ECRONICON OPEN ACCESS

EC MICROBIOLOGY
Research Article

# Production and Optimization of Biosurfactants from *Bacillus subtilis*

**Nawal Mehmood*, Mohsin Iqbal and Muhammad Asgher**

*Industrial Biotechnology Lab, Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan*

***Corresponding Author:** Nawal Mehmood, Industrial Biotechnology Lab, Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan.

**Received:** March 01, 2018; **Published:** April 03, 2018

## Abstract

Surfactants are the surface-active agents which are used widely in almost all industries including Petroleum industry, textile, polymers, plastics and food industry. The two major types of biosurfactants include non-degradable organic compounds containing synthetic surfactants and biologically produced easily biodegradable, less toxic, less irritant and humanly compatible surfactants known as biosurfactants. Interest in biosurfactants production is developing because they have ability to maintain their characteristics even in acute conditions like high pH, temperature, and salinity. The biosurfactants are better option for environment due to their biodegradability. In this study, different bacterial strains were screened on different substrates. The production of biosurfactants was optimized for enhanced production of biosurfactants by the *Bacillus subtilis* strain through response surface methodology (RSM) under central composite design (CCD). This study shows that cofficient of varience (CV) which is estimated mean value was 9.58% and goodness of fit of model $R^2$ was 0.9397 which indicates that the model is perfectly fit and reproducible while Adjusted $R^2$ was 0.9290 and predicted $R^2$ was 0.9116, (both Adj. and pred $R^2$ can be increase or decrease according to improvement of model.) In this model growth was taken at 35°C under pH 7.00 when 3% oil concentration was used in fermentation of 72 hours which results in highest production of 20.071 µl/mL. The main distinction of this research is that all work was environmental friendly and biological which not only reduces the burden of hazardous wastes but also provide a great help to reduce the environmental pollution.

***Keywords:*** *Biosurfactants; Bacillus subtilis; Waste Frying Oil; Lipopeptide; Oil Displacement*

## Introduction

Surfactants, also known as surface active molecules, can be divided into two groups on the basis of their synthesis, biosurfactants and synthetic surfactants. Synthetic surfactants are formed by the reaction of organic chemicals. On the other hand, biosurfactants can be produced by using biological machinery, excreted by microorganisms outside their cells for example fungi, yeast and bacteria. Biosurfactants have number of advantages comparatively, such as low toxicity, low irritancy, high biodegradability and human skin compatibility [1,2]. Biosurfactants preserve their characteristics under extreme temperature, pH, salinity and other environmental stress. Due to some of these advanced properties, biosurfactants have prospective use in different industries like petroleum, biopolymers, natural cosmetics, and pharmaceutical industries [3]. In recent times, due to increasing apprehension regarding environmental protection, the developed inexpensive biological processes for biosurfactants production is going to proceed [4-9].

Biosurfactants are classified into four groups. This division is based on the nature of chemical structures and types of microbial species which produce biosurfactants. These groups are as follows: glycolipids, phospholipids, lipoproteins and lipopeptide and polymeric surfactants [10]. Major components of glycolipids are carbohydrate and lipid which are linked by ester or ether groups. Those glycolipids which occur and studied abundantly are: sophorolipids, mono, -di or –trisaccharide mycolates and rhamnolipids. Phospholipids are the esters

linkage of alcohol groups on a phosphate and a lipid. Lipopeptides and lipoproteins are combinations of polypeptide and lipid chain. Polymeric surfactants are formed between fatty acid residues and saccharide units. But they are polymeric in nature [11]. The bacteria after evolution have modified themselves to manufacture and use a surface active product to feed on water-immiscible materials. It helps the bacteria of the aqueous phase to emulsify, adsorb and dissolve the material which is water-immiscible [12].

Biosurfactants are extracted from cell membrane of the bacteria or yeast. They are biodegradable and have low toxicity due to their property to reduce surface tension (ST), interfacial tension (IT), and the critical micelle concentration (CMC). Moreover, these compounds have ability to survive in a wide range of temperature and pH, they can also affect interface [13]. Additionally, they are highly suitable for petrochemical and environmental applications as compare to traditional chemicals and synthetic surfactants, because these consist of natural molecules, like glycolipids, lipopeptides, lipoproteins and fatty acids [14,15]. Bacterial strains of *Bacillus* and *Pseudomonas* genera usually produce lipopeptide biosurfactants [10]. To increase hydrocarbon degradation with biosurfactants, there are two mechanisms which can be used: Firstly, improvement of contaminants transfers in aqueous phase by interacting soluble contaminants. Secondly by reducing surface tension, the availability of hydrocarbons and their solubility may increases. These both mechanisms enable bacterial cell to contact with hydrophobic substrates [16].

It is interesting that biosurfactants come in many shapes and forms. The major criterion is that the molecule is amphiphilic so it has both hydrophilic and hydrophobic regions. These regions are precisely separated into a hydrophilic head group (charged or polar) and a hydrophobic tail, differ by chemically produced surfactants, which usually consist of linear alkyl groups. Degradation of branched tails is more difficult, so they are less used today [17]. That's why the head group forms the basis for classification into anionic, cationic, non-ionic and zwitterionic surfactants. This separation also allows chemical surfactants to aggregate to roughly spherical micelles in water above the critical micelle concentration (CMC), where the hydrophilic head groups contact with water from the surface, and shield the hydrophobic acyl chains tucked away into the micellar core.

For optimization of biosurfactant production, using Response Surface Methodology (RSM) under Central Composite Design (CCD), different parameters can be used as variables like pH, temperature, time of incubation, concentration of Carbon sources in fermentation media and also the carbon sources can be varied, like molasses, sugar whey, milk or cheese whey, waste lubricants, waste frying oil from different food industries, oil mill effluents etc. These all variables help to optimize the conditions and to obtain the high yielding specie and conditions as well. There are three methods for production and detection of biosurfactant that are drop collapse, oil spreading, and blood agar lysis [18,19].

Due to rapidly increasing petroleum use in industry, addition of hydrocarbons in the environment is massively increasing per year, either unintentionally or intentionally. Additionally, during recovery of oil, refining and transport several small spills may also occur [20]. Recently, to degrade these organic compounds, the development and implementation of novel and ecofriendly technologies are introduced. It is reported that some microbial species have effective role in the breakdown of organic compounds, because they use them as energy sources. Furthermore, to raise and improve the efficiency of bioremediation process the emerging bio compound usage is a promising method [16,20-22].

## Materials and Methods

### Place of Work

All experimental work for this research was done at Industrial biotechnology lab, University of Agriculture Faisalabad, Pakistan.

### Collection and Maintenance of Microbial Strains

Different bacterial cultures were collected from Institute of Microbiology, University of Agriculture and NIAB Faisalabad. The cultures were grown on agar-agar and nutrient-agar medium containing slants for almost 24 hours in incubator at pH 6.5 - 7 with temperature 37°C [23]. After the growth in slants, cultures were preserved at 4°C in refrigerator for subsequent use in inoculum development (Figure 1).

