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Response surface methodology for decolorization of azo dye Methyl Orange by bacterial consortium: Produced enzymes and metabolites characterization

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ABSTRACT

The use of chemometric methods such as response surface methodology (RSM) based on statistical design of experiments (DOEs) is becoming increasingly widespread in several sciences such as analytical chemistry, engineering and environmental chemistry. In the present study, the decolorization and the degradation efficiency of Methyl Orange (MO) was studied using a microbial consortium. The microbial growth of *Sphingomonas paucimobilis*, *Bacillus cereus* ATCC14579, *Bacillus cereus* ATCC11778 is well in the presence of MO (750 ppm) within 48 h at pH 7 and 30 °C. In fact, these microorganisms were able to decolorize and to degrade MO to 92%. The degradation pathway and the metabolic products formed during the degradation were also predicted using UV–vis, Fourier transform infrared (FTIR) spectroscopy and nuclear magnetic resonance (NMR) spectroscopy analysis. Under optimal conditions, the bacterial consortium was able to decolorize completely (>84%) the dye within 48 h. The color and COD removal were 84.83% and 92.22%, respectively. A significant increase in azoreductase, lignin peroxidase and laccase activities in the cells were obtained after complete decolorization. Phytotoxicity study using plants showed no toxicity of the produced products.

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1. Introduction

The textile industry wastewater is rated as the most polluting among all industrial sectors in terms of both volume and composition of the effluents [1,2]. The color in these discharged wastewater is due to synthetic dyes left unused due to industrial inefficiencies. Presently over 10,000 different dyes and pigments are used in dyeing and printing industries all over the world. Many of them are believed to be toxic and carcinogenic [3].

Almost 7×10^5 tones of dyes are produced around the world every year, and most of them are azo dyes containing one or more azo groups (R1–N=N–R2), which are extensively used as industrial raw materials [4]. It is quite undesirable to discharge azo dyes with different color into the environment due to their higher pollution and toxic intermediates produced [5]. Compared with chemical and physical methods, the biological treatment has been the main focus for the degradation of these dyes, which can produce lower costs and fewer toxic resultants [6,7]. Many microorganisms, belonging to bacteria, fungi, even yeasts proved their ability to decolorize azo dyes by bioadsorption or degradation. Among these microorganisms, bacteria and fungi which played a key roles in the treatment of wastewater containing dyes, and it has been proved that can they decolorize dyes with different types of enzymes [8].

Recently, azoreductase activity was detected in many bacteria, such as *Sphingomonas xenophaga* BN6, *Pigmentiphaga kullae* K24 and *Caulobacter subvibrioides* C7-D [9–11]. There is a little application for practical treatment although many pure cultures are available to decolorize azo dyes, which can be supported by three reasons [12]. Firstly, fungi cannot use azo dyes as sole carbon and energy source, and their growth is time consuming. Secondly, low efficiency of bacteria degrading azo dyes is achieved under aerobic conditions, because oxygen is a more efficient electron acceptor compared with azo dye. Thirdly, single strains cannot adapt the complex and variable environment conditions. Therefore, mixed microbial populations are expected to perform better than single microorganisms [13]. However, there is a little information about using fungal–bacterial consortium to decolorize azo dyes.

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Table 1
Mixture design matrix with the experimental analysis.

Assay	*Sphingomonas paucimobilis*	*Bacillus cereus* ATCC14579	*Bacillus cereus* ATCC11778	Total	COD removal (%)	Decolorization (%)	Germination (%)
1	1.00	0.00	0.00	1.00	89.67	82.80	70
2	0.00	1.00	0.00	1.00	90.40	84.83	85
3	0.00	0.00	1.00	1.00	90.18	81.84	82
4	0.50	0.50	0.00	1.00	88.43	80.21	65
5	0.50	0.00	0.50	1.00	92.22	79.86	87
6	0.00	0.50	0.50	1.00	91.57	84.83	85
7	0.33	0.33	0.33	1.00	88.98	84.06	67
8	0.66	0.16	0.16	1.00	90.11	79.67	83
9	0.16	0.66	0.16	1.00	90.25	82.40	84
10	0.16	0.16	0.66	1.00	89.71	77.67	75

Statistical optimization method (a central composite design coupled with response surface methodology (RSM)) overcomes the limitations of classical methods and was successfully employed to obtain the optimum process conditions while the interactions between process variables were demonstrated [14].

The importance and theoretical concepts behind the optimization through experimental design as well as RSM in research and development efforts have been thoroughly discussed in a number of informative articles and the sequential steps of RSM are also highlighted in subsequent sections of this study. RSM is nowadays, a promising as well as a powerful tool for multivariate optimization through sequential experimentation. Several researchers have already been using various RSM approaches to explain optimization process [15].

The present work aims to study the ability of *Sphingomonas paucimobilis*, *Bacillus cereus* ATCC14579 and *Bacillus cereus* ATCC11778 to decolorize MO. However, the selection of optimal conditions for the growth and the different proportions of the three microorganisms using the response surface methodology can ameliorate the decolorization performances of the cells for MO. The produced metabolites during the degradation were also predicted using UV–vis FTIR spectroscopy and nuclear magnetic resonance (NMR) spectroscopy analysis. These metabolites were studied for their toxicity using the plants phytotoxicity.

2. Material and methods

2.1. Dye and chemicals

The commercially used textile azo dye Methyl Orange ($C_{14}H_{14}N_3NaO_3S$, E.C. No. 2089253; λ_{max} = 466 nm) was purchased from the Sigma–Aldrich (Chemical Company, MO, USA) and used for the study without any further purification.

