ISSN: 2279 - 0594

Available Online at www.jbpr.in

Journal of Biomedical and Pharmaceutical Research, Volume 3, Issue 4, 2014, 01-07

RESEARCH ARTICLE

OPTIMIZATION OF RHAMNOLIPID BIOSURFACTNT PRODUCTION BY *STREPTOMYCES MATENSIS* (NBRC 12889^T) USING PLACKETT-BURMAN DESIGN

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Received 10 June 2014; Accepted 1 July 2014

ABSTRACT

Surfactants produced by microorganisms are known as biosurfactants. Biosurfactants are becoming important biotechnology products for industrial and medical applications due to their specific modes of action, low toxicity, relative ease of preparation and widespread applicability. In the present study, *Streptomyces matensis* (NBRC 12889^T), the biosurfactant producing Actinomycetes, was isolated from soil contaminated with poultry litter near Visakhapatnam, India. The isolated strain was grown in Kim's medium for extracellular biosurfactant production and it was identified as Rhamnolipid biosurfactant by Orcinol assay taking L-Rhamnose as standard. Further Optimization of medium constituents present in Kim's medium was done by the Plackett-Burman design. Among the seven medium constituents, three variables, namely concentration of Olive oil, $NaNO_3$ and level of inoculums were identified to cause significant effect on the biosurfactant production. By using the optimal fermentation medium, biosurfactant production was improved to 344.99 µg/ml compared to the unoptimized medium which was 315.93 µg/ml, an increase of 9.2 %. By this the Plackett-Burman design can be powerfully used to improve biosurfactant production by *Streptomyces matensis* (NBRC 12889^T) as a function of various salt compositions and levels of ingredients in the production medium.

Key words: *Streptomyces matensis*, Actinomycetes, Rhamnolipid, Fermentation, Kim's medium.

INTRODUCTION:

Microbial compounds that exhibit pronounced surfactant and emulsifying activities are classified as biosurfactants. They have primarily been used for environmental applications because of their diversity, environment-friendly nature, suitability for large-scale production and selectivity [1]. There are many advantages of the biosurfactants as compared to their chemically synthesized counterpart. Increased environmental awareness has led to serious consideration of biological surfactants as possible alternatives to synthetic surfactants [2]. Unlike the synthetic surfactants, microbially produced compounds are easily biodegradable and thus particularly suitable for environmental applications such as bioremediation and dispersion of oil spills [3].

Under certain conditions, many microorganisms can be induced to produce biosurfactants. In this sense, the study of the effect of different factors that influence the production of these compounds is of great importance. The typology, quantities and qualities of biosurfactants are influenced by the nature of the substrate, pH conditions, temperature, concentration of various salts and biomass activity [4]. The synthesis of biosurfactants depends largely on the availability of carbon sources and the balance between carbon and other limiting nutrients [5]. In this context, the present study deals with optimizing the culture conditions by using the Plackett-Burman experimental design to maximize the biosurfactant production by the isolate *Streptomyces matensis* (NBRC 12889^T).

METHODS:

Isolation of Biosurfactant Producing Actinomycetes:

Selection of soil samples: The soil sample was collected in sterile plastic bags from poultry near Visakhapatnam, India. This soil sample was found to be rich in fats and

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oils, and hence was used for the screening of biosurfactant producing organisms.

Selective Isolation of Actinomycetes:

The collected soil sample was air dried at room temperature for one week then was pre-treated at 55 ^{0}C in a hot air oven for 3 h, and then was stored at room temperature in sterile bags. This sample was labelled as PLS. One gram of soil sample was, serially diluted and 100 µl aliquot of appropriate dilution was applied to humic acid-Salts-Vitamin agar plates [6], with pH adjusted to 7.0. Humic acid was synthesised in laboratory using modified Essington method[7]. These plates were then supplemented with 50 µg/ml of Rifampicin and Cycloheximide, incubated at 28 ^{0}C for 7 days for the growth of Actinomycetes colonies. After 7 days of incubation, Actinomycetes colonies were preliminarily selected based on colony morphology, and, a small portion of the colony was streaked on the Starch-casein agar medium.

