Bioconversion of crude glycerol to biosurfactant using *Pseudomonas aeruginosa* in a bioreactor under optimized conditions

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Abstract- The microbial surface active agents or biosurfactant have shown potential in various applications because of their specific surface action, very low toxicity, good emulsifying capacity and also utilized as wetting agent, spreading agent, foaming agent in detergent, food processing, cosmetics, fertilizers and widespread in various field like medical industries, petroleum and petrochemicals. One of most efficient strain for biosurfactant production is *Pseudomonas aeruginosa* and biosurfactant production may be measured on the basis of surface tension reduction of the culture broth. This study aims to the optimization of process parameters (C/N ratio, temperature and pH) for the production of biosurfactant using *Pseudomonas aeruginosa* MTCC 7814. The statistical tool viz. CCD-RSM was used for analyzing the optimum conditions for biosurfactant production. The optimum conditions were found to be a mineral salt medium with 5.5% w/v glycerol as sole carbon-source with sodium nitrate as nitrogen source (the optimum C/N ratio was 73.8.), incubation temperature of 34.5°C and pH of 6.7. A low cost substrate was generated in the laboratory as crude glycerol via transesterification of waste soybean oil and utilized as sole carbon-source for production of biosurfactant in a 4 L bioreactor under optimized conditions. The encouraging results were obtained from the bioreactor study.

Keywords – *Pseudomonas aeruginosa,* crude glycerol, optimization, biosurfactant, bioreactor

I. INTRODUCTION

Biosurfactant, microbial surface active agents are compounds which reduced surface and interfacial tension between the two liquids or one liquid one solid. Biosurfactants are also used as emulsifier and to lower down the surface tension of water. They contain amphipathic compounds consisting of hydrophilic and hydrophobic domains. Biosurfactants are gaining interest by researchers due to their utility at commercial scale. They have unique properties like biodegradability, reduced toxicity, eco-friendly compared to synthetic surfactants etc. The chemically synthesized surfactants are non-biodegradable and may cause danger for environment due to their toxicity. The great importance of the development of research in the field of biosurfactant is mainly due to its positive attribute towards the protection of environment. They have environmental applications such as hydrocarbon remediation from soil, dispersion of oil spills and enhancement of oil recovery [1, 2]. Biosurfactant create potential uses in commercial applications like in food, pharmaceuticals, biomedical industries, cosmetic industries [3-6] and enhance oil recovery [7]. It is also used as a bio control agent in agriculture applications [8]. The most studied microorganism is *Pseudomonas aeruginosa* which is capable to produce higher yield of biosurfactant. Different types of substrate utilized for the production of biosurfactant are soybean oil [9], olive oil [10] peanut oil cake [11], coconut oil [12] etc. by different strains and environmental conditions. The biosurfactant production and enhancement of growth of microorganisms are affected by many factors. The types of biosurfactant are influenced by some parameters like substrate, pH, temperature, concentration of salts and minerals, carbon to nitrogen ratio [13].

The main problem associated with the biosurfactant production is its high production cost, which make it economically unfavorable. To reduce the cost of biosurfactant production, easily available and low cost substrate can be used such as crude glycerol obtained from the biodiesel industry during transesterification reaction. The edible or non-edible oils may be converted to biodiesel and glycerol via transesterification reaction where triglyceride reacts with alcohol (methanol/ethanol) in the presence of strong acidic or basic environment. However, it is reported that the base-catalyzed transesterification of oils proceeds faster than the acid-catalyzed reaction [14].

Furthermore the optimization of process parameters like C/N ratio, temperature and pH for the biosurfactant production using well suited microorganism and low cost substrate like crude glycerol may definitely

lower down the overall production cost. The aim of present investigation was the utilization of waste material (used vegetable oil) to produce biosurfactant. The central composite design of experiments followed by response surface methodology (RSM) was applied to optimize process parameters (C/N ratio, temperature and pH). The biosurfactant production was examined under the optimized conditions in a bioreactor using crude glycerol obtained from transesterification reaction of waste vegetable oil by *P. aeruginosa* MTCC 7814.

