RESPONSE SURFACE METHODOLOGY APPLIED TO LACCASES ACTIVITIES EXHIBITED BY *STENOTROPHOMONAS MALTOPHILIA* AAP56 IN DIFFERENT GROWTH CONDITIONS

Said Galai,[a,*] Youssef Touhami,[b] and Med Nejib Marzouki [a]

Stenotrophomonas maltophilia AAP56, laccase-producing bacteria, growing under different conditions, exhibit laccase activity that is highly affected by some environmental factors. Response surface methodology (RSM) was applied for the determination of laccase factor dependence using two substrates: ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) and DMP (2,6 Dimethoxy-phenol). RSM was carried out with a 2^4 factorial design using four variables, namely, dye (0 to 0.1 mg mL^{-1}), Cu in Med (0 to 400 µM), shaking (0 to 150 rpm), and $CuSO_4$ in assay (0 to 0.2 mM). Significant correlation between the effects of these variables on R1 (ABTS oxidase activity) and R2 (DMP oxidase activity) responses was detected. Astonishing results showed differences between these two activities with respect to copper activity dependence. Anoxic conditions exhibited a significant ability to induce the enzyme. This bacterial laccase activity (produced under optimal conditions according to RSM) was used to decolorize an azoic dye, Reactive Black 5 (RB5). It was efficient only in the presence of a redox mediator to degrade RB5 after 20 min of incubation time.

Keywords: Laccase; Decolorization; Response surface methodology; Experimental design; Reactive Black 5

*Contact information: a: Bioengineering Unit 99-26UR09, National Institute of Applied Sciences and Technology, b: Department of chemical and biological engineering, National Institute of Applied Sciences and Technology, University of Carthage, Tunis, Tunisia; * Corresponding Author: Phone: +216 71703627, +216 71703829, Fax: +216 71704329 , Address: National Institute of Applied Sciences and Technology, Department of Bioengineering, BP 676 – 1080 Tunis Cedex, Tunisia. E-mail: galai_said@yahoo.fr*

INTRODUCTION

The multicopper oxidases constitute a family of enzymes that present broad substrate specificity, oxidizing numerous aromatic phenols and amines (Fernandes et al. 2007). The list of substrates known to be oxidized by these enzymes has increased significantly in recent years: methoxy- or amino-monophenols (2,6-dimethoxy-phenol : DMP) and several non-phenolic compounds such as aromatic diamines, ABTS, 1-naphthol, hydroxyindoles, and syringaldazine are laccase substrates (Mayer 1987; Cai et al. 1993; Klonowska et al. 2002). The laccases constitute a large subfamily of multicopper oxidases, and they have been implicated in various biological activities related to cell division (Deckert et al. 1998), pigment formation (Sanchez-Amat and Solano 1997; Sanchez-Amat et al. 2001), sporulation (Hullo et al. 2001), copper resistance (Ruijssenaars and Hartmans 2004), Cu^{2+} efflux (Kim et al. 2001), and oxidation of toxic compounds (Bains et al. 2003). The ability of laccases to act as

nonspecific oxidases enzymes makes them highly useful biocatalysts for various biotechnological applications (Mayer 2002; Durán et al. 2002; Rodríguez Couto and Toca Herrera 2006; Riva 2006) such as decolorization. However, a few laccases, designated as metallo-oxidases, are able to oxidize lower valence metal ions, such as Cu^{+}, Fe^{2+}, and Mn^{2+}, with high specificity (Fernandes et al. 2007). In general, laccases exhibit four copper atoms, which play an important role in the enzyme catalytic mechanisms (Cha and Cooksey 1991). Moreover, several multicopper oxidases are involved in Cu resistance (Cha and Cooksey 1991; Lee et al. 1994; Ruijssenaars and Hartmans 2004), and they have been induced or enhanced by the presence of copper in the culture medium (Galai et al. 2009; Klonowska et al. 2001). This fact makes several laccases copper-dependant enzymes.

Many authors have implicated laccase in the aerobic or anoxic decolorization of many azoic dyes (Chen et al. 1999; Chang et al. 2001; Yu et al. 2001; Chen et al. 2003), such as Reactive Black 5 (RB5). The RB5 decolorization was brought about by the yeast *Debaryomyces polymorphus* (Yang et al. 2005), the fungus *Candida oleophila* (Lucas et al. 2006), or by the laccase from *Pleurotus sajor-caju* (Murugesan et al. 2007), and an important correlation between the azo dyes addition, and the induction of laccase activity has been reported (Yang et al. 2005; Murugesan et al. 2007).

Stenotrophomonas maltophilia AAP56 is an interesting bacterium that produces biological activities such as laccase activities (Galai et al. 2009) and textile industrial effluent decolorization (Galai et al. 2010). A correlation between bio-decolorization and syringaldazine-oxidase activity has been previously proposed (Galai et al. 2009), but no report exists for the role of dye addition or anoxic condition in *Stenotrophomonas maltophilia* laccase induction. In the aim to study the influence of some factors, eight bacterial cultures were set in different conditions. This study reports the effect of shaking, azo dye, and copper on *S.maltophilia* AAP56 laccases activities (ABTSO and DMPO) by response surface methodology (RSM). In fact, the analysis was carried out through a 2^4 factorial experimental design. RSM is an efficient method to optimize the parameters of the experiment (Murugesan et al. 2007) or to study the effect and the interaction of different factors onto the interested response (London et al. 1982). This method is greatly able to identify a few important variables from a large set (four) of candidate variables and to determine how a few variables (dye, copper in medium, copper in assay and shaking) affect the response (laccase activities).

EXPERIMENTAL

Chemicals and Culture Medium

The dye Reactive Black 5 (RB5), C.I.20505 was obtained from Sigma Aldrich. The substrate ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt) was purchased from Fluka-Biochimica and the DMP (2,6-Di-methoxy-phenol) from Sigma. The copper form used along this work was $CuSO_4$ (BioBlock). The medium used for bacterial growth was LB broth (Sigma) prepared as follows: 20 g per L of H_2O. The bacterial strain *Stenotrophomonas maltophilia* AAP56 was isolated and identified as previously described (Galai et al. 2009) in Bioengineering Laboratory (National Institute

of Applied Sciences and Technology, Tunis, Tunisia). The strain maintenance was ensured by use of 25% (v/v) glycerol fraction conserved at -80°C. For each use, the bacterium cells were cultivated into LB agar plates incubated at 25°C over night. In order to guarantee the purity of culture and the cell viability, *S. maltophilia* AAP56 has been deposited in the Colección Española de Cultivos Tipo (CECT) under accession number CECT 7853.

Culture Conditions

Laccases activities produced by *S.maltophilia* AAP56 were investigated in eight cultures (referenced by: N and NCu for the colored medium or T and TCu for the non-colored medium). Experimental conditions were described as follows: T1, culture in LB medium under shaking conditions; T2, culture in LB medium with static conditions; TCu1, culture in LB medium supplemented by 400 µM $CuSO_4$ with shaking; TCu2, culture in LB medium supplemented by 400 µM $CuSO_4$ under static conditions; N1, culture in LB medium supplemented by 0.1 mg mL^{-1} of RB5 with shaking conditions; N2, culture in LB medium supplemented by 0.1 mg mL^{-1} of RB5 with static conditions; NCu1, culture in LB medium supplemented by 400 µM $CuSO_4$ and 0.1 mg mL^{-1} of RB5 under shaking conditions; and NCu2, culture in LB medium supplemented by 400 µM $CuSO_4$ and 0.1 mg mL^{-1} of RB5 under static conditions. In general, static conditions were referenced by the suffix "2". In front, shaking conditions were referenced by the suffix "1". The incubation temperature used in all bacterial culture experiments was 25°C.