Reduced nicotinamide adenine dinucleotide (NADH), 2,2′-azinobis (3-ethylbenzthiazoline)-6-sulfonate (ABTS) and methyl sulfoxide-*d*6, 99.9 at% D (contains 0.03% v/v TMS) (DMSO *d*6) were purchased from Sigma–Aldrich Chemicals, USA. Sodium phosphate buffer (PBS), tartaric acid, acetate buffer and n-propanol were purchased from Biorad, USA. All chemicals used were of the highest purity available and of analytical grade.

2.2. Microorganisms and culture media

Three microorganisms, *S. paucimobilis*, *Bacillus cereus* ATCC14579, *Bacillus cereus* ATCC11778 were used in this study. *S. paucimobilis* was isolated in previous works of Ayed et al. [16,17] with the ability of degrading azo and triphenylmethane dyes (Congo Red, Methyl Red, Malachite Green and Crystal Violet). *Bacillus cereus* ATCC14579 and *Bacillus cereus* ATCC11778 are a reference strains. The used medium was composed in 1000 ml of distilled water: glucose (1250 mg/L), yeast extract (3000 mg/L), $MgSO_4$ (100 mg/L); $(NH_4)_2SO_4$ (600 mg/L); NaCl (500 mg/L); K_2HPO_4 (1360 mg/L); $CaCl_2$ (20 mg/L); $MnSO_4$ (1.1 mg/L); $ZnSO_4$ (0.2 mg/L); $CuSO_4$ (0.2 mg/L); $FeSO_4$ (0.14 mg/L) and it was maintained at a constant pH of 7 by the addition phosphate buffer [16,17].

2.3. Experimental design and methods

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 microbial consortium. Generally, the mixture design was used to study the relationships between the proportion of different variables and responses. Ever since Scheffe devised a single-lattice and single-core design in 1958, the mixture design has developed a variety of methods [18,19].

Response surface methodology (RSM) is usually applied following a screening study to explore the region of interest of the factors identified by the preceding study [15]. The mixture design is widely used in the formulation of food experiment, chemicals, fertilizer, pesticides, and other products. It can estimate the relationship between formulation and performance through regression analysis in fewer experiment times [20].

In this study, *S. paucimobilis*, *Bacillus cereus* ATCC14579 and *Bacillus cereus* ATCC11778 were used as mixture starters, with different proportions ranging from 0 to 100%, as shown in Table 1. Decolorization experiments were taken according to the ratio given by the experimental design, and 10% of mixed culture were inoculated into the Mineral Salt Medium (MSM) (3.0 g/L yeast extract and 1.25 g/L glucose and 750 ppm MO) at 37 °C for 10 h in shaking conditions (150 rpm) [21,22].

2.4. Statistical analysis

The statistical analyses were performed by the use of multiple regressions and ANOVA with the softwares Minitab v 14.0 and Essential Regression v 2.2. The significance of each variable was determined by applying Student's *t*-test [23,24].

The *P*-value is the probability that the magnitude of a contrast coefficient is due to random process variability. A low *P*-value indicated a "real" or significant effect.

2.5. Color and COD removal

Chemical oxygen demand (COD) was determined spectrometrically by 5B-1 Quick COD analyzer (LianHua Environmental Instrument Institute, Langzhou, PR China). The color of the influent and the effluent was monitored spectrophotometrically (Hach DR 2000). Color and COD removal were determined using the following equations [17].

$$\text{Color removal}\,(\%) = \frac{A_i - A_t}{A_i} \times 100 \quad (1)$$

where A_i was the initial absorbance and A_t was the absorbance at incubation time t.

$$\text{COD removal}(\%) = \frac{\text{initial COD}(0\,\text{h}) - \text{observed COD}(t)}{\text{initial COD}(0\,\text{h})} \times 100 \quad (2)$$

All assays were carried in triplicate.

2.6. Enzymatic activities

S. paucimobilis, *Bacillus cereus* ATCC14579 and *Bacillus cereus* ATCC11778 cells were grown in the nutrient broth (pH 6.6) incubated at 30 °C for 24 h and harvested by centrifugation at 10,000 × *g* for 20 min. These cells (ca. 12 g/L) were suspended in potassium phosphate buffer (50 mM, pH 7.4) for sonication (Sonics vibracell ultrasonic processor), keeping sonifier output at 40 amp and giving 8 strokes each of 40 s with a 2 min interval at 4 °C. This extract was used as source of enzyme without centrifugation. Similar procedures were followed for the obtained cells after complete decolorization (6 h) [25].

Lignin peroxidase (Lip) and laccase activities were assayed in cell-free extract as well as in culture supernatant. Lip activity was determined by monitoring the formation of propanaldehyde at 300 nm in the reaction mixture of 2.5 ml containing 100 mM n-propanol, 250 mM tartaric acid, 10 mM H_2O_2 [25]. Laccase activity was determined in the reaction mixture of 2 ml containing 0.1% ABTS (2,2′-azino-bis (3-ethylbenzothiazoline)-6-sulfonic acid) (ABTS; Sigma, St. Louis, USA) in 0.1 M acetate buffer (pH 4.9) and monitored by measuring the increase of optical density at 420 nm [26]. Enzyme assays were carried in triplicate at 30 °C. The blanks contained all components except the enzyme. One unit of enzyme activity was defined as a change in absorbance unit min^{-1} mg protein^{-1}. Azoreductase activity was determined by monitoring the decrease in the Methyl Orange concentration at 466 nm by the reaction mixture of 2.2 ml containing 152 mM Methyl Orange, 50 mM sodium phosphate buffer (pH 5.5) and 20 mM NADH. One unit of enzyme activity was defined as a microgram of Methyl Orange reduced min^{-1} mg protein^{-1} [27]. Malachite green reductase activity was determined as described previously by Jadhav and Govindwar [28]. All assays were carried in triplicate.