II. MATERIALS AND METHODS

2.1 Materials

P. aeruginosa MTCC 7814 obtained from Microbial Type Culture Collection & Gene Bank, Institute of Microbial Technology, CSIR, Chandigarh, India was maintained on medium, consisted of (g/L), 1.0 Beef Extract, 2.0 Yeast Extract, 5.0 Peptone, 5.0 Sodium Chloride and 15.0 Agar at 35±2°C and sub cultured monthly. All other chemicals were of standard make.

Raw Material

Waste soybean oil samples were collected from locally available hotels and fast food shops and used as such after filtration.

Conversion of glycerol from waste soybean oil

Glycerol was produced via transesterification of waste soybean oil. The triglyceride react with an alcohol (methanol; 20%) in the presence of a strong base (KOH; 1%) resulted the conversion of waste soybean oil into biodiesel and glycerol. The reaction mixture was separated using a separating funnel. Two layers were formed; the lower layer was collected as crude glycerol.

Bioreactor

The 4L bench top bioreactor (Applikon) was used for the experiment with a total working volume of 3L. The bioreactor was equipped with pH, temperature, dissolved oxygen, antifoam monitoring and control devices. Also media was in situ sterilized in the bioreactor. Aseptic sampling was done at regular intervals (2h).

2.2 Methods

Inoculum preparation

The inoculum was developed using a loop full culture from the agar slant of *P. aeruginosa* MTCC 7814 and transferred in 2 ml liquid growth media and incubated at 35±2°C the 24h.The freshly grown cells were transferred to the 10ml liquid inoculum media in test tubes and incubated at 35±2°C. After incubation the cells were transferred to 50 ml inoculum media in 250 ml Erlenmeyer flask and incubated in a rotary shaker at 150 rpm, 35±2°C for 24h. The inoculum media was consisted of the contents as mentioned above for the maintenance of the culture except Agar.

Fermentation Media

The fermentation media 100 ml in a 500ml Erlenmeyer flask was consisted of (g/L) glycerol (as per the experimental plan), 2.0 $NaNO_3$, 0.2 $MgSO_4$, 0.3 KH_2PO_4 and the pH was maintained according to the experimental plan. All the flasks were sterilized at 121°C for 15 min in an autoclave. The flasks were incubated in a rotary shaker at 150 rpm and the temperature was maintained as per the experimental plan for 72 h. However fermentation media for bioreactor study was consisted of optimized value of C/N ratio and optimized fermentation conditions. Other components were remained the same. All the optimization experiments were conducted with pure glycerol as sole carbon source whereas, crude glycerol obtained from transesterification of waste soybean oil was used for the bioreactor experiment, kept all other media constituents same in both the cases.

Analytical methods

Estimation of glycerol

The glycerol was analyzed by American society for Testing and Materials (ASTM) standard method using UV Vis spectrophotometer colorimetric method [15].

Estimation of surface tension

Estimation of surface tension was done by using Automatic Tensiometer (Kyowa Japan, DY 500).

Estimation of Critical Micelle Concentration

Determination of CMC was done by plotting the surface tension versus concentration of biosurfactant in the solution [16].

Estimation of Emulsification index

The determination of E_{24}value was done by adding of 2ml of supernatant to the 2ml of kerosene then vortexed the mixture for 2 min and allowed to settle for 24 h. The emulsification index (E_{24}) was estimated by measuring the height of the emulsion layer ($H_{emulsion}$) and total height (H_{total}) of the liquid column. The formula used for the calculation of E_{24} is given as equation (1) [17]:

$$E_{24}(\%) = H_{emulsion}/H_{total} \times 100 \quad (1)$$

Estimation of biomass

The dry cell weight technique was used to quantify microbial growth as bacterial cell density through the absorbance at 640nm using a UV-Vis spectrophotometer.

Scheme of optimization

The experiments for optimization of process parameters for the production of biosurfactant were carried out in Erlenmeyer flasks as per the experimental plan. The experimental range and levels of independent variables i.e. C/N ratio (A), pH (B) and reaction temperature (C) are laid down in Table 1. All the experiments were carried out in triplicate and mean of three readings were taken as result for a particular experiment.

