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Parameter Optimization of Surface Active Properties and Quantification of Biosurfactant Produced in Continuous Stirred Tank Bioreactor during Biodesulfurization of Diesel

Sujaya Bandyopadhyay *
Chemical Engineering Department,Jadavpur University

Ranjana Chowdhury
Chemical Engineering Department,Jadavpur University

Abstract— In the present study, production of biosurfactants, namely, 2-hydroxybiphenyl and different lipids like glycolipid ,rahmnolipid and phospholipid have been examined using Rhodococcus sp. During biodesulfurization process of diesel, substituted benzothiophenes (BTs) and dibenzothiophenes (DBTs) which are difficult to remove by hydrotreatment, get converted to 2-hydroxy biphenyl, which is a hydrotrope. Other biosurfactants like phospholipids, glycolipids and rhamnolipids are generated from the microbial secretion. Kinetics of biodesulfurization of deep desulfurized diesel using Rhodococcus sp. has been studied with special reference to removal of organo-sulfur compounds in diesel and production of 2-hydroxy biphenyl (2-HBP). Quantification of 2-hydroxy biphenyl (2-HBP) has been done by Gibbs Assay and HPLC.A continuous stirred tank bioreactor has been studied using the variation of operating parameters like initial concentration of organo-sulfur compounds, dilution rate .and recycle ratio. A statistical model was developed to optimize the behaviour of the bioreactor for the improvement of the surface active properties namely surface tension and E24 of the biosurfactant.

Keywords— optimization,surface tension,E24,biodesulfurization,bioreactor

I. INTRODUCTION

Biosurfactants are surface active, amphiphillic compounds produced either intracellularly or extracellularly by microorganisms. They contain both hydrophilic polar heads such as carbohydrates, amino acids, hydroxyl, phosphate etc as well as long chain hydrophobic tail groups. This feature facilitates their accumulation in oil-water interface leading to reduction of surface and interfacial tensions. These molecules arrange themselves into molecular assemblies, termed as micelles. Biosurfactants stabilize emulsions, show less toxicity and possess higher biodegradability compared to chemical surfactant and exhibit lower Critical Micelle Concentration (CMC)values compared to their chemical counterparts which means less surfactant is required for the earlier to achieve maximum lowering of surface tension[1,2]. Several bacteria produce biosurfactants like glycolipids, phospholipids and rhamnolipids during their growth or reactions with long chain alkanes and aromatics. These microbial surfactants show hydrophilic and lipophilic balance (HLB) value ranging from 16 to 20 which relates to the longer hydrocarbon chain length of the molecule. Microbial surfactants have gained popularity due to their effectiveness at extreme climatic conditions like temperature, pressure, pH, salinity and various applicability [1,2]. Biosurfactants may be successfully utilized in detergent, food, pharmaceutical, fertilizer, plastics, paints, building and construction, metal and mineral industries. They can also be utilized as an ingredient in insecticidal, herbicidal and fungicidal spraying compositions, in bioremediation of pollutants , dispersion of oil spill and in advanced oil recovery as a formulation of drilling mud or fluid[2]. A variety of cheap raw materials like agro wastes, oil wastes, starchy substance, lactic whey and distillery wastes have already been reported for biosurfactant production. In our present study the sources of biosurfactant is hydrotreated diesel which during the course of microbial sulfur reduction process produce biosurfactants as byproducts.

Diesel is petroleum cut in which sulfur compounds like dibenzothiophene (DBT) and multi substituted dibenzothiophene remain unconverted even after deep hydrodesulfurization due to their refractory nature. Compounds like 4-MDBT,3-MDBT,2-MDBT,1-MDBT,4,6-DMDBT,2,4-DMDBT,4M,6-EDBT are some of the examples. On the other hand, sulfur di-oxide released into the atmosphere through the combustion of diesel containing the refractory organo-sulfur compounds is one of the most critical issues of environmental concern as it is a major contributor to the generation of acid rain, increase in particulate matter in emission and various health problems. Production of ultra–low sulfur diesel (ULSD) with sulfur range of 15-10 ppm has thus become mandatory for refineries in all developed and developing countries. The limit of diesel sulfur content of developing countries like India, Brazil, Colombia etc have also been set at 50 ppm. The production of ultra-low sulfur diesel using conventional catalytic route involves high cost .The biodesulfurization of deep hydro-desulfurized diesel may serve as a cost effective route to produce ULSD. Hydrocarbon degrading micro-organisms, adapted to grow and thrive in oil containing environment, may have an important role in the biodesulfurization of hydro-treated diesel. The organisms like *Rhodococcus erythropolis, Rhodococcus globerulus ,Grodonia alkanivorans, Pseudomonas putida , Bacillus subtilis*[3-10] are some of the examples. Simultaneously with the desulfurization of petroleum cuts, these micro-organisms produce some biosurfactants with different chemical nature and molecular size as byproducts of their metabolic pathway.