2.7. UV–vis spectral analysis FTIR and NMR

Decolorization was monitored by UV–vis spectroscopic analysis, whereas biodegradation was monitored using FTIR spectroscopy. Decolorization of each dye was followed by monitoring the changes in its absorption spectrum (200–700 nm) using a Hitachi UV–Vis spectrophotometer (Hitachi U-2800) and comparing the results with those of the respective controls [17].

The produced metabolites (after decolorization of the medium) were centrifuged at 15,000 rpm for 30 min after the complete degradation of the adsorbed dye to remove any bacterium remained and metabolites were extracted from supernatant by the addition of 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 the percent (%) transmission at different wavelengths were observed. The Fourier Transform Infrared Spectroscopy (FTIR) analysis of extracted metabolites was done on Perkin Elmer, Spectrum one instrument and compared with control dye in the mid-IR region of 400–4000 cm^{-1} with 16 scan speed. The samples were mixed with spectroscopically pure KBr in the ratio of 2:200, pellets were fixed in the sample and the analysis was carried out [21,22].

2.7.1. ^{1}H-NMR spectroscopy

All ^{1}H NMR spectra were recorded on a Bruker Avance 300 spectrometer at 300.13 MHz at 300 K. The dried samples were dissolved in DMSO-*d6* and transferred to 5 mm-diameter tubes. The water was suppressed by the classical double-pulsed field gradient of echo sequence: WATERGATE. 64 scans were collected (relaxation delay, 5 s; acquisition time, 3.64 s; spectral window of 3420 Hz; 32,000 data points). A 0.3 Hz line broadening was applied before Fourier transformation and a baseline correction was performed on the spectra before integration with Bruker software.

Proton Nuclear Magnetic Resonance (NMR), ^{1}H NMR studies were used for the MO dye before bacterial consortium treatment and for the produced products. The samples were prepared after 6 h of incubation of *S. paucimobilis*, *Bacillus cereus* ATCC14579 and *Bacillus cereus* ATCC11778 in the cultivation medium containing the dye. The dried samples were dissolved in Deuterated Dimethyl Sulfoxide (DMSO *d6*) for recording the ^{1}H NMR Bruker 300 UltraShield TM 300 MHz NMR.

2.8. Toxicity tests

Phytotoxicity tests were performed in order to assess the toxicity of Methyl Orange and its produced metabolites. Tests were carried out accordingly as described previously by Ayed et al. [16,17] on two kinds of seeds which are commonly used in Tunisian agriculture: *Triticum aestivum*. The Methyl Orange and ethyl acetate extracted product (dry) were dissolved separately in distilled water and made the final concentration of 1000 mg/L. Toxicity test was done by growing the seeds (10) of each plant species separately into control dye and extracted product sample. Germination (%) was recorded after 7 days [17].

3. Results and discussion

3.1. Model establishment

Through linear regression fitting, the regression models of tow responses (COD % and decolorization %) were established (Table 1). The regression model equations are as follows:

$$Y_{\text{decolorization\%}} = 89.937\text{S1} + 90.699\text{S2} + 90.006\text{S3} + (-8.750)\text{S1} \times \text{S2} + (5.917)\text{S1} \times \text{S3} + (1.897)\text{S2} \times \text{S3}$$

$R^2 = 55.71\%; P = 0.377$

$$Y_{\text{COD removal}(\%)} = 82.73\text{S1} + 84.86\text{S2} + 80.88\text{S3} + (-12.56)(\text{S1} \times \text{S2}) + (-9.93)(\text{S1} \times \text{S3}) + 6.11(\text{S2} \times \text{S3})$$

$R^2 = 64.02\%; P = 0.512$

where S1: *S. paucimobilis*, S2: *Bacillus cereus* ATCC14579 and S3: *Bacillus cereus* ATCC11778.

3.2. Effect of the microorganisms formulation on the decolorization and COD removal of Methyl Orange

In the mixture design, the effect of the variable change on the responses (COD and color removal) can be observed on the ternary contour map. Fig. 1 shows the effect of the interaction of *S. paucimobilis*, *Bacillus cereus* ATCC14579 and *Bacillus cereus* ATCC11778 on the COD variation. The statistical significance of the ratio of mean