Table 1: Experimental range and levels of independent variables

Independent variables	Symbol	Range and levels				
		-α	-1	0	+1	+ α
C/N ratio, (w/w)	A	46.4	60	80	100	113.6
pH	B	6.1	6.5	7.0	7.5	7.8
Reaction temperature (°C)	C	28.3	30	32.5	35	36.7

#The value of α was calculated as 1.682 where $\alpha = 2^{k/4}$, (k=3, the number of variables)

Experimental design and RSM

The 2^3 rotatable central composite design (CCD) was adopted in order to fit a second order model and response surface methodology (RSM) was utilized to optimize the process parameters. The design consisted of 20 set of experiments. It included eight experiments for factorial portion (2k=8, where k is the number of independent variables, 3 in this case), six experiments for axial points (2k=6) and six replications of the centre point used to check the reliability of the data for lack of fit test [18]. The coded and actual units are given in Table 2. The second order model was selected for predicting the optima point and expressed as

$$Y=\beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (2)$$

where, Y represents response variable i.e. surface tension (mN/m). β_0 is offset value, β_1, β_2 and β_3 are coefficients of linear terms, β_{11}, β_{22} and β_{33} are coefficients of quadratic terms and β_{12}, β_{13} and β_{23} are coefficients of interactive terms. The effect of variables, C/N ratio (A), pH (B) and temperature (C) were studied on reduction of surface tension of fermentation broth. Regression analysis and graphical analysis were performed using Design Expert v.9.0.6.2 (Stat-Ease Inc. Minneapolis) software.

Statistical analysis

The statistical software package Design–Expert®9.0.6.2, Stat–Ease Inc., Minneapolis, MN, USA was used for experimental design and subsequent regression and graphical analysis of the experimental data. All experiments were done in triplicate, and the average surface tension value (mN/m) was taken as the response.

Table 2: Central composite design consisted of 20 experiments (coded and actual units).

Run no.	Variables*					
	Coded			Actual		
	A	B	C	A	B	C
1	-1	-1	-1	60	6.5	30
2	1	-1	-1	100	6.5	30
3	-1	1	-1	60	7.5	30
4	1	1	-1	100	7.5	30
5	-1	-1	1	60	6.5	35
6	1	-1	1	100	6.5	35
7	-1	1	1	60	7.5	35
8	1	1	1	100	7.5	35
9	-1.682	0	0	46.4	7.0	32.5
10	1.682	0	0	113.6	7.0	32.5
11	0	-1.682	0	80	6.1	32.5
12	0	1.682	0	80	7.8	32.5
13	0	0	-1.682	80	7.0	28.3
14	0	0	1.682	80	7.0	36.7
15	0	0	0	80	7.0	32.5
16	0	0	0	80	7.0	32.5
17	0	0	0	80	7.0	32.5
18	0	0	0	80	7.0	32.5
19	0	0	0	80	7.0	32.5
20	0	0	0	80	7.0	32.5

*Independent variables: C/N ratio (A), pH (B) and temperature (C)

III. RESULTS AND DISCUSSION

Optimization of biosurfactant production

Statistical modeling

According to the experimental plan, range and levels of independent variables, C/N ratio (A), pH (B) and reaction temperature (C) studied for the production of biosurfactant are shown in Table 1. The value of α was calculated as 1.682 where $\alpha = 2^{k/4}$, (k=3, the number of variables). The coded values of all independent variables and the experimental value of the only response variable Y (mN/m) along with predicted values are presented in **Fig. 1**. The coefficients were calculated by using Design Expert v.8.0. The quadratic model in terms of coded variables is given in equation (3).

$$Y=+30.65+3.68A-0.62B-0.63C-0.02AB-2.85AC-1.75BC+2.29A^2-0.60B^2-0.54C^2 \quad (3)$$

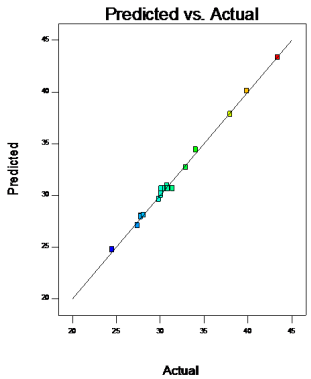

Figure 1. Presentation of experimental and predicted values of response (Y) surface tension (mN/m) using central composite design consisted of 20 experiments.

