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Stabilization of bacterial cells culture on immobilized Alginate beads and optimization of Congo red decolorization

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The paper deals with the primary objective to optimize different parameters such as concentration, temperature, *p*H, time and size of cells for the decolorization of azo dye (Congo red) by using bacterial isolates by the method of immobilized bacterial cells. *Staphylococcus aureus* immobilized beads show a maximum dye decolorization of 94% under optimum condition and found to be more efficient in dye decolorization. The optimum value for degradation is found to be 35°C, for 400 mg/L of concentration of dye at the medium *p*H of 7. The order of the reaction was found to be first order with the rate constant of 0.073 h^{-1}. The dye adsorbed on the bead surface follows Freundlich adsorption isotherm with the constant values are K = 1.368 and 1/n = 0.773. Studies have been carried out by FTIR, GC-MS analysis for confirming the biodegradation of Congo Red by the immobilized bacterial cells. The results reported warrant further investigation to establish the usefulness of these isolates for bioremediation and biodegradation application such as wastewater treatment.

Keyword: Decolorization, Azo dye, Immobilization, Kinetics, *Staphylococcus aureus*

The major pollutants in textile effluents are organic, coloured, surfactant and chlorinated compounds and salts. The residual dyes from different sources are considered as wide variety of organic pollutants introduced into the natural water resources or wastewater treatment systems. In addition, the consequence triggered by other pollutants in textile industrial wastewater and the presence of little amount of dyes in the water, that are nevertheless highly visible, seriously affects the aesthetic quality and transparency of water bodies leading to damage to the aquatic environment[1-3,5]. Dyes are toxic, mutagenic and decrease light penetration for photosynthetic activity, causing oxygen shortage and curbing downstream beneficial uses such as drinking water, irrigation and recreation[4]. The discharge of dye-containing effluents into the water environment is undesirable, not only because of their colour, but also because breakdown products of dyes that are toxic, carcinogenic or mutagenic to life forms[6-8]. One of the most complex tasks for the treatment plants of textile industries is the elimination of the colour, mainly because dyes and pigments are intended to bear biodegradation such that they persist in the environment for a long period. In addition, the high temperature of the wastewater reduces the concentrations of dissolved oxygen[9-12].

Azo dyes are important groups of artificial colorants that are widely used in printing, textiles, and pharmaceutical industries. Azo dyes are water-soluble synthetic organic compounds[13]. Azo dyes contain one, two or three azo linkages, linking phenyl, naphthyl rings that are usually substituted with some functional groups including triazine amine, chloro, hydroxyl, methyl and nitro[14]. In the past, treatment systems were mainly depended on using physical or chemical-treatment processes, occasionally in conjunction with biological treatment. The physical and chemical techniques such as flocculation combined with flotation, electro-flotation, membrane-filtration, electro-coagulation, electrochemical destruction, ion-exchange, irradiation, precipitation, ozonation, and adsorption[15-17]. Some of these techniques have been shown to be effective, although there are excess amount of chemical usage or sludge generation with obvious disposal problems, costly plant requirements or operating expenses, lack of effective colour reduction, particularly for sulfonated azo dyes; and sensitivity to a variable wastewater input[18].

Microbial degradation is one of the ways to remove the environmentally harmful compounds. Bioremediation process involves detoxification and mineralization where the waste is converted into inorganic

compounds such as carbon dioxide, water and methane[19]. Effective microorganisms are collected from wastewater, residual sites and sludges which are believed to have a resistance against hazardous compounds. This is particularly due to their tolerance capacity even at higher concentration of xenobionts[20].

Microbes use the residual product as one of their substrate and grow on them, degrading and fragmenting, which is highly valuable in case of bioremediation. *Pseudomonas aeruginosa* is used for the reclamation of oil or metal contaminated soils by producing surfactants and this strain is also used in decolourisation and degradation of dyes. *Pseudomonas* species has been characterized for complete and partial mineralization of pesticides and fungicides. *Bacillus* species have been characterized and documented for the ability to degrade aliphatic and aromatic compounds[21]. The present work concentrates on the effect of alginate beads on the longevity of the organism that enhance the effective decolourization of azo dyes.

Experimental Section

Materials preparations

Wastewater sample was collected from the common effluent treatment plant for the textile industry located in Tamilnadu, India. The effluent was collected in airtight container. The collected effluent was filtered through Whattman filter paper to remove suspended particles[22]. The *p*H was maintained at 7.0 and stored at 4°C. All the chemicals and reagents were purchased form Sigma-Aldrich Company.

Isolation of dye decolorizing bacteria

The bacterial species isolated from effluent was used in this study. The strain was grown and maintained on nutrient agar slant at 4°C. Inoculum was prepared by suspending organism from slant. The bacterial cells were grown in Erlenmeyer flasks containing 100 mL medium containing glucose (1.0 mg/L), yeast extract (0.2 mg/L), peptone (0.5 mg/L), supplemented with 500 mg/L of dye for adapting dye degradation in further studies. The cell suspensions were aseptically transferred to experimental flasks for degradation studies. The degradation studies were carried out in mineral medium containing K_2HPO_4 (1.6 g/L), Na_2HPO_4 (0.6 g/L), NH_4 NO_3 (1.0 g/L), NaCl (0.5 g/L), $MgSO_4.7H_2O$ (0.1 g/L), $CaCl_2.2H_2O$ (0.1g/L), $(NH_4)_2SO_4$(0.1g/L) respectively[23-28].

