Application of the mixture design to optimise the formulation of active consortia to decolorize azo-dye methyl red

Lamia Ayed, Besma Harbi, Abdelkrim Cheref, Amina Bakhrouf and Sami Achour

ABSTRACT

With the aid of analysis software (Minitab 14.0), the formulation of pure culture in Mineral Salts Medium (MSM) can be optimized for several responses and the best formulation can be obtained. The influence of the different mixtures of three strains in the pure culture in MSM on the flavor components in decolorization of Methyl Red (with initial total cell density fixed at OD600 = 1 and in addition of 750 ppm of dye) was studied using equilateral triangle diagram and mixture experimental design to assess color and COD removal during species evolution. The regression model on microorganism composition and main metabolites was established. The results suggested that the highest predictable specific decolorization rate and Chemical Oxygen Demand (COD) were 77.97 and 93.77%. Based on these, the response values that satisfied all expectations were optimized, and the optimal composition of the mixed consortium for the decolorization and COD removal were (Sphingomonas paucimobilis 45.20%, Bacillus sp 61.94% and Staphylococcus epidermidis 80.00%) and (Sphingomonas paucimobilis 77.03%, Bacillus sp 86.42% and Staphylococcus epidermidis 71.74%) respectively. Very high regression coefficient between the variables and the responses: decolorization and COD removal were respectively $R^2 = 0.96$ and 0.81 indicated excellent evaluation of experimental data by polynomial regression model.

Key words | azo dye, bacterial decolorization, equilateral triangle diagram, experimental design

INTRODUCTION

Approximately 10,000 different dyes and pigments are produced annually worldwide and used extensively in the dye and printing industries. Synthetic dyes are chemically diverse, with the commonly used in industry divided into those of azoic, triphenylmethane or heterocyclic/polymeric structures. Product processing methods often cause a loss of large amounts of dyes to wastewaters, representing 10 – 15% of the dyes applied. Several of these dyes are very stable to light, temperature and microbial attack, making them recalcitrant compounds, many of them are also toxic (Robinson et al. 2001).

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% (Soares et al. 2002). They are designed to convey high photolytic stability and resistance towards major oxidising agents. The release of azo dyes into the environment in effluent from textile dyeing plants has 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 (Rieger et al. 2002). In addition, some azo dyes or their metabolites may be mutagens or carcinogens (McCann & Ames 1976). Several combined anaerobic and aerobic microbial treatments have been suggested to enhance the degradation of azo dyes (O’Neill et al. 2000).

Bio-decolorization and biodegradation is an environmental-friendly and cost-competitive alternative to the chemical decomposition process (Verma & Madamwar 2005). Most studies on azo dye biodegradation have focused on bacteria and fungi, where bacteria are found to be more efficient (Stolz 2001; Eichlerová et al. 2006). Chang et al. (2000) found that mutant strain of Escherichia coli was able to decolorize azo dye C.I. Reactive red 22. Rhodopseudomonas palustris could decolorize azo dye Acid red B and Rhodobacter sphaeroides was able to decolorize Methyl orange (Song et al. 2003; Liu et al. 2006). However, the results based on a number of experiments did not only fail to reach the best combination while spend a lot of experimental materials, but will also affect the progress of the study.

The effectiveness of microbial decolorization depends on the activity of selected microorganisms. The genus Sphingomonas has recently received a lot of attention and becomes biotechnologically interesting microorganism because of its degradation capabilities for various xenobiotic substances. Members of this genus are able to degrade biphenyl, naphthalene, pyrene and dioxin, among several substances. However, up to now there are no reports on biodecolorization of anthraquinone dyes by this genus. In our laboratory, a new isolate identified as Sphingomonas xenophaga showed a strong ability to decolorize bromoamine acid (Qu et al. 2005). Many microorganisms belonging to different taxonomic groups of bacteria, fungi and algae have been reported for their ability to decolorize different dyes (Isil & Tugba 2008).