***Figure 1:*** *Collected bacterial strains and their slants.*

## Screening of Bacteria

All the native bacterial strains of *Bacillus* species were grown on medium nutrient agar, agar-agar slants and then their inoculum were prepared. 2.5g of Luria Broth L.B medium (2.5g) in 100 mL of distilled water in a flask with pH 7.0 - 7.2. Medium was sterilized in autoclave for 15 minutes at 120°C and 1 atm pressure. After bringing it to room temperature bacterial culture were transferred in inoculum under sterilized laminar air flow hood. The flasks were kept in shaker at 120 rpm at 37°C. After 72 hours in shaker, clear brown media was turned blur whitish brown liquid and bacterial growth was checked by taking optical density using UV-Visible Spectrophotometer at 600 nm [7]. The homogenous inoculum of each bacterial culture was used to inoculate the fermentation medium to produce biosurfactants.

## Liquid State Fermentation for Biosurfactant Production

For fermentation, the experiments were carried out in 250 ml baffled Erlenmeyer flasks containing 50 ml medium. The composition of the basal medium was ($gL^{-1}$): $NaNO_3$14, $KH_2PO_4$ 2, $K_2HPO_4$ 4, KCl 0.2, $MgSO_4.7H_2O$ 1, $CaCl_2$ 0.02, $FeSO_4.7H_2O$ 0.024, yeast extract (YE) 0.02 and 0·5 ml of a trace-element solution containing ($gL^{-1}$): 0.26 $H_3BO_3$, 0.5 $CuSO_4.5H_2O$, 0.5 $MnSO_4.H_2O$, 0.06 $MoNa_2O_4.2H_2O$, and 0.7 $ZnSO_4.7H_2O$. Finally, waste frying oil (obtained from public caters) was added in concentration of 4% and 0.2% molasses was used as an additional carbon source. Medium was sterilized at 120°C at 1 atm for 15 minutes in autoclave. The final pH of the medium was adjusted to 7·2. Inoculum were then added in each flask and incubated at 30°C on a rotary shaker (150 rpm) for 5 - 7 days to get maximum production and maximum biosurfactant producing strain was screened [24].

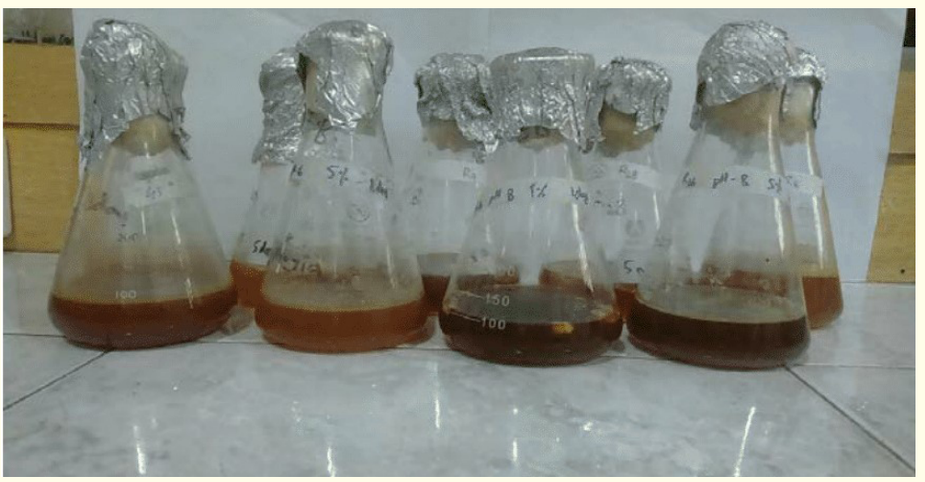

***Figure 2:*** *Fermented flask containing product and bacterial culture.*

Waste frying oils were kindly offered by the public catering in University of agriculture Faisalabad. The waste frying oil was of dark-brown color due to the presence of free fatty acids was allowed to precipitate for 48 hours and then upper foam and lower solids were discarded and only central portion of oil was used as major carbon source.

### Extraction and Chemical Characterization of Biosurfactant

The biosurfactants produced by *Bacillus* strains were extracted by the method described by Pornsunthorntawee., *et al.* [25]. After removing the bacterial cells by centrifugation, the obtained supernatant was acidified to pH 2.0 using 5M HCl solution, and the acidified supernatant was left overnight at 4°C for complete precipitation of the biosurfactants. After centrifugation at 8500 rpm for 20 minutes, the precipitates were dissolved in distilled water at pH 7.0, followed by biosurfactant extraction with a solvent having a 65:15 chloroform-to-methanol ratio at room temperature [7]. Then the organic phase was transferred to a rotary evaporator. It removed the solvent and a viscous honey-colored biosurfactant product was obtained. The components in the extracted biosurfactant were then tested by oil displacement test to confirm that whether the product is biosurfactant. The collected biosurfactants were characterized using Surfactin (Sigma, USA) as standard.

### Optimization of Biosurfactant Production

The parameters for enhanced biosurfactant production by the selected bacterial strains were optimized through Response Surface Methodology (RSM) under Central Composite Design (CCD) [7]. Optimization of condition for biosurfactant production from *Bacillus subtilis* was done by using statistical design named Design Expert 7.0.0 trial. Response Surface Methodology (RSM) under Central Composite Design (CCD) was used for this and selected four variables that were temperature, pH, concentration of oil in percentage and time of incubation. *Bacillus subtilis* have the ability to survive in extreme temperatures that is from 30 - 50°C with 5°C change so the range was 30°C, 35°C, 40°C, 45°C and 50°C. pH ranges from 6 - 8 i.e. 6, 6.5, 7, 7.5 and 8. Time of incubation is the time duration in which fermentation occurs and the biosurfactant were produced by the bacteria in a specific media of fermentation it usually takes days and nights so we take the time of incubation in hours so in this experiment time of incubation was 24 hours to 120 hours its range was 24 hr, 48 hr, 72 hrs, 96hrs and 120 hours. Oil concentration range was 1 - 5% (Table 1).

| Independent variables | Coded levels | |
|---|---|---|
| **Ranges** | **-1** | **+1** |
| pH | 6 | 8 |
| Temperature (°C) | 30 | 50 |
| Oil Concentration (%) | 1 | 5 |
| Incubation period (h) | 24 | 120 |

***Table 1:*** *Variables used for the whole experimental design.*

Biosurfactants have the ability that they are not degraded at extremes of temperature so the bacteria in hot water springs or in extreme low temperature if produce biosurfactant, would not be degraded and remains as were produced without changing their properties. In the same way pH also have no adverse effect on biosurfactants. These factors may affect the growth of bacteria but have no effect on properties of biosurfactants. Those bacteria which gives extracellular products are usually responsible for production of biosurfactant under some specific conditions and substrates. So, for this research a complete design of trials considering four variable factors was made (Table 2).