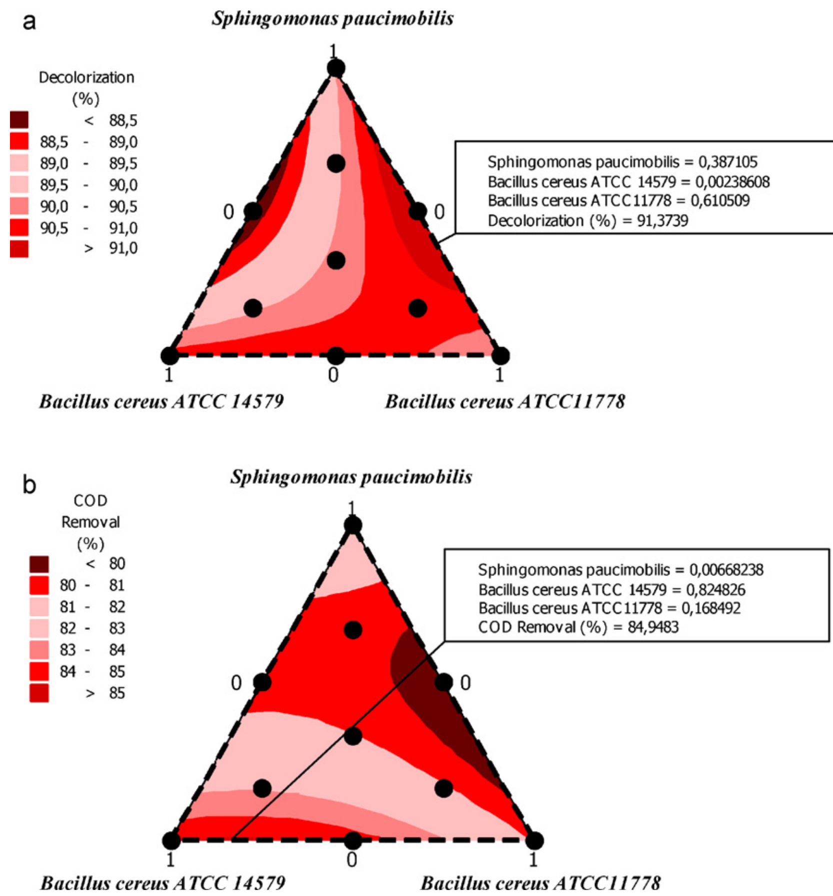

Fig. 1. Mixture contour plots between the variables (*Sphingomonas paucimobilis*, *Bacillus cereus* ATCC14579, *Bacillus cereus* ATCC11778) contents for color (a) and COD removal (b).

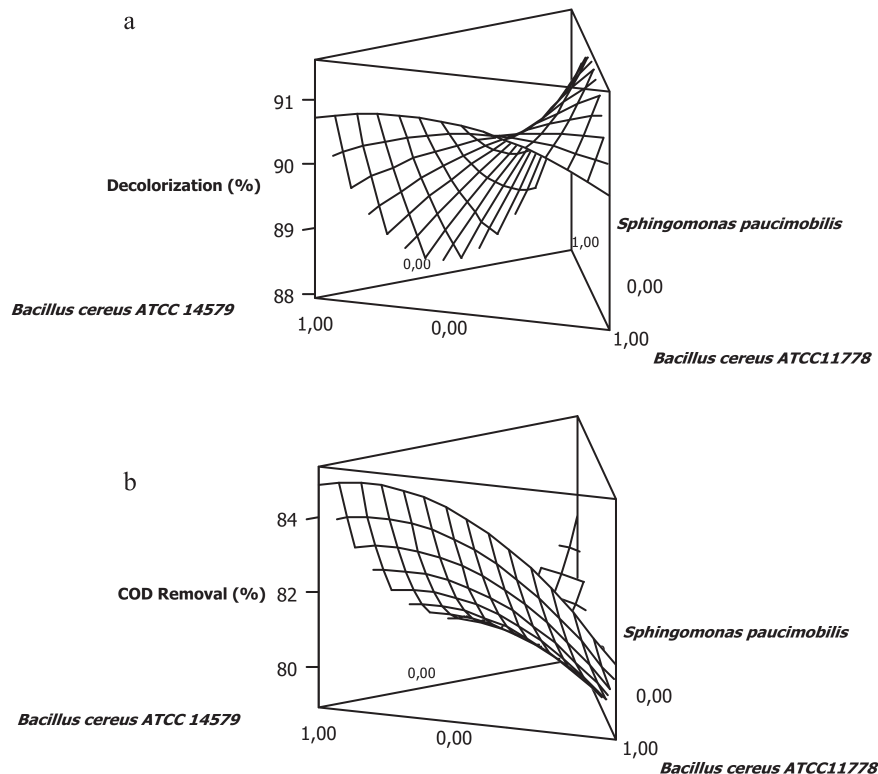

Fig. 2. Response surface plot and its contour plot of color (a) and COD removal (b) *Sphingomonas paucimobilis*, *Bacillus cereus* ATCC14579, *Bacillus cereus* ATCC11778 contents.

Table 2
Analysis of variance of COD% (ANOVA) for the selected linear and interactions model for Methyl Orange.

Source	Degrees of freedom	Sum of square	Sum of adjusted squares	Adjusted average squares	F-ratio	P-value (significance)
Regression	5	29.39	29.39	5.87	1.01	0.51
Linear regression	2	14.52	8.73	4.36	0.75	0.53
Quadratic regression	3	14.86	14.86	4.95	0.85	0.53
Residual error	4	23.36	23.36	5.84		
Total	9	52.76				

square variation due to regression and mean square residual error was tested using analysis of variance ANOVA. ANOVA is a statistical technique which subdivided the total variation in a set of data into component parts associated with specific sources of variation for the purpose of testing hypotheses on the parameters of the model.

Only the obtained results for color and COD removal were presented in this work for clarity of purpose. According to the ANOVA analysis (Tables 2 and 3), the regression adjusted average squares and the linear regression adjusted average squares were 5.87861 and 4.36751 respectively which allowed the calculation of the Fisher ratios (F-value) for assessing the statistical significance. The model F-value (1.01) implies that most of the variation in the response can be explained by the regression equation. The associated P-value is used to prove whether F-ratio is large enough to indicate statistical significance. A P-value is more than 0.1 (i.e. $\alpha = 0.05$ or 95% confidence) indicated that the model cannot be considered statistically significant. The adjusted coefficient (R^2) reached 55.71% and 64.02%, indicating that the quadratic models had a good fit with the target ratio formula [29].

The P-value for the obtained regression was $P = 0.512$ and $P = 0.377$. For the decolorization and COD removal was more than 0.1 and means consequently that at least one of the terms in the regression equation have a significant correlation with the response variable. The ANOVA test showed a term for a residual error, which measures the amount of the variation in the response data left unexplained by the model [30].

In order to confirm the obtained experimental results of 97.19% of decolorization and 99.05% of COD removal, a mixture contour plot (Fig. 1) and surface plot (Fig. 2) were plotted by MINITAB® 14 Software Programme. The higher decolorization (100%) and COD removal (98%) yields were obtained when *S. paucimobilis*, *Bacillus cereus* ATCC14579 and *Bacillus cereus* ATCC11778 proportions were 0.023%, 0.459% and 0.516%; and 0.006%, 0.985% and 0.008%, respectively for color and COD removal.

