Chemical Engineering Journal 162 (2010) 495–502

Contents lists available at ScienceDirect

Chemical Engineering Journal

journal homepage: www.elsevier.com/locate/cej

Use of active consortia of constructed ternary bacterial cultures via mixture design for Congo Red decolorization enhancement

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ARTICLE INFO

Article history:
Received 11 March 2010
Received in revised form 24 May 2010
Accepted 26 May 2010

Keywords:
Azo dye
Bacterial decolorization
Response surface
Mixture design
Phytotoxicity

ABSTRACT

Azo dyes are widely used in textile dyeing which contains toxic and carcinogens compounds. So they must be treated before their discharge into the receptor medium. In this aim, this work is focused on the aerobic decolorization of water soluble azo dye Congo Red (CR) by bacterial consortium (*Sphingomonas paucimobilis*, *Bacillus* sp. and *Staphylococcus epidermidis*). The effect of different combinations of these three strains cultivated in the pure culture in mineral salts medium (MSM) on the decolorization of Congo Red (cell density fixed at OD600 = 1 with the addition of 750 ppm of the dye) was studied using equilateral triangle diagram and mixture experimental design to assess color and COD removal during species growth. Using software analysis (Minitab 14.0), the formulation of pure culture in MSM can be optimized for several responses and the best formulation can be obtained. The results suggested that the highest decolorization and chemical oxygen demand (COD) rates were 100% and 98%, respectively. Very high regression coefficient between the variables and the responses: decolorization and COD removal were R^2 = 32.69% and R^2 = 52.25%, respectively indicating an excellent evaluation of experimental data by polynomial regression model. FTIR (Fourier transform infrared) and UV–vis analysis confirmed the biodegradation of Congo Red by the bacterial consortium. The produced metabolites were extracted and their phytotoxicity study suggested their low toxicity.

1. Introduction

Azo dyes are the most commonly used in textile, cosmetic, paper-making, and food industry [1–4]. Over 7×10^5 t and approximately 10,000 different dyes and pigments are produced annually worldwide, about 10% of which may be found in wastewater [5].

Azo dyes are synthetic organic compounds widely used in textile dyeing. This chemical class of dyes, which is characterised by the presence of at least one azo bond (N=N) bearing aromatic rings, dominates the worldwide market of dyestuffs with a share of about 70% [5]. They are designed to convey high photolytic stability and resistance towards major oxidising agents [6]. The release of azo dyes into the environment in effluent from textile dyeing plants become a major concern in wastewater treatment, since they are highly recalcitrant to conventional wastewater treatment processes. The recalcitrance of azo dyes has been attributed to the presence of sulfonate groups and azo bonds, two features generally considered as xenobiotic [7]. In addition, some azo dyes or their metabolites may be mutagens or carcinogens [8]. As a result, it is of great interest to develop effective means to treat dye-bearing wastewater. Biotreatment usually has the advantage of low cost and high efficiency.

Several combined anaerobic and aerobic microbial treatments have been suggested to enhance the degradation of azo dyes [9]. Microbiological decolorization is an environmental-friendly and cost-competitive alternative to the chemical decomposition process [10]. Most studies on azo dye biodegradation have focused on bacteria and fungi [11,12]. Chang et al. [13] showed that mutant strain of *Escherichia coli* was able to decolorize azo dye C.I. Reactive red 22 to 8.2 mg dye g cell^{-1} h^{-1} and *Rhodobacter sphaeroides* was able to decolorize Methyl orange [14,15]. However, the results based on a number of experiments did not only fail to reach the best combination while spending a lot of experimental materials, but will also affect the progress of the study.

In this research, we used the experimental design (Minitab 14.0) to optimize the formulation of the predominant strains isolated from textile wastewater plant, in order to effectively explain the biodegradation of Congo Red. After biodegradation, the chemical

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doi:10.1016/j.cej.2010.05.050

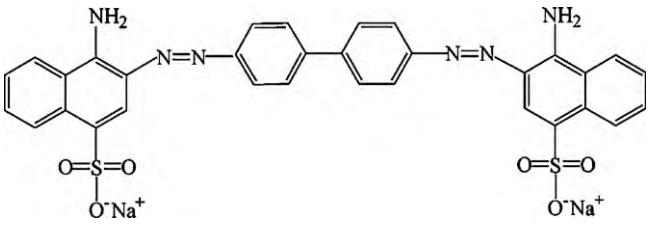

Fig. 1. Chemical structure of Congo Red (λ_{max} (nm) = 486).

oxygen demand (COD) and color removal were measured. The relationships between the different combinations and products were analyzed through the Minitab to select the optimal bacterial combination.

2. Material and methods

2.1. Chemicals

Congo Red ($C_{32}H_{22}N_6O_6S_2Na_2$, C.I. No. 22120) (Fig. 1) was purchased from Sigma–Aldrich. All chemicals used were of the highest purity available and of analytical grade.