Enzyme Extraction

The bacterial cells were harvested by centrifugation at 23,660 g for 5 min at 4°C, and the resultant bacterial pellet was immediately washed with 1 mL of phosphate buffer (0.1 M pH7.0) and centrifuged again at 13,000 rpm for 5 min at 4°C to collect the bacterial cells. The pellet was conserved in ice and re-suspended in 0.5 mL of phosphate buffer (0.1 M pH7.0). Protein extraction was carried out by sonication for 5 min at 40% amplitude and a 0.7 sec cycle with the sonicator LABSONIC®M B.BRAUN Biotech International. To obtain the cell extract, the centrifugation was done at 13,000 rpm for 20 min at 4°C to pull down the cell fragment. The supernatant was taken carefully in the cool tube. The protein concentration was determined by the Bradford method using bovine serum albumin as standard (Sigma Aldrich) (Bradford 1976).

Laccase Assays

To measure the laccase activity, two substrates were used, DMP and ABTS, to respectively measure DMPO (DMP oxidase) activity and ABTSO (ABTS oxidase) activity. The standard conditions of these two assays can be described as follows: DMPO activity was measured at 37°C in 96 well plates. The substrate oxidation was monitored at 468 nm during 5 min. The total volume of reaction mixture was 250 µL containing 0.1 M phosphate buffer, pH 5.0, 2 mM DMP dissolved in the same phosphate buffer, and an appropriate amount of enzyme. ABTSO activity was made similarly at 37°C in a 250 µL well. The assay mixture was composed by 0.1 M phosphate buffer, pH 5.0, 0.5 mM ABTS, and crude extract. During 5 min the absorbance was steady at 420 nm in a SkanIt

spectrophotometer. Some of these assays were supplemented by 0.2 mM $CuSO_4$, as previously planned in the experimental design (Tables 1 and 2).

Table 1. Factorial Experimental Design (2^4) Setting by Four Factors Applied to RSM

	Factor1 : *X1*	**Factor2 : *X2***	**Factor3 : *X3***	**Factor4 : *X4***	**Response1 : *Y1***	**Response2 : *Y2***
	Dye	Cu in Med	Shaking	$CuSO_4$ in assay	ABTSOact (mU mg^{-1})	DMPOact (mU mg^{-1})
1	-1	-1	+1	-1	0	0
2	-1	-1	+1	+1	0	20.2
3	-1	-1	-1	-1	0	0
4	-1	-1	-1	+1	0	38.78
5	-1	+1	+1	-1	6.46	0
6	-1	+1	+1	+1	67.4	48.63
7	-1	+1	-1	-1	63.28	2.38
8	-1	+1	-1	+1	50.47	54.77
9	+1	-1	+1	-1	0	0
10	+1	-1	+1	+1	0	9.89
11	+1	-1	-1	-1	0	0
12	+1	-1	-1	+1	0	21.8
13	+1	+1	+1	-1	2.27	1.96
14	+1	+1	+1	+1	40.82	28.86
15	+1	+1	-1	-1	85.9	4.53
16	+1	+1	-1	+1	51.71	32.65
Cp 1	-0.43	23.02	-8.4	3.28	7.74 (***a***)	
Cp 2	-4.6	5.19	-4.08	15.42		4.75 (***a***)

(***a***) This coefficient was calculated as follows: Standard Deviation/√16

Each test was complementarily monitored relative to a control assay, which contained the same compound except for the cell extract. One unit of enzyme activity (U) was defined as the amount of enzyme that catalyzed the oxidation of 1 µmol of substrate per min under the standard assay conditions. To calculate the specific laccase activities, the following formulas were applied respectively for ABTS and DMP oxidase activities,

$$\text{ABTSO (mU mg}^{-1}\text{)} = dDO_{420} * 10^3 / 0.025 * [\text{Protein concentration}] * \varepsilon_{ABTS} \quad (1)$$

$$\text{DMPO (mU mg}^{-1}\text{)} = dDO_{468} * 10^3 / 0.025 * [\text{Protein concentration}] * \varepsilon_{DMP} \quad (2)$$

where dDO is the unit of absorbance observed per min of time reaction; [Protein concentration] is the protein cell extract concentration (mg mL^{-1}) of each sample; ε_{ABTS} is

the molar extinction coefficient of ABTS equal to 36000 M^{-1} cm^{-1}, and ε_{DMP} is the molar extinction coefficient of DMP equal to 14800 M^{-1} cm^{-1}.

Table 2. Range and Level of Independent Variables Chosen to Study Its Impact in Response Y1 and Y2

Factor	Unit	Variables	+1	-1
X1	(mg mL^{-1})	RB5	0.1	0
X2	(µM)	Cu in Med	400	0
X3	(rpm)	Agitation	150	0
X4	(mM)	$CuSO_4$ in Assay	0.2	0

Decolorization Measurement

Color removal was determined in centrifuged (23,660 g for 5 min) culture samples appropriately withdrawn at different growth times. Both absorbance readings at visible maximum peak (595 nm) and scanning the spectrum between 380 and 750 nm were performed using a SkanIt spectrophotometer with 96 wells plates.

Regarding the RB5 decolorization monitored with enzymatic extract (such as experiment 8, Table 1), the assay was performed in a reaction mixture containing an appropriate amount of crude extract, 0.5 mM RB5, a predefined concentration of redox mediator, and 0.2 mM $CuSO_4$ in 0.1 M phosphate buffer pH 5.0, incubated at 37 °C for 1 h. The mediator effect was assayed at 1 mM ABTS, 10 mM acetosyringone (ASGN), 10 mM syringaldehyde (SGA), and 10 mM 1-hydroxybenzotriazole (HoBT). The dye degradation was monitored by the decrease in absorbance at 595 nm. The decolorization rate was calculated as the percentage of remaining absorbance per hour. Scanning the absorbance spectrum between 380 and 800 nm was performed using a SkanIt spectro-photometer with 96 wells plates.

Factorial Experimental Design and RSM

Response surface methodology (RSM) is an efficient technique to evaluate the interaction between different experimental factors at different levels and experimentally obtained results. This optimization process involves three major steps: (i) finalizing statistically designed experiments, (ii) estimating the coefficients in a mathematical model, and (iii) calculating the predicted response, the residuals between the two responses and checking the adequacy of the model. Two levels for the estimation of the parameters in a mathematical model were designed, as seen in Table 1. To study the laccase factor dependence, a 2^4 factorial experimental design was selected. The two activities ABTSO and DMPO, designated respectively as Y1 and Y2, were considered as response 1 and response 2. This type of design was implemented using the software

STATGRAPHICS Centurion XVI.I with four variables at two levels (Table 1): Concentrations of dye (0 and 0.1 mg mL^{-1}), copper concentration in the bacterial growth medium (0 and 400 µM), copper sulfate in the enzymatic assay (0 and 0.2 mM), and two different shaking conditions (0 and 150 rpm) were chosen as the critical variables and designated respectively per X1, X2, X4, and X3.