Kodama et.al. [11] reported that 2-hydroxybiphenyl (2-HBP) and methyl substituated 2-HBPs were produced during biodesulfurization of hydrotreated diesel using *Rhodococcus Sp.* This bacterial strain follows Kodama 4S pathway. This microorganism also secretes some other biosurfactants like phospholipids, rhamnolipids, glycolipids etc during biodesulfurizsation of hydrotreated diesel. The presence of biosurfactant eases the uptake of water-immiscible hydrocarbons and sulfur nutrients by the microorganisms, suspended in aqueous medium, through the reduction of interfacial tension. Though different sources of biosurfactant production were reported but process optimization and economy are important to make biosurfacrtant competitive with its chemical counterparts.

In the present study, *Rhodococcus sp* (NCIM 2891) was selected for the production of biosurfactant through biodesulfurizsation of hydrotreated diesel. The aim of this study was to evaluate the effectiveness of biodesulfurizion process of hydrotreated diesel and production of biosurfactants both in batch mode in Erlenmeyer flasks and in a continuous chemostat using *Rhodococcus sp*. The effects of inlet substrate concentration, dilution rate and recycle ratio on the surface active properties of the biosurfactants were investigated. Total amount of lipids produced in the aqueous phase over 4 days of continuous operation was extracted and was quantified at each dilution rate. The surface properties like surface, interfacial tension, emulsification index and CMC value were measured.TLC and FTIR were done to compare the biosurfactants with standard one. A statistical model was developed to simulate the behavior of the bioreactor for the improvement of the surface active properties namely surface tension and E24 of the biosurfactant.

II. EXPERIMENTAL

2.1. Materials

Beef extract (E. Merck), peptone (E. Merck), NaCl (Ranbaxy), methanol (E. Merck), acetone (E. Merck), dibenzothiophene (Aldrich Chemical), N_2 (Prakash traders), chloroform (E. Merck), NaOH (E. Merck), pentane (E. Merck), hexadecane (E. Merck), HCl (E. Merck), isopropyl alcohol (Process chemical industries) ,di ethyl ether (E. Merck) ,2- hydroxybiphenyl (Fluka), acetonitrile, acetic acid, Gibb's Reagent(2,6 dichloro quinine-4-chloroimide)(E.Merck),Moris reagent(Merck,India) ,Silica gel GF 254(Merck),Micro-organism *Rhodococcus sp.* (NCIM 2891) (NCIM,Pune) have been used during the present investigation.

2.2. Composition of the Growth Medium for Microorganisms

Basis: 1 dm^3

Beef extract: 10 g, NaCl (AR): 5 g, peptone (for bacteriology): 10 g.

2.3. Diesel Used

Hydrodesulfurized diesel samples were purchased from outlets of Indian oil Corporation (IOC), Kolkata, India

III. ANALYTICAL METHODS

3.1 Sulfur analysis

X-ray fluorescence (XRF), Energy dispersive X-ray fluorescence (EDXRF), Gas Chromatography using flame photometric detector and CHNSO analyser (2400 series-II, Perkin Elmer, U. S. A.) were used to determine the concentration of sulfur in the diesel samples before and after the microbial treatment.

3.2 Analysis of 2-Hydroxybiphenyl (HBP) using HPLC

The concentration of 2-hydroxy-biphenyl (2-HBP) in the treated diesel was determined using HPLC (Perkin Elmer Series 200) equipped with a reverse phase NOVA PAK C18 column(250 mm,5 µm pore diameter,column pressure 82.5 bar)(particle size 60A^o,300mm x3.9 mm) using acetonitrile (50% v/v) as mobile phase . 50% acetonitrile and 50% HPLC grade water was passed for first one hour for washing the column. Flow rate of mobile phase was maintained at 0.5 ml/min. Then hexadecane solvent was injected and hold for five minutes. Finally 10 µL sample was injected after twenty times dilution. Analysis was done at 215 nm using an UV detector.