| Run | A: pH | B: Temperature (°C) | C: Incubation Time (Hours) | D: Oil Conc. (%) |
|---|---|---|---|---|
| 1 | 7.00 | 40 | 72 | 3 |
| 2 | 7.00 | 40 | 72 | 3 |
| 3 | 7.00 | 45 | 72 | 3 |
| 4 | 7.00 | 40 | 48 | 3 |
| 5 | 6.00 | 30 | 24 | 5 |
| 6 | 6.00 | 50 | 120 | 5 |
| 7 | 6.00 | 50 | 24 | 1 |
| 8 | 8.00 | 50 | 24 | 5 |
| 9 | 7.00 | 40 | 72 | 3 |
| 10 | 8.00 | 50 | 120 | 5 |
| 11 | 7.00 | 40 | 96 | 3 |
| 12 | 8.00 | 50 | 120 | 1 |
| 13 | 8.00 | 30 | 24 | 1 |
| 14 | 6.00 | 50 | 120 | 1 |
| 15 | 8.00 | 30 | 24 | 5 |
| 16 | 6.00 | 30 | 24 | 1 |
| 17 | 6.50 | 40 | 72 | 3 |
| 18 | 7.50 | 40 | 72 | 3 |
| 19 | 8.00 | 30 | 120 | 1 |
| 20 | 7.00 | 35 | 72 | 3 |
| 21 | 6.00 | 50 | 24 | 5 |
| 22 | 8.00 | 30 | 120 | 5 |
| 23 | 7.00 | 40 | 72 | 2 |
| 24 | 7.00 | 40 | 72 | 3 |
| 25 | 7.00 | 40 | 72 | 3 |
| 26 | 6.00 | 30 | 120 | 5 |
| 27 | 7.00 | 40 | 72 | 3 |
| 28 | 7.00 | 40 | 72 | 4 |
| 29 | 8.00 | 30 | 120 | 1 |
| 30 | 6.00 | 50 | 24 | 1 |

***Table 2:*** *Central Composite Design for Optimization of Biosurfactant Production.*

## Results and Discussion

For the production of biosurfactant, waste and environmental non-friendly substrates were used in this research to reduce them to environment friendly product which is of great importance and use in daily life as well in major industries. The results indicate that this product which was taken show the complete significance and also show positive result towards the oil displacement test. The optimization of product was done with a statistical design Response Surface Methodology using Central Composite design in which triplicate of 30 trials were carried out in different conditions. The results of these trials reveal that the maximum growth of bacteria and production of

biosurfactant was taken after 72 hours of fermentation when the fermentation media was supplemented with 3% concentration of waste frying oil, pH was maintained to 7.00 while process was provided with 40oC temperature. In these conditions, the maximum product was observed. Here it is important to note that after 72 hours of incubation almost maximum supplements were used and maximum product was obtained although some of the nutrients were still present in the solution but the decline phase of bacteria starts after 72 hours of incubation because of limited supply.. But beside the maximum quantity, product was also taken from 30oC - 50oC temperature, 6 - 7 pH and 24 hrs-120 hrs but in variable amounts because *Bacillus subtilis* is resistant to high temperature and extremes of pH.

### Screening of Biosurfactant Producing Bacteria

Among 12 samples of bacterial isolates one strain of *Bacillus subtilis* was found to efficiently utilizing waste frying oil and produce biosurfactant. Different strains of *Bacillus* species like *B. subtilis, B. cereus, B. licheniformis, B. thuringiensis, B. megaterium, B. anthracis* etc. were observed and used for screening the best growing and high yield giving bacteria. These bacteria were not given as there name but mentioned as A, B, C etc. in table 3. A medium containing waste frying oil as carbon was used along with molasses as an additional source. A mineral salt medium used for growing biosurfactant producers was initially supplemented with glucose to initiate biomass production. Table 3 shows the production of biosurfactants using waste frying oil in liquid state fermentation. The results were collected after fermentation and by doing oil displacement test for each strain.

### Optimization of Biosurfactant Production by *Bacillus subtilis*

From all bacteria, high yielding bacteria was selected and characterized that was *Bacillus subtilis*. RSM was proceeded under Central Composite Design (CCD) to optimize the production process to get maximum yield and find the best conditions in which bacteria gives best product yield. Table 4 shows the whole experimental design in which four variables were used to optimize the production, 30 experimental runs were made using different conditions.

| Run | A: pH | B: Temperature (°C) | C: Incubation Time (Hours) | D: Oil Conc. (%) | Biosurfactant (µl/mL) |
|---|---|---|---|---|---|
| 1 | 7.00 | 40 | 72 | 3 | 20.069 |
| 2 | 7.00 | 40 | 72 | 3 | 20.067 |
| 3 | 7.00 | 45 | 72 | 3 | 20.009 |
| 4 | 7.00 | 40 | 48 | 3 | 18.987 |
| 5 | 6.00 | 30 | 24 | 5 | 10.899 |
| 6 | 6.00 | 50 | 120 | 5 | 13.951 |
| 7 | 6.00 | 50 | 24 | 1 | 10.753 |
| 8 | 8.00 | 50 | 24 | 5 | 16.001 |
| 9 | 7.00 | 40 | 72 | 3 | 20.070 |
| 10 | 8.00 | 50 | 120 | 5 | 17.961 |
| 11 | 7.00 | 40 | 96 | 3 | 19.681 |
| 12 | 8.00 | 50 | 120 | 1 | 16.365 |
| 13 | 8.00 | 30 | 24 | 1 | 14.096 |
| 14 | 6.00 | 50 | 120 | 1 | 10.275 |
| 15 | 8.00 | 30 | 24 | 5 | 14.979 |
| 16 | 6.00 | 30 | 24 | 1 | 13.946 |
| 17 | 6.50 | 40 | 72 | 3 | 16.189 |
| 18 | 7.50 | 40 | 72 | 3 | 18.964 |
| 19 | 8.00 | 30 | 120 | 1 | 16.872 |
| 20 | 7.00 | 35 | 72 | 3 | 20.071 |
| 21 | 6.00 | 50 | 24 | 5 | 13.986 |
| 22 | 8.00 | 30 | 120 | 5 | 17.998 |
| 23 | 7.00 | 40 | 72 | 2 | 19.937 |
| 24 | 7.00 | 40 | 72 | 3 | 20.068 |
| 25 | 7.00 | 40 | 72 | 3 | 20.069 |
| 26 | 6.00 | 30 | 120 | 5 | 10.999 |
| 27 | 7.00 | 40 | 72 | 3 | 20.069 |
| 28 | 7.00 | 40 | 72 | 4 | 19.905 |
| 29 | 8.00 | 30 | 120 | 1 | 13.005 |
| 30 | 6.00 | 50 | 24 | 1 | 16.905 |