Screening for Biosurfactant Activity:

Seven day old Actinomycetes were inoculated into 500 ml Erlyn mayor flasks containing 100 ml of Kim's medium [8], containing 3% olive oil as the sole carbon source. Then, the broth cultures were incubated at 30±2 ^{0}C on a reciprocal shaker at 120 rpm for 5 days. This culture broth was then tested for the production of extracellular biosurfactants.

Oil Displacement Method:

Forty ml of distilled water was added to a petridish (15cm diameter), followed by the addition of 10µl of crude oil to the surface of the water, 10µl of sample was added onto the centre of this oil film. The diameters of the oil displaced zone on the surface was measured and compared with the zones produced by an un-inoculated medium as negative control, and sodium laryl sulphate as positive control. This test was carried out in triplicates [9].

Orcinol Assay:

Orcinol Assay method was used for direct assessment of the amount of Rhamnolipid biosurfactant present in the sample. 400 µl of cell-free supernatant was taken, and its pH was adjusted to 2.0 by adding 2N HCl. Addition of HCl results in the separation of Rhamnolipid. Then 750 µl of diethyl ether was added to this mixture to extract Rhamnolipid into an organic layer. This procedure of Solvent addition and extraction were repeated twice. Ether fractions were dried by evaporation. Then, 400 µl of pH 8.0 adjusted phosphate buffer was added to the remaining precipitate. Then, 300 µl of this precipitate was measured out, and 2.7 ml of Orcinol was added to it. Then, the test tubes with this precipitate were boiled in water for 20 min. Then, these test tubes were kept in darkness for 35 min, to cool it down to room temperature. Then, the Optical density of this precipitate was measured at 421 nm against blank. The concentrations of the Rhamnolipid were calculated using standard graph, and taking L-rhamnose as standard [10].

Time Course of Biosurfactant Production:

The time course of biosurfactant production was followed in batch cultures at optimum conditions. This experiment was designed for 10 days starting from the log phase to the stationary phase under submerged culture conditions [11]. The resultant cell-free supernatant was removed by filtration, followed by cold centrifugation at 10,000 rpm at 4 ^{0}C for 20 min. The supernatant then was analyzed for the biosurfactant production by Orcinol assay.

Identification of the Strain:

The Identification of the strain PLS-1, isolated from poultry litter soil sampled near Visakhapatnam, India, was done by 16S r-RNA sequencing, biochemical, morphological and cultural characterizations [12].

Optimization of the Production of Biosurfactant by Plackett-Burman Design:

An 8-Run Plackett-Burman design was applied to reflect the relative importance of various fermentation factors involved in the production of biosurfactant by PLS-1 [13]. For each variable, a high (+) and a low (-) levels were tested. The examined variables in this experiment and their levels were shown in Table 1. Eight different trials were performed in duplicates. The main effect of each variable was determined by the following equation:

Exi = (Mi+ – Mi-) / N

Where, Exi is the variable main effect, and Mi+, Mi- are the emulsification activity in the trials, where the independent variable was present in high and low concentrations, respectively, and N is the number of trials divided by 2. The statistical t-value for equal unpaired samples was calculated using STATISTICA-7, to determine the variable significance [14]. This design was applied with nine different fermentation conditions as shown in Table 2 with the 9th row representing the basal control. All experiments were performed in duplicates and the averages of the results of Orcinol assay were taken. The data from the experiment was used to calculate the effects and to determine the statistical significance of those effects. The variables with p-value < 0.05 were considered to have a significant effect on the biosurfactant production.