Immobilization of bacterial cells

Sodium alginate, 4% (w/v), solution was prepared in 25 mM Phospate–acetate buffer (*p*H 7.5) bystirring for 2 hours at room temperature and was stored at 4°C. The inoculum was centrifuged at 10000 rpm for 15 min and supernatant was discarded. The pellet (3 g/L) was taken and mixed aseptically into sodium alginate solution and dropped into 0.3M $CaCl_2$ solution. After adding for 90 min the calcium alginate beads were collected, washed thoroughly with acetate buffer (*p*H 7.5) to remove excess calcium from the beads and stored in same buffer at 4°C[29].

Experimental design for dye degradation studies using CCD

Central composite design (CCD) was adopted for optimizing the physical parameters for dye degradation using immobilized bacterial cells was proposed using Design Expert 7.0.0 (Trial version, State-Ease Inc, Minneapolis). The three variables temperature (A), *p*H (B) and initial Congo red concentration (C) was optimized. Each variable was studied at three different levels (-1, 0, +1) as shown in Table 1. All the variables at a central coded value were considered as zero. 2^3 factorial central composite experimental designs leading to a set of 20 experimental runs was used to optimize the parameter for dye degradation.Regression analysis was performed to estimate the response function as a second-order polynomial[30].

$$Y = \beta_o \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X^2{}_i + \sum_{i=1,i<j}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_{ij}$$

where, Y is the response (dye degradation) and β_i, β_j, β_{ij} are the coefficients estimated from regression.

Dye degradation studies

Optimization experiments were carried out in 250 mL Erlenmeyer flask containing 100 mL of minimal medium inoculated with immobilized cells and maintained according to the experimental conditions. The flask was incubated for 36 hr at 36°C. The effect of concentration was studied by varying the concentration from 100 mg/L to 1000 mg/L. The rate of substrate degradation at bead surface depends on the diffusivity within the porous matrix [4,16,23].

$$-r_A = \frac{4}{3}\pi r^3 k\, C_d$$

where,r_A is the rate of degradation at bead surface (g dye/ g microorganism time)

Table 1 — Design table for RSM

Std	Run	Factor 1 A:Temperature	Factor 2 B:pH	Factor 3 C:Concentration	Response 1 % of degradation	
		C		mg/l	Actual	Predicted
20	1	35	7	500	94	91.8
18	2	35	7	500	92	91.8
2	3	40	6	250	73	71.4
1	4	30	6	250	88	90.1
16	5	35	7	500	94	91.8
9	6	26.59104	7	500	76	74.2
15	7	35	7	500	92	91.8
13	8	35	7	79.55179	84	82.4
3	9	30	8	250	81	81.7
10	10	43.40896	7	500	62	63.7
4	11	40	8	250	69	70.5
12	12	35	8.681793	500	84	82.9
6	13	40	6	750	69	68.4
5	14	30	6	750	71	69.6
19	15	35	7	500	90	91.8
17	16	35	7	500	89	91.8
8	17	40	8	750	80	78.0
14	18	35	7	920.4482	70	71.5
7	19	30	8	750	70	71.7
11	20	35	5.318207	500	81	82.0
Std. Dev.		2.298406		R-Squared	0.971704	
Mean		80.45		Adj R-Squared	0.946238	
C.V. %		2.856938		Pred R-Squared	0.945228	
PRESS		288.9509		Adeq Precision	17.30667	

k is the rate constant ($time^{-1}$)
r is the radius of bead (cm)
C_d is the concentration of the dye (g dye / L)

Samples of cell free supernatant collected at regular interval of time and centrifuged at 5000 rpm for 10 min the supernatant was assayed for concentration of the dye and pellet for biomass estimation. Residual concentration of the dye was analyzed spectrophotometrically. The concentration of dye was calculated from OD values using standards. Jalandoni-Buan and Decena-Soliven, 2004 [1] estimated by Schimadzu UV-Visible spectrophotometer (UV 1800) at the maximum wavelength for the dye (λ_{max} = 530 nm) in the visible region on a UV visible spectrophotometer. The efficiency of the isolates to degrade/decolorize the dye was expressed as,

$$Percentage\ Degradation = \left\{\frac{A_o - A_f}{A_o}\right\} \times 100$$

where, A_o be the initial concentration and A_f be the residual concentration of the dye.

Identification of metabolites

The culture medium containing the degradation products of dye was centrifuged and the supernatant was extracted with equal volume of methanol, and the extract was dried over Na_2SO_4. The extract was concentrated in a rotary evaporator. This extract was subjected to GC-MS analysis. GC-MS technique was performed using GC Shimadzu QP2010 system. The column used was Elite 1 fused silica capillary column. Mass spectra were recorded under scan mode in the range of 40-1000 m/z.

