Acclimatization of strain in high hydrocarbon condition and Optimization of media composition and other factors using one-factor-at-a-time strategy and biostatistical analysis for biosurfactant production

Section A: Acclimatization of microbial strain at high hydrocarbon condition and its Optimization using one-factor-at-a-time strategy for biosurfactant production.

3.1. Introduction

Biosurfactants are surface-active metabolites produced by microorganisms when grown on water miscible or oily substrates. They either remain adherent to Microbial cell surfaces or are secreted in the culture broth. They possess the characteristic property of reducing the surface and interfacial tensions using the same mechanisms as chemical surfactants (Gharaei-Fathabad 2011).

For bacteria growing on hydrocarbons, the growth rate can be limited by the interfacial surface area between water and oil (Sekelsky et al. 1999). When the surface area becomes limiting, biomass increases arithmetically rather than exponentially. Stated briefly, emulsification is a cell-density-dependent phenomenon: that is, greater the number of cells, higher the concentration of extracellular product. The concentration of cells in an open system, such as an oil-polluted body of water, never reaches a high enough value to effectively emulsify oil. Furthermore, any emulsified oil would disperse in the water and not be more available to the emulsifier-producing strain than to competing microorganisms (Ron et al. 2002).

In number of studies it was found that the genetically modified microorganisms will survive less in environment condition, like when they were introduce into the natural

condition of soil, because expression of inserted gene require extra energy that could reduce their environmental fitness (Viebahn et al. 2009). Survival chances could become more lesser of genetically modified microorganisms when they were introduce under stress environmental condition such as crude oil contaminated soil. To overcome this problem one of the best approach is to adapt or acclimatize the microbial strain under stress environmental (hydrocarbon) condition for the production of biosurfactant. It has been reported that yeast shows higher production of biosurfactant than bacteria this is due to presence of a rigid cell wall. In prokaryotic cells, the membrane may be damaged at high concentrations of biosurfactant. Among yeasts, *Candida sp*. has been widely used for Biosurfactant production when grown on water-immiscible substrates (Kim et al. 1999; Asfora Sarubbo et al. 2006).

Biosurfactants are microbial amphiphilic polymers and polyphilic polymers that tend to interact with the phase boundary between two phases in a heterogeneous system, defined as the interface. For all interfacial systems, it is known that organic molecules from the aqueous phase tend to immobilize at the solid interface. There they eventually form a film known as a conditioning film, which will change the properties (wettability and surface energy) of the original surface (Banat et al. 2000) in an analogy to organic conditioning films, biosurfactants may interact with the interfaces and affect the adhesion and detachment of microorganism (Muthusamy et al. 2008).

Originally, biosurfactants attracted attention as hydrocarbon dissolution agents in the late 1960s, and their applications have been greatly extended in the past five decades as an improved alternative to chemical surfactants (carboxylates, sulphonates and sulphate

acid esters), especially in food, pharmaceutical and oil industry (Neu et al. 1990; Neu 1996).

In this chapter, Acclimatization of *C. tropicalis* MTCC230 under high hydrocarbon (Kerosene, Petrol, Mustard oil) as carbon source along with glucose for the production of biosurfactant. After acclimatization, different nitrogen source (NH_4Cl, $NaNO_3$, NH_4NO_3), effect of microelements for the production of biosurfactants was studied and determine the emulsification index from different hydrocarbon source.

3.2.Material and Methods

Media and Culture Conditions

The strain *Candida tropicalis* MTCC 230 was used in this study. *Candida tropicalis* MTCC 230 was purchased from the Institute of Microbial Technology (IMTECH) Chandigarh, India. The culture was maintained in a medium with the following composition (g/l): malt extract (3), yeast extract (3), peptone (5), glucose (10), agar 20 g and distilled water 1 liter at pH 6.2 (Accorsini et al. 2012; Khopade et al. 2012). Culture medium component such as malt extract, peptone and yeast extract were purchased from Hi-media, Mumbai, India and glucose from Merck, India.

The production of the biosurfactant was carried out in submerged state of fermentation with media containing the following (g/l): NH_4Cl (2), Na_2HPO_4 (2.2), KH_2PO_4 (0.14), $MgSO_4.7H_2O$ (0.6), $FeSO_4.7H_2O$(0.02), NaCl (0.01), yeast extract (0.02), $CaCl_2$ (0.04), trace elements (g/l) ($ZnSO_4.7H_2O$ 1.7, $MnSO_4.4H_2O$ 0.85, H_3BO_3 0.56, $CuSO_4.5H_2O$ 0.8, $Na_2MoO_4.2H_2O$ 0.16, $CoCl_2.6H_2O$ 0.45, EDTA 1.0, $NiCl_2.6H_2O$ 0.005 and KI 0.6) 0.1 ml/L, carbon source (glucose & hydrocarbon) 2% was used in

fermentation, carried out at 34ºC temperature in a rotary shaker with 200 rpm at pH 6.2 for 72 h (Kuyukina et al. 2001; Khopade et al. 2012). Different concentrations of hydrocarbons (petrol, kerosene oil and mustard oil) and glucose were used as the 2 % carbon source.

Acclimatization of Candida tropicalis* MTCC 230 *under high hydrocarbon condition

C. tropicalis MTCC230 was serially acclimatized from low to high concentrations of hydrocarbons, in this, the lower hydrocarbon concentration acted as a seed culture for the higher hydrocarbon concentration. There is a parallel relationship between the hydrocarbon utilization, cell growth, and biosurfactant production. We are using total 2% carbon source, in 100ml media firstly 1.75% glucose + 0.25% hydrocarbons (kerosene, petrol, mustard oil) were used then slowly increase the concentration hydrocarbon and decrease the concentration of glucose as per, accordingly 1.5% glucose + 0.5% hydrocarbons (kerosene, petrol, mustard oil) then 0.75% glucose + 0.75% hydrocarbons (kerosene, petrol, mustard oil) then 0.5% glucose + 1.5% hydrocarbons (kerosene, petrol, mustard oil). Acclimatization of *Candida tropicalis* MTCC 230 was carried out under high hydrocarbon condition.

Emulsification Activity

The production of biosurfactant was estimated in terms of the Emulsification index value i.e., $\%E_{24}$ at different time intervals. The $\%E_{24}$ is the height of the emulsion layer, divided by the total height of the liquid, multiplied by 100 as shown in Eq. 1 (Saikia et al. 2012; Donio et al. 2013). Emulsification index (E_{24}) was measured after 24 h.

$$\%E_{24} = \frac{\text{Height of emulsified layer (cm)}}{\text{Height of total liquid (cm)}} \times 100 \qquad \text{……..(1)}$$

Recovery

The microbial cells were harvested from the culture broth by centrifugation at 12,000g for 20 min. The supernatant was acidified with 6N hydrochloric acid solute on to pH 2.0. The precipitated biosurfactant was allowed to settle at 4ºC overnight. The precipitated biosurfactant was collected by centrifugation at 12,000g for 20 min and the acidified biosurfactant was neutralized using 1N NaOH (pH 7) solution. After the acid precipitation solvent extraction was done for obtaining the crude biosurfactant, in solvent extraction an equal volume of ethyl acetate was added into the supernatant and the organic phase was dried on a rotary vacuum evaporator (Bao et al. 2014; Balan et al. 2017).