Table 1: Affect of Independent variables on biosurfactant production levels

Factor (g/l)	Symbol	Level		
		+1	0	-1
Olive oil (% v/v)	Oo	32	30	28
$NaNO_3$	Na	1.2	1	0.8
KH_2PO_4	Kh	0.12	0.1	0.08
$MgSO_4.7H_2O$	Mg	0.12	0.1	0.08
$CaCl_2$	Ca	0.12	0.1	0.08
Yeast extract	Ye	0.22	0.2	0.18
Level of inoculum (% v/v)	Li	102	100	98

RESULTS AND DISCUSSION:

Isolation and Screening of Biosurfactant Producing Actinomycetes:

A total of fifteen strains of Actinomycetes were isolated from the poultry littered soil named PLS. All these isolates were initially screened for extracellular biosurfactant production grown on Kim's medium containing olive oil as sole source of carbon [8]. Out of these fifteen strains, only PLS-1 showed positive result for showed positive result for drop displacement test [9]. As per the results observed for these four isolates, the drop displaced zone for PLS-1 showed maximum displaced area with a diameter of 7.5 cm**,** indicating a good Rhamnolipid biosurfactant production. These results are shown in the Figure 1.

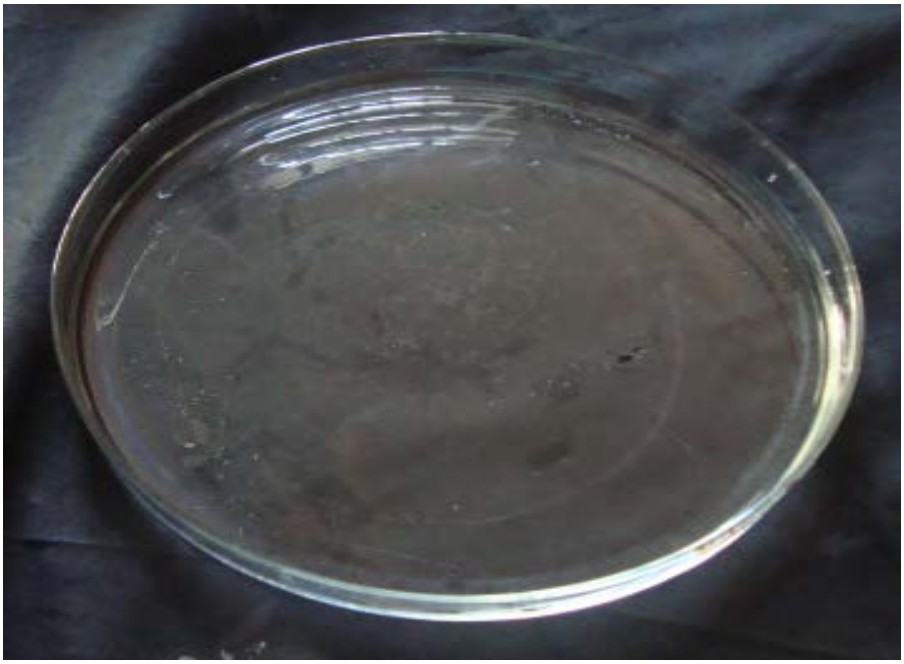

Figure 1 Drop displaced zone of isolate PLS-1

Identification Assay for Rhamnolipid:

In the present study, Orcinol assay was conducted using PLS-1 isolate for the conformation of Rhamnolipid [10]. This assay had confirmed that the isolate PLS-1 is a potential producer of glycolipids. The types of glycolipid were found to be Rhamnolipid in nature. Therefore, this isolate PLS-1 was selected for quantification of Rhamnolipid by taking L-Rhamnose as standard.

Characterization and Identification of Strain PLS-1:

The strain PLS-1 showed good growth in Kim's medium using olive oil as sole source of carbon at temperature range 30±2 in 7 days. Outer surface of colonies was perfectly round initially, but later they developed thin wavy mycelium. The aerial and substrate mass colour is white. By morphology shown in Figure 2,16S rDNA, and by analysing the phylogenetic tree sequencing, the

isolated strain was found to be *Streptomyces matensis* NBRC 12889^T by IMTECH Chandigarh, India