To fit the response function and experimental data, regression analysis was performed and second order model for the response (Y) was evaluated by analysis of variance (ANOVA), which is presented in Table 3. The regression for the response was statistically significant at 95% of confidence level. The Model F-value of 377.47 and low %CV value of 1.33 implies the model is highly significant. A poor F-value, 0.42of the lack of fit test (Table 3) confirming the reliability of the experimental data. A reasonable agreement was found between "Pred R-Squared" and "Adj R-Squared" values. "Adeq Precision" measures the signal to noise ratio and a ratio greater than 4 is desirable. In the present case a very high signal to noise ratio of 62.682 indicates adequate signal and chance of noise in the values is very less. The goodness of fit of the model was checked by the determination coefficient (R^2). In this case, the value of the determination coefficient (R^2=0.9954) indicates that only 0.46 % of the total variations are not explained by the model.

Table 3: Analysis of variance (ANOVA) for the response variable (surface tension).

Source	Sum of Squares	Degree of freedom DF	Mean square	F value	p-value
Model	377.47	9	41.94	238.95	0.0001*
A-C/N ratio	185.09	1	185.09	1054.51	0.0001*
B-pH	5.18	1	5.18	29.54	0.0003
C- Temperature	5.38	1	5.38	30.65	0.0002
AB	3.200E-003	1	3.200E-003	0.018	0.8953
AC	64.98	1	64.98	370.20	0.0001*
BC	24.57	1	24.57	139.98	0.0001*
A^2	75.34	1	75.34	429.22	0.0001*
B^2	5.16	1	5.16	29.42	0.0003
C^2	4.18	1	4.18	23.81	0.0006
Residual	1.76	10	0.18		
Lack of Fit	0.52	5	0.10	0.42	0.8185
Pure Error	1.24	5	0.25		
Cor Total	379.23	19			

*p< 0.05, 5% significance level.

From the ANOVA it is clear that A, B, C, AC, BC, A^2, B^2, C^2 are significant model terms. The interactive effect of the variables involved on the response variable i.e. surface tension of the broth is shown in Fig. 2, 3 and 4. The highly parabolic nature of the contours in Fig. 2 (interaction between C/N ratio and temperature) and Fig. 3 (interaction between temperature and pH value) states that the reduction of surface tension i.e. the production of biosurfactant is highly dependent on the interactive behavior on the respective parameters. However dependency of the interaction of pH and C/N ratio on the response variable was found to be very less and shown in the Fig. 4 (contours are more circular in nature).

The interaction between C/N ratio and temperature on surface tension of the broth while pH was selected as 7 is shown in Fig 2. From the figure and experimental data it can be interpreted that minimum and maximum surface tension of the broth were found to be 24.53 and 43.39 mN/m respectively by conducting fermentation experiments at pH 7. The range of variation of C/N ratio and temperature were 46.36 to 113.64 g/g and 28.3 to 36.7°C respectively.

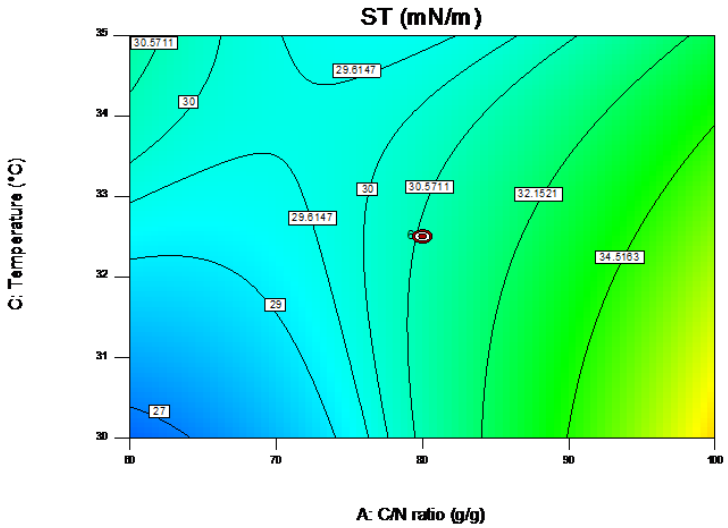

Figure 2. Effect of interaction of parameters C/N ratio and temperature on the response variable (surface tension of the broth) at pH 7.