3.3. Result and Discussion

Biosurfactants have wide applications in different fields, but its production is limited, due to inability of microorganisms to degrade the hydrocarbon. In this study three hydrocarbon Kerosene, Petrol and mustard oil is used as carbon source along with glucose. When hydrocarbon used as sole carbon source for *Candida tropicalis* MTCC 230 no growth was observed but when we use hydrocarbon along with glucose, showing diauxic growth, so we tried to increase hydrocarbon as per decreasing the glucose simultaneously. Initially hydrocarbon concentration is taken less and then concentration of hydrocarbon is slowly step wise increase with decrease glucose concentration and determined their effect on growth curve of *Candida tropicalis* MTCC 230. Fermentation was carried out at 34°C in a rotary shaker with agitation speed of 200 rpm for 72hrs.

Growth curve of Candida tropicalis under different concentration of hydrocarbon along with glucose

For petrol oil : When petrol oil used along with glucose at different concentration as shown in Figure 3.1 Maximum exponential phase and stationary phase was achieved at 0.5% glucose + 1.5% petrol. When further, we increase in petrol percentage no cell growth was obtained in diauxic growth.

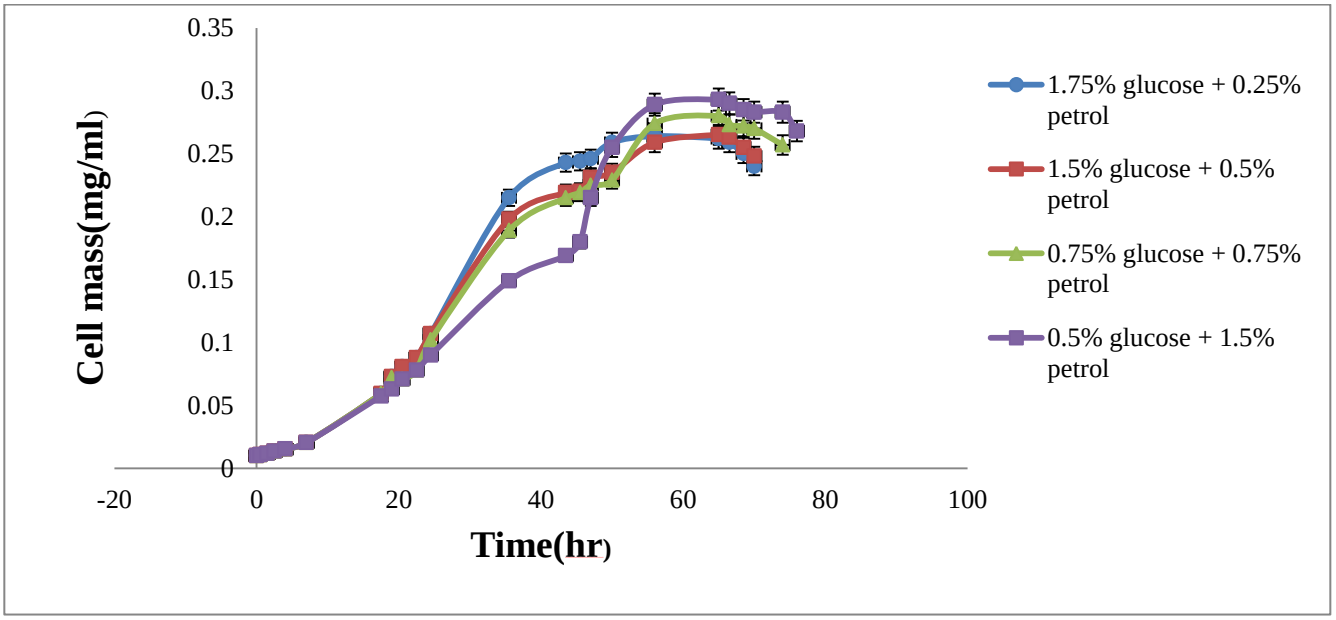

Figure 3.1. Growth curves of *Candida tropicalis MTCC230* under different concentration of glucose and petrol

kerosene oil: When kerosene oil used along with glucose at different concentration as shown in Figure 3.2. Exponential phase and stationary phase was very less achieved at 0.5% glucose + 1.5% kerosene oil as compare to petrol oil. Further increase of kerosene oil will lead to low growth of *C. tropicalis* MTCC230 that was unsatisfactory.

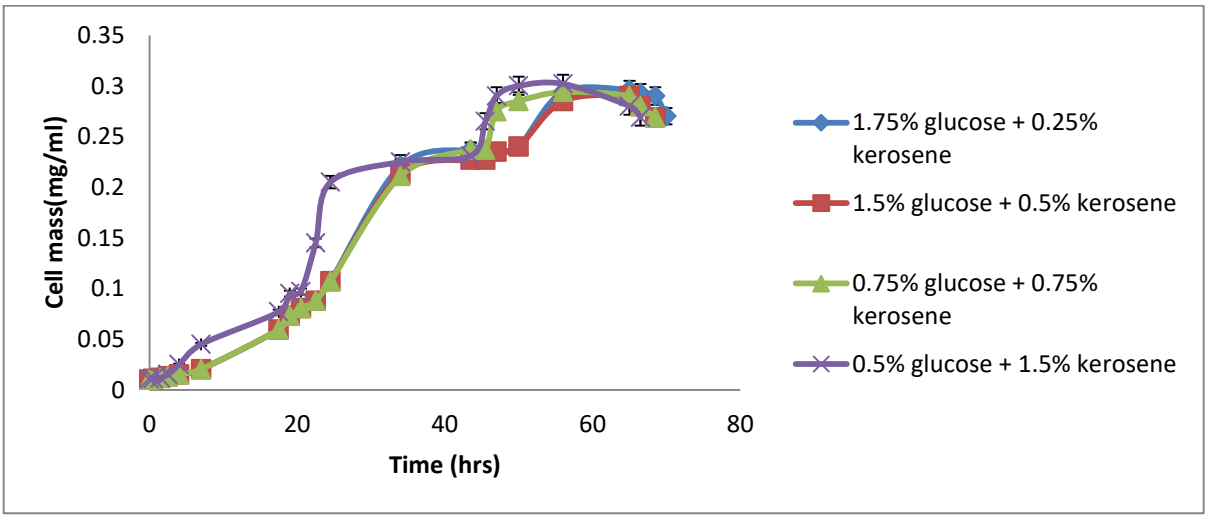

Figure 3.2. Growth curves of *Candida tropicalis MTCC230* under different concentration of glucose and kerosene oil.