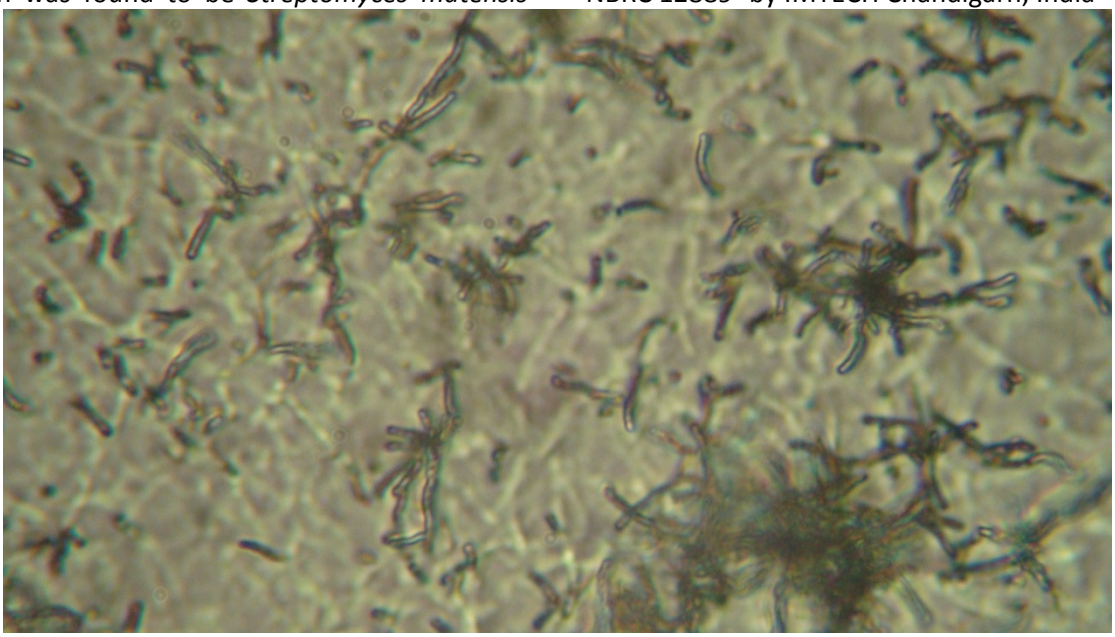

Figure 2: Aerial Mycelia of Strain PLS-1 observed at 400X magnification

Time Course of Biosurfactant Production:

The biosurfactant production was dependent on the growth of culture in the fermentation medium. From the 3rd day of growth, surfactant concentration started to increase, reaching its maximum after about 5th day, and there was a decrease in surfactant concentration. This indicated that the biosurfactant production had occurred predominantly in the exponential growth phase, the results are shown in Figure 3 [11].