In this research, we used the mixture design in the experimental design (Minitab 14.0) to optimize the formulation of the predominant strains isolated from Textile waste water plant. After biodegradation, the Chemical Oxygen Demand (COD) and percentage of decolorization were measured. The relationships between the different combinations and products were analyzed through the Minitab to select the optimal bacterial combination.

MATERIALS AND METHODS

Microbial strain

The strains were microcapsules of Sphingomonas paucimobilis (14 × 10⁷ cfu), Bacillus sp (4.2 × 10⁸ cfu), Staphylococcus epidermidis (2.6 × 10⁹), which were isolated from Textile Waste Water plant in KsarHellal, Tunisia.

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 (λ₆₀₀ nm = 1) prepared in Mineral Salt Medium (MSM) at 37°C, 150 rpm (New Brunswick Scientific Shaker, Edison, NJ) was used as the inoculum.

Design of the experiment

In this study, Sphingomonas paucimobilis, Bacillus sp and Staphylococcus epidermidis were used as mixture starters, ranging from 0 to 100%, as shown in Table 1. Decolorization experiments were taken according to the ratio given by the experiment design, and 10% mixed microcapsules were inoculated into MSM solution (3.0 g/l yeast extract and 1.25 g/l glucose and 750 ppm MR) at 37°C for 10 h with shaking (150 r/min). COD removal and percentage of decolorization were determined after biodegradation.

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 is 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 (Wang et al. 2006; Muteki et al. 2007).

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 (Plackett & Burman 1946; Ghanem et al. 2000). 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.
Acclimatization

The acclimatization was performed by gradually exposing *Sphingomonas paucimobilis*, *Bacillus sp* and *Staphylococcus epidermidis* to the higher concentrations of MR (Kalme et al. 2006). This 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) and finally the organism was provided with MR solution as sole source of nutrient. Acclimatization experiments were carried out at optimum temperature (Kalme et al. 2006; Ayed et al. 2009).

Analytical methods

Absorbance of the supernatant withdrawn at different time intervals were measured at the maximum absorption wavelength for the dye MR (\( \lambda_{\text{max}} = 435 \text{ nm} \)) in the visible region on a Shimadzu double beam spectrophotometer (UV 1601). The initial absorbance of dye with MSM solution was used as a reference for dye change and decolorization noted as 0% removal. The decolorization and COD removal were calculated according to the following formulation (Equation 1 and Equation 2) (Ayed et al. 2009).

\[
\% \text{ Decolorization} = \frac{(1 - F)}{I} \times 100 \quad (1)
\]

\[
\text{COD removal(\%)} = \frac{\text{initialCOD(0h)} - \text{observedCOD(t)}}{\text{initialCOD(0h)}} \times 100 \quad (2)
\]

Phytotoxicity and microbial toxicity studies

Phytotoxicity tests were conducted to assess the impact of the treated colored water on vegetation once it is thrown to the ecosystem as well as to explore the possible reuse of the treated solution in irrigation fields such as parks, golf-courses, etc. We have assessed the toxicity of the untreated and treated samples at the concentration 750 ppm. Tests were carried out according to the ISO (1993) on two kinds of seeds commonly used in Indian agriculture: *Sorghum bicolor* and *Triticum aestivum*. The microbial toxicity was carried out using *Sphingomonas paucimobilis* on Mueller and Hinton agar plate having composition 1% peptone, 0.5% NaCl, 0.3% yeast extract and 2.5% agar.

RESULTS AND DISCUSSION

Model establishment

Table 1 lists the experimental values of the contents of *Sphingomonas paucimobilis*, *Bacillus sp* and *Staphylococcus epidermidis* in decolorized dye using mixture starter. Through linear regression fitting, the regression models of two responses (COD % and decolorization %) were