Kitchen waste oil (mustard oil): In case of kitchen waste oil (mustard oil) when used along with glucose at different concentration as shown in Figure 3.3. Exponential phase and stationary phase was very less achieved at different concentration of kitchen waste oil (mustard oil). When we use above the concentration of 0.75% glucose + 0.75% Kitchen waste oil (mustard oil) the growth was not obtained in case of *C. tropicalis MTCC230.*

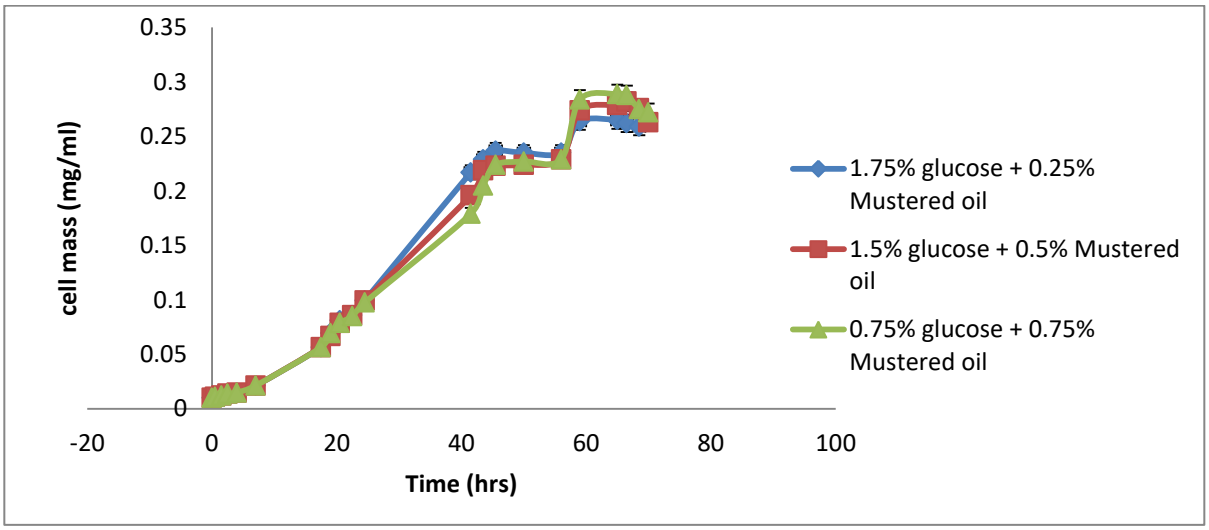

Figure 3.3. Growth curves of *Candida tropicalis* MTCC230 under different concentration of glucose and Kitchen waste oil (mustard oil).

Among all oils (petrol, kerosene, mustard oil) *Candida tropicalis* MTCC230 gets best acclimatized in petrol and showing maximum exponential phase and stationary phase that was achieved at 0.5% glucose + 1.5% petrol.

Emulsification index of biosurfactant produced by different hydrocarbon source

The emulsification index of biosurfactant was calculated that was produced by different hydrocarbon sources (petrol, kerosene oil, mustard oil). Highest emulsification index was obtained when petrol was used as a carbon source as compare to kerosene and mustard oil (Figure 3.4 & 3.5). The Emulsification index shows the Emulsifying activity of biosurfactant, higher the Emulsification indexes higher the biosurfactant production. This would help to select the best hydrocarbon and was used as a carbon source for the production of biosurfactant. Figure 3.4 & 3.5, shows that the highest emulsification index

was shown by biosurfactant produce from acclimatized *Candida tropicalis MTCC230* in high petrol used as a carbon source along with glucose.

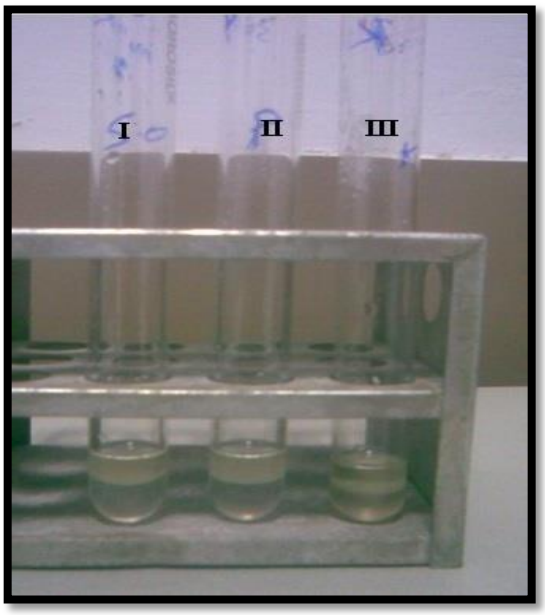

Figure 3.4. Emulsification activity when different hydrocarbon used as carbon source for biosurfactant production (I- Kitchen waste oil, II-petrol, III-kerosene oil).

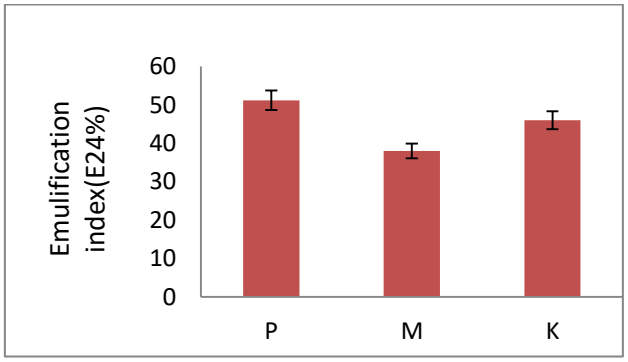

Figure 3.5. Emulsification index (E24%) shown by different hydrocarbons sources (P-Petrol, M-Mastured oil, K-Kerosene oil).

Optimization of pH

Six production media were prepared at different pH 6, 6.5, 7, 7.5, 8, and 8.5 respectively. 0.75% glucose + 1.25% hydrocarbons (petrol oil) used as a carbon source and submerged state fermentation was carried out for 72 hrs. After 72hrs, absorbance was taken for each pH at 600nm to estimate the maximum cell mass. At 6.8 pH maximum cell mass was obtained as shown in Figure 3.6. This reveals that 6.8 was the optimum pH for acclimatized *C. tropicalis* MTCC230 growth.

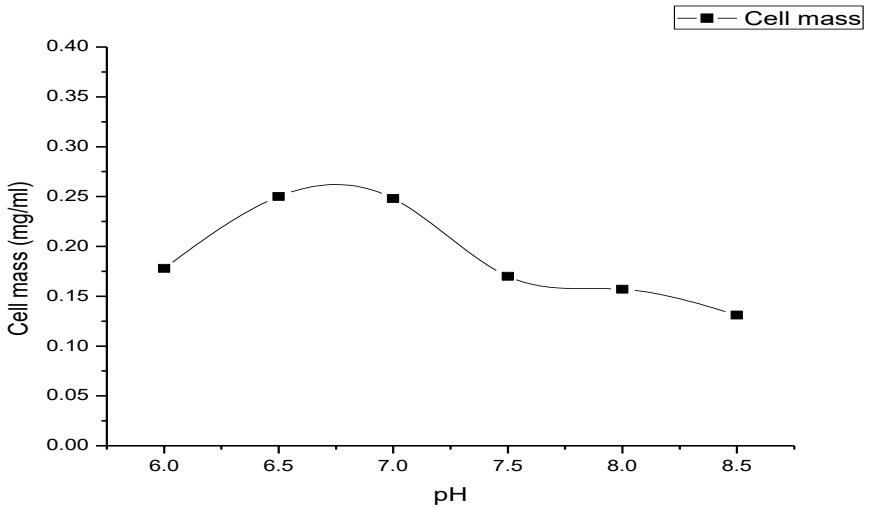

Figure 3.6. Optimization of pH

Optimization of Temperature

Six production media are prepared at different temperature 30ºC, 32ºC, 34ºC, 36ºC, 38ºC, and 40ºC respectively. 0.75% glucose + 1.25% hydrocarbons (petrol oil) used as a carbon source and submerged state fermentation was carried out for 72 hrs. After 72hrs, absorbance was taken for each temperature at 600nm to estimate the maximum cell mass. At 34^{o}C maximum cell mass was obtained as shown in Figure 3.7. This shows that at 34^{o}C was the optimum temperature for acclimatized *C. tropicalis* MTCC230 growth.