***Table 4:*** *Central Composite Design under Response Surface Methodology for Optimization of Biosurfactant Production.*

## ANOVA for Response Surface Model of Biosurfactants

If probability or P value is smaller, significance of the modal will be increased. For a significant modal Probe > F would be less than 0.05. For a significant modal lack of fit should not be significant. Values of "Prob > F" is 0.0021 which is less than 0.0500 indicate model terms are significant. Table 5 shows the results of ANOVA for response surface biosurfactants, In this case A, B, AC, BC, BD, $A^2$ are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve model.The "Lack of Fit F-value" of 8.833 implies the Lack of Fit is not significant. There is only 0.01% chance that a "Lack of Fit F-value" this large could occur due to noise. The Model F-value of 22.89 implies the model is significant. There is only 0.01% chance that a "Model F-Value" this large could occur due to noise (Table 5).

| Source | Sum of Squares | df | Mean Square | F-Value | P-value Prob > F | |
|---|---|---|---|---|---|---|
| Model | 308.68 | 14 | 22.05 | 22.89 | 0.0021 | Significant |
| A- pH | 73.17 | 1 | 73.17 | 75.96 | 0.0001 | |
| B-Temprature | 0.69 | 1 | 0.69 | 0.72 | 0.0108 | |
| C-Time of incubation | 2.33 | 1 | 2.33 | 2.42 | 0.1406 | |
| D-Conectration of oil | 1.25 | 1 | 1.25 | 1.30 | 0.2724 | |
| AB | 0.63 | 1 | 0.63 | 0.65 | 0.4320 | |
| AC | 4.59 | 1 | 4.59 | 4.77 | 0.0453 | |
| AD | 0.045 | 1 | 0.045 | 0.046 | 0.8327 | |
| BC | 1.02 | 1 | 1.02 | 1.06 | 0.0188 | |
| BD | 7.09 | 1 | 7.09 | 7.36 | 0.0160 | |
| CD | 1.12 | 1 | 1.12 | 1.16 | 0.2987 | |
| $A^2$ | 10.48 | 1 | 10.48 | 10.88 | 0.0049 | |
| $B^2$ | 0.61 | 1 | 0.61 | 0.64 | 0.4377 | |
| $C^2$ | 0.14 | 1 | 0.14 | 0.14 | 0.7118 | |
| $D^2$ | 0.35 | 1 | 0.35 | 0.36 | 0.5578 | |
| Residual | 14.45 | 15 | 0.96 | | | |
| Lack of Fit | 14.45 | 10 | 1.44 | 3.178 | 0.1001 | Non Significant |
| Pure Error | 8.833E-006 | 5 | 1.767E-006 | | | |
| Cor Total | 323.13 | 29 | | | | |

***Table 5:*** *ANOVA for Response Surface Quadratic Model.*

## Analysis of Varience of Biosurfactant Production

The comparison of response variability values with the variables under experiment and their particular interaction was calculated by the coefficient of determination $R^2$. The best analysis of fitting of the model was calculated by $R^2$ its values ranges from 0 - 1 and most best predicted value is near to 1 [5].

The "Pred R-Squared" of 0. 9116 is in reasonable agreement with the "Adj R-Squared" of 0.9290."Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. our ratio of 10.730 indicates an adequate signal. This model can be used to navigate the design space (Table 6).

| Std. Dev. | 1.4 | R Squared | 0.9397 |
|---|---|---|---|
| Mean | 15.39 | Adj R-Squared | 0.9290 |
| C.V. % | 9.58 | Pred R-Squared | 0.9116 |
| PRESS | 95.93 | Adeq Precision | 10.730 |

***Table 6:*** *Table for analysis of Varience of biosurfactants.*

Closer the value of $R^2$ to 1.0 stronger will be the model and pedicted efficiency of response is better so the value 0.9397 indicates that model is of good fitness and is significant as 0.9397 is approaching unity. The cofficient of varience (CV) is the ratio of standered error of estimated mean value which is expressed in percentage. A model is considered as reproduceabale if CV is less than 10% here in my results it is 9.58 % which means that this model is reproduceable [5].

## Final Equation in Terms of Coded Factors

Biosurfactant =+19.79+2.11 * A +0.20 * B +0.38 * C+0.28 * D+0.20 * A * B +0.54 * A * C+0.053 * A * D-0.25 * B * C+0.67 * B * D+0.26 * C * D -7.94 * $A^2$+1.92 * $B^2$-0.91 * $C^2$ +1.44 * D2

## Final Equation in Terms of Actual Factors

Biosurfactant= -338.90680 +111.53158* pH -1.71470* Temprature -8.74294E -004 * Time of incubation -3.73963* Conectration of oil +0.019812* pH *Temprature +0.011164* pH *Time of incubation +0.026375* pH * Conectration of oil -5.27083E-004* Temprature * Time of incubation +0.033281* Temprature * Conectration of oil +2.75130E-003*Time of incubation * Conectration of oil -7.93580* $pH^2$+0.019182* $Temprature^2$ -3.93143E-004* Time of $incubation^2$ +0.36055 * $Conectration\ of\ oil^2$.

## Regression Coefficient for Biosurfactants production from *Bacillus subtilis*

In case of regression coefficient model, the positive and negative coefficient of any factor indicates that it has significant effect on protease enzyme production. The positive values of linear coefficient indicates that production of protease enzyme increased with initial increase in any factor. While the negative linear coefficient for pH, temperature, fermentation time and oil concentration indicated that at higher levels of these factors protease activity decreased.

The t and p values are usually applied to analyze the significance of each coefficient. Greater t test value and lower the p value depicts greater significance of corresponding coefficient [26]. P value P ≤ 0.01 predicts that model terms are highly significant (Table 7).

| Factor | Coefficient | SE Coefficient | P- value | T- value |
|---|---|---|---|---|
| Intercept | 19.79 | 0.27 | 0.0021 | 73.29 |
| A- pH | 2.11 | 0.24 | 0.0001 | 8.79 |
| B-Temperature | 0.20 | 0.24 | 0.01.8 | 0.83 |
| C- Incubation Time | 0.38 | 0.24 | 0.1406 | 1.58 |
| D- Oil Concentration | 0.28 | 0.24 | 0.2724 | 1.16 |
| AB | 0.20 | 0.25 | 0.4329 | 0.80 |
| AC | 0.54 | 0.25 | 0.0453 | 2.16 |
| AD | 0.053 | 0.25 | 0.8327 | 0.21 |
| BC | -0.25 | 0.25 | 0.0188 | -1 |
| BD | 0.67 | 0.25 | 0.0160 | 2.68 |
| CD | 0.26 | 0.25 | 0.2987 | 1.04 |
| $A^2$ | -7.94 | 2.41 | 0.0049 | -3.29 |
| $B^2$ | 1.92 | 2.41 | 0.4377 | 0.79 |
| $C^2$ | -0.91 | 2.41 | 0.7118 | -0.37 |
| $D^2$ | 1.44 | 2.41 | 0.5578 | 0.59 |

***Table 7**: Estimated regression coefficients for biosurfactants production by Bacillus subtilis in LSF.*

## Oil Displacement Test

Oil displacement test is used for characterizing the biosurfactant with the help of its surface activity by measuring the clear zone which was formed when solution containing biosurfactant product was dropped on the thin oil layer which was spread on the water surface. More the clear zone, better is the biosurfactant with more surface tension reduction ability [27]. Test was performed by adding 40 mL of distilled water in a petri dish and then added crude oil in the center of petri dish to form a thin layer of oil on the water and after few minutes of spreading oil, the solution containing biosurfactant was added. The maximum clear zone diameter was observed under light and measured. Test was conducted at room temperature. This test was just a clear identification of whether product is present in the obtained solution or not.