2.2. Microbial strains

The strains of *Sphingomonas paucimobilis* (14×10^7 cfu), *Bacillus* sp. (4.2×10^8 cfu), *Staphylococcus epidermidis* (2.6×10^6 cfu), were isolated from the activated sludge of the textile wastewater treatment plant in KsarHellal, Tunisia. Cultivable Gram-negative and Gram-positive bacteria on Mac-conkey plates were isolated and identified. Colonies were selected according to routine phenotypic tests: morphological characteristics (shape, size, surface texture, color and opacity), Gram strain, respiration/fermentation, catalase in order to identify the bacterial species, strain's biochemical Api 20NE and Api ID32 Staph (Table 1) (bio-Mérieux, 69280 Marcy-l'Etoile/France).

The culture was cultivated and maintained by weekly transfers on to nutrient agar slants. For production experiments, the culture was revived in nutrient broth (pH 7.0) and freshly prepared 3 h old culture ($\lambda_{600\,nm} = 1$) in MSM which contained (per liter of distilled water): $MgSO_4$: 0.1; $(NH_4)_2SO_4$: 0.6; NaCl: 0.5; K_2HPO_4: 1.36; $CaCl_2$: 0.02; $MnSO_4$: 1.1 mg/l; $ZnSO_4$: 0.2 mg/l; $CuSO_4$: 0.2 mg/l; $FeSO_4$: 0.14 mg/l. The culture was shacked at 150 rpm (New Brunswick Scientific Shaker, Edison, NJ) and at 37 °C. 10% of bacteria were used as the inoculum.

2.3. Acclimatization

The acclimatization was performed by gradually exposing *S. paucimobilis*, *Bacillus* sp. and *S. epidermidis* to higher concentrations of Congo Red (CR) [16]. These bacteria were grown for 24 h at 30 °C in 250 ml Erlenmeyer flasks containing in g/l: yeast extract: 3.0 and glucose: 1.25 (pH 7.0). During the investigation, nutrient broth concentration was decreased from 90% (w/v) to 0% (w/v). Acclimatization experiments were carried out at optimum temperature [16,17].

2.4. Analytical methods

Absorbance of the supernatant withdrawn at different time intervals were measured at the maximum absorption wavelength for the dye CR (λ_{max} = 486 nm) using a Shimadzu double beam spectrophotometer (UV 1601). The chemical oxygen demand was carried out with O'Dell and James [18] micro method, on samples extracted from biodegradation tests of Congo Red at 0.1 and 0.5 g/l

Table 1
Physiological characteristics, APi 20NE and ID32 Staph phenotypical profile of strains.

Tests	Api 20NE
Gram strain and morphology	Gram-negative rods
Oxidase activity	+
Catalase activity	+
Mobility	+
Respiration/fermentation	AS/NF
Reduction of nitrate to nitrite	–
Indol	–
Fermentation of glucose	–
Arginine	–
Urease	–
Hydrolysis of esculine	+
Hydrolysis of gelatine	–
Production of β-galactosidase	+
Assimilation of glucose	–
Arabinose	+
Mannose	+
Maltose	–
N-acetyl-glucosamine	+
Maltose	+
Gluconate	+
Caprate	–
Adipate	–
Malate	+
Citrate	–
Phenyl acetate	–
Identification	*Sphingomonas paucimobilis*
Tests	**Api ID32 Staph**
Gram strain and morphology	Gram-positive cocci
Urease	+
Arginine deshydrolase	–
Ornithine decarboxylase	–
Hydrolysis of esculine	–
Fermentation of glucose	+
Fructose	+
Mannose	+
Maltose	+
Lactose	+
Trehalose	–
Mannitol	–
Raffinose	–
Reduction of nitrate to nitrite	+
Production of acetone	+
Production of β-galactosidase	–
Arginine arylamidase	–
Phosphatase alkaline	+
Pyrrolydonyl arylamidase	–
Novobiocine	–
Saccharose	+
N-acetyl-glucosamine	–
Turanose	–
Arabinose	–
β-Glucoronidase	–
Ribose	–
Cellobiose	–
Identification	*Staphylococcus epidermidis*

concentrations. The initial absorbance of the dye was used as a reference. The decolorization and COD removal were calculated according to the following equations [17,19,20]:

$$\text{color removal}\,(\%) = \frac{I - F}{I} \times 100 \quad (1)$$

where I is the initial concentration of CR and F is the concentration of CR at time t (min).

$$\text{COD removal}\ (\%) = \frac{\text{initial COD} - \text{observed COD}(t)}{\text{initial COD}} \times 100 \quad (2)$$

Table 2
Mixture design matrix with the experimental.