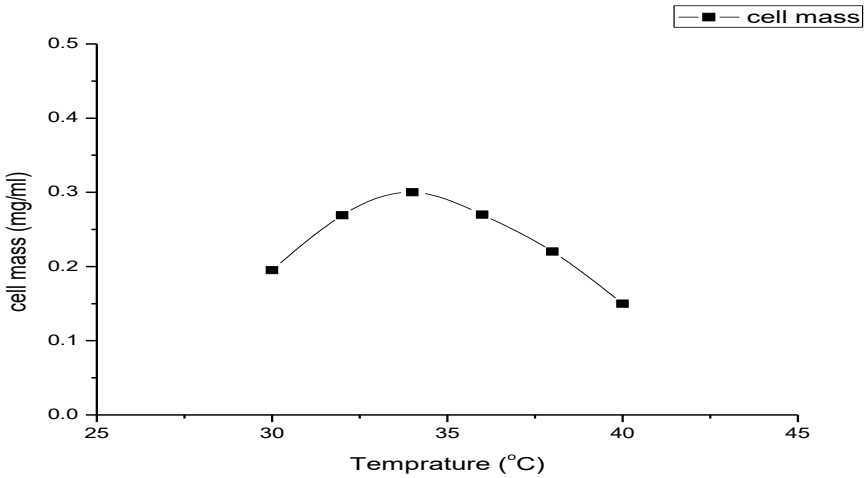

Figure 3.7. Optimization of temperature

Effects of nitrogen sources on biosurfactant production

Candida tropicalis MTCC230 is able to use nitrogen sources such as ammonia or nitrate. However, in order to obtain high concentrations of biosurfactant it is necessary to have restrained conditions of this macro-nutrient. Sodium nitrate and ammonium nitrate were two best sources of nitrogen for microbial growth and biosurfactant synthesis (Abdel-Mawgoud et al. 2008; Meyer 2010).

In this study different nitrogen sources such as ammonium chloride, ammonium nitrate and sodium nitrate were used for biosurfactant production from an acclimatized *Candida tropicalis MTCC230*. Biosurfactant production was calculated in terms of the Emulsification index (% E_{24}) in case of using ammonium chloride, ammonium nitrate and sodium nitrate (Table 3.1). Ammonium chloride shows the highest Emulsification index (% E_{24}) then ammonium nitrate and sodium nitrate. So, this result revealed that the ammonium chloride is the best nitrogen source for biosurfactant production from an acclimatized *Candida tropicalis MTCC230*. Fermentation was carried out at 34°C in a rotary shaker with

agitation speed of 200 rpm for 72hrs. All the emulsification indexes were performed in triplicate.

Table 3.1. Effect of different nitrogen source on emulsification index

Nitrogen source	**Emulsification index (E24%)**
NH_4Cl	51
$NaNO_3$	48
NH_4NO_3	42

Effects of Micro and Trace elements on the Biosurfactant Production

Micro and Trace elements in the culture medium plays an important role in the biosurfactant production and microbial growth. Iron, manganese, and magnesium are co-factors of enzymes involved in the synthesis of surfactin (Biourfactant) (Gudiña et al. 2015). In this study the effect of multiple metal cations used on biosurfactant production was examined. When they were used individually and all together added into the medium their effects on both microbial growth and biosurfactant yield were examined (Wei et al. 2007).

When no supplements (Micro and Trace elements) were present in the production media the minimum emulsification index (E_{24}%) was observed. When Iron and magnesium used individually in production media, emulsification index (E_{24}%) was good but when all the metal cations and trace elements were used all together in the production media the highest emulsification index (E_{24}%) was obtained as shown in Table 3.2. This reveals that

the role of micro and trace elements play a major role for biosurfactant production from an acclimatized *C. tropicalis* MTCC230.

Table 3.2. Effect of Microelements on biomass and emulsification index

Additive	**Biomasses (g/l)**	**Emulsification index (E_{24}%)**
No supplements	1.05	24
$MgSO_4$	1.56	36
$CaCl_2$	1.41	34
$FeSO_4$.7H20	1.59	40
Trace Elements	1.61	31
All Metals	2.70	51

3.4. Conclusion

Candida tropicalis MTCC230 get acclimatized under high concentration of hydrocarbons, for petrol (0.5%glucose+1.5%petrol) for kerosene oil (0.5%glucose+1.5% kerosene) and for mustard oil (0.75% glucose+0.75% mustard oil). Best microbial growth profile was obtained when 0.5% glucose + 1.5% petrol oil was used as carbon for *Candida tropicalis* MTCC230 and also showing highest emulsification index. Estimation of emulsification index is the direct method to determine the biosurfactant production when immiscible substrates were used as a carbon source. Hydrocarbons tested served as

substrates for emulsification by the biosurfactant, petrol oil was the best substrate while waste kitchen oil (mustard oil) was the poorest. The optimum pH and temperature was 6.8 and 34^0C respectively, for highest cell growth under acclimatized hydrocarbon condition.

Ammonium chloride, ammonium nitrate and sodium nitrate were used as a nitrogen source for biosurfactant production from an acclimatized *Candida tropicalis MTCC230*, among these nitrogen sources ammonium chloride shows the highest Emulsification index (% E_{24}) this reveals that ammonium chloride served as a best nitrogen source for an acclimatized *Candida tropicalis* MTCC230 to produce emulsifier for solubilization of an immiscible carbon source. Micro and trace elements play a major role for biosurfactant production from an acclimatized *C. tropicalis* MTCC230. When they were used all together in the production media the highest microbial growth and emulsification index (E_{24}%) was obtained. Among different carbon and nitrogen sources, ammonium chloride and petrol were found to give maximum emulsifying activity. The highest experimental emulsifying index after optimizing the parameters (carbon source, nitrogen source, temperature, pH and microelements) was found to be 54%. The optimum values of these parameters were selected for further experiment.

Section B: Optimization of media composition and other factors for biosurfactant production using biostatistical analysis (Response Surface Methodology).

3.5. Introduction

Biosurfactant have wide application in chemical, food, medical, cosmetic, agriculture and pharmaceutical industries, In-spite of these applications they cannot compete with the chemical surfactants due to their high production costs and the low yield during large scale production, this problem may be overcome by process optimization.

Process optimization is a tedious process due to involvement of multivariable process parameters. One of the classical and old methods of media optimization is by changing one variable at a time keeping others at a fixed level but this method is a laborious, time and cost consuming method and also the chances of error are high as it is manually optimized. To overcome this problem, Statistical optimization is widely used for media optimization. One of the technique, Response Surface Methodology (RSM) which is used to explain the combined effects of all the factors in a fermentation process. It is a collection of experimental strategies, mathematical methods and statistical inference (Elibol, 2004; Liu et al. 1998; Tanyildizi et al. 2005). Response surface methodology (RSM) is the most relevant multifactorial techniques used in analytical optimization; it has been used extensively in media optimization due to the decreases in the number of experiments and the cost of production (Abbasi et al., 2013; Sen, 1997).