3.3. Analysis of 2-Hydroxybiphenyl (HBP) using Gibbs assay

Gibbs reagent or 2,6 dichloroquinone 4-chlroimide was dissolved in ethanol (10 mg/mL).Solution of 2-HBP was prepared in hexadecane with concentration ranging from 1-10 mg/L. pH of the sample was maintained at 8 .0 with Na_2CO_3.Gibbs reagent was added to the biphenyl solution .The reaction mixtures with different HBP concentrations were incubated for 60 min at 30 ^oC. It was centrifuged at 4000 rpm for 20 min. The blue colour developed was assayed under spectrophotometer (SPECTRASCAN UV 3600-Chemito) at a wavelength of 610nm.A standard curve was created using 2-HBP concentration vs. spectrometry result. A linear correlation was obtained. Concentration of 2-HBP in treated diesel was determined using this standard curve after development of color and doing spectrophotometric analysis. Thus Gibbs assay [6] was used to verify the concentration of 2-Hydroxy-biphenyl in the treated diesel.

3.3 Continuous mode experiments in a Chemostat

The experiment was conducted in a 2.5 dm^3 B.Braun chemostat with working volume of 1.5 dm^3. Sterilized Hydrotreated diesel and the sterile sulfur free aqueous medium were used as sulfur source and nutrient broth for the microorganisms respectively. The chemostat was then inoculated by adding 10% v/v inoculum i.e., the enriched bacterial strain under aseptic conditions inside a laminar flow chamber. After inoculation the chemostat was equipped with an air compressor and a stirrer .The stirring and aeration rate were maintained at 80rpm and 25 L per hour respectively.

Both the dilutuion rate and the recycle ratio were varied. The inlet sulfur concentration in feed diesel was varied from 100 to 540 ppm. Temperature of the chemostat was maintained at 28^{o}C.

3.4. Characterization of Biosurfactants.

Measurement of Surface Tension, Interfacial tension and Measurement of Emulsification Index (E24) was done by same procedure followed by Bandyopadhyay et.al.[12]

Significance and measurement of Critical Micelle concentration (CMC)

A surfactant solution has a surface populated with adsorbed molecules in a state of lower free energy than those in the bulk solution. As a result less work is needed to create unit surface area on a surfactant solution compared with that needed to create same for the pure solvent. In other words, the adsorption of surfactant lowers the surface tension of the liquid solvent. With the following assumptions namely,

- surfactant is a single component
- surface excess of solvent is zero
- activity of surfactant molecule is equal to concentration

Gibb's energy equation can be written as

$$-d\gamma = \Gamma RTd\ln C \qquad (2)$$

$$\text{Or, } \Gamma = \frac{1}{RT}\left(-\frac{d\gamma}{d\ln C}\right) \qquad (3)$$

Where Γ is excess surface, γ is surface tension and C is the surfactant concentration. As CMC is reached further addition of surfactant causes the formation of aggregates or micelles .Essentially all the additional surfactant aggregates into micelles and beyond this point surface tension usually remains constant.

By plotting surface tension against logarithm of surfactant concentrations, the critical micelle concentration is determined by noting the onset of constancy of surface tension with respect to surfactant level.

3.5. Extraction and determination of lipid content of cell free aqueous medium

Bligh and Dyer procedure [13] has been followed for lipid extraction. According to this method choloroform (20 mL), methanol (10 ml) and 10 ml cell free aqueous reaction broth were mixed thoroughly using a homogenizer for 5 to 7 minutes. The mixture was taken for centrifugation for 10 minutes. Two layers were separated with bottom layer containing lipids dissolved in chloroform. These steps were repeated for three times so that all lipid parts can be transferred in the chloroform layer. Finally this layer was taken through filtration in a preweighed round bottom flask. Chloroform was evaporated under vacuum. Final weight of lipid was determined by the difference of final and initial weights of the flask. Thin layer chromatography (TLC) was done to separate polar lipids from non polar lipids and free fatty acids.

3.6. Vibrational spectroscopy in the infrared region-FT-IR analysis*:*

The cell free liquid sample and standard sample was analyzed by FT-IR spectra measurement, carried out by a spectrometer model VERTEX 70 FTIR, with a spectral range from 700 to 4000 cm^{-1}.