Through this factorial design, the mathematical model was defined by matrix calculus for the two study responses as follows,

$$Y = a_0 + a_1.X1 + a_2.X2 + a_3.X3 + a_4.X4 + a_{12}.X1.X2 + a_{13}.X1.X3 + a_{14}.X1.X4 + a_{23}.X2.X3 + a_{24}.X2.X4 + a_{34}.X3.X4 + a_{123}.X1.X2.X3 + a_{124}.X1.X2.X4 + a_{134}.X1.X3.X4 + a_{234}.X2.X3.X4 + a_{1234}.X1.X2.X3.X4 \quad (3)$$

where Y is a predicted response; a_0 is a constant; a_1, a_2, a_3, a_4 the linear coefficients; $a_{12}, a_{13}, a_{14}, a_{23}, a_{24}, a_{34}$ are cross coefficients; and $a_{123}, a_{124}, a_{134}, a_{234}, a_{1234}$ are cross-interacted coefficients.

The parietal coefficient (C_p) was calculated through 16 experiment responses for each of 4 factors and 11 factor-interactions as follows,

$$Cp = \sum (\alpha_i * Y_i) / 16 \quad (4)$$

where α_i is a value of the level (+1 or -1) and Y_i is a respective value of the response.

RESULTS

Factorial Design and Mathematical Models considered in the RSM

Factorial design was applied to four significant variables (*X1*, *X2*, *X3*, and *X4*) into two response types (*Y1* and *Y2*) as described in Tables 1 and 2. The data resulting from 16 experiments of the effect of four variables (Dye, Cu in medium, shaking, and $CuSO_4$ in assay) on laccases activities (ABTSO and DMPO) were used to obtain two mathematical models. These equations were obtained from Eq. 3 through the study of the parietal coefficient (*Cp*) of each factor and the interaction between factors. The fitted model was designed to explain the relationship between the independent variables and dependent responses. The mathematical expressions of ABTSO (Y1) and DMPO (Y2) activities with variables coded by *X1*, *X2*, *X3*, and *X4* are shown below,

$$\boldsymbol{Y1} = 23.02 + 23.02*\boldsymbol{X_2} - 8.4*\boldsymbol{X_3} - 8.4*\boldsymbol{X_2}*\boldsymbol{X_3} + 9.15*\boldsymbol{X_3}*\boldsymbol{X_4} + 9.15*\boldsymbol{X_2}*\boldsymbol{X_3}*\boldsymbol{X_4} \quad (5)$$

$$\boldsymbol{Y2} = 16.52 - 4.6*\boldsymbol{X_1} + 5.19*\boldsymbol{X_2} - 4.08*\boldsymbol{X_3} + 15.42*\boldsymbol{X_4} - 4.58*\boldsymbol{X_1}*\boldsymbol{X_4} + 4.08*\boldsymbol{X_2}*\boldsymbol{X_4} \quad (6)$$

where ***Y1*** is ABTSO, ***Y2*** is DMPO, $\boldsymbol{X_1}$ is Dye RB5, $\boldsymbol{X_2}$ is Cu in Medium, $\boldsymbol{X_3}$ is shaking condition, and $\boldsymbol{X_4}$ is $CuSO_4$ in the assay mixture.

Then the predicted responses were calculated, and the residuals value between the experimental and the theoretically were estimated and represented in Table 3 for the ABTSO and Table 4 for the DMPO activity. The estimated responses seem to have a functional relationship with the experimental one.

Table 3. Comparison Table between Predicted and Experimental Values for the Response Y1 : ABTSO Activity

	Y1 (pre)	Y1 (Exp)	Residual 1
1	0	0	0
2	0	0	0
3	0	0	0
4	0	0	0
5	10.94	6.46	-4.48
6	47.54	67.4	19.86
7	81.14	63.28	-17.86
8	44.54	50.47	5.93
9	0	0	0
10	0	0	0
11	0	0	0
12	0	0	0
13	10.94	2.27	-8.67
14	47.54	40.82	-6.72
15	81.14	85.9	4.76
16	44.54	51.71	7.17

Table 4. Comparison Table between Predicted and Experimental Values for the Response Y2 : DMPO Activity

	Y2 (pre)	Y2 (Exp)	Residual 2
1	4.07	0	-4.07
2	27.77	20.2	-7.57
3	4.09	0	-4.09
4	35.93	38.78	2.85
5	1.85	0	-1.85
6	46.31	48.63	2.32
7	6.31	2.38	-3.93
8	54.47	54.77	0.3
9	4.11	0	-4.11
10	9.41	9.89	0.48
11	4.05	0	-4.05
12	17.57	21.8	4.23
13	1.89	1.96	0.07
14	27.95	28.86	0.91
15	6.27	4.53	-1.74
16	36.11	32.65	-3.46

Adequacy of the ABTSO Model

The results of analysis of variance (ANOVA) are shown in Table 5-A, which indicates that the predictability of the model was significant at the 95% confidence level. The predicted response fit well with those of the experimentally obtained response. A coefficient of determination (R^2) value of 93% showed that the equation was highly reliable. A *P* value of less than 0.05 indicates that the model was statistically significant. The model was presumed to be adequate for prediction within the range of variables chosen. Figure 1-A shows observed responses versus those from the statistical model (Eq. 5).