Our objective in this chapter was to optimize the production of biosurfactant by using biostatistically based experimental design, this technique is not only less time consuming but also shows the interactive effect of different parameters. To the best of our knowledge, there have been no reports on the application of statistical methods for the

optimization of biosurfactant production when *Candida tropicalis* MTCC230 is grown on two carbon sources of which one is water immiscible and another one is water-miscible. With the help of this study we can enhance the production of biosurfactant by achieving the acclimatized strain of *Candida tropicalis* MTCC230 in high hydrocarbon concentration media.

3.6. Materials and Methods

Selection of the Optimum variables

The classical methods of media optimization is by changing one variable at a time keeping others at a fixed level as it is manually optimization. Best microbial growth profile was obtained when 0.5% glucose+1.5% petrol oil was used as carbon for *Candida tropicalis* MTCC230 and also showing highest emulsification index. The optimum pH and temperature was 6.8 and 34^0C respectively, for highest cell growth under acclimatized hydrocarbon condition. Ammonium chloride served as a best nitrogen source for an acclimatized *Candida tropicalis MTCC230* to produce emulsifier for solubilization of immiscible carbon source. Micro and trace elements play a major role for biosurfactant production from an acclimatized *C. tropicalis* MTCC230. When they were used all together in the production media the highest microbial growth and emulsification index (E_{24}%) was obtained.

The highest experimental emulsifying index after optimizing the parameters (carbon source, nitrogen source, temperature, pH and microelements) was found to be 54%. The optimum values of the parameters were selected for further experiments. So, from these results six variables were obtained (Hydrocarbon, glucose, pH, temperature, Ammonium

chloride, Micro elements) and these were used for further response surface methodology (RSM) technique for process optimization.

Response Surface Methods

Response surface methodology (RSM) was developed by Box and collaborators in the 50s (Gilmour, 2006). It is a collection of mathematical and statistical techniques based on the fit of a polynomial equation to the experimental data. Response surface methodology (RSM) was applied in two stages, first to identify the significant factors for the production of biosurfactant using Plackett–Burman design criteria and later the significant nutrients resulted from Plackett–Burman design were optimized by using a central composite design. The main goal of response surface is to efficiently hunt for the optimum values of the variables such that the response is maximized. *Factors or independent variables* are experimental variables that can be changed independently of each other. Typical independent variables comprise the pH, temperature, reagents concentration, flow rate, and elution strength etc. (Bezerra et al. 2008). In this study six variables namely hydrocarbon, glucose, ammonium chloride, micro elements, pH and temperature were used.

Plackett–Burman Design

Plackett-Burman design (PBD) is a classical method used for screening large variables. It is a small-sized two-level factorial experimental design programmed to identify critical physicochemical parameters from N number of variables in $N+1$experiments. It gives information only on the effects of single factors, but not on interactions effects between and among the variables (Ekpenyong et al. 2017). The main

effect was calculated as the difference between the average of measurements made at high level (+1) and low level (-1) (Rajendran et al. 2007). Each variable was tested at two levels namely a high level denoted by (+1) and a low level denoted by (-1) as listed in Table 3.3. Six variables were screened by conducting twelve experiments and the experimental design. Table 3.3 show the factors and their levels used in the experimental design and Table 3.4 shows the detail of the design, through the interactive process.

Table 3.3. Six Medium Factors and Their Levels used in Plackett-Burman Design

Variable code	Variables	Low level -1	Low level +1
X1	Hydrocarbon	60%	80%
X2	Glucose	20%	40%
X3	Ammonium Chloride	1.5(g/l)	2.5(g/l)
X4	Micro Element	1.5(g/l)	2.5(g/l)
X5	Temperature	30(g/l)	36(g/l)
X6	pH	6	8

Table 3.4. Matrix of Plackett-Burmam Design and results of evolution of factors affecting Biosurfactant production by *Candida tropicalis* MTCC 230

Run No.	X1	X2	X3	X4	X5	X6	E_{24} Activity	Predicted value
1.	80	40	1.5	2.5	30	8	48	48.1667
2.	80	40	1.5	2.5	30	6	46	45.8333
3.	80	40	2.5	1.5	36	8	53	52.8333
4.	80	20	1.5	1.5	36	8	59	58.1667
5.	80	20	2.5	1.5	30	6	43	38.8333
6.	80	20	2.5	2.5	36	6	47	44.1667
7.	60	20	1.5	1.5	30	6	40	36.1667
8.	60	20	1.5	2.5	36	8	43	43.8333
9.	60	20	2.5	2.5	30	8	24	26.8333
10.	60	40	1.5	1.5	36	6	44	47.8333
11.	60	40	2.5	1.5	30	8	36	33.1667
12.	60	40	2.5	2.5	36	6	35	36.1667

Central Composite Designs

Central composite design (CCD) has been widely used statistical method based on the multivariate nonlinear model for the optimization of process variables and also used to determine the regression model equations and operating conditions from the appropriate experiments. It is also useful in studying the interactions of the various parameters affecting the process (Sadhukhan et al. 2016). Central composite design (CCD) was applied to determine the optimum concentration of the four most significant variables screened from Plackett–Burman design criteria. Four independent variables namely hydrocarbon (X1), ammonium chloride (X2), micro elements (X3) and temperature (X4) each independent variable had 3 levels which were –1, 0, and +1. A total 31 different combinations were chosen in random order according to a CCD configuration for four variables. The variables of the experiments were coded according to the following equation:

$$\text{Coded value} = \frac{\text{Actual Level - (Higher level + lower level) /2}}{\text{(Higher level + lower level) /2}}$$

The variables and their levels selected for the biosurfactant production were: hydrocarbon (60 - 100 %), ammonium chloride (0.5 - 2.5 g/l), microelements (0.5 - 2.5 g/l), and temperature (31.5 - 37.5ºC). The data obtained were fitted to a second-order polynomial equation:

$$Y = \beta_o + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij}\, x_i\, x_j \ldots..$$

Where, Y is the predicted response; β_o is the offset term; β_i is the linear effect; β_{ii} is the square effect; and β_{ij} is the interaction effect. X_i is *i*th independent variable. The second order polynomial coefficients were calculated using the Minitab software version 15.

The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). This analysis included Fisher's F-test (overall model significance), its associated probability p(F), correlation coefficient R, determination coefficient R^2 which measures the goodness of fit of regression model. It also includes the Student's t-value for the estimated coefficients and the associated probabilities p(t). For each variable, the quadratic models were represented as contour plots (2D). The optimal combination was determined from the contour plots.

Using the CCD method, a total of 32 experiments with various combinations of hydrocarbon, ammonium chloride, microelements and temperature were conducted. Table 3.5 shows the levels of the variables and design matrix to investigate this work.