Result and Discussion

Morphological analysis and immobilization

About 10 strains was isolated among them the two strain shows the remarkable ability in decolorizing the dye. These strains used for extensive studies and were identified based on morphological characteristics and 16S rRNA sequences were initially analyzed at NCBI server were *Proteus mirabilis*(Gram negative bacilli) and *Staphylococcus aureus* (Gram positive cocci).

Immobilization

These isolated organisms were purified into pure culture and each were immobilized using calcium alginate method (Fig. 1). All the analysis was performed using this immobilized culture. The surface texture and immobilised beads were screen under Scanning Electron Microscope (SEM) and reported in Fig. 2.

Kinetics of dye degradation

The rate of biodegradation of dye was calculated by testing the kinetic data with first-order model. Staphylococcus aureus immobilized beads showed a better result that *Proteus mirabilis*. Hence the

$$-r_A = \frac{-dC_d}{dt} = k\,C_d$$

Integrating the above rate equation between the limits 0 to t from C_{d0} to C_d, the above kinetic expression becomes

$$\int_{C_{do}}^{C_d} \frac{(-dC_d)}{C_d} = k \int_0^t dt$$

$$\ln C_d = \ln C_{d0} - kt$$

where, C_d is the concentration of dye at time t and k (h^{-1}) is the first-order rate constant. From the plot of $\ln C_d$ versus t, it was observed that this model fits the data well. The k value was found to be 0.073 h^{-1} and R^2 being 0.967 (Fig. 3). The biosorption equilibria of an azo dye (congo red) were examined in this study using immobilized beads as the biosorbent. It was found that the Freundlich isotherm model fitted well to the biosorption equilibrium data. It expressed an empirical equation for representing the isothermal variation of adsorption of a quantity of dye adsorbed by unit mass of solid adsorbent.

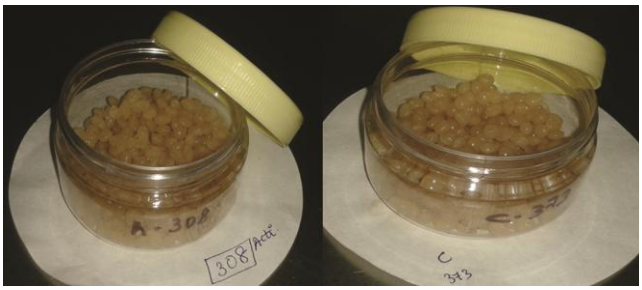

Fig. 1 — Immobilized beads of *Proteus mirabilis and Staphylococcus aureus*

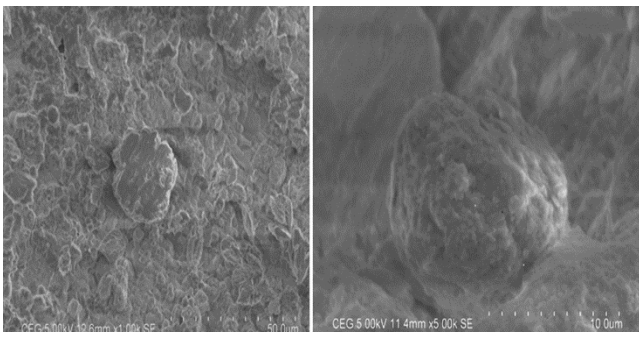

Fig. 2 — SEM image of immobilized bead surface at the resolution of 50 µm and 10 µm

$$\frac{x}{m} = k\,C^{\frac{1}{n}}$$

where, $\frac{x}{m}$ is the amount of adsorbate adsorbed by the unit mass of adsorbent, k and n are constant whose values depend upon adsorbent at temperature, the values of k and 1/n are found to be 1.368 and 0.773 respectively(Fig. 4).

Effects of physiochemical conditions

Temperature has strong effect on degradation as shown in Fig. 5 (a). The percentage degradation of dye increased from 50.4% to 92% as the temperature increased from 20°C to 35°C. Further increase in temperature decreased degradation percentage from 92% to 80%. This might be due to inhibition of enzyme secretion at higher temperature.

The effect of *p*H on dye degradation is shown in Fig. 5(b). Percentage degradation decreased as the medium *p*H deviated from neutral condition. The percentage degradation of dye increased from 60%