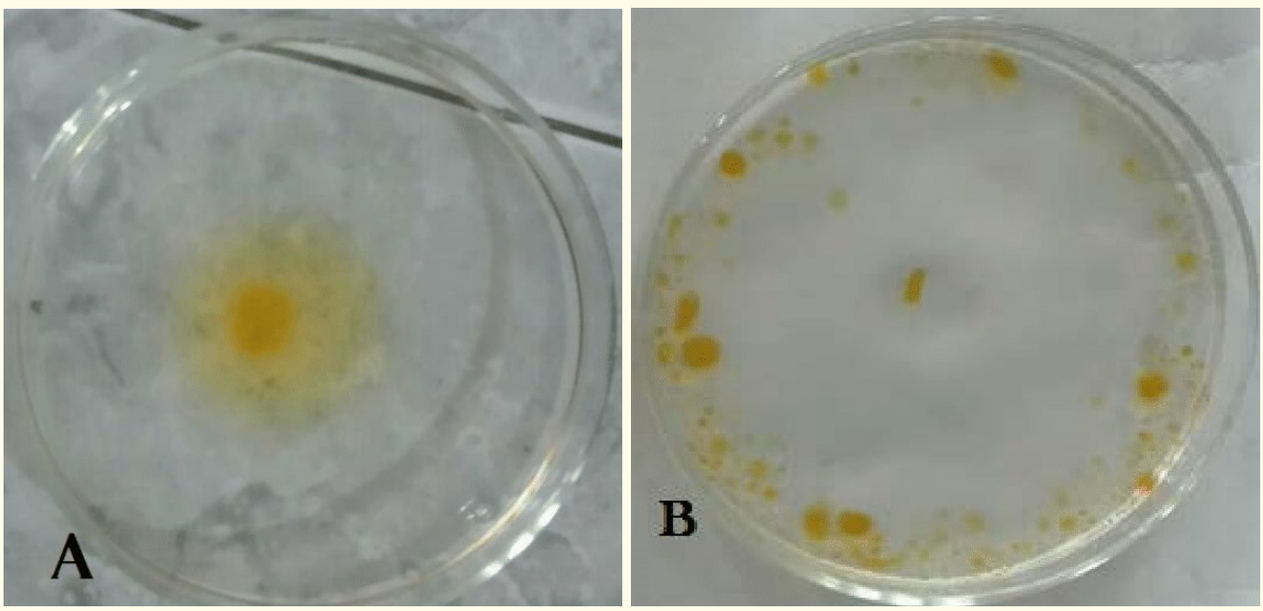


***Figure 3:** A- Oil suspended in water at the center of petri dish, B- After adding biosurfactant on suspended oil.*

## Interaction Among Variables for Optimization of Biosurfactant Production

The relationship of independent variables and dependent variables can be studid graphically by Contour response surface (RS) plots as well as by 3D Response surface plots which can demonstrate the relation among response and variables graphically, by three dimensional 3D response surafce plots optimum circumstances can be explained.

## pH and Temprature

The Biosurfactants production synthesized by *Bacillus subtilis* was influenced by both of these factors. The cooperative effect of temperature and pH was significant. The optimum pH and temperature was pH 7 - 8 and temperature 40oC. This graph shows the yeild of 20.021 ul/mL as a mean of the yeild was taken but the maximum yeild 20.071 was observed only at the darkest points shown as holes on the plot, this was observed when time of incubation was 72 hours, Concentration of oil was 3%, pH was 7 and temprature was 40$^{0}$C (Figure 4).

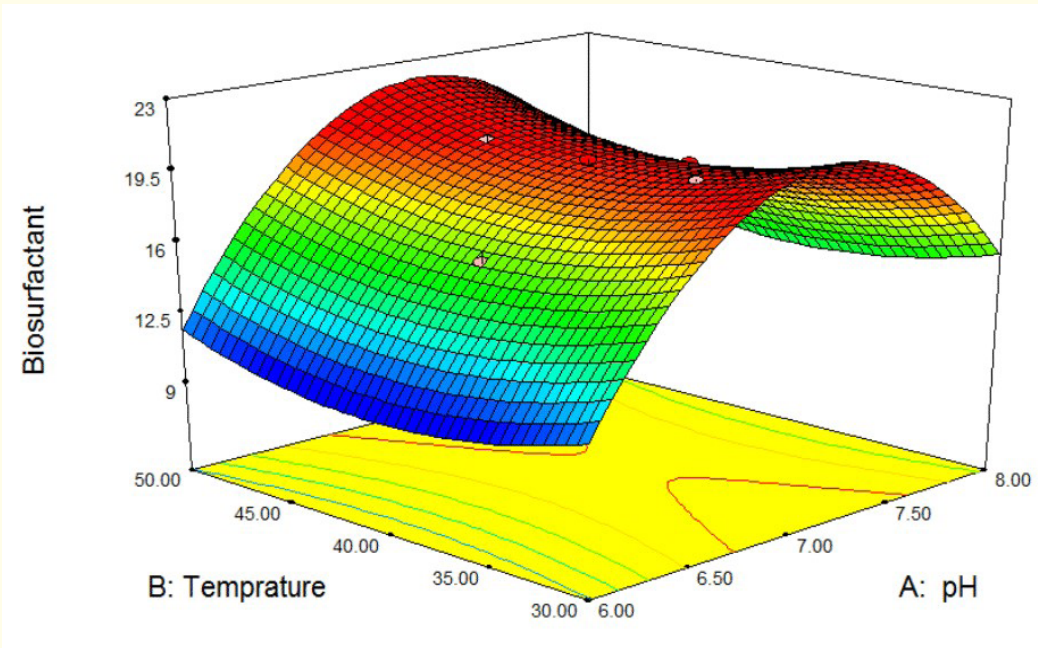


***Figure 4:*** *3D plot show the interacation of Temprature and pH.*

## pH and Time of incubation

For the biosurfactant responcen surface in terms of process variables pH and time of incubation it is easy to see while examining the figures that optimum production was near to 72 hours of fermentation and at 7 pH, here the maximum production of biosurfactant was taken 18.098 ul/mL beside the holes shown in graph are the highest yeilds of near 20.071 ul/mL. By studing contour plot it was seen that process was more sensitive to pH. If we see there is direct relation between pH and time of incubation upto a certain limit after which fluctuations in results were seen (Figure 5).