Table 3.5. Selected parameter range for biosurfactant production

Variable code	Variables	-2	-1	0	+1	+2
X1	Hydrocarbon	60	70	80	90	100
X3	Ammonium Chloride	0.5	1.0	1.5	2.0	2.5
X4	Micro Element	0.5	1.0	1.5	2.0	2.5
X5	Temperature	31.5	33.0	34.5	36	37.5

3.7. Results and Discussion

Plackett–Burman Design (PBD) for Screening Important Medium Factors for Biosurfactant Production

Plackett–Burman design was used to identify the effect of six medium components: hydrocarbon, glucose, ammonium chloride, micro elements, temperature, and pH for biosurfactant production. The effects of these components are shown in Table 3.6.

Table 3.6. Analysis of Plackett-Burman Design on optimization of culture medium in shake flask culture

Term	Effect	Coef	SE Coef	T value	P
Constant	-	43.167	0.7226	59.73	0.000
X1	12.333	6.167	0.7226	8.53	0.000
X2	1.000	0.500	0.7226	0.69	0.520
X3	-7.000	-3.500	0.7226	-4.84	0.005
X4	-5.333	-2.667	0.7226	-3.69	0.014
X5	1.333	0.667	0.7226	0.92	0.399
X6	7.333	3.667	0.7226	5.07	0.004

S = 2.50333 PRESS = 180.48
R-Sq = 96.48% R-Sq (pred) = 79.71% R-Sq (adj) = 92.25%

The effects of hydrocarbon, ammonium chloride, microelements and temperature were +12.333, -7.000, -5.333 and 7.333, respectively, and all have confidence levels 96.48 %. Hence, they were considered as the most significant factors that affect the biosurfactant production.

Other had effects of lower confidence levels (P>0.05) and were considered insignificant. Significant and non-significant factors are shown in Pareto chart Figure 3.8, the largest effects (most important factors) are presented in the upper portion and then progress down to the smallest effects (least important factors). Hydrocarbon, ammonium chloride, microelements and temperature considered as a significant factor whereas pH and glucose are non-significant.

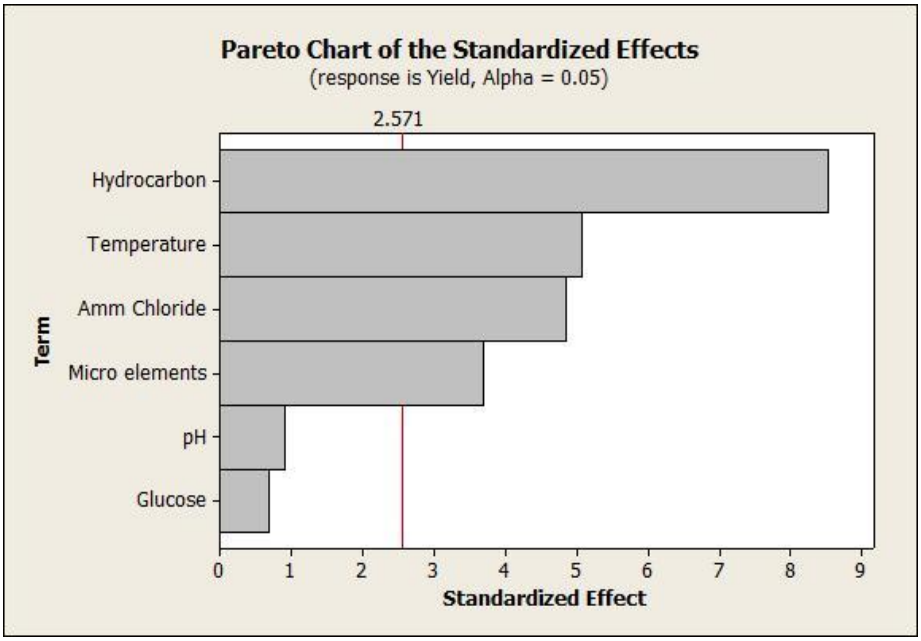

Figure 3.8. Pareto chart of six-factor effects on Biosurfactant production. Upper four factors beyond the red line shows the significant effect on production (P<0.05) and the factors below the red line shows the non-significant factor (P>0.05).

Central Composite Design (CCD) and Response Method

Thirty-one experiments were carried out according to the CCD as shown in Table 3.7 were explained by the following second-order polynomial equation (Shukla et al. 2013). Response results shown in Table 3.6 were analyzed using Minitab 15.0 software.

The t-test and P values were used to identify the effect of each factor on biosurfactant production as shown in Table 3.8. Hydrocarbon, ammonium chloride, microelements and temperature, interaction of the four selected variables had a significant effect on biosurfactant yield (P>0.05), as well as quadratic terms of hydrocarbon, ammonium chloride, microelements and temperature. The fit of the model was checked by the coefficient of determination R^2. R^2 was calculated to be 99.96 %. The application of multiple regression analysis methods yielded the following regression Eq. 2 to the experimental data.

$Y(E_{24}) = -4091.73 + 14.80X1 + 149.31X2 + 127.4X3 + 190.62X4 - 0.10X1X1 - 48.11X2X2 - 39.81X3X3 - 2.73X4X4 - 0.00X1X2 + 0.01X1X3 + 0.02X1X4 - 1.45X2X3 + 0.28X2X4 + 0.22X3X4.$ …… (eq.2)

Table 3.7. CCD matrix employed for Hydrocarbon, ammonium Chloride, Microelements, and Temperature independent variables

Run No.	X1	X2	X3	X4	Emulsifying Activity	Predicted response
1.	60	1.5	1.5	34.5	54.9	54.4333
2.	90	2.0	1.0	33.0	55.0	54.4250
3.	80	1.5	1.5	34.5	97.1	97.0429
4.	80	1.5	1.5	37.5	84.8	84.9167

5.	90	2.0	2.0	33.0	68.3	68.0417
6.	80	1.5	1.5	34.5	97.2	97.0429
7.	90	1.0	1.0	33.0	41.1	41.5417
8.	90	1.0	2.0	33.0	56.7	56.6083
9.	80	1.5	0.5	34.5	43.2	42.7167
10.	80	0.5	1.5	34.5	36.0	36.3500
11.	70	1.0	1.0	33.0	37.8	37.6917
12.	90	2.0	1.0	36.0	66.7	67.5583
13.	70	2.0	2.0	33.0	63.5	63.8917
14.	90	2.0	2.0	36.0	82.1	81.8250
15.	80	1.5	1.5	34.5	97.0	97.0429
16.	80	1.5	1.5	31.5	60.0	59.9500
17.	70	2.0	1.0	36.0	62.9	62.6083
18.	70	1.0	2.0	36.0	64.1	64.2917
19.	90	1.0	1.0	36.0	54.6	53.8250
20.	80	1.5	1.5	34.5	97.0	97.0429
21.	90	1.0	2.0	36.0	70.0	69.5417
22.	100	1.5	1.5	34.5	63.0	63.5333
23.	70	2.0	2.0	36.0	76.7	76.5750

24.	80	1.5	1.5	34.5	97.0	97.0429
25.	80	2.5	1.5	34.5	61.8	61.5167
26.	70	1.0	2.0	33.0	53.0	52.4583
27.	80	1.5	1.5	34.5	97.0	97.0429
28.	70	1.0	1.0	36.0	48.3	48.8750
29.	70	2.0	1.0	33.0	49.8	50.5750
30.	80	1.5	1.5	34.5	97.0	97.0429
31.	80	1.5	2.5	34.5	71.2	71.7500