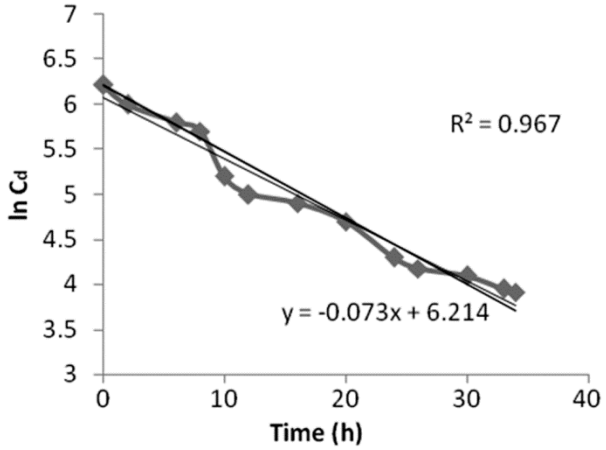

Fig. 3 — First order kinetics

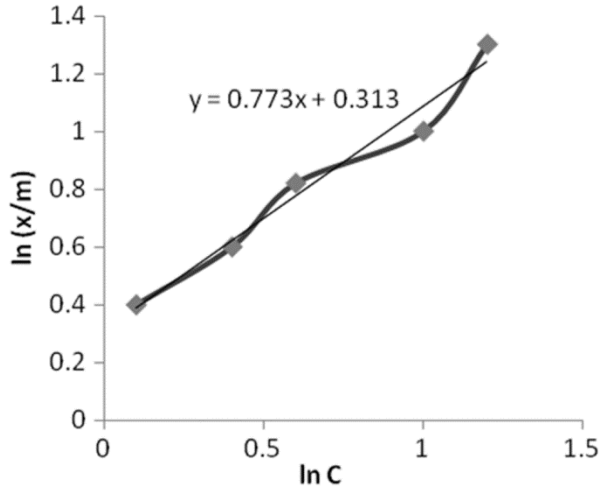

Fig. 4 — Freundlich adsorption isotherm

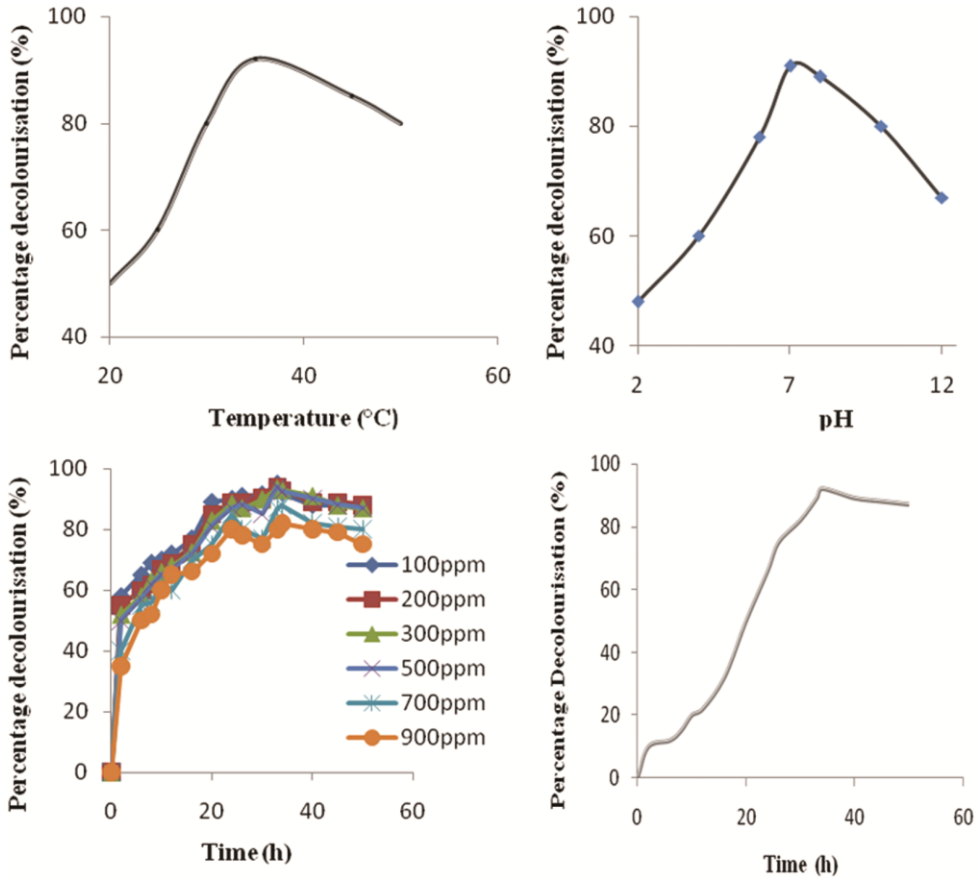

Fig. 5 — Optimization of physiochemical parameters (a) Temperature (b) *p*H (c) initial concentration of dye (d) time.

to 80% as *p*H increased from 4 to 6 and reached maximum of 92.5% at *p*H 7. The increase in *p*H from neutral condition significantly affected the biochemical reactions required for dye degradation.

The effect of initial concentration was studied from 100 mg/L to 1000 mg/L as shown in Fig. 5(c). The percentage degradation of dye increased from 60% to 93% with increase in initial concentration of dye from 100 mg/L to 500 mg/L. Further, increase in concentration degradation decreased from 92% to 87% substantially resulting from intense substrate inhibition at high concentration.

Decrease in percentage decolorization was observed at lower time 36 h as well as higher time 48 h for the organisms (Fig. 5d). It is thought that metabolites formed during the process of decolorization by cultures may significantly increase the time for degradation up to 36 h and further it starts decreases.

