^{-1})	0	120
X_5	NaCl (g/l)	0.1	1
X_6	$FeSO_4$ (g/l)	0.01	0.5
X_7	KH_2PO_4 (g/l)	0.07	0.7
X_8	Na_2HPO_4 (g/l)	0.45	4.5
X_9	$NaNO_3$ (g/l)	0.65	6.5
X_{10}	$MgSO_4$ (g/l)	0.01	0.2
X_{11}	Yeast extract (g/l)	0.05	1

Table 2. Analysis of media components as per placket- Burmann design for biosurfactant production

Variables	Medium components	Effect	SE	t(X_i)	*p*- value	Significance level
X_1	Orange peel (g/l)	8.10	2.26	3.58	0.0043	Significant
X_2	Temperature (°C)	0.13	2.26	0.05	0.96	Non-significant
X_3	pH	3.68	2.26	1.62	0.1335	Non-significant
X_4	Aeration (rev min^{-1})	-0.39	2.26	0.17	0.8681	Non-significant
X_5	NaCl (g/l)	5.00	2.26	2.21	0.0492	Significant
X_6	$FeSO_4$ (g/l)	-3.84	2.26	1.69	0.1191	Non-significant
X_7	KH_2PO_4 (g/l)	6.37	2.26	2.81	0.0170	Significant
X_8	Na_2HPO_4 (g/l)	-2.87	2.26	1.26	0.2337	Non-significant
X_9	$NaNO_3$ (g/l)	5.06	2.26	2.23	0.0475	Significant
X_{10}	$MgSO_4$ (g/l)	-3.50	2.26	1.54	0.1518	Non-significant
X_{11}	Yeast extract (g/l)	-4.44	2.26	1.96	0.0758	Non-significant

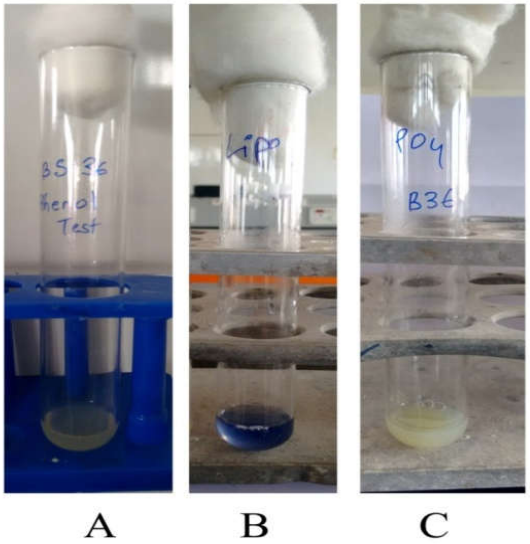

Figure 3. Qualitative identification of biosurfactant (A) Phenol-H_2SO_4test, (B) Biuret test and (C) phosphate test

From the PBD, surface tension results, calculated main effects at a confidence level of 95 % are summarized in Table 2. Within the range of the tested variables, the factors that appeared to be of positive effects (+1 level) are orange peel, KH_2PO_4, $NaNO_3$, sodium chloride, pH and temperature. Presence of high levels of orange peel, KH_2PO_4, $NaNO_3$, sodium chloride, pH and temperature in the growth medium affects surfactant production positively. On the other hand, the presence of $FeSO_4$, Na_2HPO_4, $MgSO_4$, yeast extract and aeration at their lowest levels would result in high surfactant production. Orange peel is the most significant factor with positive effect for biosurfactant production with 29.10% contribution followed by KH_2PO_4 (18.01%), $NaNO_3$ (11.37 %), sodium chloride (11.08%) within the tested levels.On the basis of the calculated *t-test*, orange peel, KH_2PO_4, $NaNO_3$ and sodium chloride were the most significant variables affecting surfactant production.