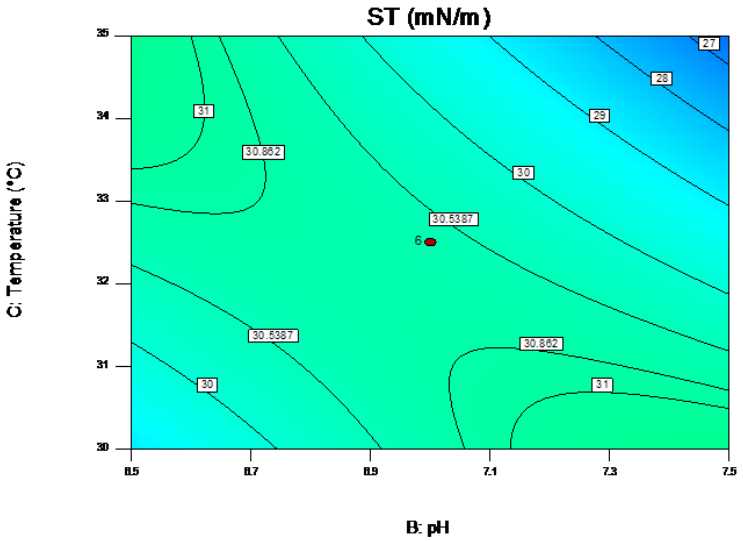

Figure 3. Effect of interaction of parameters temperature and pH on the response variable (surface tension of the broth) at C/N ratio 80 g/g.

The effect of interaction of pH and temperature on surface tension of fermentation broth is shown in **Fig. 3**, when C/N ratio was selected at 80 g/g as the center point. From the figure and experimental data it is evident that as the pH increases, the response also increases significantly i.e. surface tension decreases but it is not much dependent on the variation of temperature individually.

Interaction between C/N ratio and pH is shown in **Fig. 4**, while temperature was kept constant at 32.6°C. More circular nature of contours shown in the figure signifies that the production of biosurfactant or reduction in surface tension of the broth is least dependent on interactive behavior of these two parameters.

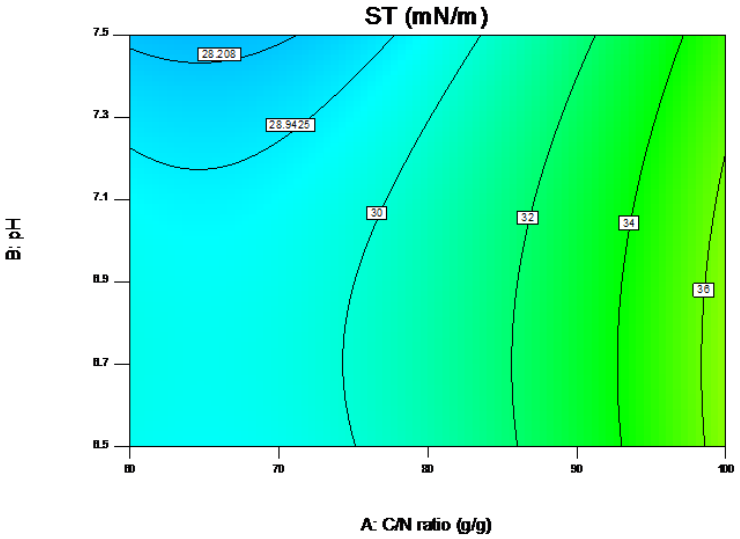

Figure 4. Effect of interaction of process variables pH and C/N ratio on the response variable (surface tension of the broth) at temperature 32.6°C.