The mixture surface plots (Fig. 2), which are a three-dimensional graph, are represented using decolorization and COD removal based on the simultaneous variation of *S. paucimobilis*, *Bacillus cereus* ATCC14579 and *Bacillus cereus* ATCC11778 in the consortium composition ranging from 0 to 100% for each strain. The mixture surface plot also described individual and cumulative effect of these three variables and their subsequent effect on the response [31].

The residual values (i.e. observed minus predicted values of Y_{COD}) can then be plotted in a normal probability plot for color removal ($Y_{decolorization}$) (Fig. 3a). All points from this residual plot lies close to the straight line confirming the conjecture that effects other than those considered in the model may be readily explained by random noise. Adequacy of the model was also checked by means of constructing the normal plot of the residuals for COD removal (Fig. 3b). Once again, all points from this residual plot lies close to the straight line confirming the conjecture that affects other than those considered in the model may be readily explained by random noise [32].

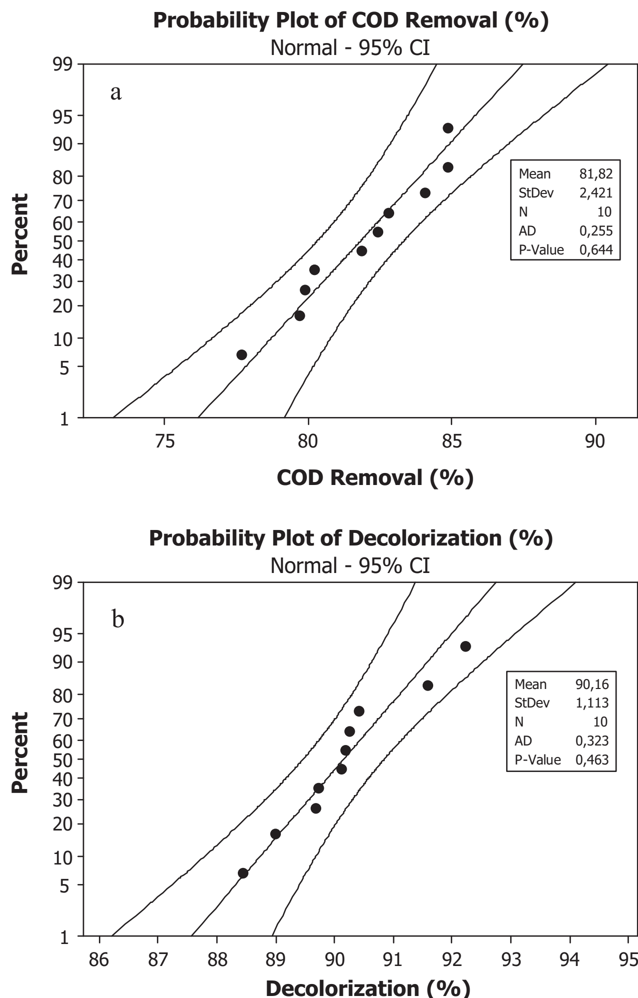

Fig. 3. Normal probability plot of the residuals; COD (a) and decolorization (b) removal a model at optimal treatment conditions.

Table 3
Analysis of variance of % decolorization (ANOVA) for the selected linear and interactions model for Methyl Orange.

Source	Degrees of freedom	Sum of square	Sum of adjusted squares	Adjusted average squares	F-ratio	P-value (significance)
Regression	5	7.13	7.13	1.42	1.42	0.37
Linear regression	2	1.27	0.39	0.19	0.20	0.83
Quadratic regression	3	5.86	5.86	1.95	1.95	0.26
Residual error	4	4.01	4.01	1.00		
Total	9	11.14				

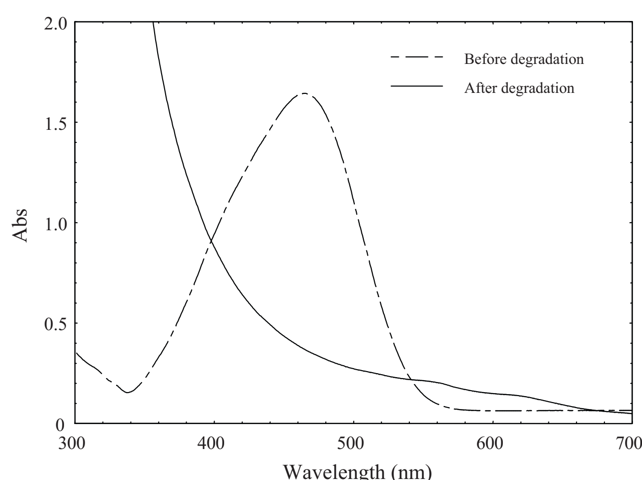

Fig. 4. UV–vis spectra of Methyl Orange (750 ppm) biodegraded by bacterial consortium (*Sphingomonas paucimobilis*, *Bacillus cereus* ATCC14579, *Bacillus cereus* ATCC11778) before and after degradation. Temperature = 30 °C, pH = 7.0.

3.3. UV–vis, FTIR and NMR

Fig. 4 shows a typical time dependent UV–vis spectrum of MO solution during biodegradation. The absorbance peaks corresponding to the dye biodegradation. Their decrease indicating that the dye has been removed. The MO spectrum in visible region exhibits a main peak with a maximum absorbance at 466 nm (Fig. 4). The decrease of absorbance of MO showed a rapid degradation of the dye. According to Chen et al. [33], the biodecolorization of dyes can be due to the adsorption to the biomass or the biodegradation. If the dye removal is attributed only to the biodegradation, either the major visible light absorbance peak will disappear or a new peak will appear. As shown in Fig. 4 the main absorbance peak approximately disappeared within 48 h. In addition an extra absorbance peaks appeared in the decolorized solution, probably resulting from the absorbance of metabolites or degraded fragments of the dye molecules [34]. These results indicated that the decolorization by the consortium may be largely attributed to biodegradation. To confirm this result MO dye and the produced metabolites were analyzed by FTIR and NMR spectra.