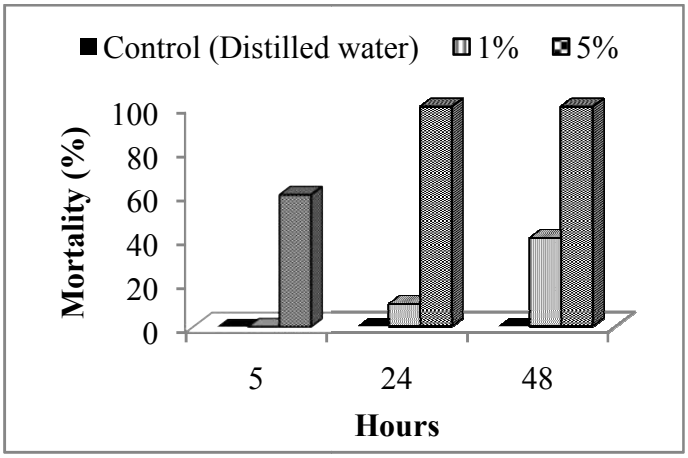

Figure 4. Mortality of rice moth treated with *B. amyloliquefaciens* BS36

Among the significant factors, increase in concentration of orange peel and 45°C temperature showed increase in biosurfactant production indicating direct relationship between these two factors for high yield of surfactant (Figure 2). According to the data obtained from the Plackett- Burman experimental results, the medium predicted to be near optimum should be of the following composition (g/100ml):Orange peel, 0.25; sodium chloride, 0.01; $FeSO_4$, (0.05); KH_2PO_4, 0.7; Na_2HPO_4(0.045); $NaNO_3$ (0.65); $MgSO_4$,(0.02); yeast extract, (0.005); pH, 9 incubating at 45°C shaking condition for 24 hrs. Predicted media increased bio surfactant yield by1.5 fold (28.17 mN/m) within 24 hrs compared to basal media (41.88 nM/m). Nawawi *et al.* (2010) reported a direct correlation with K_2HPO_4 and inversely proportional relationship with $FeSO_4$, $MgSO_4$, and $NaNO_3$ and biosurfactant production. Korayem *et al.* (2015) identified treated molasses, peptone, Tween 80, incubation period and inoculum size as significant factors affecting biosurfactant production in *Streptomyces* Spp. were biosurfactant production elevated from 31.74% (unoptimized media) to 42.68% (optimized medium). Based on the

optimization experiments, concluded that the biosurfactant production by *B. subtilis* SPB1 was enhanced to 1.65-fold over the original production determined by the conventional one-factor-at-a-time optimization method.

Characterization of the biosurfactant: In order to characterize the nature of biosurfactant, Phenol-H_2SO_4test, Biuret test and phosphate test were performed with cell free supernatant of *B. amyloliquefaciens* BS36. On addition of $CuSO_4$, violet colour formed indicating the presence of lipopeptides containing biosurfactants, whereas it showed no change in colour for Phenol-H_2SO_4 test and phosphate test (Figure 3). The results on preliminary identification revealed that the biosurfactant produced by BS36 was lipopeptide. Zouari *et al.* (2014) reported lipopeptide biosurfactant production using agro- industrial residues by *B. subtilis* SPB1. Lipopeptide biosurfactant with a hydrophobic moiety of octadecanoic acid methyl ester and a peptide part was reported by *Brevibacterium aureum* MSA13 (2010).

Insecticidal activity of *B. amyloliquefaciens* BS36 biosurfactant on *Corcyra cephalonica:* In the present study, *B. amyloliquefaciens* BS36 crude culture filtrate showed highest mortality rate (100%) of rice moth at a 5 % concentration after 24 hours (Figure 4). With 1% concentration mortality also increased with increase in time intervals after treatment.Two hours of exposure had no effect, however mortality observed at high concentration of biosurfactant. Our finding in the present study suggests that *B. amyloliquefaciens* BS36have high potential as source of active compound for insecticides.

Conclusion

The results of the current study give a basis for large scale fermentation of *B. amyloliquefaciens* BS36 for biosurfactant production. The study also suggests the effectiveness of statistical tools in bioprocess optimization with a large gain of cost and time.

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