IV. RESULTS AND DISCUSSION

In a previous work the viability of production of biosurfactant during biodesulfurisation of diesel was demonstrated. Since the aqueous to diesel ratio may influence production of biosurfactant , this effect has been investigated in this research study by varying the aqueous to diesel ratio in the range of 20:80 to 0:100.

In figures 1a to 1c values of cell concentration, % conversion of sulfur, values of surface tension of aqueous and diesel layers of cell-free supernatant of 48 hour sample of batch type experiments have been column plotted as a function of diesel to aqueous ratio. From the analysis of the plots it appears that cell growth, biodesulfurization , i.e. conversion of diesel sulfur and production of biosurfactant, as reflected through lowering of surface tension of oil and aqueous phases, occur at all ratios of diesel to aqueous phase. However, the best performance has been observed at the oil to aqueous phase ratio of 80:20 from the perspective of all parameters. Thus further investigation in the chemostat in continuous mode, diesel to aqueous phase ratio has been maintained at 80:20.The trends of the column plots may be explained by the fact that as the diesel proportion is increased, the availability of sulfur compounds, the energy source of the microorganism, as well as hydrocarbons – one of the carbon sources for growth, increases resulting in the increased trend in cell concentration up to the ratio of 80:20.As the diesel concentration increases beyond 80%, availability of oxygen may decrease at the interfacial zone due to formation of thick oil layer[12,14]. As a consequence, there are declines in the values of biomass concentration, the sulfur conversion as well as the lowering of surface tension.

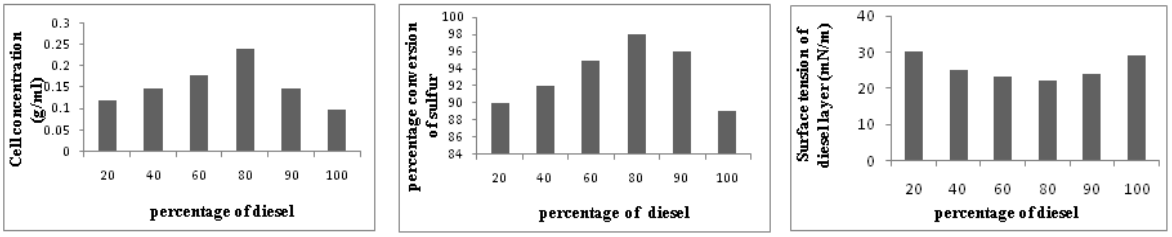


Fig 1a, 1b and 1c representing column plots of cell concentration, % conversion of sulfur, values of surface tension of diesel layers of cell-free supernatant of 48 hour sample of batch type experiments vs.percentage of diesel

CHNSO analysis to determine sulfur reductions

From the analysis done using CHNSO analyser it was shown that the sulfur content has come down from 350 ppm to 30 ppm after biodesulfurization (shown in table 1).Percentage of nitrogen, hydrogen, carbon and oxygen were also determined for both of the oil samples.

TABLE 1
CHNSO analysis

Name of sample	N%	C%	H%	O%	S%
Diesel	1.3	64.6	10.5	23.6	0.035
Treated Diesel	1.06	51.9	8.4	38.6	0.003

Identification and quantification of 2-hydroxybiphenyl in treated diesel using HPLC:

Organo sulfur compound DBT gets converted to 2-hydroxybiphenyl through multi-enzymatic pathway (Kodama 4S pathway). 2-hydroxybiphenyl was identified and quantified using HPLC at residence time 5.72 minutes. HPLC Chromatogram of biodesulfurized diesel identified and quantified 2-HBP which have been represented in figure 2 .It was compared with standard 2-HBP in acetone solution (1:20) (blue line) .This also help to quantify 2-HBP in biodesulfurized diesel .

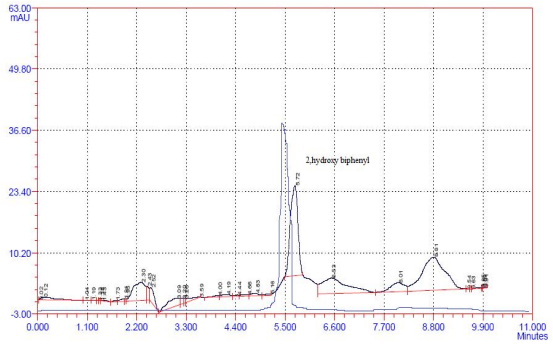


Fig2 HPLC chromatogram of treated diesel and 2-HBP standard

Identification and quantification of 2-hydroxybiphenyl in treated diesel Gibbs Assay

A standard linear curve was obtained by plotting the OD values after the colour development as discussed in the earlier section and concentration of standard 2-HBP in acetone solution**.** OD values of the unknown solution where the 2-HBP concentration has to be find out were obtained as 0.32 and 0.58 which represents concentration as 380 ppm and 800 ppm respectively.