Table 1 | Mixture design matrix with the experimental

<table>
<thead>
<tr>
<th>Experiment</th>
<th>A: <em>Sphingomonas paucimobilis</em></th>
<th>B: <em>Bacillus sp</em></th>
<th>C: <em>Staphylococcus epidermidis</em></th>
<th>Total COD removal (%)</th>
<th>Decolorization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.00000</td>
<td>0.00000</td>
<td>0.00000</td>
<td>1.00000</td>
<td>68.75</td>
</tr>
<tr>
<td>2</td>
<td>0.00000</td>
<td>1.00000</td>
<td>0.00000</td>
<td>1.00000</td>
<td>70</td>
</tr>
<tr>
<td>3</td>
<td>0.00000</td>
<td>0.00000</td>
<td>1.00000</td>
<td>1.00000</td>
<td>62.5</td>
</tr>
<tr>
<td>4</td>
<td>0.50000</td>
<td>0.50000</td>
<td>0.00000</td>
<td>1.00000</td>
<td>92.5</td>
</tr>
<tr>
<td>5</td>
<td>0.50000</td>
<td>0.00000</td>
<td>0.50000</td>
<td>1.00000</td>
<td>67.5</td>
</tr>
<tr>
<td>6</td>
<td>0.00000</td>
<td>0.50000</td>
<td>0.50000</td>
<td>1.00000</td>
<td>76.1875</td>
</tr>
<tr>
<td>7</td>
<td>0.33333</td>
<td>0.33333</td>
<td>0.33333</td>
<td>1.00000</td>
<td>86.875</td>
</tr>
<tr>
<td>8</td>
<td>0.66667</td>
<td>0.16667</td>
<td>0.16667</td>
<td>1.00000</td>
<td>76.5625</td>
</tr>
<tr>
<td>9</td>
<td>0.16667</td>
<td>0.66667</td>
<td>0.16667</td>
<td>1.00000</td>
<td>93.5625</td>
</tr>
<tr>
<td>10</td>
<td>0.16667</td>
<td>0.16667</td>
<td>0.66667</td>
<td>1.00000</td>
<td>89.5</td>
</tr>
</tbody>
</table>
established. The regression model equations are as follows:

\[ Y_{\text{decolorization\%}} = 45.20 \times A + 61.94 \times B + 80.00 \times C \]

\[ R^2 = 96.40\%; \quad P = 0.029 \]

\[ Y_{\text{COD\%}} = 77.03 \times A + 86.42 \times B + 71.74 \times C \]

\[ R^2 = 81.88\%; \quad P = 0.269 \]

where A: *Sphingomonas paucimobilis*; B: *Bacillus* sp and C: *Staphylococcus epidermidis*.

The statistical significance of the ratio of mean square variation due to regression and mean square residual error was tested using analysis of variance. 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 results obtained for decolorization rates \((Y_{\text{decolorization}})\) was presented herein for clarity of purpose. According to the ANOVA (Table 2), the regression adjusted average squares was \((454.356)\) and the linear regression adjusted average squares \((454.356)\) allowed the calculation of the Fisher ratios \((F\)-value\) for assessing the statistical significance. The model \(F\)-value \((0.641)\) 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 is more than 0.1 (i.e. \(a = 0.05 \) or 95% confidence) indicates that the model is not to 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 (Zhou et al. 2007).

The \(P\)-value for the regression obtained \(R^2 = 96.40\%; \quad P = 0.029\) for decolorization was less 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 (Xudong & Rong 2008).

### Effect of formulation on the percentage of decolorization and COD removal of methyl red

The mixture design is now used widely in the formulation experiment of food, chemicals, fertilizer, pesticides, and other products. It can estimate the relationship between formulation and performance through regression analysis in fewer experiment times (Zhang et al. 2006). Zhang et al. (2006) studied the formulation of plant protein beverage using the mixture design, obtaining the optimized combination of walnut milk, peanut milk, and soy milk. In the

### Table 2 | Analysis of variance of % decolorization (ANOVA) for the selected linear plus interactions model for methyl red