On the basis of model, the optimum values of the parameters were calculated by setting the first order derivatives of the equation (3) (dY/dA, dY/dB and dY/dC) as zero. The optimum values of the variables A, B and C in coded and actual form thus obtained are given in **Table 4**.

Table 4: Solution for optimum conditions

Parameters	Coded value	Actual Value
C/N (g/g)	-0.31	73.8
pH	-0.64	6.7
Temperature (°C)	+0.79	34.5

Validation of model and significance of study

Three sets of experiments were conducted at the optimum conditions and the mean value of the surface tension was found to be 33.04 mN/m. The same was theoretically evaluated from equation 3 for optimum values of A, B, C and was found to be 92.1% to that of the theoretical value. The closeness of the theoretical value to that of experimental value validates the model.

Transesterification of waste soybean oil for crude glycerol production

The crude glycerol obtained via transesterification reaction of waste soybean oil (WSB). The maximum conversion of glycerol was found to be 70 wt% from the transesterification of WSB at 60°C after 40 min. Above 60°C the conversion of glycerol was found to be lower (data not shown). Also it was observed that the rate of glycerol production was increased with time. After 40 min the increase in conversion of glycerol was negligible (data not shown). The glycerol thus produced by transesterification reaction was used for the production of biosurfactant under optimized conditions.

Biosurfactant production in bioreactor under optimized conditions

Bioreactor study was carried out in a 4 L Applikon bioreactor with 3 L working volume under the optimized conditions as expansion of the flask level experiments. The crude glycerol obtained via transesterification reaction of waste soybean oil (WSB) was used as sole carbon source. The fast growing *Pseudomonas aeruginosa* MTCC 7814 cells were used to produce biosurfactant via fermentation. The glycerol concentration, surface tension and biomass concentration were measured at regular interval. Also CMC and E_{24} values were calculated.

After 64 h of fermentation, biomass concentration was found to be maximum, 3.168 g/L and it was observe that the stationary phase was ended at this point. The residual glycerol concentration was found to be 4.247 g/L after 64 h fermentation time, after that insignificant utilization of glycerol was observed. The surface tension, critical mass concentration and emulsifying index of the fermented broth were found to be 27.65 mN/m, 19.60 mg/l and 76% respectively, after 64 h fermentation time.

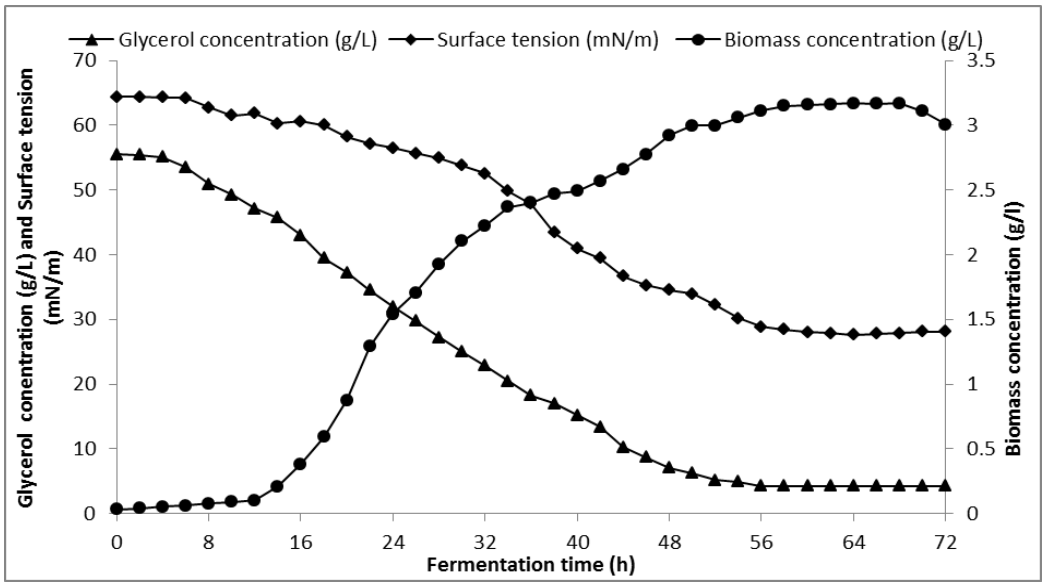

Figure 5. Fermentation profile for the bioconversion of crude glycerol to biosurfactant in a bioreactor using *Pseudomonas aeruginosa* MTCC 7814, under optimized conditions.