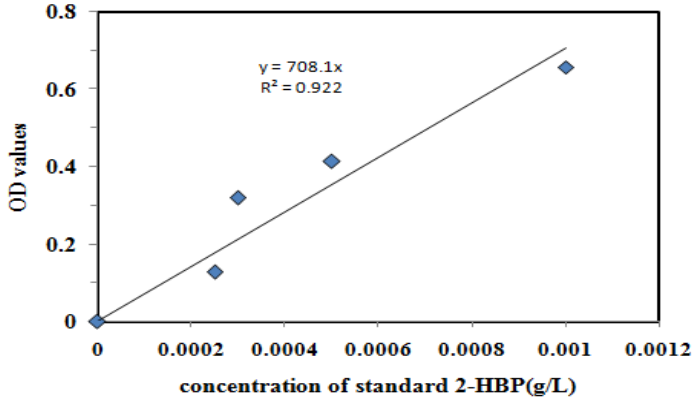


Fig3: Plot used in Gibbs assay for determination of concentration of 2-HBP

FT-IR analysis of microbially treated diesel and other biosurfactant:

The biodesulfurised diesel, 2-HBP and natural surfactant soap nut extracts were compared and characterized for the presence of functional group and figure 4 has shown the FT-IR (VERTEX 70 FT-IR) superimposed spectra. Band assignments of IR spectrum of treated diesel oil, 2-HBP which were summarized in table 4, indicate that they contain similar type and number of atomic groupings and structures). In the superimposed FTIR spectrum, O-H stretching spectrum of 2-HBP, Ritha and treated diesel appeared at 3000-3200 cm^{-1} wave number. Other spectrum of C-H, C=O, C=C stretching, C-H bonding are comparable for all three samples. It can be concluded that they have similarity in their functional groups which are responsible for their surfactant properties.

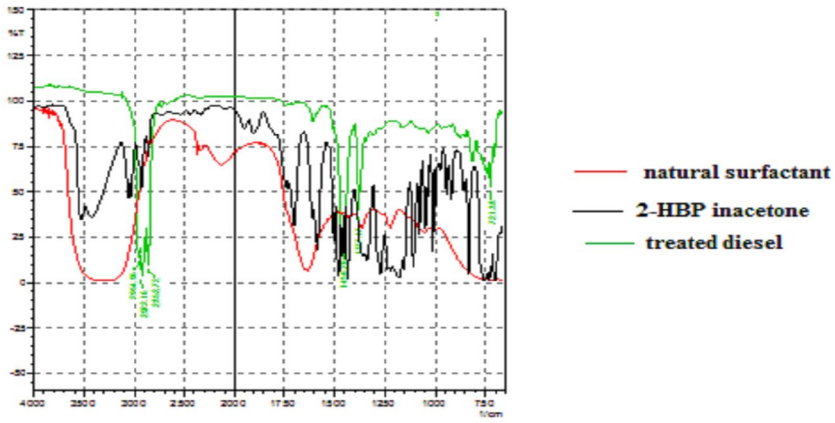


Fig4 superimposed FTIR spectrum comparing natural surfactant, 2-HBP in acetone solution and treated diesel

STATISTICAL ANALYSIS-RESPONSE SURFACE METHODOLOGY FOR REGRESSION AND OPTIMIZATION

Quadratic and 2F1 models were established on the basis of the results of experiments recommended by CCD (table 2) tables to represent the relationship between the responses, i.e, surface tension and emulsification index of the biosurfactants and the multiple independent variables namely dilution rate, recycle ratio and inlet sulfur concentration. The proposed models in terms of coded factors based on the regression coefficients for minimization of surface tension and maximization of E24 are as follows,

$$Y = 28.99 + 0.98A - 0.47B - 8.78C - 0.78A*B - 0.78A*C + 0.18B*C + 0.31A^2 + 0.58B^2 + 7.8C^2 \quad (4)$$

$$E = 45.93 - 1.01A + 0.56B + 13.26C + 0.46A*B \quad (5)$$

Where Y is surface tension (dynes/cm) and E is the emulsification index (%), A is the dilution rate (D) (per hour), B is the recycle ratio(R) and C is the inlet sulfur concentration (S_0) (mg/l).The quadratic effects of A, B and C are found to be significant (P<0.0001).