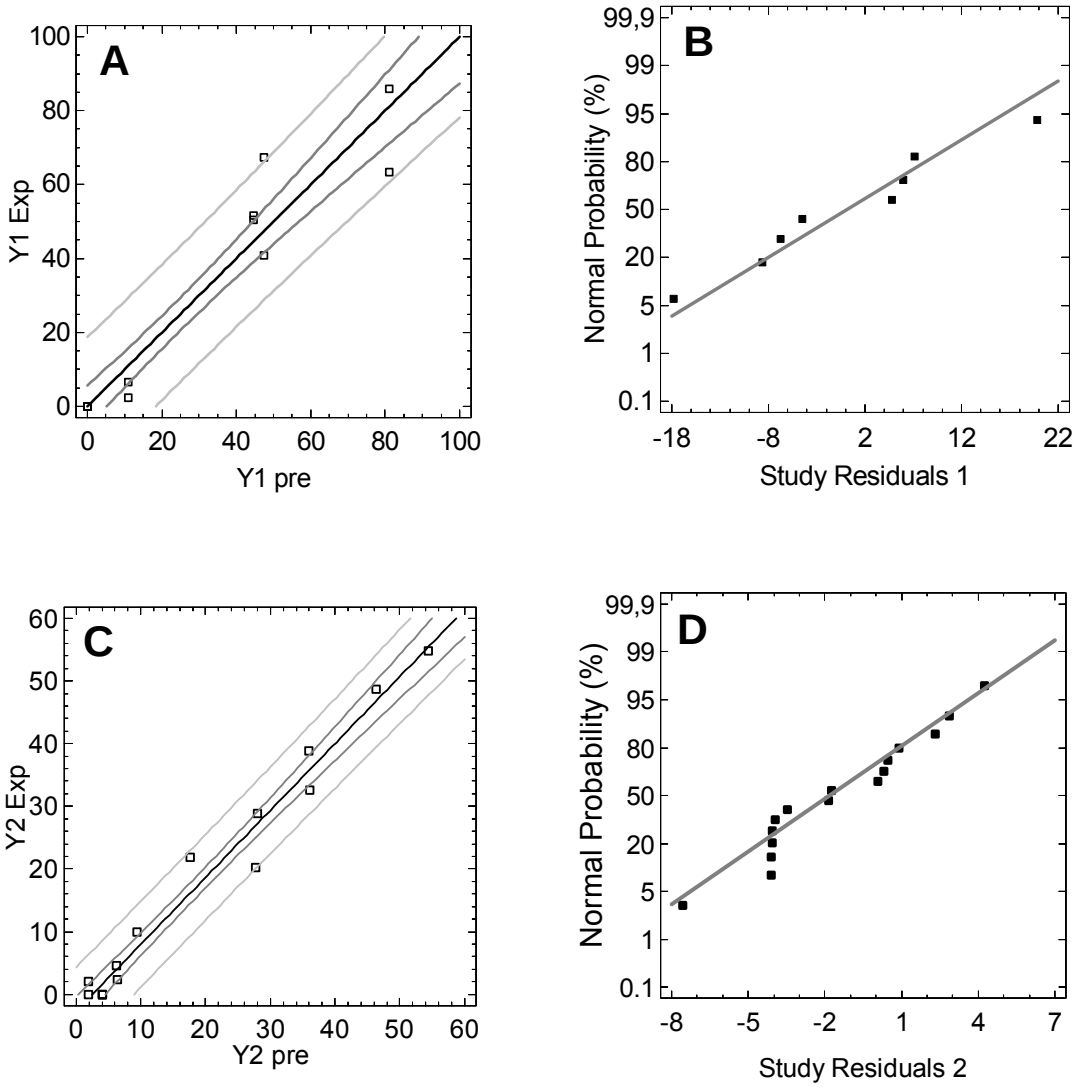


Fig. 1. Statistical studies of the model fitted for the response Y1 **(A)** and Y2 **(C)**. The regression curves were set between predicted theoretical responses and experimental obtained value. Statistical analysis was by normality plot of the residual 1 **(B)** and residual 2 **(D)**.

The correlation between the two values seems to be very high; it has been expressed by the adjusted model equation as follows,

$$Y1_{Exp} = -0.00334434 + 1.00012 * Y1_{pre} \quad (7)$$

where $Y1_{Exp}$ is the experimentally obtained value and $Y1_{pre}$ is the theoretically predicted response.

The coefficient of determination (1.000) indicates that the predicted data of the response from the empirical model was in good agreement with the experimentally obtained data. It is important to confirm the fitted model to make sure that it gives sufficient approximation to the actual test. The residuals from the Table 4 play an important role in judging model adequacy. By constructing a normal probability plot of the residuals, a check was made for the normality assumption. As shown in Fig. 1-B, the normality statement was satisfied, as the residual plot approximated a straight line.

Table 5. (A) ANOVA Table for the Response Y1: ABTSO Activity

Source	*Sum of Squares*	*Degree of freedom*	*Mean Squares*	*F-value*	*Probability*
Model	13418,9	1	13418,9	195,08	0,0000
Residual	963,028	14	68,7877		
Total (Corr.)	14382,0	15			

Correlation coef.: 0,96594 ; R^2: 93,3039 % ; R^2(adj): 92,8256 % ; STD: 8,29384 ; abs. Error: 4,7168

Table 5. (B) ANOVA Table for the Response Y2: DMPO Activity

Source	*Sum of Squares*	*Degree of freedom*	*Mean Squares*	*F-value*	*Probability*
Model	5274,24	1	5274,24	553,28	0,0000
Residual	133,458	14	9,53268		
Total (Corr.)	5407,7	15			

Correlation coef.: 0,987583; R^2: 97,5321 % ; R^2(adj): 97,3558 % ; STD: 3,0875; abs. Error: 2,36405

Adequacy of the DMPO Model

The results of the adjustment, as a linear model, was well described by the relation between Y2 (Exp) and Y2 (pre) by the model adjusted equation as follows,

$$Y2_{Exp} = -2.7168 + 1.06857 * Y2_{pre} \quad (8)$$

where $Y2_{Exp}$ is the experimental obtained value and $Y2_{pre}$ is the theoretically predicted response.

As the value of the probability in the table of the ANOVA (Table 5-B), is lower than 0.05, there was a statistically significant relationship between the experimental results Y2 (Exp) and the predicted values Y2 (pre), with a degree of confidence of 95%.

The statistics of R^2 indicate that the adjusted model explained 97.53% of variability in Y2 (Exp). The coefficient of determination in the adjusted model was 0.987, which indicates a strong relation between the study responses (experimental and predicted). The estimated typical error was indicated by the standard deviation of the residues, which was equal to 3.087. The average absolute error of 2.364 is the average value of the residues. These parameters were used to define a limit of prevision and confidence, as shown in Fig. 1-C. The point plot seems to be statistically included into the prevision domain. In terms of adequacy of the fitted model, Fig. 1-D shows the normality plot of the residual (Table 4), which demonstrates that the normality assumption was satisfied, as the residual plot approximated a straight line.

Response Surface Methodology Design

It is necessary to obtain adequate models before beginning the RSM design. The fitted models were previously validated by statistical studies and were used in response surface methodology. In fact, the studied regression, residuals, and normal probability (Fig. 1) demonstrate that the two mathematical models can be considered as good ones. Moreover, the experimental response was very reliable. The relationship between the experimental and the predicted value was statistically satisfied to validate the fitted model designed for responses Y1 and Y2.

Response surface methodology is widely used in applied microbiology and biochemistry. In fact, RSM was successfully applied for optimizing conditions of RB5 degradation by fungal laccase (Murugesan et al. 2007), for evaluation of bioconversion experimental condition of citronellal (Cheynier et al. 1983), for the finalizing parameters for bio-bleaching a wheat pulp by bacteria-producing laccase (Gursharan et al. 2008), and for factors effect on the assay of γ-glutamyl-transferase enzyme (London et al. 1982). The method has been used generally to show the combined effect of two factors (in different levels) on a response.

The resulting surface can be informative in terms of the effect of the different level of factors onto the response through the contour plot. In the present case, the factors that affected the two responses were different, and showed that the more significant interactions were detected through the study of the parietal coefficient. Regarding ABTSO activity, the more important interactions were between X2/X3 and between X3/X4. However, DMPO activity showed two interesting interactions between X1/X4 and between X2/X4. Then, two surfaces were designed for each response using STATGRAPHICS Centurion XVI.I, as shown in Figs. 2 and 3 with the contour plot.