Assay	*Sphingomonas paucimobilis*	*Bacillus* sp.	*Staphylococcus epidermidis*	Total	COD removal (%)	Decolorization (%)
1	1.00000	0.00000	0.00000	1.00000	94.3750	41.5370
2	0.00000	1.00000	0.00000	1.00000	98.2250	68.3062
3	0.00000	0.00000	1.00000	1.00000	94.5125	90.4554
4	0.50000	0.50000	0.00000	1.00000	94.1875	46.1538
5	0.50000	0.00000	0.50000	1.00000	94.0625	46.1558
6	0.00000	0.50000	0.50000	1.00000	95.5125	92.2592
7	0.33333	0.33333	0.33333	1.00000	95.0500	90.4938
8	0.66667	0.16667	0.16667	1.00000	99.0750	97.0927
9	0.16667	0.66667	0.16667	1.00000	97.4875	97.1986
10	0.16667	0.16667	0.66667	1.00000	95.3875	94.3767

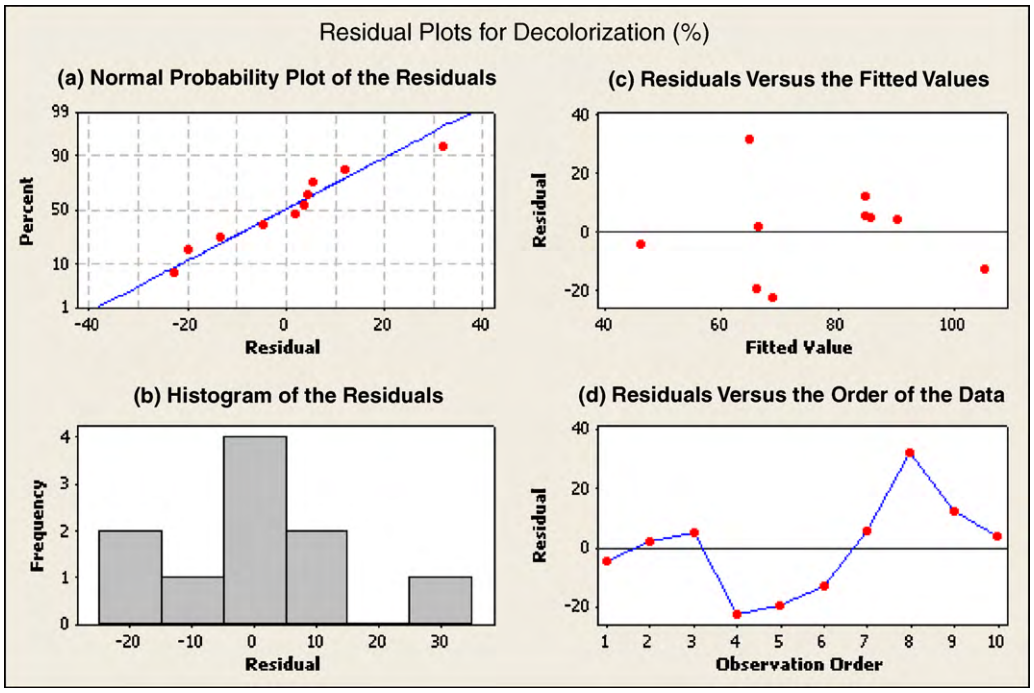

Fig. 2. (a) Normal probability plot of the residuals; (b) histogram of the residuals; (c) residual versus the fitted value; (d) residual versus the order of the data.

2.5. Experimental design

The D-optional method in the experimental design, provided by the software Minitab (Ver. 14.0, U.S. Federal Government Commonwealth of Pennsylvania, USA), was used to optimize the formulation of the above microbial consortium strains. Generally, the mixture design was used to study the relationships between the proportion of different rate of bacteria (Table 1) and responses (decolorization and COD removal). Since Scheffe devised a single-lattice and single-core design in 1958, the mixture design has developed a variety of methods [21,22].

In this study, *S. paucimobilis*, *Bacillus* sp. and *S. epidermidis* were used as mixture starters, ranging from 0 to 100%, as shown in Table 2. Decolorization experiments was taken according to the ratio given by the experiment design, and 10% of mixed culture which were inoculated into the MSM solution (3.0 g/l yeast extract and 1.25 g/l glucose and 750 ppm of CR) at 37 °C for 10 h in shaking conditions (150 rpm).

The *P*-value is the probability that the magnitude of a contrast coefficient is due to random process variability. A low *P*-value indicates a "real" or significant effect. The significance of each variable was determined by applying the Student's *t*-test [23,24]. The statistical analyses were performed by use of multiple regressions and ANOVA with the softwares Minitab v 14.0 and Essential Regression v 2.2.

2.6. UV–vis spectral analysis FTIR

Decolorization was monitored by UV–vis spectroscopic analysis, whereas biodegradation was monitored using FTIR spectroscopy.

Table 3
Analysis of variance of COD% (ANOVA) for the selected linear plus interactions model for Congo Red.