The decolorization of MO occurred after 2 days of incubation at 30 °C under aerobic conditions. There have been some reports which suggest that the decolorization of sulfonated azo dyes occur under aerobic conditions after 2 months [35]. Another reports indicated that azo dyes are essentially non-degradable by bacteria under aerobic conditions [36]. Kulla et al. described a degradative pathway for sulfonated azo dyes by *Pseudomonas* strains previously adapted to grow on the corresponding carboxylated azo dyes [37]. In this work the experiments showed that the microbial consortium is able to decolorize MO under aerobic conditions within 48 h.

Others works have reported that anaerobic cleavage of the azo linkage by the reductase enzymes is the initial step of the biodegradation of the azo dyes [38]. The aerobic degradation of MO observed here suggests, in accordance with Kulla et al., which proved that despite the presence of oxygen, the initial degradation step appears to be a reduction of the azo linkage by an oxygen-insensitive azo reductase [37].

The comparison of FTIR spectrum (Fig. 5) between the control dye and the extracted metabolites clearly indicated the biodegradation of the parent dye compounds by the consortium. The absorption bands in the control dye spectrum represented the stretching vibrations of S═O at 624 cm^{-1} and 1202 cm^{-1} as well as a stretching vibration at 1041 cm^{-1} for C–N. The stretching vibration of C–O band showed the absorption at 1121 cm^{-1}. 3682 cm^{-1} represented the O–H stretching vibration. 3788 cm^{-1} represented the presence of free NH group from parent dye structure. The stretching vibration of C–H was reported at 2921 cm^{-1}, whereas

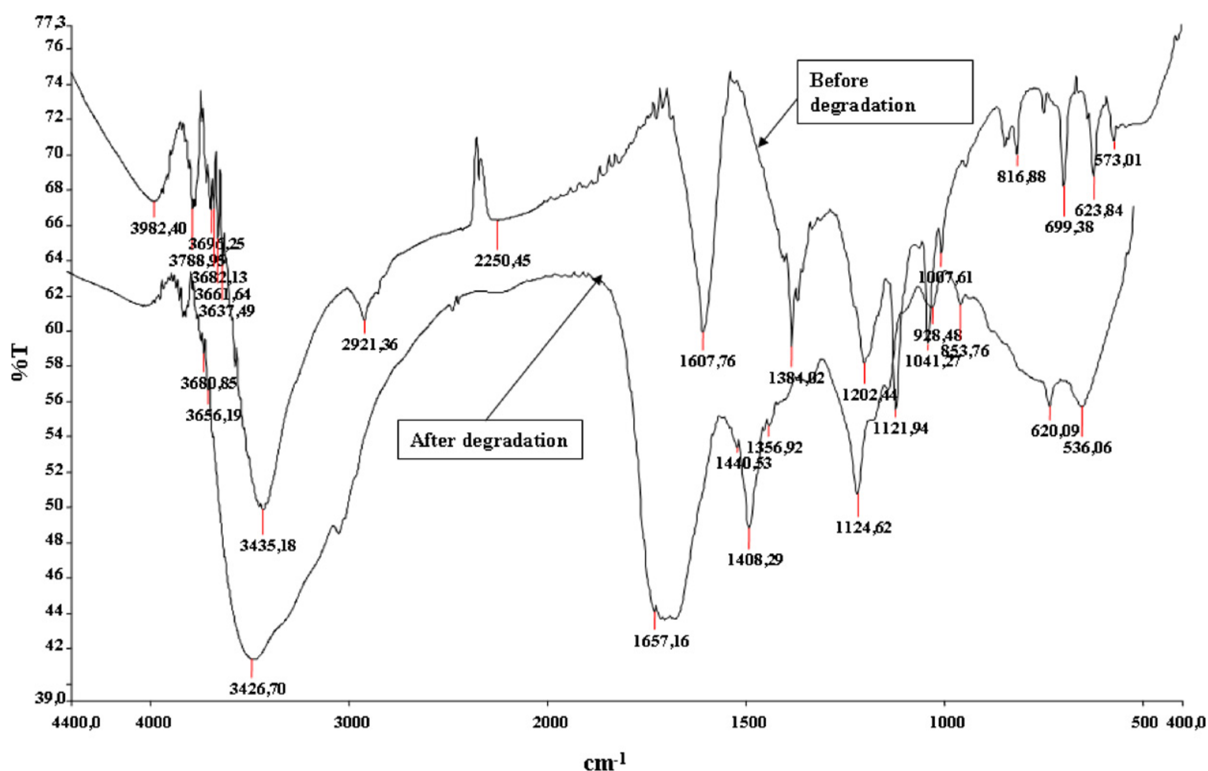

Fig. 5. FTIR spectra of Methyl Orange before and after degradation (48 h). (*Sphingomonas paucimobilis*, *Bacillus cereus* ATCC14579, *Bacillus cereus* ATCC11778.) Temperature = 30 °C, pH = 7.0.