Effect of carbon sources on dye decolorization

Several bacterial strains that decolourize azo dyes aerobically require organic carbon source, as they cannot utilize dye as the growth substrate. The results obtained clearly indicated that the highest rate of decolorization was observed with glucose followed by fructose and maltose whereas lactose and sucrose did not support appreciable decolorization.

Regression analysis

Multiple regression analysis was carried out considering full quadratic model equation on the responses to evaluate the adequacy of fit and results are reported in Table 2. The coefficient of determination for the model equation are R^2 = 0.971, adjusted R^2=0.946 and Predicted R^2= 0.945 for the responses were reported. These values suggested that the predicted values are linear relation with experiment values.

Interaction effects of parameters

As the temperature (A) and *p*H (B) increases, percentage degradation increases up to its optimum level. As the temperature increases from 30°C to 35°C and *p*H increases from 6 to 7 an increase in percentage degradation was observed. After that a decrease in percentage degradation was observed with increase in temperature and *p*H. The maximum

Table 2 — ANOVA for response surface quadratic model

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Model	1814.123	9	201.5693	38.15669	< 0.0001	significant
A-Temperature	132.5404	1	132.5404	25.08966	0.0005	
B-pH	1.198306	1	1.198306	0.226837	0.6441	
C-Concentration	145.2945	1	145.2945	27.50398	0.0004	
AB	28.125	1	28.125	5.324011	0.0437	
AC	153.125	1	153.125	28.98628	0.0003	
BC	55.125	1	55.125	10.43506	0.0090	
A^2	943.6752	1	943.6752	178.636	< 0.0001	
B^2	158.7612	1	158.7612	30.0532	0.0003	
C^2	399.2789	1	399.2789	75.58276	< 0.0001	
Residual	52.82671	10	5.282671			
Lack of Fit	31.99338	5	6.398676	1.535682	0.3247	not significant
Pure Error	20.83333	5	4.166667			
Cor Total	1866.95	19				

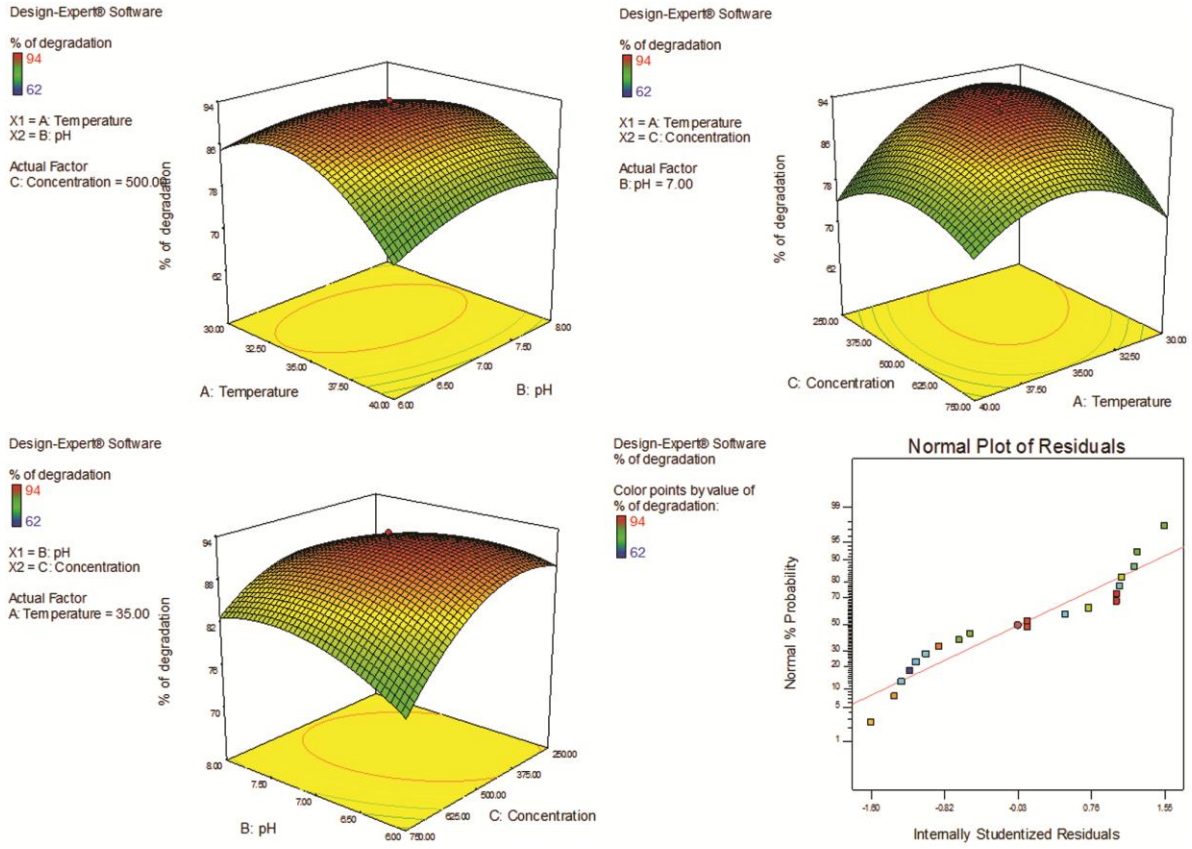

Fig. 6 — Interactive effect of parameters on percentage degradation Graphical comparison of experimental and predicted values.