Source	Degrees of freedom	Sum of square	Sum of adjusted squares	Adjusted average squares	*F*-Ratio	*P*-Value (significance)
Regression	5	9.6694	9.6694	1.93387	0.39	0.836
Linear regression	2	8.5929	9.2080	4.60401	0.93	0.467
Quadratic regression	3	1.0765	1.0765	0.35883	0.07	0.972
Residual error	4	19.9056	19.9056	4.97641		
Total	9	29.5750				

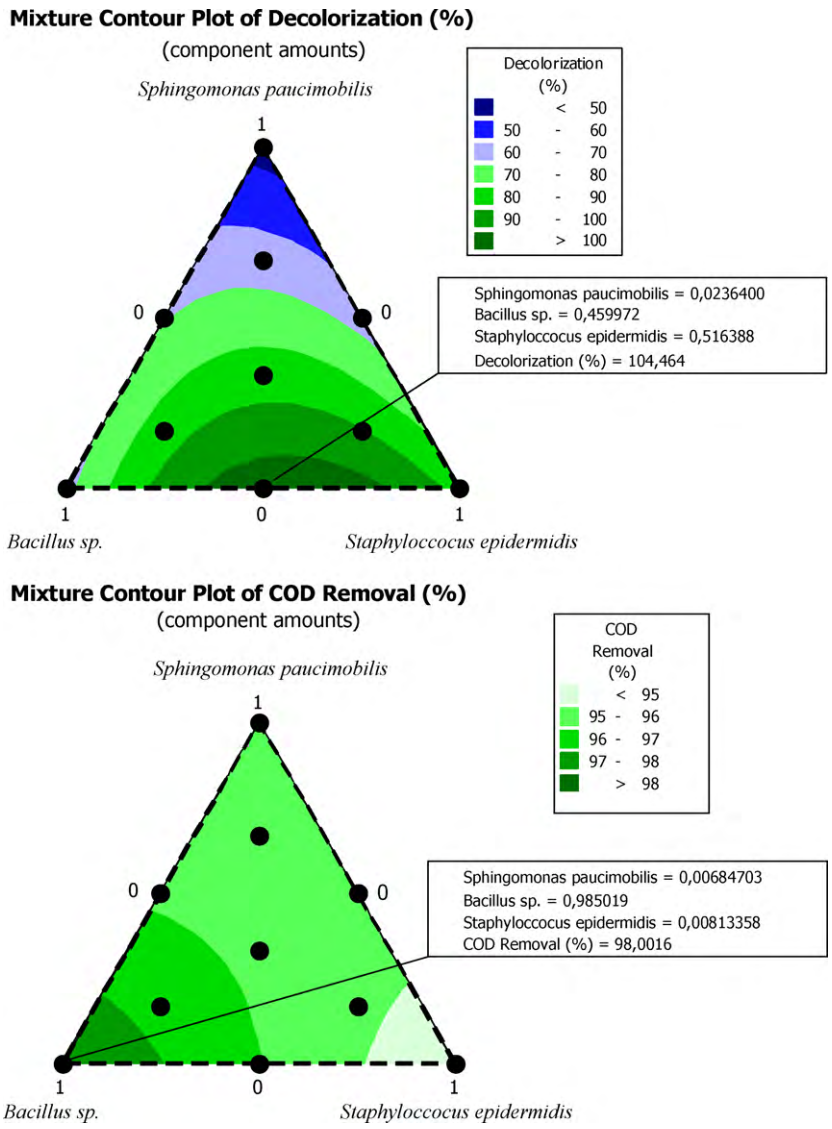

Fig. 3. Mixture contour plots between the variables (*Sphingomonas paucimobilis*, *Bacillus* sp. and *Staphylococcus epidermidis* contents) for decolorization and COD removal.

The decolorization of the dye was followed by monitoring, changes in its absorption spectrum (200–700 nm) using a Hitachi UV–Vis spectrophotometer (Hitachi U-2800). The obtained results were compared to those of the respective controls. The produced metabolites during the dye biodegradation (after decolorization of medium) were centrifuged at 15,000 rpm for 30 min after complete degradation of adsorbed dye to remove any remained bacterium. Metabolites were extracted from supernatant by adding equal volume of ethyl acetate; the samples were used for UV–vis spectral analysis. FTIR analysis was carried out using Perkin Elmer 783 Spectrophotometer (Nicolet Analytical Instruments, Madison, WI) and changes in % transmission at different wavelengths were observed. The samples were mixed with spectroscopically pure KBr in the ratio 2:200, pellets were fixed in the sample and the analysis was carried out [19,20].

2.7. Phytotoxicity and microbial toxicity studies

Phytotoxicity tests were conducted to assess the impact of the treated colored water on vegetation. We have assessed the toxicity of the untreated and treated samples at the concentration of 750 ppm. Tests were carried out according to the ISO (1993) [25] on two kinds of seeds: *Sorghum bicolor* and *Triticum aestivum*. The microbial toxicity was carried out using *S. paucimobilis* on Mueller and Hinton agar plate having composition 1% peptone, 0.5% NaCl, 0.3% yeast extract and 2.5% agar [19].

3. Results and discussion

3.1. Model establishment

Through linear regression fitting, the regression models of two responses (COD (%) and decolorization (%)) were established. The regression model equations are as follows:

$$Y_{\text{decolorization\%}} = 95.142\,S1 + 98.077\,S2 + 94.140\,S3 + (-3.068)S1 \times S2 + (3.409)S1 \times S3 + (-0.320)S2 \times S3, \quad R^2 = 32.69\%;\ P = 0.567$$

$$Y_{\text{COD}} = 46.23\,S1 + 66.403\,S2 + 85.769\,S3 + 50.022(S1 \times S2) + 0.165(S1 \times S3) + 117.848(S2 \times S3), \quad R^2 = 52.25\%;\ P = 0.836$$

where S1: *S. paucimobilis*; S2: *Bacillus* sp.; and S3: *S. epidermidis*.