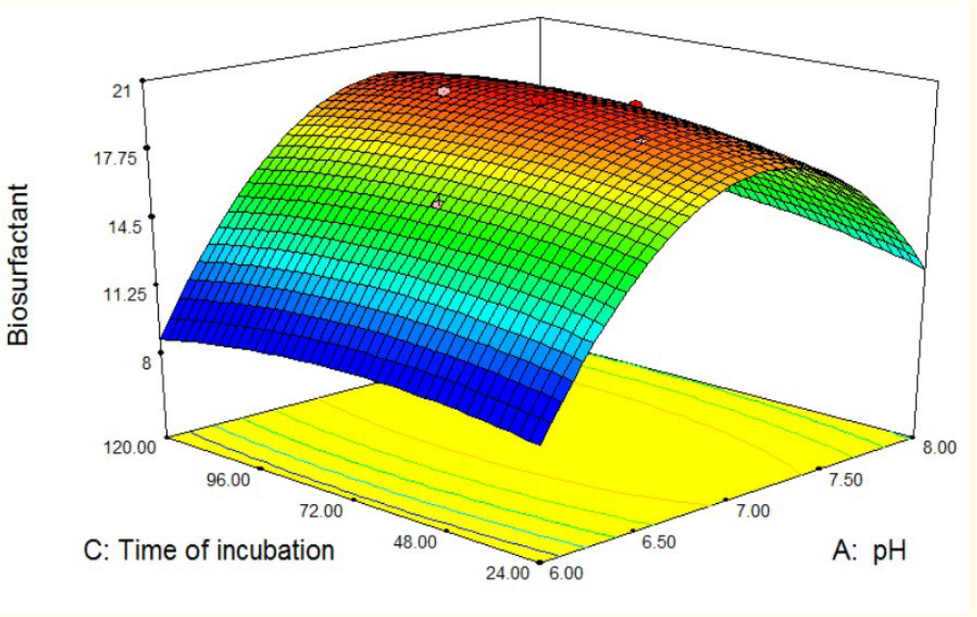


***Figure 5:*** *3D plot between pH and Time of incubation.*

### pH and concentration of oil

For the biosurfactant responcen surface in terms of process variables pH and concentration of oil it is easy to see while examining the figures that optimum production is near to 3% of oil concentration used in fermentation media provided as carbon source and at 7 pH, here the maximum production of biosurfactant was taken as 19.663 ul/mL (Figure 6).

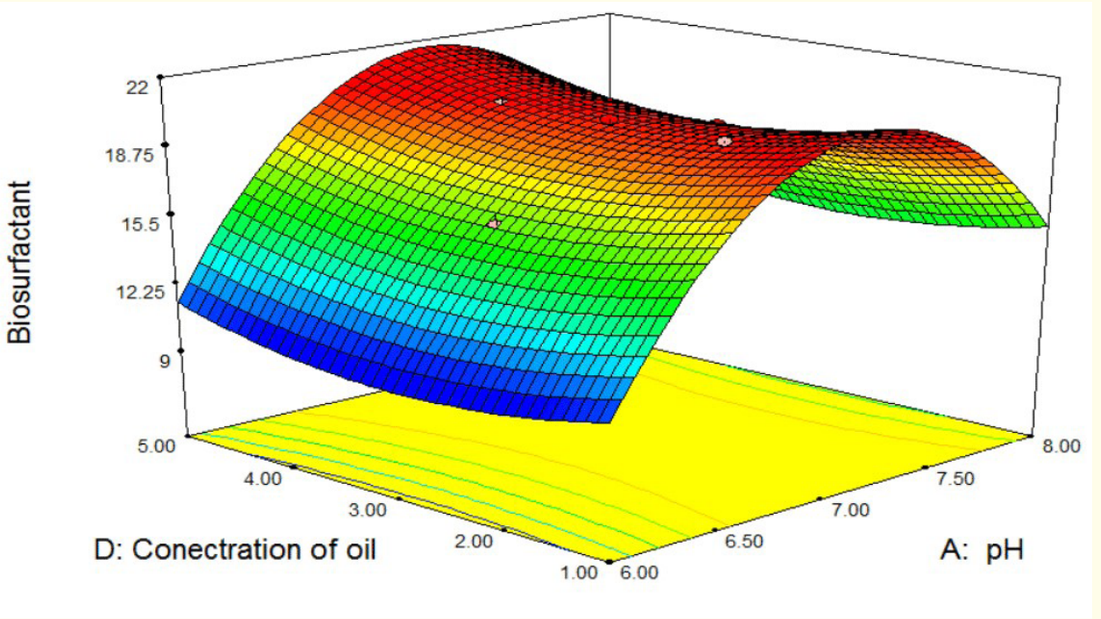


***Figure 6:*** *3D plot between pH and concentration of oil.*

### Temprature and time of incubation

For the biosurfactant responcen surface in terms of process variables temprature and time of incubation, it is easy to see while examining the figures that optimum production is near to 72 hours of fermentation and at 40oC, here the maximum production of biosurfactant was 20.195 ul/mL. By studing contour plot it was seen that process was more sensitive to temprature (Figure 7).