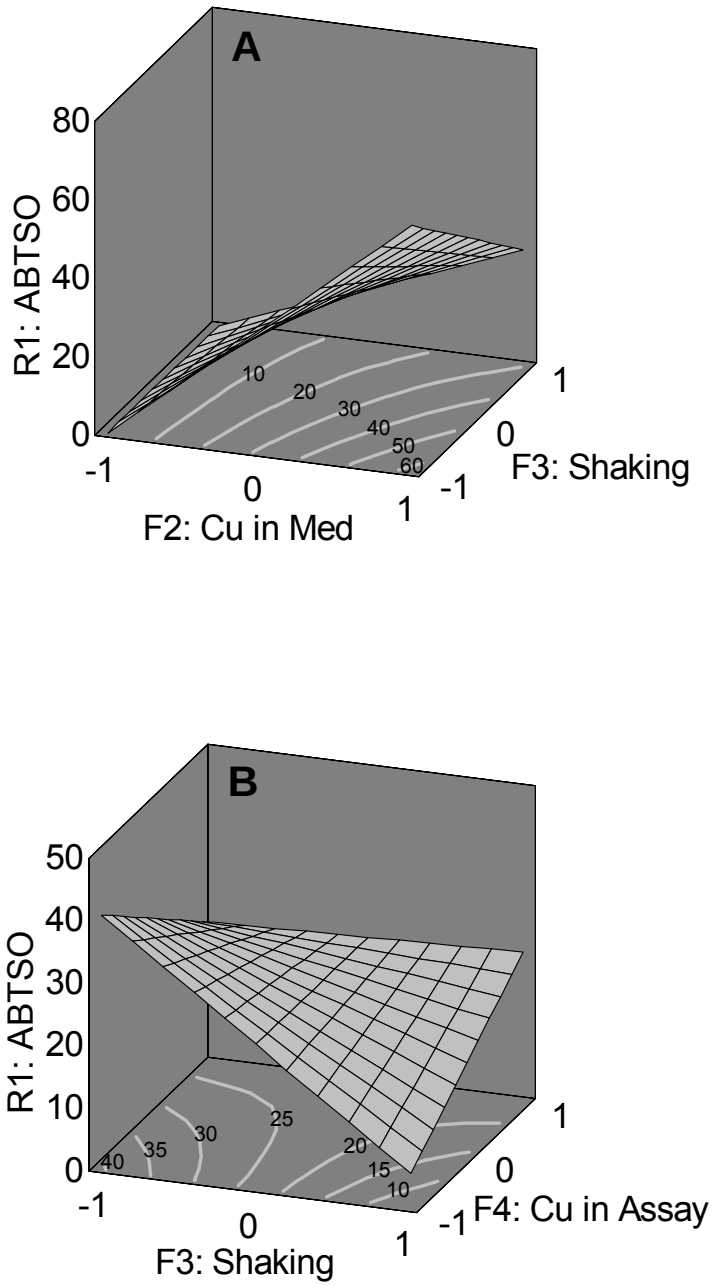


Fig. 2. Response surface plot showing **(A)** the effect of shaking and copper addition in the medium, **(B)** the effect of shaking and copper addition in the assay mixture on the ABTSO activity

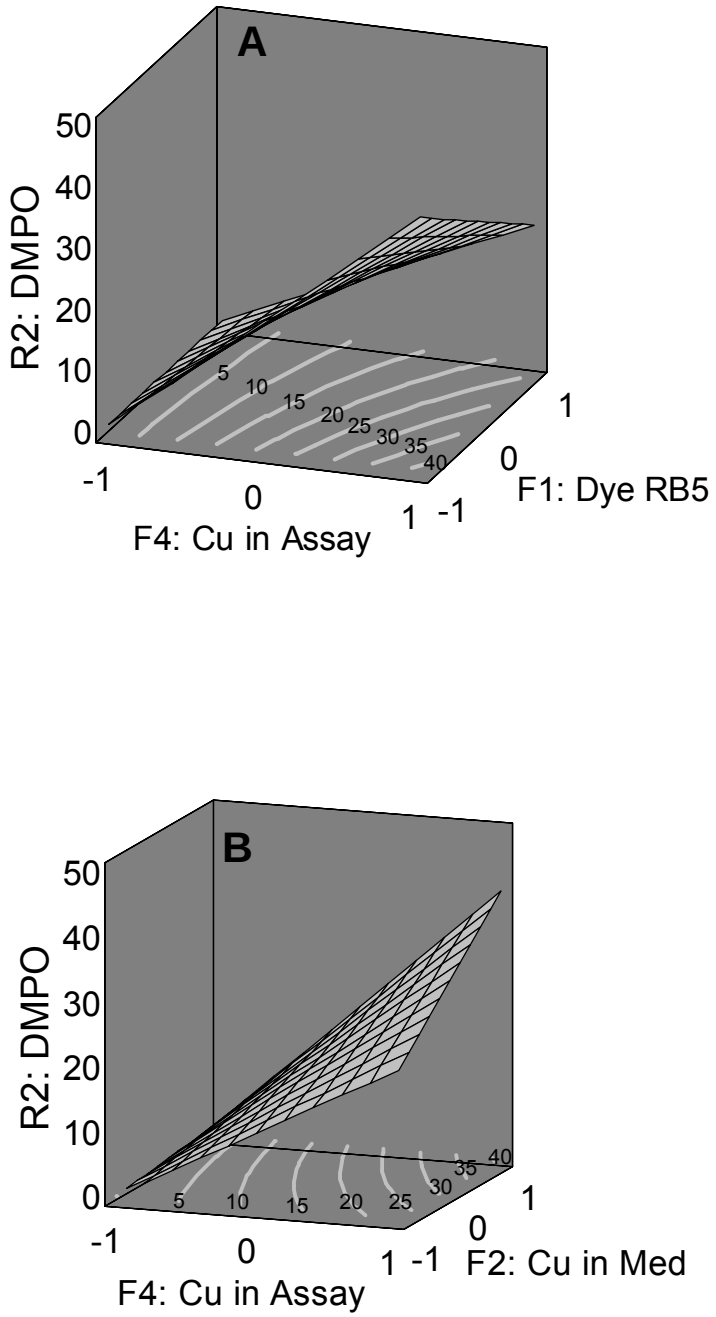


Fig. 3. Response surface plot showing **(A)** the effect of copper supplementation in the assay and dye addition in the medium, **(B)** the effect of copper addition in the growth media and in the assay mixture on the DMPO activity

Analysis of Laccase Factor Dependence

Laccases activities analysis showed a different factor attribution on the oxidation of the phenolic substrate DMP and diammonium salt substrate ABTS. In fact, Table 1 shows that the most influential parameters were X2 and X3 in the case of ABTSO and X2 and X4 in the case of DMPO. However, through the parietal coefficient, the interaction seems to have been significant between X2/X3 and X3/X4 in the case of the response ABTSO and between X1/X4 and X2/X4 in the case of DMPO activity.

Regarding the first response ABTSO activity, the parietal coefficient (*Cp*) defined, for each factor separately (Table 1), demonstrates that the most important parameters that affected the response Y1 were Cu in medium (Factor 2) and shaking (Factor 3). Studying the interaction between X2/X3, and its correlation with the Y1, indicates that the two factors had an important effect (Fig. 2-A). The optimal conditions for these parameters were 0 rpm for the shaking (X3) and 400 µM of $CuSO_4$ (X2) in the culture medium. The interaction between the concentration of copper in the medium and the shaking condition of the culture had a great effect on the ABTSO activity produced by the bacteria.

However, Factors 3 and 4 seem to have had an independent effect, which means that the interaction between these two factors cannot seriously affect ABTSO production by bacteria. These two parameters had a distinct effect on the Response 1, as shown by the contour plot of the surface (Fig. 2-B). In fact, the optimal parameter for the ABTSO activity was no shaking in the culture condition and no addition of copper in the assay mixture. The enhancement of Factor 3 (from 0 to 150 rpm) can affect seriously the ABTSO activity, but the increase of concentration of copper in the enzymatic assay did not have a great effect (Fig. 2-B). It can be concluded that the optimal conditions to obtain a maximal response for ABTSO activity are 0 rpm for shaking (X3), 400 µM of copper in the LB medium (X2), and no copper addition in the assay mixture (X4).