Table 4
Analysis of variance of % decolorization (ANOVA) for the selected linear plus interactions model for Congo Red.

Source	Degrees of freedom	Sum of square	Sum of adjusted squares	Adjusted average squares	*F*-Ratio	*P*-Value (significance)
Regression	5	2582.37	2582.37	516.474	0.88	0.567
Linear regression	2	1756.38	859.96	429.979	0.73	0.537
Quadratic regression	3	825.99	825.99	275.329	0.47	0.721
Residual error	4	2359.75	2359.75	589.936		
Total	9	4942.11				

3.2. Effect of formulation on color and COD removal

Recently, the mixture design is widely used. It can estimate the relationship between formulation and performance through regression analysis in fewer experiment times [26]. In the mixture design, the effect of the change of variables on the responses can be observed on the ternary contour map. Fig. 2 shows the effect of the interaction of *S. paucimobilis*, *Bacillus* sp. and *S. epidermidis* on the COD removal. The statistical significance (ANOVA analysis) of the ratio of mean square variation due to the regression and mean square residual error was tested using analysis of variance.

Only obtained results for decolorization and COD removal were presented for clarity of purpose. According to the ANOVA analysis (Tables 3 and 4), the regression adjusted average square and the linear regression adjusted average square were 9.6694 and 9.2080, respectively which allowed the calculation of the Fisher ratios (*F*-value) for assessing the statistical significance. The model *F*-value (0.39) implies that most of the variation in the response can be explained by the regression equation. The associated *P*-value is used to judge whether *F*-ratio is large enough to indicate statistical significance. A *P*-value more than 0.1 (i.e. $\alpha = 0.05$ or 95%) indicated that the model could not be considered statistically significant. The non-significant value of lack of fit (>0.05) revealed that the quadratic model is statistically significant for the response and therefore it can be used for further analysis [27].

The *P*-value for the regression obtained $R^2 = 32.69\%$; $P = 0.567$ for decolorization was more than 0.1 and means consequently that at least one of the term in the regression equation has significant correlation with the response variable. The ANOVA test also shows a term for residual error, which measures the amount of variation in the response data left unexplained by the model [28].

In the mixture design, the effect of variable changes on the responses can be observed on the ternary contour map. In this study three variables could be compared. Fig. 3 (mixture contour plot) and Fig. 4 (surface plot) showed the effect of the interaction of *S. paucimobilis*, *Bacillus* sp. and *S. epidermidis* on the decolorization and COD removal. In order to confirm the experimental results that 97.19% of decolorization was obtained and 97.48% of COD was removed, a mixture contour plot (Fig. 3) and surface plot (Fig. 4) were plotted by MINITAB® 14 Software Programme, where maximum values percentage of decolorization (100%) and COD removal (98%) which was predicted if the *S. paucimobilis*, *Bacillus* sp. and *S. epidermidis* proportion were (0.023%, 0.459% and 0.516%) and (0.006%, 0.985% and 0.008%).

The lines of contour plots (Fig. 3.) predict the values of each response at different proportion of *S. paucimobilis*, *Bacillus* sp. and *S. epidermidis*. These values are similar to the experimental values.

The mixture surface plots (Fig. 4), presented by a three-dimensional graph using color and COD removal based on the simultaneous variation of *S. paucimobilis*, *Bacillus* sp. and *S. epi-*

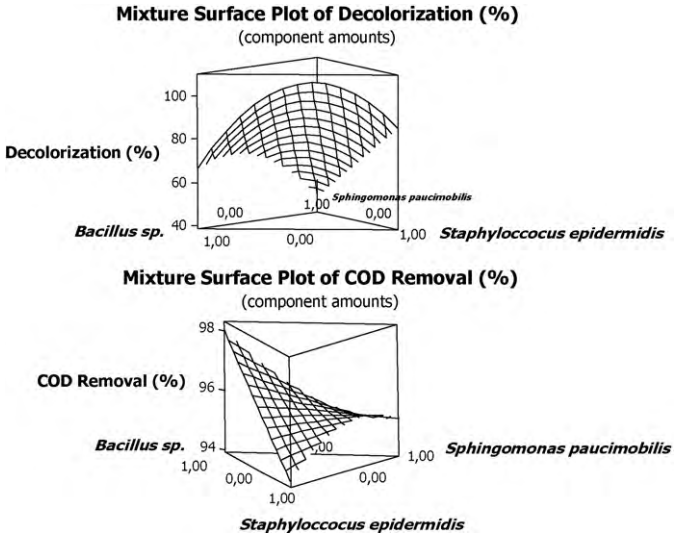

Fig. 4. Response surface plot and its contour plot of decolorization (*Sphingomonas paucimobilis*, *Bacillus* sp. and *Staphylococcus epidermidis* contents).