percentage degradation of 94% was found to be *p*H 7 and temperature was found to be 35°C. As the initial concentration increases (C) from 250 to 400 mg/L and temperatures (A) from 30 to 35°C, an increase in percentage degradation was observed. After that a decrease in percentage degradation was observed with increase in temperature and initial concentration. At the maximum percentage degradation was 94%, *p*H was found to be 7 and temperature was found to be 35°C. The initial concentration (C) and *p*H (B) increases, the percentage degradation also increases up to its optimum level. After that a decrease in percentage degradation was observed with increase in initial concentration and *p*H. At the maximum percentage degradation was 94%, *p*H was found to be 7 (Fig. 6).

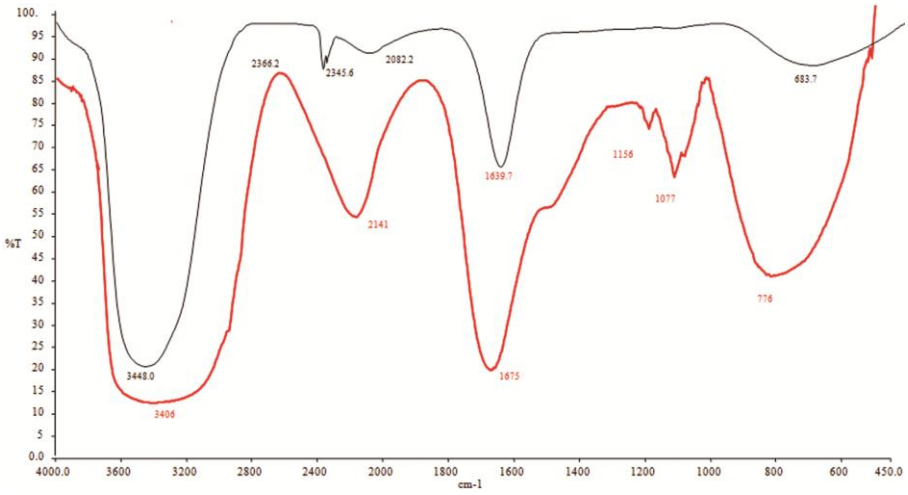

Fig. 7 — FT-IR before and after degradation

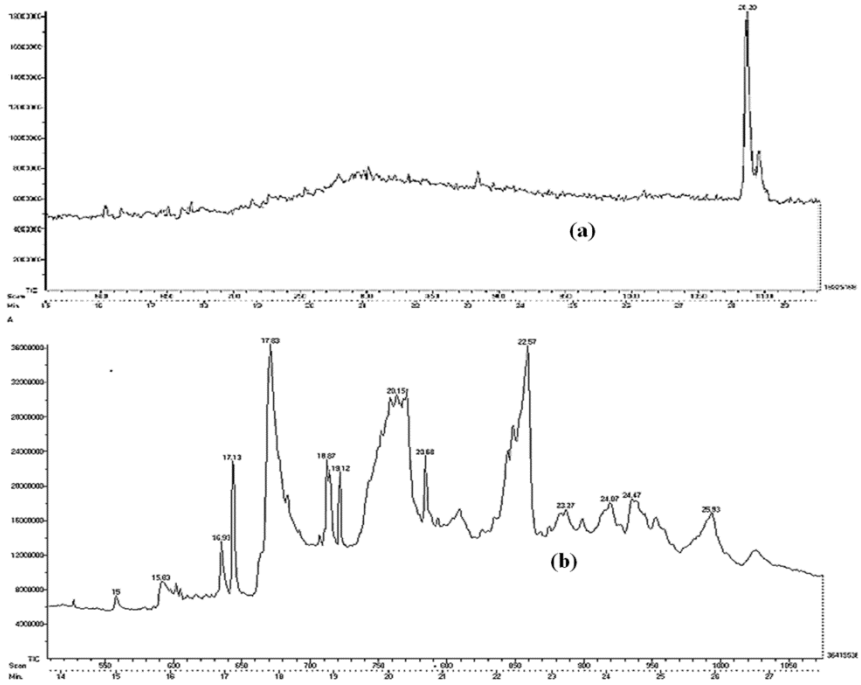

Fig. 8 — GC Curve for the dye solution (a) crude extract of dye (b) degradation products.

FTIR analysis of breakdown products

The bio-transformed metabolites were characterized by FTIR. The results of FTIR of Congo red and the sample obtained after decolourisation experiments show various peaks The dye before decolourisation experiments display peaks at 3406, 2141, 1675, 1156, 1077, 776 $cm^{-1}$for - NH (primary or secondary amine) stretching, -C=N, alkenes stretch, -N=N- stretching, -S=O, respectively. The IR spectra of degradation product displays six peak at 3448.0, 2366.2, 2345.6, 2082.2, 1639.7, 683.7 cm^{-1} (Fig. 7) show that the broad peak at 3406 cm^{-1} shifted as short 3448 cm^{-1} peak and formation of narrow sharp peak of 2366, 2345 cm^{-1} indicates that the vibration in secondary amine group the formation of alkenes. The peaks at 2141, 1675 cm^{-1} were disappeared and the peaks observed at 2082, 1639 cm^{-1} indicates the azo bond was undergoing certain changes. The disappearance of peak at 1156, 1077, 776 cm^{-1} due to the vibration of aromatic amines or may be due to vibration of carboxylic acids and -S=O stretch. The result confirms that the organisms are also capable to degrade the dye.