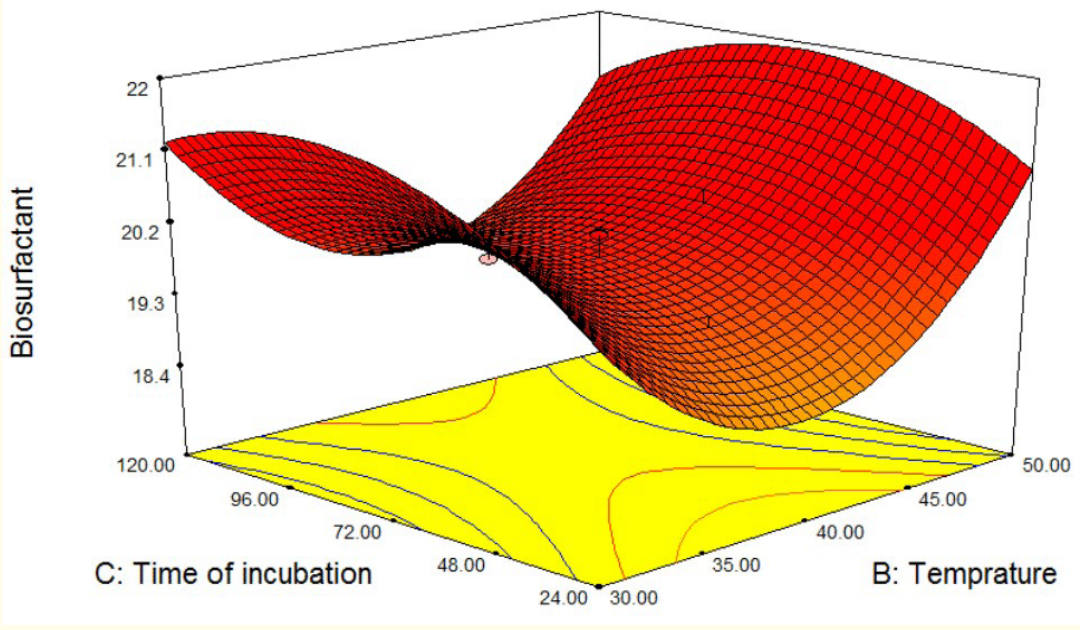


***Figure 7:*** *3D plot between Temprature and Time of incubation.*

## Temprature and concentration of oil

For the biosurfactant responcen surface in terms of process variables temprature and concentratiuon of oil it is easy to see while examining the figures that optimum production is near to 3% oil concentartion provided in process of fermentation and at $40^0$C temprature, here the production of biosurfactant which was expected to be maximum of 20.071 ul/mL can be raised upto 20.52 ul/mL to 21.027 ul/mL (Figure 8).

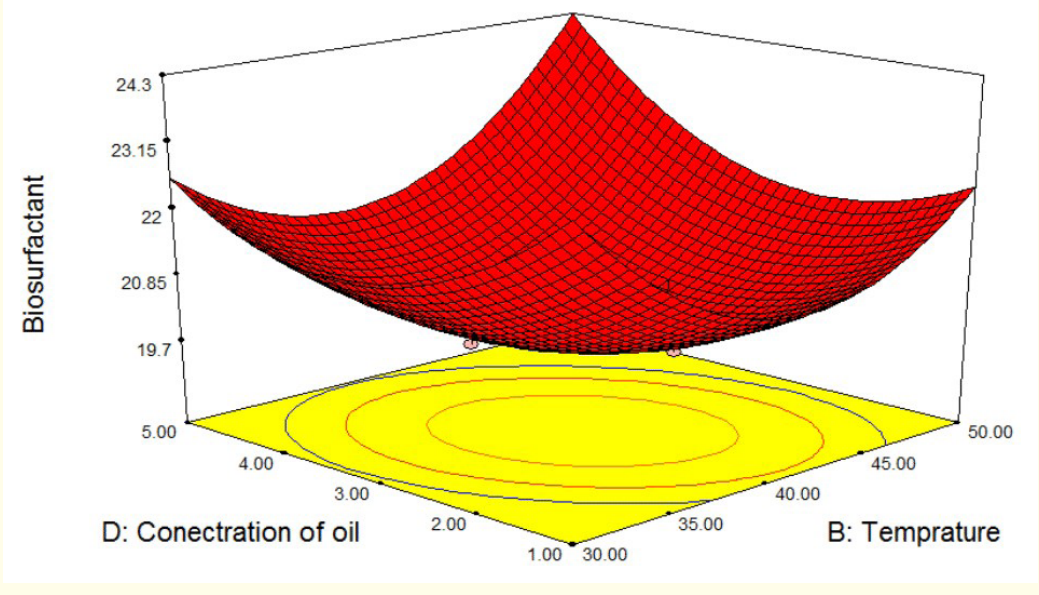


***Figure 8:*** *3D plot between Temprature and Concentration of oil.*

### Time of incubation and concentration of oil

For the biosurfactant responcen surface in terms of process variables time of incubation and concentratiuon of oil, it is easy to see while examining the figures that optimum production is near to 3% oil concentartion provided in process of fermentation and in 72 hours of fermentation, here the production of biosurfactant was taken nearest to the maximum production of biosurfactants comapritively. In these relative conditions 20.061 - 19.542 ul/mL was taken which is nearest to 20.071 ul/mL (Figure 9).

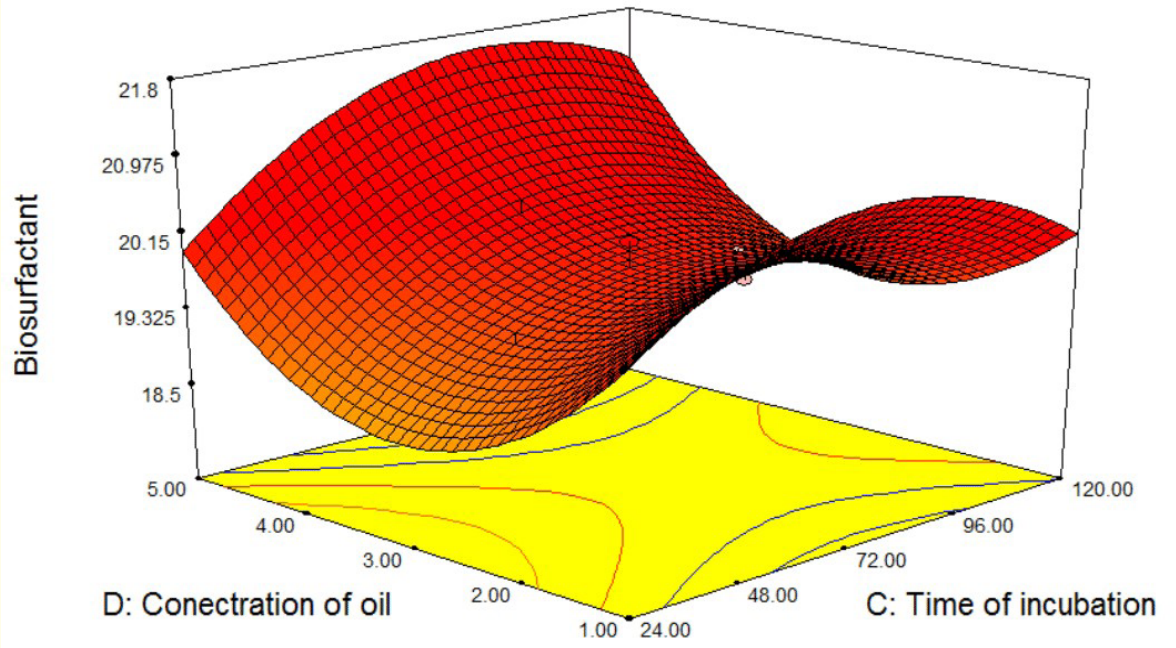


***Figure 9:*** *3D plot between Time of incubation and concentration of oil.*

Results of above research indicated that the strain of *Bacillus subtilis* was efficient comparatively in lipopeptide biosurfactant production and hydrocarbon emulsification. Biosurfactants are of different types and different species produce different kind of biosurfactants for instance Rhamnolipid type of biosurfactants are produced by *Pseudomonas* species while Lipopeptide type of biosurfactants are produced by *Bacillus* species [12]. Lipopeptides and lipoproteins are combinations of polypeptide and lipid chains. Polymeric surfactants are formed between fatty acid residues and saccharide units but are polymeric in nature [11]. Different waste products of industries can be used for the production of biosurfactants, the carbon source given in this research was waste frying oil which was taken from university caters. Waste frying oil supplements increased the biomass and lipopeptide production to several folds [28]. These results suggested the use of a low cost renewable carbon source like waste frying oils from food industry as well from public caters and other agro wastes for the biosurfactant production is very appreciable [24], which could be applied for bioremediation of hydrocarbon-contaminated sites and enhanced oil recovery [14].