The "Pred R-Squared" of 0.9322 is in reasonable agreement with the "Adj R-Squared" of 0.9554. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. A ratio of 36.651 indicates an adequate signal which signifies this model can be used to navigate the design space.The ANOVA table has been provided as table 3. Dilution rate has an antagonistic effect while recycle ratio and inlet sulfur concentrations have synergistic effect. Figures 5a- Figures 5e show the 3D plots representing the combined effects of S_0 and R; S_0 and D; and R and D respectively on the response variables, namely, surface tension and emulsification index of the biosurfactants. Process optimization has been done using the Design Expert software .It identifies the set of values of independent variables namely S_0 ,R and D resulting in desirable values of the response variables namely surface tension and emulsification index. Optimum values of surface tension and emulsification index are 28 dynes/cm and 60.2 at S_0=540 ppm, D=0.01/hour, and R= 0.4 with desirability of 0.72.

TABLE2
Face centered central composite design

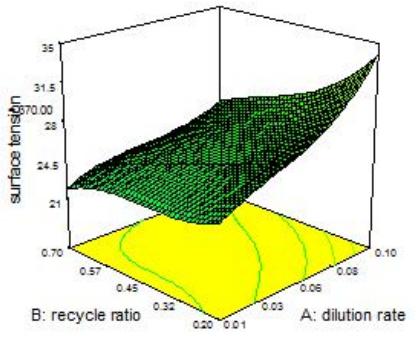
Run no	*Factor1* *A:dilution rate(/h)*	*Factor2* *B:recycle ratio*	*Factor3* *C:inlet sulphur concentration(ppm)*	**Response 1** **Surface tension(dynes/cm)**	*Response 2* *Emulsification Index(E24)*
1	*0.1*	*0.2*	*370*	*38*	*43.89932*

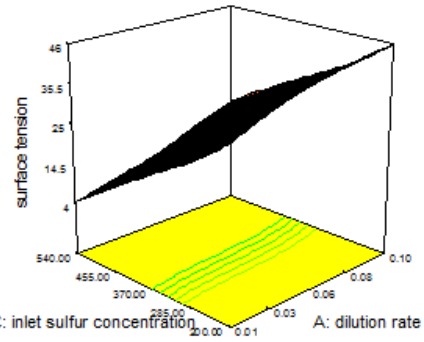
2	0.0775	0.45	370	28	45.42711
3	0.01	0.45	200	44	33.68491
4	0.0775	0.45	285	36	38.79722
5	0.1	0.45	370	28	44.92125
6	0.0325	0.575	455	28	53.23478
7	0.055	0.7	540	28	59.7548
8	0.055	0.2	540	28	58.63068
9	0.0325	0.45	370	28	46.43882
10	0.055	0.575	285	35	39.58411
11	0.0325	0.325	455	28	52.90265
12	0.1	0.7	540	28	59.20296
13	0.055	0.45	455	28	52.56285
14	0.1	0.45	200	48	31.66148
15	0.0775	0.45	455	28	52.057
16	0.055	0.2	370	28	45.37091
17	0.01	0.2	200	45	33.58271
18	0.01	0.2	540	28	60.10226
19	0.1	0.7	200	48	32.68341
20	0.055	0.45	370	28	45.93297
21	0.055	0.2	200	48	32.11113
22	0.01	0.45	370	28	46.94468
23	0.055	0.7	370	28	46.49503
24	0.1	0.45	540	28	58.18103
25	0.055	0.325	370	30	45.65194
26	0.055	0.45	540	29	59.19274
27	0.1	0.2	200	48	30.63955
28	0.0775	0.575	455	28	52.453
29	0.1	0.7	370	30	45.94319
30	0.0325	0.575	285	35	39.975
31	0.01	0.7	200	46	33.7871
32	0.055	0.325	455	28	52.28182
33	0.0325	0.45	455	28	53.06871
34	0.055	0.45	200	45	32.67319
35	0.01	0.45	540	28	60.20445
36	0.01	0.7	540	28	60.30665
37	0.0325	0.325	285	35	39.64287
38	0.0775	0.325	285	36	38.40123
39	0.055	0.575	370	28	46.214
40	0.0775	0.575	285	36	39.19322
41	0.055	0.7	200	45	33.23526
42	0.0325	0.575	370	28	46.60489
43	0.0775	0.325	455	28	51.661
44	0.055	0.325	285	36	39.02205
45	0.0775	0.575	370	28	45.82311
46	0.055	0.45	285	36	39.30308
47	0.0325	0.45	285	34	39.80894
48	0.0325	0.325	370	28	46.27276