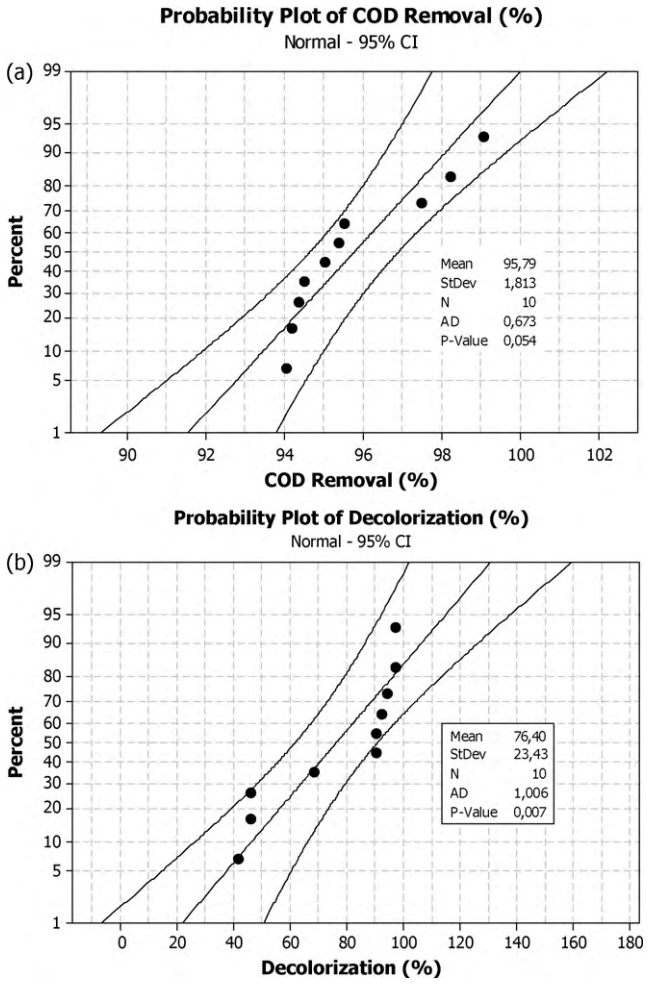

Fig. 5. Normal probability plot of the residuals: COD (a) and decolorization (b) removal. A model at optimal treatment conditions.

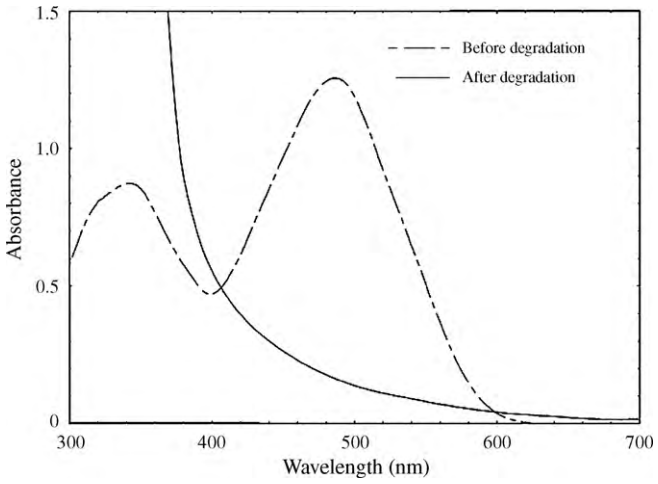

Fig. 6. UV–vis spectra of Congo Red (750 ppm) biodegraded by bacterial consortium (*Sphingomonas paucimobilis*, *Bacillus* sp. and *Staphylococcus epidermidis*) before and after degradation. Temperature = 30 °C; pH = 7.0.

dermidis composition from 0 to 100% for each strain. The mixture surface plot also described the individual and cumulative effect of these three variables (strain) and tested their subsequent effect on the response (color and COD removal) [29].

The normal probability plot of the residuals for COD and decolorization is shown in Fig. 5a and b. All data points lie close to a straight line and within the 95% confidence interval lines. These results indicate that the calculated residuals follow a normal distribution with mean values near zero. According to the above observations, it can be concluded that there is a good agreement between the experimental values and the mathematical model developed and the observed differences (i.e. the residuals) may be readily explained as random noise [30].

3.3. Interpretation of residual graph

The normal probability plot, Fig. 2(a), showed that the distribution of residual value which is defined as the difference between the predicted (model) and observed (experimental) (Table 2) are forming a straight line and residual value are normally distributed on the both side of the line. They indicated that experimental points are reasonably aligned with predicted value. The histogram, presented in Fig. 2(b), of the residuals showed the distribution of the residuals for all the observations. The one long tail in the plot indicated skewness in the data whereas one bar is far from the others, these points was outlined. The plot between individual residual values and in the fitted value showed that all the residuals are scattered randomly about the zero and one or two points are outliners (Fig. 2(c)).