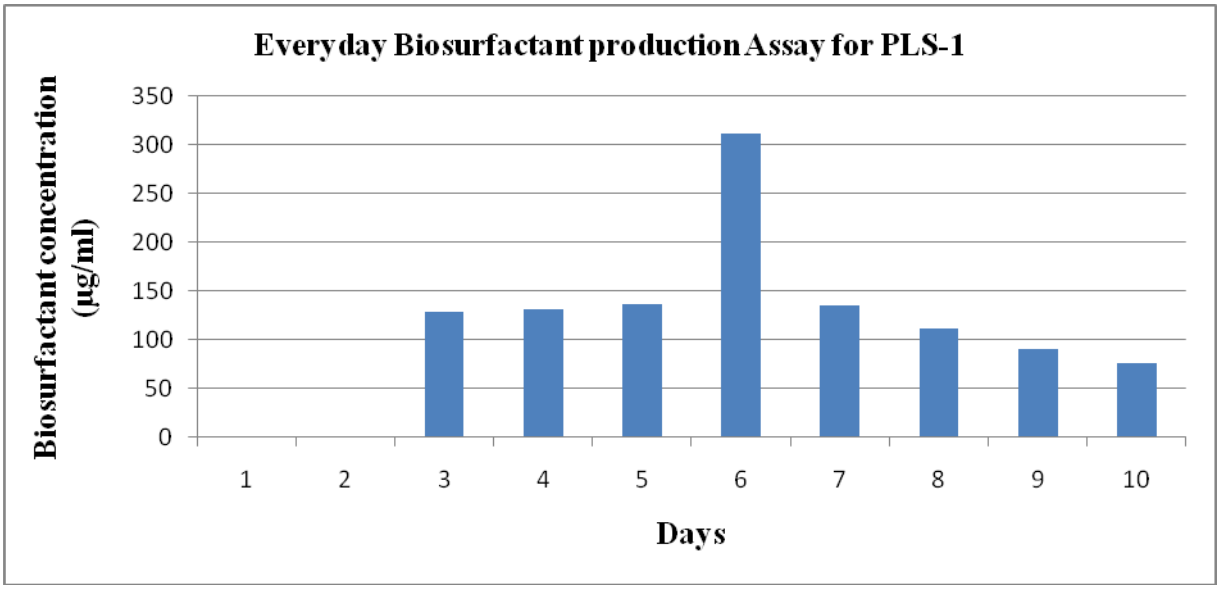

Figure 3: Everyday Biosurfactant production Assay for PLS-1 isolate

Optimization of biosurfactant production by Plackett-Burman design:

Plackett-Burman design is one of the screening designs used for identifying the important factors from among many potential factors. In the traditional method, screening for each category of the sources is done at an arbitrarily selected level of each source, one category at a time, while keeping the other ingredients constant. The difficulty is the selection of the categories, levels and the ingredients to keep constant. But in the Placket-Burman design, the data generated are used to select a few compounds in each category, based on highest product

promotion. Different levels of the selected compounds are then evaluated to achieve optimum level. The interactive effects among the sources of different categories are ignored completely. So, statistical experimental designs are powerful tools for searching the key factors rapidly from a multivariable system and minimizing the error in determining the effect of the ingredients. Therefore, results are achieved in an economical manner [15, 16].

Table 2: Results of the Plackett-Burman experimental design for seven factors

Runs	Oo	Na	Kh	Mg	Ca	Ye	Level of Inoculum	Biosurfactant concentration (µg/ml)
1	+1	-1	-1	-1	-1	+1	+1	321.74
2	-1	-1	-1	+1	+1	+1	-1	298.02
3	-1	-1	+1	+1	-1	-1	+1	308.67
4	-1	+1	-1	-1	+1	-1	+1	311.09
5	+1	+1	+1	+1	+1	+1	+1	344.99
6	-1	+1	+1	-1	-1	+1	-1	315.93
7	+1	+1	-1	+1	-1	-1	-1	321.74
8	+1	-1	+1	-1	+1	-1	-1	313.03
9	0	0	0	0	0	0	0	315.93

In this study, Plackett-Burman design was employed to evaluate the significant effect on seven different culture elements on the production of biosurfactant by *Streptomyces matensis* (NBRC 12889^T) using a basal medium. The main effect, t-values and p-values were estimated for each independent variable on biosurfactant production as shown in Table 3, and graphically represented in Figure 4. The results indicated that the presence of high levels of olive oil, $NaNO_3$, KH_2PO_4, Yeast extract, level of inoculum in the growth medium affects biosurfactant production positively.