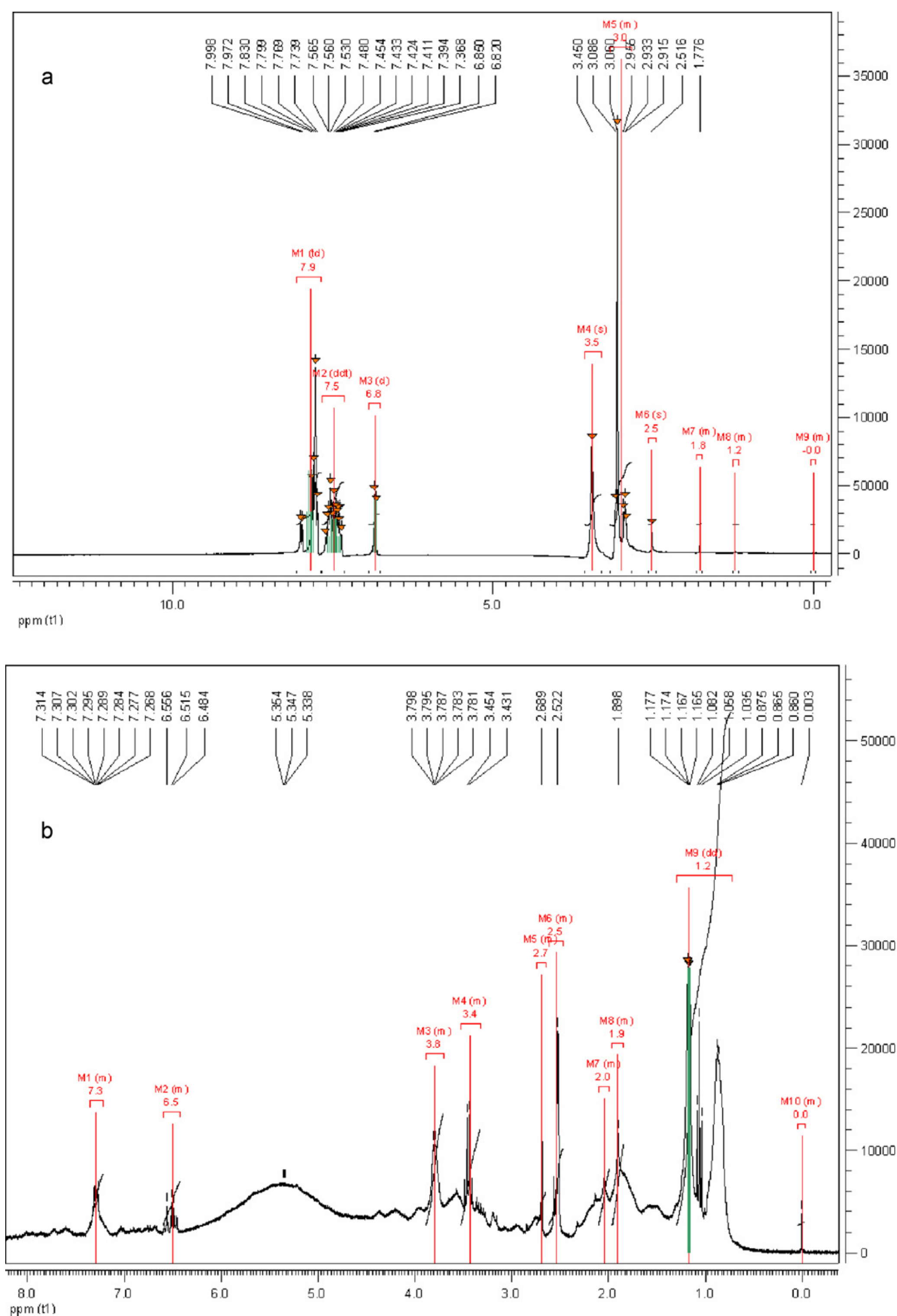

Fig. 6. ^{1}H NMR spectra of Methyl Orange before (a) and after degradation (b). (*Sphingomonas paucimobilis*, *Bacillus cereus* ATCC14579, *Bacillus cereus* ATCC11778.) Temperature = 30 °C, pH = 7.0.

the band at 1607 cm^{-1} represented –N=N– stretching of azo group.

The FTIR spectrum of 48 h extracted metabolites showed a significant change in the band positions compared to control dye spectrum.

After 48 h extracted metabolite bands at 1657 cm^{-1} pointed towards the formation of aromatic compounds as benzaldehyde and benzoic acid. A new band observed at 1440 cm^{-1} represented C–H deformation of alicyclic CH_2, whereas another band at 3426 cm^{-1} was observed for OH stretch vibration. The formation of hydrocarbon aliphatic compounds could be explained by the absorption band at 3000 cm^{-1}.

The ^{1}H NMR spectrum of MO before degradation (Fig. 6a) showed signals between 6.6 ppm and 8.0 ppm corresponding to the

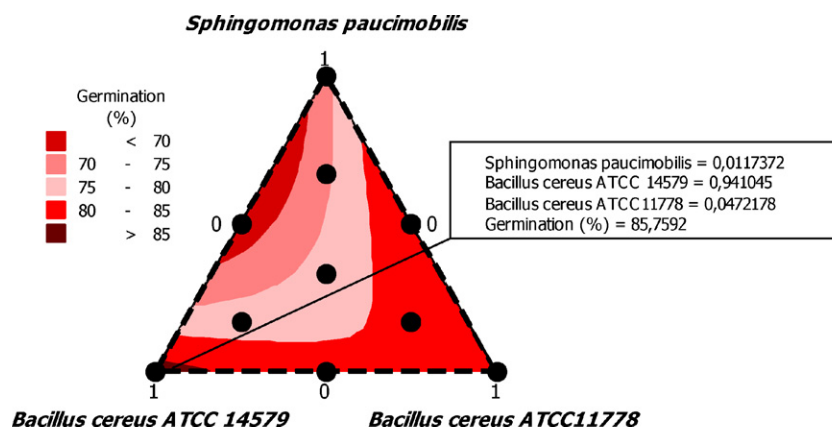

Fig. 7. Mixture contour plots between the variables (*Sphingomonas paucimobilis*, *Bacillus cereus* ATCC14579, *Bacillus cereus* ATCC11778) contents for germination of *T. aestivum*.