Table 3.8. Regression coefficients and their significance for response surface quadratic

Term	Coef	SE Coef	T	P
Constant	-4091.73	64.1182	-63.815	0.000
Hydrocarbon	14.80	0.3690	40.097	0.000
Amm. Chloride	149.31	7.0071	21.309	0.000
Microelement	127.45	7.0071	18.188	0.000
Temperature	190.62	3.3309	57.227	0.000
Hydrocarbon*Hydrocarbon	-0.10	0.0011	-90.607	0.000
Amm. chloride*Amm.chloride	-48.11	0.4200	-114.533	0.000
Microelement*Microelement	-39.81	0.4200	-94.773	0.000

Temperature*Temperature	-2.73	0.0467	-58.587	0.000
Hydrocarbon*Amm. Chloride	-0.00	0.0281	- 0.000	1.000
Hydrocarbon*Microelement	0.01	0.0281	0.534	0.601
Hydrocarbon*Temperature	0.02	0.0094	1.959	0.068
Amm. chloride*Microelement	-1.45	0.5616	-2.582	0.020
Amm. chloride*Temperature	0.28	0.1872	1.514	0.150
Microelement*Temperature	0.22	0.1872	1.158	0.264

S = 0.561553 PRESS = 28.8986
R-Sq = 99.96% R-Sq (pred) = 99.75% R-Sq (adj) = 99.92%

Where, Y is the yield of biosurfactant production, and X1, X2, X3 and X4 are the coded values of hydrocarbon, ammonium chloride, microelements and temperature, respectively. Analysis of variance for the biosurfactant production obtained from this design is given in Table 3.9.

Table 3.9. ANOVA of regression model

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	11527.6	11527.62	823.40	2611.14	0.000
Linear	4	3273.7	1381.16	345.29	1094.97	0.000
Square	4	8249.4	8249.42	2062.35	6540.05	0.000
Interaction	6	4.5	4.55	0.76	2.40	0.075
Residual Error	16	5.0	5.05	0.32	*	*
Lack-of-Fit	10	5.0	5.01	0.50	80.90	0.000
Pure Error	6	0.0	0.04	0.01	*	*
Total	30	11532.7	*	*	*	*

DF, Degree of freedom; SS, sum of squares; MS, mean square

ANOVA gives the value of the model and can explain whether this model adequately fits the variation observed in biosurfactant production with the designed parameter. The goodness of fit of the model was checked by determination coefficient (R^2). In this case, the value of the R^2 (0.9996) for Eq. 2 indicates that the sample variation of 99.96 % for biosurfactant was attributed to the independent variables, and only 0.04 % of the total variation cannot be explained by the model. Figure 3.9 A–F show the 2D contour plots of biosurfactant production for each pair of significant factor by keeping the other two factors constant. The 2D contour plots are the graphical representation of the regression equation.

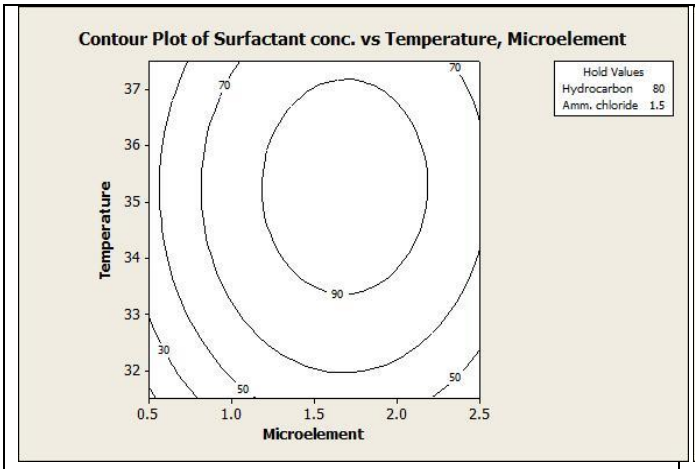

Fig A. contor plot between temperature and microelements keeping the hold values of hydrocarbon 80% and ammonium chloride 1.5(g/l)

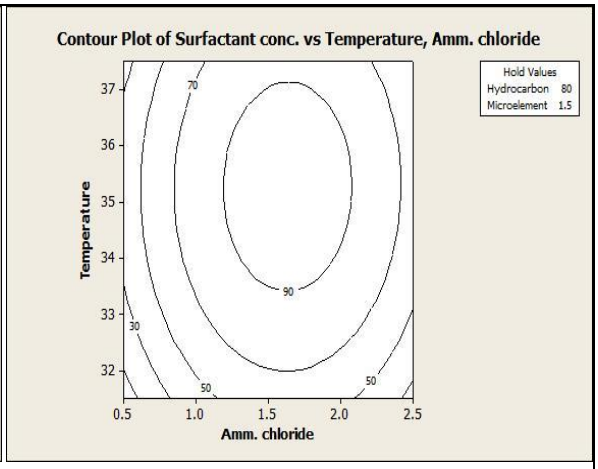

Fig B.contor plot between temperature and ammonium chloride keeping the hold values of hydrocarbon 80% and microelements 1.5(g/l).

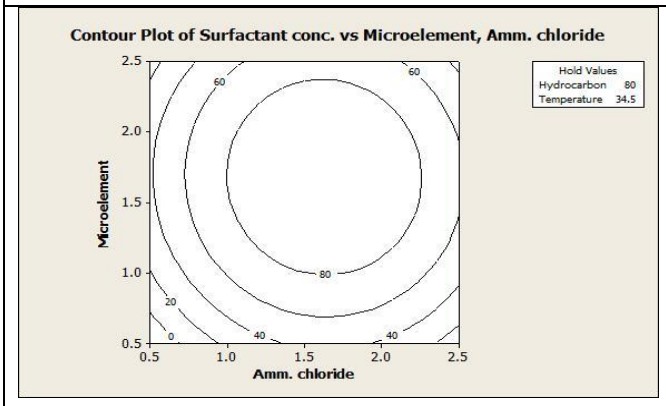

Fig C. contor plot between microelements and ammonium chloride keeping the hold values of hydrocarbon 80% and temperature 34.5ºC

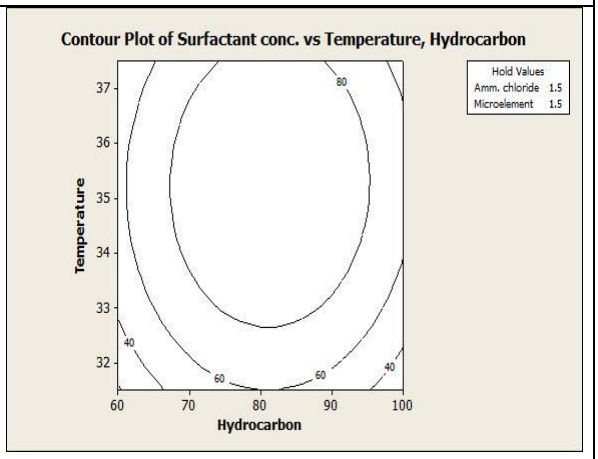

Fig D.contor plot between temperature and hydrocarbon keeping the hold values of ammonium chloride 1.5(g/l) and microelements 1.5(g/l)