<table>
<thead>
<tr>
<th>Source</th>
<th>Degrees of freedom</th>
<th>Sum of square</th>
<th>Sum of adjusted squares</th>
<th>Adjusted average squares</th>
<th>(F)-ratio</th>
<th>(P)-value (significance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>6</td>
<td>7,334.00</td>
<td>7,334.00</td>
<td>1,222.33</td>
<td>13.38</td>
<td>0.029</td>
</tr>
<tr>
<td>Linear regression</td>
<td>2</td>
<td>908.71</td>
<td>61.19</td>
<td>30.59</td>
<td>0.33</td>
<td>0.739</td>
</tr>
<tr>
<td>Residual error</td>
<td>3</td>
<td>274.00</td>
<td>274.00</td>
<td>91.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>7,608.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 3 | Analysis of variance of COD\% (ANOVA) for the selected linear plus interactions model for methyl red

<table>
<thead>
<tr>
<th>Source</th>
<th>Degrees of freedom</th>
<th>Sum of square</th>
<th>Sum of adjusted squares</th>
<th>Adjusted average squares</th>
<th>(F)-ratio</th>
<th>(P)-value (significance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>2</td>
<td>165.99</td>
<td>165.995</td>
<td>82.995</td>
<td>0.58</td>
<td>0.585</td>
</tr>
<tr>
<td>Linear regression</td>
<td>2</td>
<td>165.99</td>
<td>165.995</td>
<td>82.995</td>
<td>0.58</td>
<td>0.585</td>
</tr>
<tr>
<td>Residual error</td>
<td>7</td>
<td>1,001.34</td>
<td>1,001.34</td>
<td>143.048</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>1,167.33</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
mixture design, the effect of the change of variables on the responses can be observed on the ternary contour map. Figure 1 shows the effect of the interaction of *Sphingomonas paucimobilis*, *Bacillus sp* and *Staphylococcus epidermidis* on the decolorization of MR; Figure 2 shows the effect of the interaction of *Sphingomonas paucimobilis*, *Bacillus sp* and *Staphylococcus epidermidis* on the variation of COD. The statistical significance of the ratio of mean square variation due to regression and mean square residual error was tested using analysis of variance (Table 3).

**Interpretation of residual graph**

The normal probability plot, Figure 1(a), shows that the distribution of residual value which is defined as the difference between the predicted (model) and observed

![Residual plots for COD (%)](image)

**Figure 1** (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.

![Mixture contour plot of decolorization (%) (component amounts)](image)

**Figure 2** Mixture contour plots between the variables (*Sphingomonas paucimobilis*, *Bacillus sp* and *Staphylococcus epidermidis* contents) for decolorization and COD removal.
(experimental) are forming a straight line and residual value are normally distributed on the both side of the line indicating that experimental point are reasonably aligned with predicted value. The histogram, presented in Figure 1(b), of the residuals shows the distribution of the residuals for all the observations. The one long tail in the plot indicating 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 shows that all the residuals are scattered randomly about the zero and one or two points are outliers (Figure 1(c)).

This last plot of Figure 1(d) is the residual value and the order of the corresponding observations. The plot is useful when the order of the observations may influence the results which can occurs 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 one with another.
Effect of formulation on the decolorization and COD removal

In the mixture design, the effect of variables change on the responses can be observed on the ternary contour map. In our study three variables can be compared. Figure 2 illustrate the effect of the interaction of *Sphingomonas paucimobilis*, *Bacillus* sp and *Staphylococcus epidermidis* on decolorization and COD removal is presented in Figure 3. The Figure 4 shows the effect of the interaction of *Sphingomonas paucimobilis*, *Bacillus* sp and *Staphylococcus epidermidis* on the decolorization and COD removal.

The mixture contour plots between the variables such as *Sphingomonas paucimobilis*, *Bacillus* sp and *Staphylococcus epidermidis* are given in Figure 2. The lines of contour plots predict the values of each response at different proportion of *Sphingomonas paucimobilis*, *Bacillus* sp and *Staphylococcus epidermidis*. These values are more or less same to the experimental values.

The mixture surface plots (Figure 3), which are a three-dimensional graph, was represented using decolorization and COD removal were represented based on the simultaneous variation of *Sphingomonas paucimobilis*, *Bacillus* sp and *Staphylococcus epidermidis* consortium composition from 0 to 100% for each space. The mixture surface plot also describing individual and cumulative effect of these three test variable and test their subsequent effect on the response.