With regards to the second response (DMPO activity), the study of parietal coefficient (*Cp*) demonstrated that the significant, influential factors were Factor 2 (Cu in Med) and Factor 4 ($CuSO_4$ in assay). However, analyzing the interactions through parietal coefficient, two additional important ones arise: between X1/X4 and X2/X4. The interaction between Factors 1 and 4 showed that the optimal conditions were 0.2 mM $CuSO_4$ in the assay and 0 mg mL^{-1} of dye (Fig. 3-A). The addition of the RB5 in the culture medium did not seriously affect the DMPO activity, but the increase of the copper concentration in the reaction mixture enhanced the response Y2 (Fig. 3-A). The second interaction between the copper in the medium (Factor 2) and the one added in the assay (Factor 4) seems to have been important (Fig. 3-B). The optimal values were 400 µM of Cu in the LB media and 0.2 mM of $CuSO_4$ in the reaction mixture.

Decolorization of RB5 with Laccase Produced by *S. maltophilia* AAP56

The difference shown between the ABTSO and DMPO activities is made clear by the experimental design and response surface methodology. Then, the enzymatic reaction of the enzymes from *S.maltophilia* AAP56 with ABTS and DMP as laccase substrate seems to be different. Study of laccase production by *S.maltophilia* AAP56 showed both ABTSO and DMPO activities to be detectable in the experiment 8 with high values (Tab. 1). This means that the bacteria were cultured in anoxic conditions and in presence of

copper in the growth medium. The enzymatic extract obtained from these conditions was applied to decolorize RB5 *in vitro* in presence of some redox mediators (Table 6). These assays show that the decolorization cannot be possible in absence of copper in the assays. The enzyme responsible for the RB5 biodegradation is highly dependent on the copper addition in the assay. RSM showed that the DMPO activity was related to Factor 4 (copper in the assay) in contrast to the ABTSO activity, which showed that the Factor 4 was not an influenced factor. These results suggest that the DMPO activity is greatly implicated *in vitro* decolorization of RB5. The best redox mediator detected through these experiments was the ABTS, showing a high decolorization rate (98%) such as represented in Fig. 5-A.

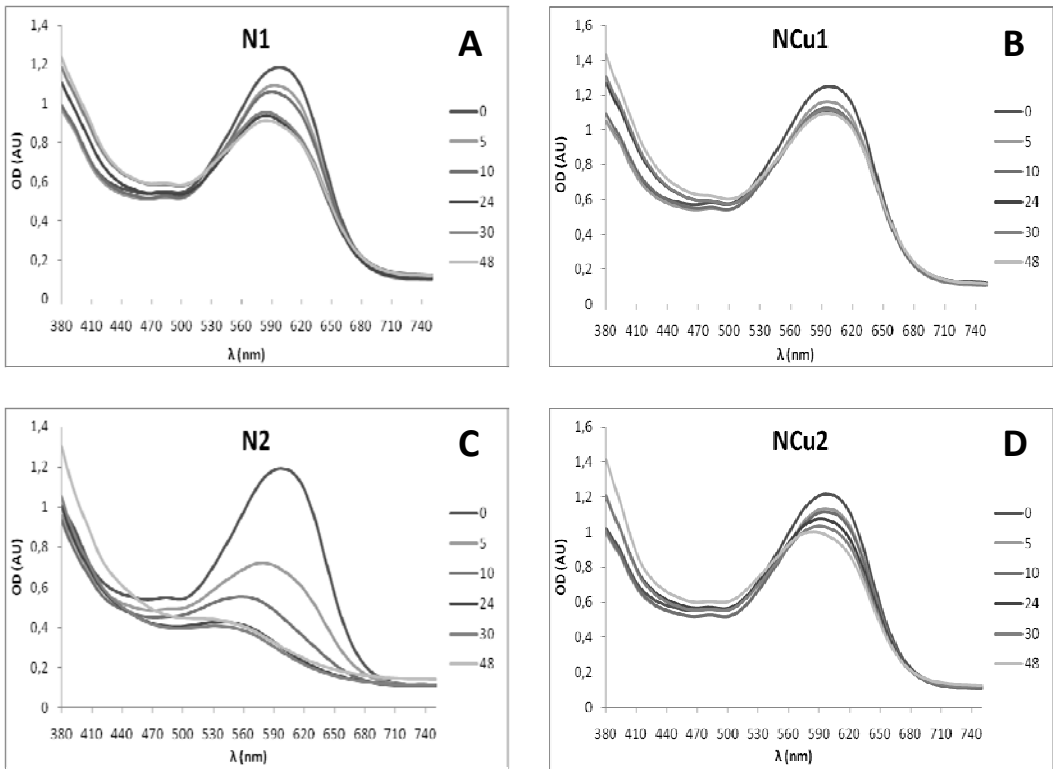
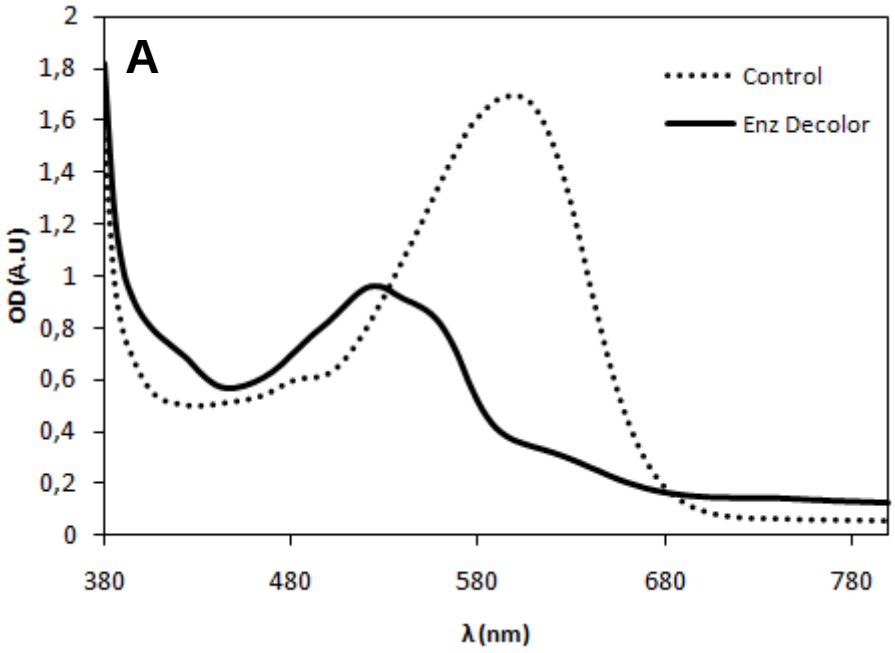


Fig. 4. Spectrum of RB5 steady from 380 to 750 nm in the medium supernatant of bacterial growth at different conditions: N1, N2, NCu1, and NCu2. Respectively, the culture of *S. maltophilia* AAP56 was set in LB medium **(A)** supplemented by 0.1 mg mL^{-1} of RB5 under shaking conditions; **(B)** supplemented by 0.1 mg mL^{-1} of RB5 with static conditions; **(C)** supplemented by 400 µM $CuSO_4$ and 0.1 mg mL^{-1} of RB5 with shaking conditions; **(D)** supplemented by 400 µM $CuSO_4$ and 0.1 mg mL^{-1} of RB5 under static conditions. The kinetic decolorization of RB5 was represented in the four figures (A, B, C and D) from top to bottom, respectively, from 0 to 48 h.