Identification of degradation pathway

The extract containing the degradation products of was subjected to GCMS analysis (Figs. 8, 9). The

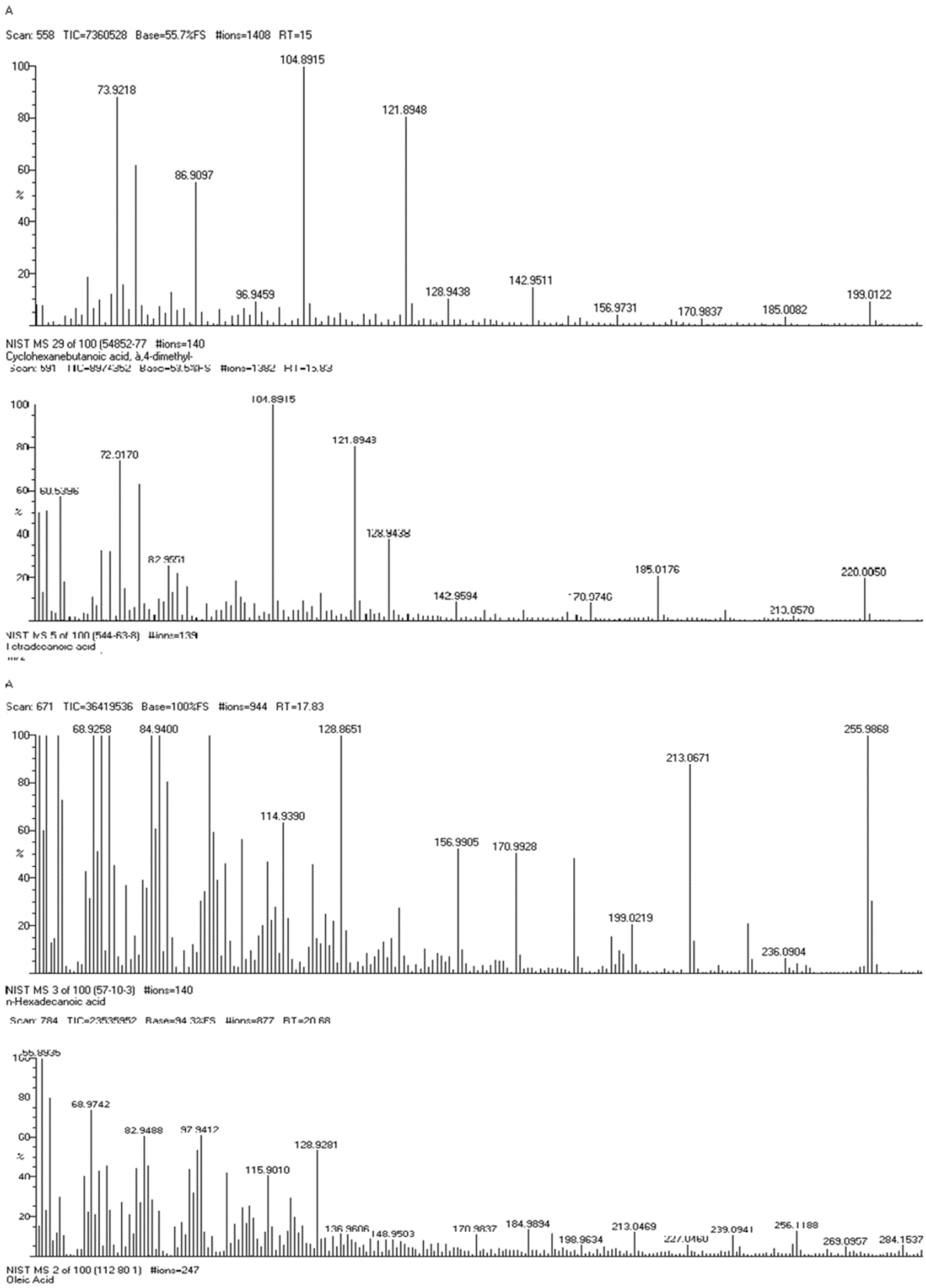

Fig. 9 — Mass spectra of the intermediate products.

result shows that the presence of fatty acids might have been formed by series of reaction such as azo reduction, desulfonation, oxidative deamination. The first step in the degradation pathway may be the reduction of azo group by azo reductase yields the formation of aromatic amines. These aromatic amines need oxygen for further breakdown to initiate metabolic degradation result in the formation of alcohols and acids. The formation of fatty acids such as cyclohexane butononic acid, Tetradecanonic acid (Myristic acid), oleic acid, Hexadecanonic acid (Palmitic acid) whose presence was confirmed from the spectra. These fatty acids undergo β-oxidation to form acetyl coA that enter into the pathway of tricarboxylic (TCA) cycle.