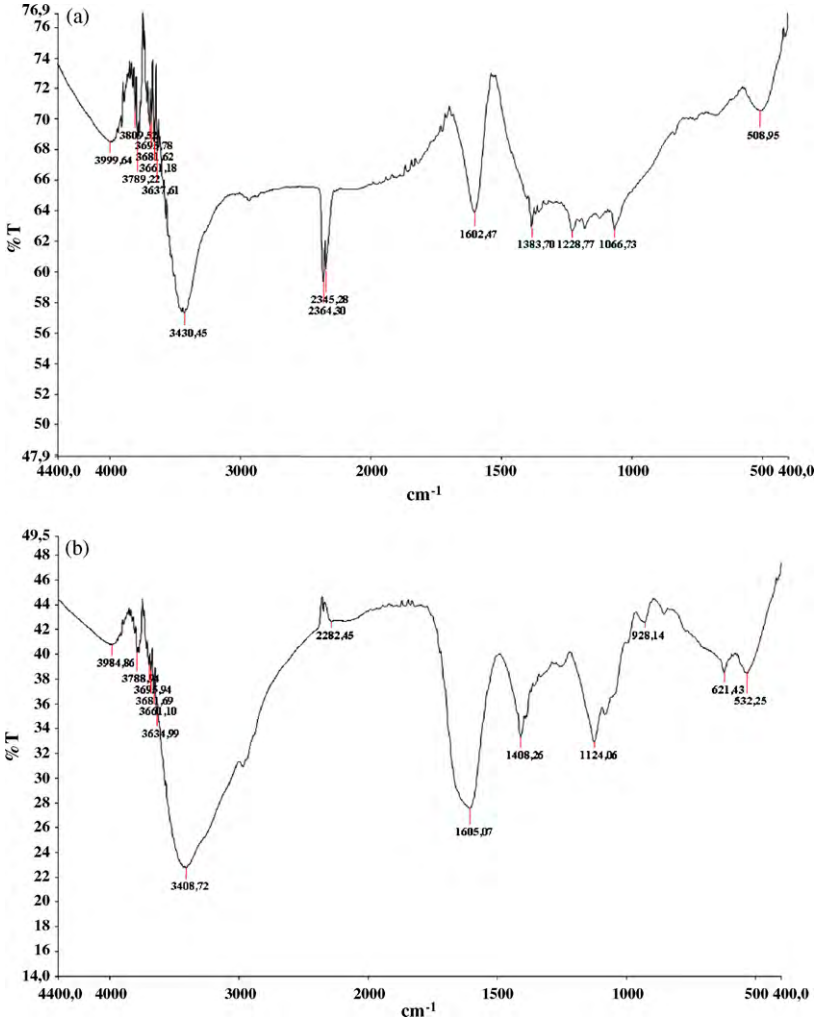

Fig. 7. FTIR spectra of Congo Red (a) and its degradation metabolites (b) (6 h). (*Sphingomonas paucimobilis*, *Bacillus* sp. and *Staphylococcus epidermidis*) Temperature = 30 °C; pH = 7.0.

Table 5
Phytotoxicity study of Congo Red and its degradation product.

Parameters studied	*Triticum aestivum*			*Sorghum bicolor*		
	MSM solution	Congo Red (750 ppm)	Extracted metabolites (750 ppm)	MSM solution	Congo Red (750 ppm)	Extracted metabolites (750 ppm)
Germination (%)	100	30	90	100	40	85
Shoot (cm) (±SD)	3.93 ± 0.12	0.05 ± 0.15	5.9 ± 0.29	2.48 ± 0.18	0.13 ± 0.02	6.8 ± 0.35
Root (cm) (±SD)	3.83 ± 0.12	0.02 ± 0.04	6.0 ± 0.37	4.37 ± 0.34	0.14 ± 0.05	6.2 ± 0.03

Values are mean of germinated seeds of three experiments. SD (±), standard deviation.

The plot of Fig. 2(d) is the residual value and the order of the corresponding observations. This plot is useful when the order of the observations may affect the results which can occur when data are collected in a line sequence. This plot can be helpful to a designed experiment in which the runs are not randomized. For residual activity data, the residuals appear to be randomly scattered about zero. No evidence exists that the regression terms are correlated each other.

3.4. Decolorization and biodegradation analysis

The decolorization was monitored by UV–vis spectroscopic analysis, whereas the biodegradation was monitored using FTIR spectroscopy. The decolorization of the dye was followed by monitoring, changes in the absorption spectrum (200–700 nm) and comparing the results, to those of the respective controls. The UV–vis spectrum of Congo Red after 6 h was presented in Fig. 6. This figure showed the decrease in optical density. The observed peaks at (486 nm) initial time (0 h) decreased without any shift in λ_{max} up to complete decolorization of the medium.

The comparison of FTIR spectrum using extracted metabolites and control dyes, clearly indicated the biodegradation of the dye by the mixed culture. Furthermore, we noted that FTIR spectra of Congo Red obtained before and after decolorization experiments showed various peaks. The FTIR spectra of the control (Fig. 7a) displays two bands at 3789.22 and 3430.45 cm^{-1} for =C–H and O–H stretch vibration, respectively. The band obtained at 1602.47 cm^{-1} corresponds to C=C stretch vibration. The bands obtained at 1383.7, 1228.77 and 1066.73 cm^{-1} indicated CH_3 symmetric bend, C–O stretch vibration and S–O stretch vibration, respectively.