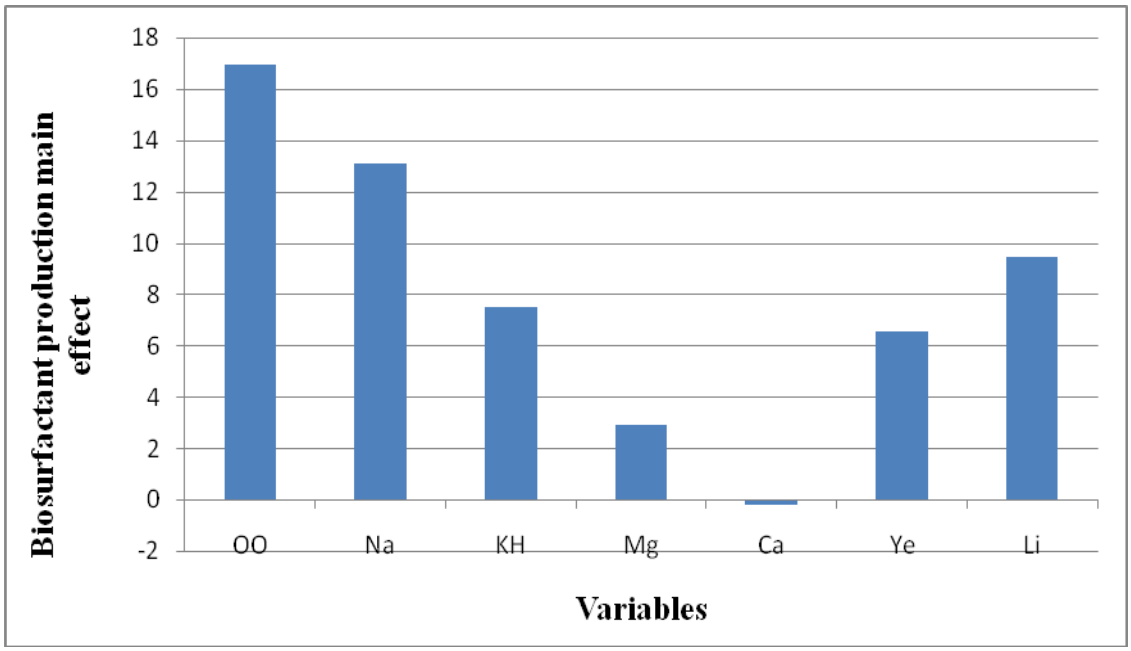

Figure 4: Fermentation conditions affecting Biosurfactant production

Standard t-values obtained from statistical methods (Cochran and Snedecor, 1989).

In addition, the presence of $MgSO_4$, $CaCl_2$ at their lowest levels would result in high biosurfactant production. The same was confirmed from the Pareto graph shown in Figure 5, which indicates higher effects were presented in the upper portion and then progress down to the lower effects.

Table 3: Statistical analysis - effects of medium constituents on biosurfactant production

Variables	Medium components (g/l)	Effect	Standard error	*t*-value	*p*- value
X_1	Olive oil (ml)	16.947	0.647	26.17	0.024
X_2	$NaNO_3$	13.072	0.647	20.18	0.031
X_3	KH_2PO_4	7.507	0.647	11.59	0.054
X_4	$MgSO_4.7H_2O$	2.907	0.647	4.49	0.139
X_5	$CaCl_2$	-0.237	0.647	-0.36	0.776
X_6	Yeast extract	6.537	0.647	10.09	0.062
X_7	Level of inoculums (ml)	9.442	0.647	14.58	0.043

*t-value significant at 1% level = 3.70; 5% level = 2.45; 10% level = 1.94; 20% level = 1.37.