Table 6. Decolorization Assays with Laccase Activity from *S.maltophilia* AAP56 in Presence of Different Redox Mediators

Redox mediators	HoBT (10mM)		SGA(10mM)		ASGN (10mM)		ABTS (1 mM)	
$CuSO_4$ (0.2 mM)	**+**	**-**	**+**	**-**	**+**	**-**	**+**	**-**
Decolorization rate (% h^{-1})	9	0	26	0	44	0	98	0

* Enzymatic extract (used for these assays) was obtained from the culture monitored in anoxic condition and in presence of 400µM copper sulfate in LB medium (TCu2).



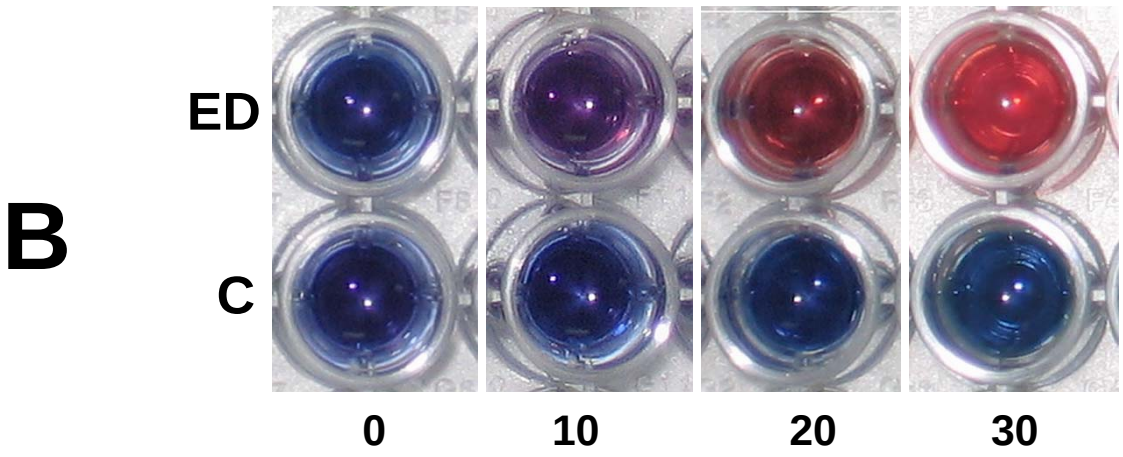


Fig. 5. (A) Spectrum of one-hour RB5 decolorization with enzymatic extract in presence of 1 mM ABTS and 0.2 mM copper sulfate. Control was monitored in the same condition (1 mM ABTS and 0.2 mM copper sulfate) in absence of enzymatic extract. **(B)** Illustrations of kinetics of RB5 decolorization at 0 min, 10 min, 20 min, and 30 min. (ED) Reaction carried out with enzymatic extract in presence of 1 mM ABTS and 0.2 mM copper sulfate. (C) Control was monitored in the same condition (1 mM ABTS and 0.2 mM copper sulfate) in absence of enzymatic extract.

DISCUSSION

The response surface methodology is very efficient for determining the optimal condition of laccase production (Singh et al. 2009) or the factor interaction effect on the enzymatic assay (London et al. 1982). This study is the first report through RSM to: determine the bacterial laccase anoxic dependence of and to make comparison between two substrates (DMP, ABTS) commonly used for screening laccase activities. The present results, obtained through RSM, lead to the conclusion that ABTSO activity is inducible by copper in the anoxic condition, but it is not copper demanding for its oxidative activity. This conclusion confirms that the laccase activity produced by *S.maltophilia* AAP56 is a copper inducible one (Galai et al. 2009). However, the anoxic conditions were not previously assayed with a bacterial laccase (Galai et al. 2009). This is the first report on the intracellular ABTS oxidase activity produced in anoxic condition by *S.maltophilia* AAP56. Copper sulphate is widely used as a promising enhancer for laccase production, but no report has existed that considers the anoxic condition. Several authors describe the role of copper in laccase induction, such as its utilization for the bacteria *Streptomyces psammoticus* under shaking condition (175 rpm) to produce an extracellular laccase (Niladevi and Prema 2008). As well, the CotA laccase from *Bacillus subtilis* is strongly dependent on the presence of copper and oxygen in the culture media (Hullo et al. 2001). γ-*Proteobacterium* JB, an alkali-tolerant soil isolate, produced laccase constitutively (Singh et al. 2007), but it has been reported that the laccase production can be enhanced by 100 μM $CuSO_4$ and at 210 rpm as the shaking condition (Singh et al. 2009). Although, *Bacillus* sp. HR03, a melanogenic soil bacterium, produced constitutively both a laccase and tyrosinase activities (Mabrouk and Yusef 2008), and *Marinomonas mediterranea* MMB1 produced a pluripotent polyphenoloxidases without copper induction but in an aerobic condition (Solano et al. 2001, Fernandez et al. 1999). Moreover, Fig. 2-B shows that the anoxic ABSTO activity was not enhanced with copper when $CuSO_4$ was added to the assay mixture. This fact makes this laccase activity unique compared to the one previously described by Galai et al. (2009). The response Y2 (DMPO activity) was not clearly affected by deletion of the copper in the medium but was seriously turned down by the decrease of the concentration of the $CuSO_4$ in the enzymatic assay. DMPO is a highly copper-dependant laccase compared to the ABTSO activity. DMP and ABTS were widely used to screen the laccase activity without any differences. Through the study of biological activities produced by *S.maltophilia* AAP56 in eight conditions culture (T1, T2, TCu1, TCu2, N1, N2, NCu1 and NCu2), a considerable difference of laccase copper dependence was observed when the DMP or ABTS are used as substrates.

However, focusing on the quantitatively production of laccases, we showed differences comparing some bacterial species. *Pseudomonas putida* was able to produce 11 mU mg^{-1} (McMahon et al. 2007), *Bacillus sp.* HR03 exhibited a DMPO-specific activity equal to 50 mU mg^{-1} (Dalfard et al. 2006), and *Sinorhizobium meliloti* CE46 purified laccase-like enzyme was characterized by 266 U mg^{-1} of ABTSO activity (Castro-Swinski et al. 2002). In comparison with *S.maltophilia* AAP56 laccase (approximate average value is 52 mU mg^{-1} for each substrate when consider experiment 8), which was obtained in cell extract, we observed that it was in the normal level of laccase

production by bacteria species. However, a purification process is needed to improve the specific activity of the enzyme.

RB5 decolorization was observed in bacterial growth medium. Figure 4-C shows that substantial RB5 color removal (80%) was observed in the anoxic condition and in absence of copper in the medium (only in condition N2). On the other hand, RSM demonstrated that there was no induction effect of dye on the laccase activity (in the two cases: aerobic and anoxic conditions). The DMPO activity that was obtained in experiment 12 (21.88 mU mg^{-1}) did not significantly contribute to the bacterial decolorization observed in the N2 condition. Hence, the others cases (N1, NCu1, and NCu2) show that the bacterial growth was unable to decolorize the azoic dye in these conditions (Fig. 4) in spite of high laccase activities. Although some authors make connection between laccase activity induction and bacterial decolorization (Ayed et al. 2010), in this work no connection between these two metabolic processes could be discerned.