The FTIR spectrum of 6 h (Fig. 7b) of extracted metabolites showed significant changes in the positions of bands compared with the control spectrum dye.

In 6 h extracted metabolites, bands at 3430.45, 1602.47, 1384.7, 1228.77 and 1066.73 cm^{-1} shifted to 3408.72, 1408.26, 1124.06 (=C–H in plane deformation vibration), 928.14 (C–H deformation) and 621.43 cm^{-1} respectively.

The sharp band at 621.43 cm^{-1} for mono-substituted acetylene benzene derivative indicated the aromatic nature of Congo Red. The other details of the changes in FTIR spectra after hydrolysis are listed in Fig. 7. Most of the changes in spectrum are related to the hydrogen bonding in starch molecules, indicating the hydrolysis treatment significantly changed the inter/intra-molecular hydrogen bonding of starch molecules.

3.5. Phytotoxicity and microbial toxicity study

Fig. 8 shows phytotoxicity analysis of the Congo Red dye before and after degradation. The relative sensitivity towards the dyes and degradation products in relation to *S. bicolor* and *T. aestivum* was studied (Table 5). The plumule length and radical indicated less toxicity of the degradation product to the plants. Parshetti et al. [31] showed that germination of *T. aestivum* was less with Congo Red treatment as compared to its degradation product. Hence phytotoxicity studies revealed that biodegradation of dyes by a microbial

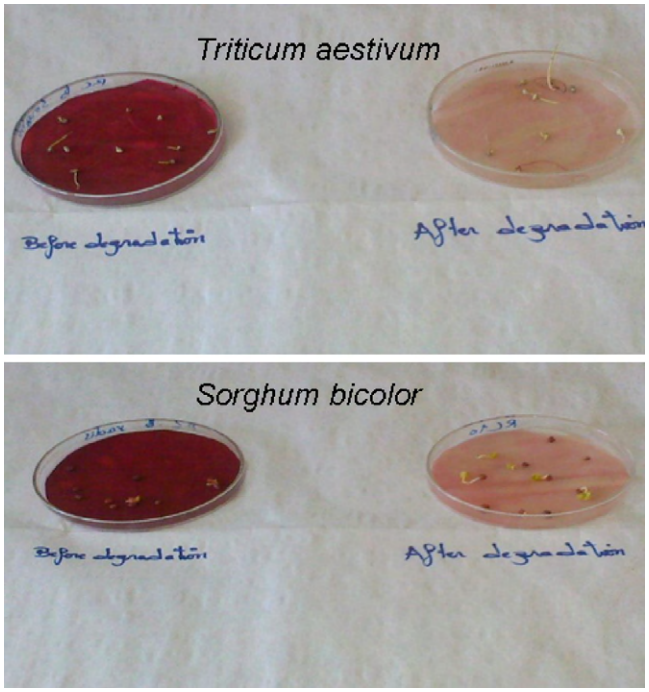

Fig. 8. Phytotoxicity study in *Sorghum bicolor* and *Tritium aestivum* before and after decolorization of Congo Red (750 ppm) by a bacterial consortium (*Sphingomonas paucimobilis*, *Bacillus* sp. and *Staphylococcus epidermidis*).

culture, resulted in their detoxification. Thus treated effluent can be used for ferti-irrigation. An inhibition zone was observed with control dyes using microbial consortium. In fact, this inhibition zone was 0.9 ± 0.0 cm, 0.99 ± 0.047 cm and 0.83 ± 0.007 cm obtained at the concentration of 500 ppm by *S. paucimobilis*, *Bacillus* sp. and *S. epidermidis*, respectively and 1.25 ± 0.01 cm, 1.18 ± 0.04 cm and 1.45 ± 0.03 cm, respectively at the concentration 750 ppm. The degradation products did not showed an inhibition growth. These findings suggest the no-toxic nature of these formed products. Previous works showed a similar toxicity between Malachite Green and Crystal Violet and their degradation products (leucomalachite and leucocrystal violet) [32].

4. Conclusion

In this paper, the mixture design method was used for the optimization of bacteria combination for the decolorization in biodegradation processes to explore a new optimization method of mixed culture. This study showed that the decolorization by mono and combined mixed cultures *S. epidermidis* and *Bacillus* sp. contributed for the major decolorization activity of the dye biotreatment. Moreover, *Bacillus* sp. contributed for the majority of COD removal.

The establishment of the regression model and the analysis of the interaction between the variables showed that the mixture design was proved to be effective for the optimization of mixed

decolorizing starter involving several species. This method will cut down the development cycle of novel culture starter and improve the accuracy of the experimental design.

The UV spectral data and FTIR confirmed the observed experimental results on COD and color removal. Congo Red was degraded into non-toxic compound by the studied microbial consortium. The results suggested that the highest decolorization and chemical oxygen demand (COD) rates were 100% and 98%, respectively. Very high regression coefficient between the variables and the responses: decolorization and COD removal were R^2 = 32.69% and R^2 = 52.25%, respectively.

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