## Conclusion

In this research lipopeptide biosurfactant was produced by *Bacillus subtilis* using waste frying oil as supplement during liquid state fermentation. Product was obtained by using a complete protocol in which a honey colored product was obtained which give positive results with one of the most satisfying test i.e. oil displacement test. A good range of biosurfactant was observed in this research which would be helpful to apply on large scale so as to meet the need in bulk. Hence the production of surfactant through biological machinery

will not only be cost effected but also if industry of biosurfactants is flourished, it will help to reduce the environmental pollution and the waste of industries would be used in useful way.

## Bibliography

1. Banat IM., *et al*. "Potential commercial applications of microbial surfactants". *Applied Microbiology and Biotechnology* 53.5 (2000): 495-508.

2. Cameotra SS and RS Makkar. "Recent applications of biosurfactants as biological and immunological molecules". *Current Opinion in Microbiology* 7.3 (2004): 262-266.

3. Desai JD and IM Banat. "Microbial production of surfactants and their commercial potential". *Microbiology and Molecular Biology Reviews* 61.1 (1997): 47-64.

4. Abalos A., *et al*. "Physicochemical and antimicrobial properties of new rhamnolipids produced by Pseudomonas aeruginosa AT10 from soybean oil refinery wastes". *Langmuir* 17 (2001): 1367-1371.

5. Fox SL and GA Bala. "Production of surfactant from Bacillus subtilis ATCC 21332 using potato substrates". *Bioresource Technology* 75.3 (2000): 235-240.

6. Morita T., *et al*. "Microbial conversion of glycerol into glycolipid biosurfactants, mannosylerythritol lipids, by a basidiomycete yeast, Pseudozyma antarctica JCM 10317 T". *Journal of Bioscience and Bioengineering* 104.1 (2007): 78-81.

7. Nitschke M and GM Pastore. "Production and properties of a surfactant obtained from Bacillus subtilis grown on cassava wastewater". *Bioresource Technology* 97.2 (2006): 336-341.

8. Nitschke M., *et al*. "Selection of microorganisms for biosurfactant production using agro-industrial wastes". *Brazilian Journal of Microbiology* 35.1-2 (2004): 81-85.

9. Wei YH., *et al*. "Rhamnolipid production by indigenous Pseudomonas aeruginosa J4 originating from petrochemical wastewater". *Biochemical Engineering Journal* 27.2 (2005): 146-154.

10. Healy MG., *et al*. "Microbial production of biosurfactants". *Resources, Conservation and Recycling* 18.1-4 (1996): 41-57.

11. Marchant R and IM Banat. "Biosurfactants a sustainable replacement for chemical surfactants". *Biotechnology Letters* 34.9 (2012): 1597-1605.

12. Santos DKF., *et al*. "Biosurfactants: multifunctional biomolecules of the 21st century". *International Journal of Molecular Sciences* 17.3 (2016): 401.

13. Rufino DR., *et al*. "Characterization and properties of the biosurfactant produced by Candida lipolytica UCP 0988". *Electronic Journal of Biotechnology* 17.1 (2014): 34-38.

14. Al-Bahry SN., *et al*. "Biosurfactant production by Bacillus subtilis B20 using date molasses and its possible application in enhanced oil recovery". *International Biodeterioration and Biodegradation* 81 (2013): 141-146.

15. Al-Sulaimani H., *et al*. "Microbial biotechnology for enhancing oil recovery: current developments and future prospects". *Journal of Biotechnology, Bioinformatics and Bioengineering* 1.2 (2011): 147-158.

16. Ayed HB., *et al*. "Enhancement of solubilization and biodegradation of diesel oil by biosurfactant from Bacillus amyloliquefaciens AN6". *International Biodeterioration and Biodegradation* 99 (2015): 8-14.

17. Scott MJ and MN Jones. "The biodegradation of surfactants in the environment". *Biochimica et Biophysica Acta (BBA)-Biomembranes* 1508.1-2 (2000): 235-251.

18. Mohsin I., *et al*. "Development of Bacillus subtilis Mutants for Overproduction of Protease". *Journal of Microbial and Biochemical Technology* 9.4 (2017): 174-180.

19. Youssef NH., *et al*. "Comparison of methods to detect biosurfactant production by diverse microorganisms". *Journal of Microbiological Methods* 56.3 (2004): 339-347.

20. Dadrasnia A and SB Ismail. "Bio-enrichment of waste crude oil polluted soil: amended with Bacillus 139SI and organic waste". *International Journal of Environmental Science and Development* 6.4 (2015): 241-245.

21. Ławniczak Ł., *et al*. "Contributions of biosurfactants to natural or induced bioremediation". *Applied Microbiology and Biotechnology* 97.6 (2013): 2327-2339.

22. Roy AS., *et al*. "Bioremediation potential of native hydrocarbon degrading bacterial strains in crude oil contaminated soil under microcosm study". *International Biodeterioration and Biodegradation* 94 (2014): 79-89.

23. Anandaraj B and P Thivakaran. "Isolation and production of biosurfactant producing organism from oil spilled soil". *Journal of Bioscience and Technology* 1.3 (2010): 120-126.

24. Zhu Y., *et al*. "Reuse of waste frying oil for production of rhamnolipids using Pseudomonas aeruginosa zju. u1M". *Journal of Zhejiang University-Science A* 8.9 (2007): 1514-1520.

25. Pornsunthorntawee O., *et al*. "Isolation and comparison of biosurfactants produced by Bacillus subtilis PT2 and Pseudomonas aeruginosa SP4 for microbial surfactant-enhanced oil recovery". *Biochemical Engineering Journal* 42.2 (2008): 172-179.

26. Arunkumar T., *et al*. "Application of response surface methodology (RSM)- CCD for the production of laccases using submerged fermentation". *International Journal of Pharma and Bio Sciences* 5.5 (2014): 429-438.

27. Rodrigues LR., *et al*. "Physicochemical and functional characterization of a biosurfactant produced by Lactococcus lactis 53". *Colloids and Surfaces B: Biointerfaces* 49.1 (2006): 79-86.

28. Rodrigues. "Optimization and characterization of biosurfactant production by Bacillus subtilis isolates towards microbial enhanced oil recovery applications". *Fuel* 111 (2013): 259-268.

**Volume 14 Issue 5 May 2018**