Process optimization curve

In order to confirm the experimental results that 77.97% decolorization when 66.23% of COD was removed and 93.77% COD removal when 53.56% decolorization, a response overlaid contour (Figure 4) and surface optimization curve (Figure 5) were plotted by MINITAB® 14 Software Programme where minimum maximum values percentage of decolorization and COD removal which were fixed (80–90%) and (62–78%). The results thus obtained were shown in Figure 5 (y value given in Figure 5) and were predicted if the *Sphingomonas paucimobilis* proportion was 0.58% (curve value), *Bacillus* sp was 0% (curve value) and *Staphylococcus epidermidis* was 94.19% (curve value) to remove 77.97% of color of dye and to remove 93.77% of COD.

Phytotoxicity and microbial toxicity study

Thus, it was of concern to assess the phytotoxicity of dye Methyl Red before and after degradation (Figure 6). The relative sensitivity towards the dyes and degradation

<table>
<thead>
<tr>
<th>Parameters studied</th>
<th>Triticum aestivum</th>
<th>Sorghum bicolor</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSM solution</td>
<td>Methyl red (750 ppm)</td>
<td>Extracted metabolites (750 ppm)</td>
</tr>
<tr>
<td>Germination (%)</td>
<td>100</td>
<td>45</td>
</tr>
<tr>
<td>Shoot (cm) (± SD)</td>
<td>3.93 ± 0.15</td>
<td>0.06 ± 0.15</td>
</tr>
<tr>
<td>Root (cm) (± SD)</td>
<td>3.83 ± 0.11</td>
<td>0.03 ± 0.15</td>
</tr>
</tbody>
</table>

*Figure 6* | Phytotoxicity study in *Sorghum bicolor* and *Triticum aestivum* before and after decolorization of methyl red (750 ppm) by a bacterial consortium (*Sphingomonas paucimobilis*, *Bacillus* sp. and *Staphylococcus epidermidis*).
products in relation to *S. bicolor* and *T. aestivum* was studied (Table 4). The length of plumule and radical indicates less toxicity of the degradation product to the plants. Ayed et al. (2009) also showed that germination of *Triticum aestivum* was less with Malachite Green treatment as compared to its degradation product. Hence phytotoxicity studies revealed that biodegradation of dyes by a microbial culture, resulted in its detoxification. Thus treated effluent can be used for ferti-irrigation. Zone of inhibition was observed with control dyes with microbial consortium (*Sphingomonas paucimobilis*, *Bacillus* sp. and *Staphylococcus epidermidis*) strains studied was respectively in cm (0.7 ± 0.0, 0.86 ± 0.027, 0.73 ± 0.004) at concentration 500 ppm and (1.15 ± 0.01, 1.68 ± 0.04 and 1.55 ± 0.03) at concentration 750 ppm. Whereas degradation products did not show growth inhibition. These findings suggest non-toxic nature of the product formed. Previous reports showed Malachite Green and Crystal Violet degradation into leucomalachite and leucocrytal violet there are equally toxic to malachite green and crytal violet (Ayed et al. 2009).

Phytotoxicity study of Methyl Red and its extracted metabolites formed after biodegradation, values are mean of germinated seeds of three experiments, SD (±), Standard deviation.

**CONCLUSION**

In this paper, the mixture design method was used for the multi-objective optimization of bacteria with the decolorization in biodegradation process as responses to explore a new optimization method of mixed starter. This study suggested that decolorization by mono and constructed mixed cultures showed that *Staphylococcus epidermidis* accounted for the majority of decolorization activity for biotreatment of dye pollutants. Moreover, *Bacillus* sp. and *Sphingomonas paucimobilis* accounted for the majority of COD removal. Through the establishment of the regression model and the analysis of the interaction between the variables, and by combining the optimization functions of the mixture design software, 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.

**REFERENCES**