Discussion

The economically viable fermentation process for biosurfactant production mainly depends on utilization of low cost substrate along with the development of cheaper overall process [19]. In the present investigation emphasis was given on the production of biosurfactant by utilization of low cost or waste materials as inexpensive carbon-source for the production of biosurfactant. The main factors affecting the biosurfactant production were found to be C/N ratio, temperature and pH. Some Carbon sources like waste frying oil [20], waste coconut oil [21] and vegetable oils[21] were found as reasonably good substrates for biosurfactant production by *Pseudomonas* and *Serratia marcescens* species. A significant cost effective alternative of carbon-source for biosurfactant production is glycerol obtained by transesterification of low cost substrate like waste vegetable oils. The C/N ratio was found to be a major factor which influenced the biosurfactant production [22-24] and its ratio can be varied according to strain and carbon-source [25]. It is reported that the values of pH and temperature varied from 4to8 and 25 to 30°C respectively. *Y. lipolytica* was used for biosurfactant production and studied the influence of initial pH and the effect of temperature [26-27]. It was observed from various studies that the production of sophorolipid biosurfactant reached maximum at 27°C and mannosyleryl production at 25°C [28]. In the present investigation the optimum results achieved at 73.8 g/g, 6.7 and 34.5°C for C/N ratio, pH and temperature respectively by *P. aeruginosa* MTCC7814 which was in good agreement with the existing research works.

In the transesterification of vegetable oils, a triglyceride reacts with an alcohol in the presence of a strong acid or base, producing a mixture of fatty acids alkyl esters (biodiesel) and glycerol. The crude glycerol obtained from transesterification of waste sunflower oil was investigated [29]. Some investigators worked on transesterification of different seed oils [30]. The process was conducted at 50°C, 240 rpm for 60 min and 60-70 wt % crude glycerol was reported depending on the types of seed oils. In the present investigation the maximum conversion of glycerol was found to be 70 wt% from the transesterification of waste soybean oil at 60°C after 40 min [31].

The biosurfactant used to reduce the surface tension of water. The low CMC value defines that the less amount of biosurfactant is required to reduce the surface tension to a certain level. The reported value of CMC in the literature ranged from 10 to 234 mg/l and surface tension from 25 to 31mN/m [32-34]. In the present investigation

the CMC and surface tension of the biosurfactant by *P. aeruginosa* MTCC 7814 after 64 h of fermentation time using bioreactor under optimized conditions were 19.60 mg/l and 27.65mN/m respectively. The capacity of biosurfactant was determined by emulsifying index via formation of emulsion on different hydrophobic substrate. The high value of emulsifying index was reported in the literature [35]. Some hydrocarbon showed poor emulsification might be due to the insufficient oxygen supply in the flask during culture growth or possible inhibitory effect on bacterial metabolism due to nutrient transport deficiency. The emulsifying index of other substrate used as a carbon sources like sunflower frying oil, crude oil and glucose were 63%, 66.6%, 46% respectively [36-38]. The comparable result of emulsifying index was obtained as 76% in the present investigation using crude glycerol as sole carbon-source by *Pseudomonas aeruginosa*.

IV. CONCLUSION

P. aeruginosa MTCC 7814 capable of producing biosurfactant from crude glycerol used as sole carbon source. Crude glycerol, a low cost substrate obtained from transesterification of waste vegetable oils. The crude glycerol is economically beneficial and inexpensive waste material generated from biodiesel industry. The biosurfactant production achieved under optimized conditions using controlled environment of bioreactor showed better and enhanced values of output parameters like surface tension, critical mass concentration and emulsifying index of the fermented broth. The surface tension of broth was reduced from 71 to 27.65 mN/m within 64 h of fermentation time along with 19.60 mg/l CMC and 76% E_{24} values. The results are encouraging and the data may be used for further scale-up process.

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