Table 4
Enzymatic activity of control (0 h) and the induced state (48 h decolorization).

Enzyme assay	Control	Induced		
		S1	S2	S3
Azo reductase[a]	1.59 ± 0.01	5.3 ± 0.02	3.56 ± 0.01	3.86 ± 0.03
Laccase[c]	0.0196 ± 0.009	0.0307 ± 0.010	0.024 ± 0.004	0.027 ± 0.001
MG reductase[b]	8.91 ± 0.31	32.70 ± 0.9	21.86 ± 0.8	22.86 ± 0.7
Lignin peroxidase[c]	10 ± 0.3	12 ± 0.97	11.66 ± 0.08	11.96 ± 0.08

S1: *Sphingomonas paucimobilis*, S2: *Bacillus cereus* ATCC14579 and S3: *Bacillus cereus* ATCC11778.
[a] μmol of Methyl Orange reduced/mg of protein min^{-1}.
[b] μg of MG reduced/mg of protein min^{-1}.
[c] Enzyme activity – units/mg of protein min^{-1}.

protons of the two aromatic rings. A singlet at 3.06 ppm is attributed to the two methyl groups attached to the nitrogen atom.

After degradation (Fig. 6b), the ^{1}H NMR spectrum of the extracted metabolites without any purification showed the disappearance of the singlet at 3.06 ppm corresponding to the two methyl groups mentioned above. This result showed that the tertiary amine has been biodegradated. In the same spectrum a broad signal appeared at 5.34 ppm relative to hydroxyl groups. This result in concordance with the absorption band observed at 3426 cm^{-1} in the FTIR spectrum. An important number of signals were observed in the high field region (0.8–2.5 ppm) of the ^{1}H NMR spectrum indicating the formation of hydrocarbon aliphatic compounds. This result was supported by the FTIR spectrum showing an absorption band at 3000 cm^{-1}. Other overlapped signals between 3 and 4 ppm currently attributable to oxygenated methines and/or methylenes. In the low field region (6.4–7.6 ppm), we also observed other absent signals in the case of MO before biodegradation, corresponding to aromatic derivatives.

3.4. Enzymatic analysis

The enzymatic activities in *S. paucimobilis*, *Bacillus cereus* ATCC14579 and *Bacillus cereus* ATCC11778 cells before and after complete decolorization of Methyl Orange in plain distilled water were represented in Table 4. The initial step of the biodegradation of azo compounds is a reductive cleavage of the azo group by azo reductase which cleaves the azo bridge [39].

The term azo dye reduction may involve different mechanisms or locations like enzymatic, non-enzymatic, mediated, intracellular and various combinations of these mechanisms and locations. Oxidative biodegradation takes place upon action of enzymes such as peroxidases and laccases. The involvement of fungal peroxidases and laccases for the oxidation of sulfonated azo dyes has been reported earlier [39]. Bacterial extracellular azo dyes oxidizing peroxidases have been characterized in *S. paucimobilis*, *Bacillus cereus* ATCC14579 and *Bacillus cereus* ATCC11778. Lignin peroxidase catalyzes the oxidative breakdown of the azo dye Methyl Orange. In this study, the induction of lignin peroxidase, azo reductase, and MG reductase strongly indicated that Methyl Orange can be degraded and reductively cleaved into the simple metabolites.

3.5. Phytotoxicity study

Thus, it was of concern to assess the phytotoxicity of the dye Methyl Orange before (germination 0%) and after degradation. The control test was the distilled water (germination 100%). The relative sensitivity towards the dyes and degradation products in relation to *T. aestivum* was studied (Table 1). The germination indicated a less toxicity of the degradation products to the plants. Fig. 7 shows the effect of the interaction between *S. paucimobilis*, *Bacillus cereus* ATCC14579 and *Bacillus cereus* ATCC11778 on germination. In order to confirm the experimental results that showed 87% of germination was obtained proportionally to 92.22% of color and 79.86% of COD removal. A mixture contour plot (Fig. 7) was plotted by MINITAB® 14 Software Program. The maximum percentage of germination (85.75%) was obtained if the *S. paucimobilis*, *Bacillus cereus* ATCC14579 and *Bacillus cereus* ATCC11778 proportions were 0.011%, 0.94% and 0.072%, respectively. Ayed et al. [16,17] showed that germination of *Triticum aestivum* was less with Malachite Green and Crystal Violet treatment as compared to its degradation products. Hence phytotoxicity studies revealed that the biodegradation of wastewater containing dyes by a microbial culture resulted by its complete detoxification. These treated effluents can be used for ferti-irrigation. However these findings suggested the non-toxic nature of the formed products. Previous works showed that the Malachite Green and Crystal Violet degradation into leucomalachite and leucocrystal violet are equally toxic to the initial compound [16].

4. Conclusions

The developed consortium showed a better decolorization yields as compared to pure cultures, which proved a complementary interaction among various isolated bacteria. The consortium achieved significantly a higher reduction in color (92.22%) and COD removal (84.83%) in less time (48 h) by using the components of the Mineral Salts Medium for the growth. The biodegradation of the azo dye (MO) was achieved by the developed consortium using *S. paucimobilis*, *Bacillus cereus* ATCC14579 and *Bacillus cereus* ATCC11778. The observation of azoreductase, Laccase and Lip activities during the decolorization suggested their involvement in the degradation process. Physicochemical monitoring results together with phytotoxicity showed the success of the consortium for reducing the toxicity of the toxic industrial dye. However, the phytotoxicity study showed that Methyl Orange was degraded into non-toxic compounds by the microbial consortium.

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