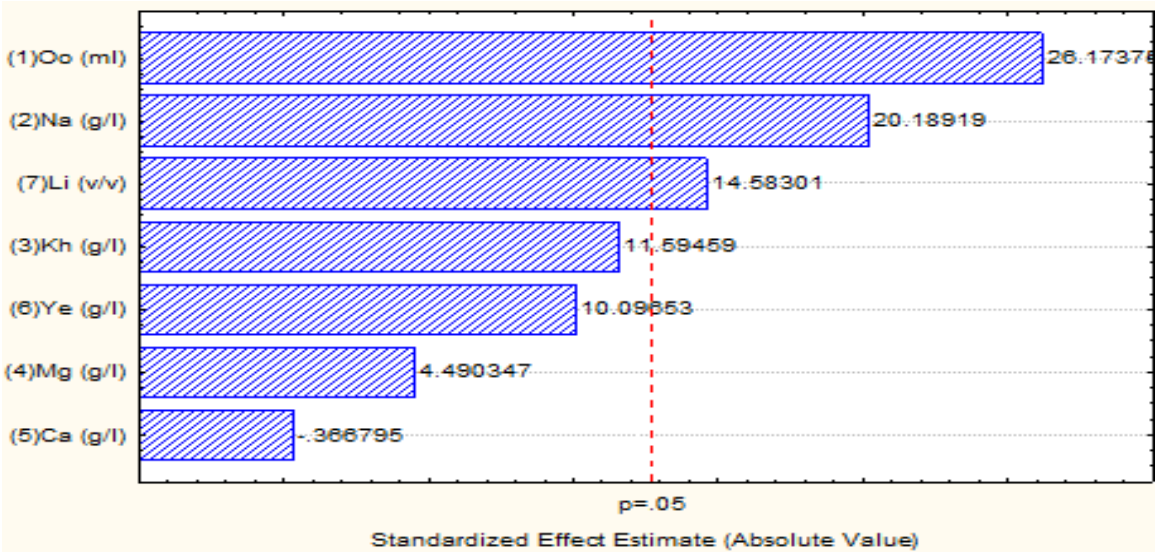

Figure 5: Pareto chart showing the effect of different media components (variables)

On the basis of the calculated t-test, olive oil, $NaNO_3$ and level of inoculums were the most significant variables affecting the biosurfactant production. Their interactions are shown in Figure 6 (a, b, c) which had shown increase of biosurfactant production with the increase of olive oil, $NaNO_3$, and level of inoculum, indicating an indirect relationship between these three factors for high yield of biosurfactant.

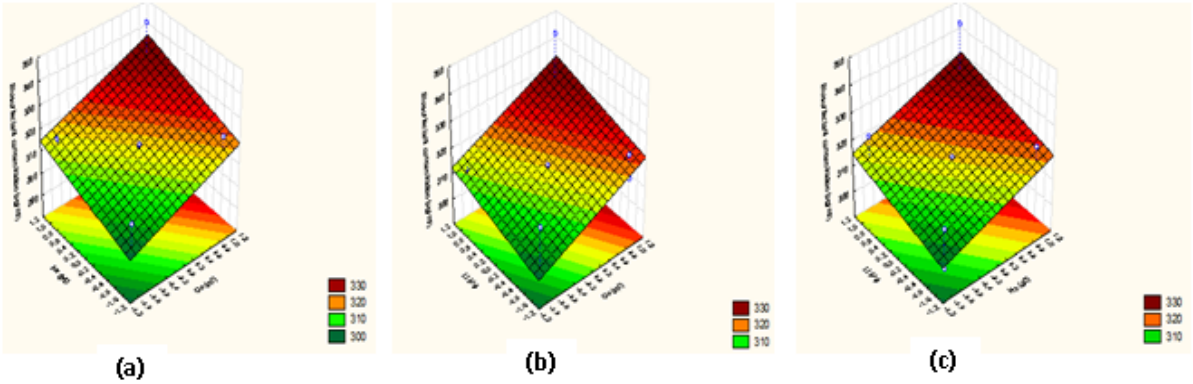

Figure 6 Interaction between (a) Olive oil and $NaNO_3$ (b) Olive oil and Level of inoculum (c) $NaNO_3$ and Level of inoculum

CONCLUSIONS:

The present study had proven that statistical designs, especially the Plackett-Burman design can be powerfully used to improve biosurfactant production by *Streptomyces matensis* (NBRC 12889^T) as a function of various salt compositions and levels of ingredients in the production medium. The Plackett-Burman design, was determined the variation of independent variables on the production of biosurfactant Rhamnolipid. Further work is necessary, subjecting the variables identified in the present study to response surface methodology to conform the optimized production medium for high yield of biosurfactant production by *Streptomyces matensis*.

CONFLICT-OF-INTERESTS NOTIFICATION:

The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENTS:

The author expresses her gratitude to Prof. G. Girija Sankar and Prof. T. Prabhakar (A.U. College of Pharmaceutical Sciences) for their immense help and valuable advises for this research.

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