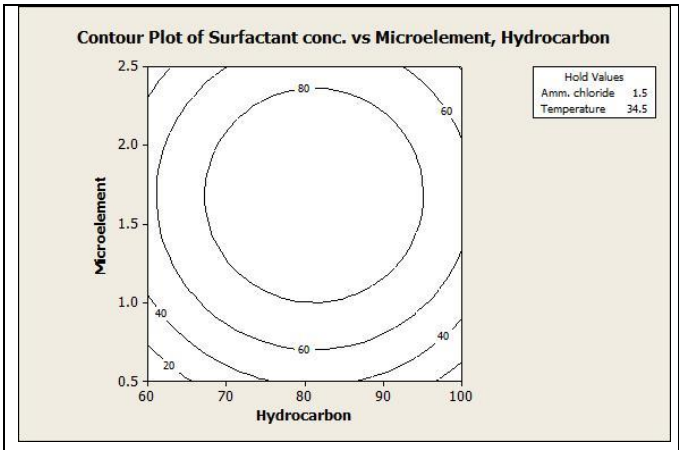

Fig E.contor plot between microelements and hydrocarbon keeping the hold values of ammonium chloride 1.5(g/l) and temperature 34.5ºC,

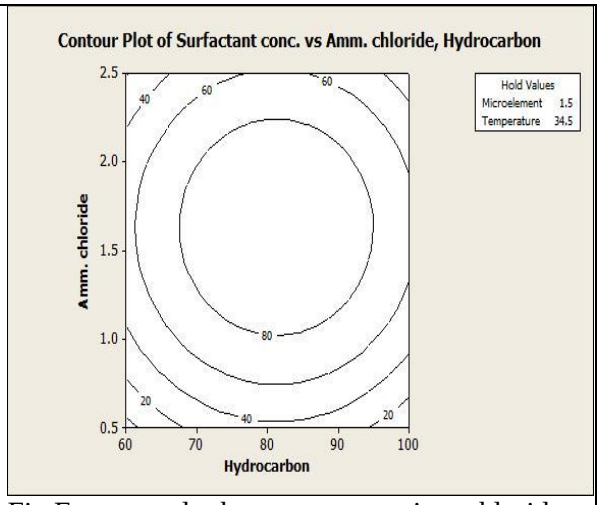

Fig F. contor plot between ammonium chloride and hydrocarbon keeping the hold values of microelements 1.5(g/l) and temperature 34.5ºC.

Figure 3.9. 2D Contour plot of Biosurfactant production in terms of E_{24} [Effect of Temperature and Microelements (A), Temperature and amm.Chloride (B), Microelements and amm.Chloride (C), Temperature and Hydrocarbon (D), Microelements and Hydrocarbon (E), amm.Chloride and Hydrocarbon (F)].

3.8. Conclusions

Among different carbon and nitrogen sources, petrol and ammonium chloride showing the highest $\%E_{24}$. *C. tropicalis* MTCC 230 was found to give the maximum cell mass and high $\%E_{24}$ during the experimental optimization of parameters, i.e., the highest $\%E_{24}$ = 54 % was obtained.

The optimization technique RSM was applied for media optimization in order to enhance the biosurfactant yield by acclimatized *C. tropicalis* MTCC 230. The present study using RSM with CCD enabled us to find the importance of factors at different levels. The RSM, including an experimental design, regression analysis, and ANOVA was an effective method for medium optimization of biosurfactant production. Acclimatized *C. tropicalis*

MTCC 230 showing diauxic growth pattern where hydrocarbon was obtained the significant factor and glucose was the non-significant factor.

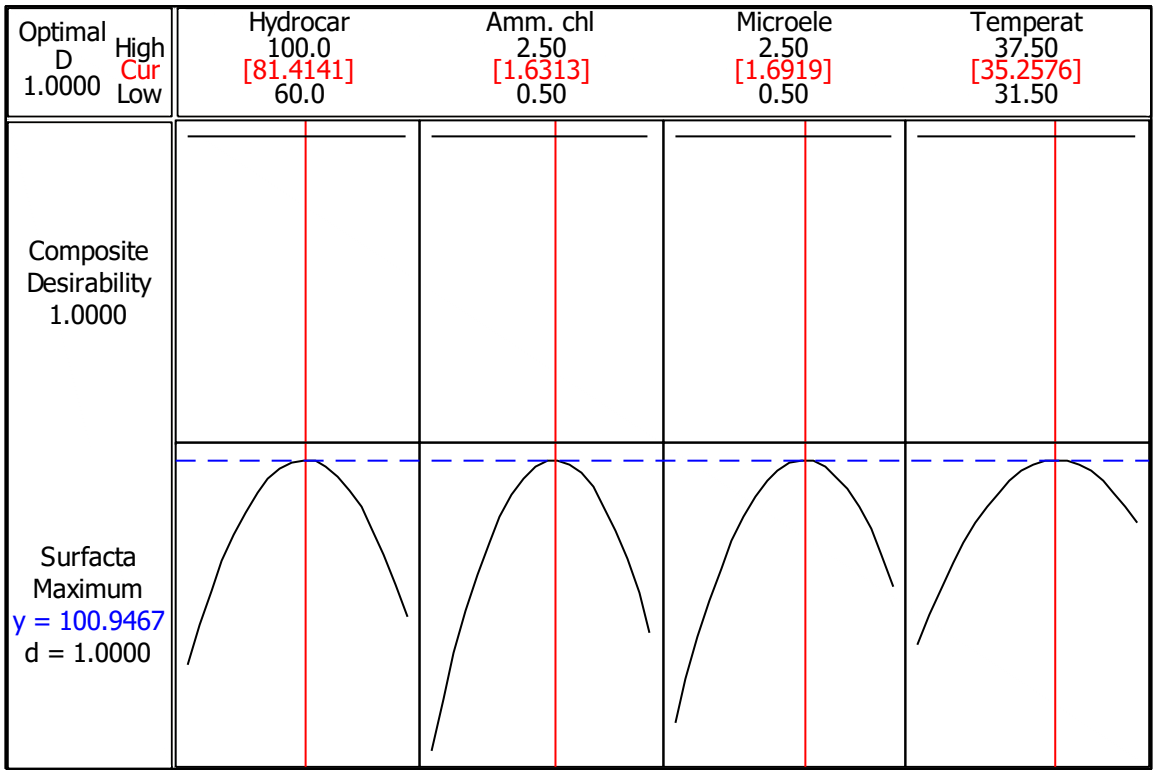

Figure 3.10. Optimum combinations of different parameters for biosurfactant production

The optimum combination of different parameters for biosurfactant production, obtained from contour plots for hydrocarbon, ammonium chloride, microelements and temperature were 81.41 %, 1.63(g/l), 1.69(g/l) and 35.25 ºC, respectively (Figure 3.10). The optimized media enhanced biosurfactant production in terms of emulsifying activity and hydrocarbon consumption two fold. The result of this study could be used to design and enhance the biosurfactant production and also help to develop the acclimatized strain in high hydrocarbon concentration so that the strain can be used in bioremediation of hydrocarbon contaminated soil and water bodies.