Conclusion

The bio-decolorization process studied a feasible and economical method of treating colored effluents. The dye is degradable under aerobic conditions with a concerted effort of immobilized bacterial cells. *Staphylococcus aureus* immobilized beads showed more and stability and the physical parameters had significant effect on dye decolourization that are optimized by using Response Surface Methodology. The optimum value for the degradation was found to be 35°C, for 400mg/L of concentration of dye at the medium *p*H of 7. The order of the reaction was found to be first order with the rate constant of 0.073 h^{-1}. The dye adsorbed on the bead surface follows Freundlich adsorption isotherm with the constant values are K = 1.368 and 1/n = 0.773. Studies were carried out by FTIR, GC-MS analysis for confirming the biodegradation of Congo red by the immobilized bacterial cells. Further the pilot model that showed an effective decolorization potential could be used in the industrial scale.

References

1 Jalandoni-Buan A C, Decena-Soliven A L A, Cao E P, Barraquio V L & Barraquio W L, *Philippine J Sci*, 139 (2010) 71.

2 Telke A A, Joshi S M, Jadhav S U, Tamboli D P & Govindwar S P, *Biodegradation*, 21 (2010) 283.

3 Brahimi-Horn M C, Lim K K, Liang S L & Mou D G, *J Indus Microbiol Biotechnol*, 10 (1992) 31.

4 Cripps C, Bumpus J A & Aust S D, *Appl Environ Microbiol*, 56 (1990) 1114.

5 Sundman G, Kirk T K & Chang H M, Journal of the Technical Association of the Pulp and Paper Industry (1981)

6 Glenn J K & Gold M H, *Appl Environ Microbiol*, 45 (1983) 1741.

7 Groff K A & Kim B R, *J Water Pollut Control Fed*, 61 (1989) 872.

8 Lade H, Kadam A, Paul D & Govindwar S, *EXCLI J*, 14 (2015) 158.

9 Haug W, Schmidt A, Nörtemann B, Hempel D C, Stolz A & Knackmuss H J, *Appl Environ Microbiol*, 57 (1991) 3144.

10 Horitsu H, Takada M, Idaka E, Tomoyeda M & Ogawa T, *Appl Microbiol Biotechnol*, 4 (1977) 217.

11 Idaka E, Ogawa T, Horitsu H & Tomoyeda M, *Color Technol*, 94 (1978) 91.

12 Karunya A, Rose C & Nachiyar C V, *World J Microbiol Biotechnol*, 30 (2014) 915.

13 Knapp J S, Newby P S, *Water Res*, 29 (1995) 1807.

14 Kulla H G, *Aerobic bacterial degradation of azo dyes. Microbial degradation of xenobiotics and recalcitrant compounds*. (Academic Press, London), 1981, 387.

15 McKay G, Waste color removal from textile effluents. American Dyestuff Reporter, 68 (1979) 29

16 Tatarko M & Bumpus J A, *Water Res*, 32 (1998) 1713.

17 Banat I M, Nigam P, Singh D & Marchant R, *Bioresour Technol*, 58 (1996) 217.

18 Nigam P, Singh D & Marchant R, *An investigation of the biodegradation of textile dyes by aerobic and anaerobic microorganisms. In Environmental Biotechnology*, (Springer, Netherlands), 1995, 278.

19 Ogawa T & Yatome C, *B Environ Contam Tox*, 44 (1990) 561.

20 Ohmomo S, Itoh N, Watanabe Y, Kaneko Y, Tozawa Y& Ueda K, *Agric Biol Chem*, 49 (1985) 2551.

21 Pasti-Grigsby, P A Goszczyanski S, Crawford RL & Crawford DL, *Appl Environ Microb*, 58 (1992) 3598.

22 Raghukumar C, Chandramohan D, Michel F C & Redd C A, *Biotechnol Lett*, 18 (1996) 105.

23 Ramasany R, Muhammad Ahmed H A & Karthik S S, *J Urban Environ Eng*, 6 (2012).

24 Schliephake K, Mainwaring D E, Lonergan G T, Jones I K & Baker W L, *Enzyme Microb Technol*, 27 (2000) 100.

25 Senan R C & Abraham T E, *Biodegradation*, 15 (2004) 275.

26 Shah M P, Patel K A, Nair S S & Darji A M, *OA J Biotechnol*, (UK), 1 (2013) 2.

27 Watanabe Y, Sugi R, Tanaka Y& Hayashida S, *Agric biol chem*, 46 (1982) 1623.

28 Wuhrmann K, Mechsner K L & Kappeler T H, *Appl Microbiol Biotechnol*, 9 (1980) 325.

29 Yatome C, Ogaw T, Hishida H & Taguchi T, *Color Technol*, 106 (1990) 280.

30 Zimmermann T, Kulla H G & Leisinger T, *The FEBS Journal*, 129 (1982) 197.