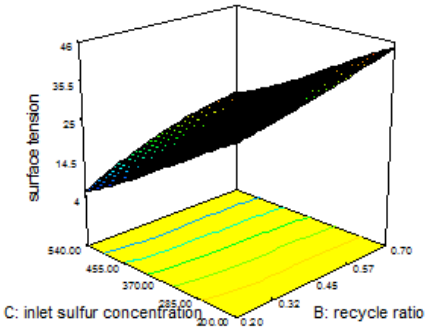
49	*0.055*	*0.575*	*455*	*28*	*52.84389*
50	*0.01*	*0.7*	*370*	*29*	*47.04687*
51	*0.1*	*0.2*	*540*	*28*	*57.1591*
52	*0.0775*	*0.325*	*370*	*28*	*45.03111*
53	*0.01*	*0.2*	*370*	*28*	*46.84249*

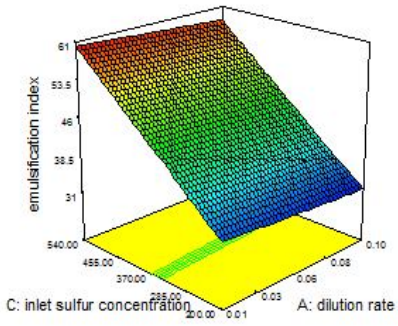
TABLE3
Analysis of variance table for Response Surface Quadratic Model

ANOVA for Response Surface Quadratic Model					
Analysis of variance table [Partial sum of squares - Type III]					
Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**
Model	2407.396	9	267.488	124.816	< 0.0001
A-dilution rate	21.511	1	21.511	10.038	0.0028
B-recycle ratio	4.900	1	4.900	2.286	0.1378
C-inlet sulfur concentration	1733.611	1	1733.611	808.944	< 0.0001
AB	7.843	1	7.843	3.660	0.0624
AC	7.843	1	7.843	3.660	0.0624
BC	0.397	1	0.397	0.185	0.6690
A^2	0.746	1	0.746	0.348	0.5583
B^2	2.685	1	2.685	1.253	0.2692
C^2	484.491	1	484.491	226.075	< 0.0001
Residual	92.151	43	2.143		
Total	2499.547	52			









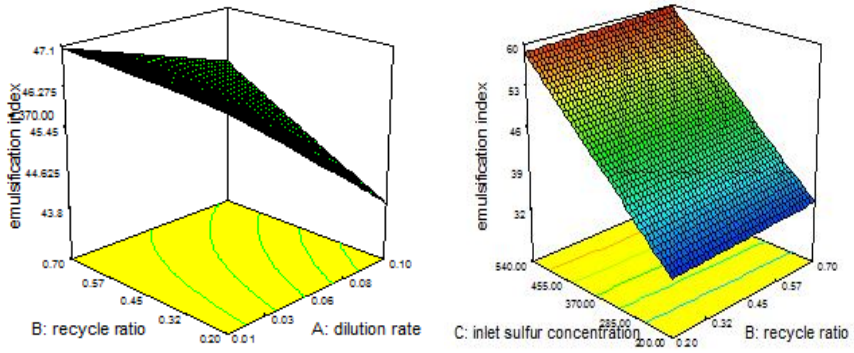


Fig 5a-5e:3D plots representing the relationship among surface tension and emulsification index and the multiple independent variables namely dilution rate, recycle ratio and inlet sulfur concentration.

V.CONCLUSIONS

A novel statistical model was developed to optimize the behaviour of the bioreactor for the improvement of the surface active properties namely surface tension and E24 of the biosurfactant. Quantification of 2-hydroxy biphenyl (2-HBP) has been done by Gibbs Assay, FTIR and HPLC. Analysis was made on cell concentration, percentage conversion of sulfur, values of surface tension of diesel layers as a function of percentage of diesel.Significance of CMC was elaborated.

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