However, the *in vitro* decolorization obtained by *S.maltophilia* AAP56 laccase needs the use of redox mediators such as HBT, ABTS, ASGN, and HoBT. RB5 is a high redox potential dye, which is not oxidized by a high redox potential laccase like those from *Pycnoporus cinnabarinus* and *Trametes villosa* (Lucas et al. 2006). It needs the use of redox mediators like acetosyringone (ASGN) and syringaldehyde (SGA) (Mayer 1987). In general, the bacterial laccases have low redox potentials compared to those of fungi (Yu et al. 2001). Moreover, the bacterial laccases can be used in neutral and alkaline pH, in contrast with fungal ones, which require acidic media (Yu et al. 2001). *Streptomyces psammoticus* laccase is able to decolorize some anthraquinonic dyes in the presence of ABTS and HoBT, but the decolorization rate is very low (Maier et al. 2004). For the bacterial laccase CotA, Brissos et al. (2009) used ABTS to screen the decolorization capacity of these laccases from *Bacillus subtilis*. However, the reaction needs 24 h to fully oxidized the azoic dye (Pereira et al. 2009). Then, the advantages of *S.maltophilia* AAP56 laccase are that it is able to decolorize RB5 in less than 20 min in presence of 1 mM ABTS (Fig. 5-B). The *S.maltophilia* AAP56 laccase shows an interesting decolorization activity, able to be applied in the bioremediation process, biobleaching of paper pulp, or decolorization of industrial effluent.

CONCLUSIONS

The response Y2 is closely related to the factor X4. In fact, the optimal conditions for the DMPO activity are 0 mg mL^{-1} of RB5, 400 µM of Copper sulphate in growth medium, and 0.2 mM of $CuSO_4$ in the enzymatic assay. Shaking condition had no significant effect on DMPO activity. Yet, the factors X1 and X2 did not have a clear effect, in comparison with that of factor X4. The enzymatic protein responsible for DMPO activity seems to be a copper dependant one to oxidase the phenolic substrates and, consequently, it is not an inducible enzyme by copper addition in the medium. However, Solano et al. (2001) report that the DMPO activity is closely related to the blue copper enzyme required the copper as an inducer or a regulatory ion such as CueO produced by *E.coli* (closely in presence of copper) or CopA produced by *Pseudomonas*

syringae and *Xanthomonas campestris* (in excess of copper). These enzymatic proteins required, also, copper to oxidase DMP, as the enzyme studied in this work. However, ABTSO activity (Y1) is inducible by 400 µM of copper and not highly affected by the absence of copper in the assay mixture. Then, the factor X2 is the more effective one on this activity. This work indicates that the two activities ABTSO and DMPO produced by *S. maltophilia* AAP56 are deeply different, and the choice to use ABTS or DMP as a substrate to screen the laccase activities must to be carefully considered.

Knowledge of influent parameters on the *S. maltophilia* AAP56 laccase activities through the present design is necessary to give information of the effect of the presence or absence of some factor (screening factors). This paper is the first approach concerning this issue. We have planned to refine these results through a subsequent experimental design, which will be able to determine the optimal conditions that would be needed to obtain high laccases activities comparing to those reported in the literature.

ACKNOWLEDGMENTS

The authors gratefully thank Pr. Antonio Sanchez Amat (Department of Genetics and Microbiology, Faculty of Biology, University of Murcia, Spain) for a generous gift of the Reactive Black 5 and the DMP substrates and for his positive discussion that made it possible to prepare this manuscript. This work was supported by the Tunisian Ministry of Higher Education, Scientific Research and Technology (Financial project of Bioengineering Unit 99 UR/09-26).

LITERATURE CITED

Ayed, L., Khelifi, E., BenJannet, H., Miladi, H., Cheref, A., Achour, S., and Bakhrouf, A. (2010). "Response surface methodology for decolorization of azo dye Methyl Orange by bacterial consortium: Produced enzymes and metabolites characterization," *Chemical Engineering Journal* 165, 200-208.

Bains, J., Capalash, N., and Sharma, P. (2003). "Laccase from a nonmelanogenic, alkalotolerant *γ-proteobacterium* JB isolated from industrial wastewater drained soil," *Biotechnol. Lett.* 25, 1155-1159.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantification of protein utilizing the principle of proteinedye-binding," *Anal Biochem* 72, 248-254.

Brissos, V., Pereira, L., Munteanu, F. D., Cavaco-Paulo, A., and Martins, L. O. (2009). "Expression system of CotA-laccase for directed evolution and high-throughput screenings for the oxidation of high-redox potential dyes," *Biotechnol. J.* 4, 558-563.

Cai, W., Martin, R., Lemaure, B., Leuba, J. L., and Petiard, V. (1993). "Hydroxy-indoles: A new class of laccase substrates," *Plant Physiol Biochem.* 31, 441-445.

Castro-Sowinski, S., Martinez-Drets, G., and Okon Y. (2002) "Laccase activity in melanin-producing strains of Sinorhizobium meliloti," *FEMS Microbiology Letters* . 209, 119-125.

Cha, J., and Cooksey, D. A. (1991). "Copper resistance in *Pseudomonas syringae* by periplasmic and outer membrane proteins," *Proc Natl Acad Sci USA* 88, 8915-8919.

Chang, J. S., Chou, C., Lin, Y., Ho, J., and Hu, T. L. (2001). "Kinetic characteristics of bacterial azo-dye decolorization by *Pseudomonas luteola*," *Water Research* 35, 2041-2850.

Chen, K. C., Huang, W. T., Wu, J. Y., and Houng, J. Y. (1999). "Microbial decolorization of azo dyes by *Proteus mirabilis*," *Journal of Industrial Microbiology and Biotechnology* 23, 686-690.

Chen, K. C., Wu, J. Y., Liou, D. J., and Hwang, S. J. (2003). "Decolorization of textile dyes by newly isolated bacterial strains," *Journal of Biotechnology* 101, 57-68.

Cheynier, V., Feinberg, M., Chararas, C., and Ducauze, C. (1983). "Application of response surface methodology to evaluation of bioconversion experimental conditions," *Appl and Environm Microbiol* 45(2), 634-639.

Dalfard, A. B., Khajeh, K., Soudi, M. R., Naderi-Manesh, H., Ranjbar, B., and Sajedi, R. H. (2006). "Isolation and biochemical characterization of laccase and tyrosinase activities in a novel melanogenic soil bacterium," *Enzyme and Microbial Technology* 39, 1409-1416.

Deckert, G., Warren, P. V., Gaasterland, T., Young, W. G., Lenox, A. L., Graham, D. E., Overbeek, R., Snead, M. A., Keller, M., Aujay, M., Huber, R., Feldman, R. A., Short, J. M., Olson, G. J., and Swanson, R. V. (1998). "The complete genome of the hyperthermophilic bacterium *Aquifex aeolicus*," *Nature* 393, 353-358.

Durán, N., Rosa, M. A., D'Annibale, A., and Gianfreda, L. (2002). "Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: A review," *Enzyme and Microbial Technology* 31, 907-931.

Fernandes, A. T., Soares, C. M., Pereira, M. M., Huber, R., Grass, G., and Martins, L. O. (2007). "A robust metallo-oxidase from the hyperthermophilic bacterium *Aquifex aeolicus*," *FEBS Journal* 274, 2683-2694.

Fernandez, E., Sanchez-Amat, A., and Solano, F. (1999). "Location and catalytic characteristics of a multipotent bacterial polyphenol oxidase," *Pigment Cell Res* 12, 331-339.

Galai, S., Limam, F., and Marzouki, M. N. (2009). "A new *Stenotrophomonas maltophilia* strain producing laccase. Use in decolorization of synthetics dyes," *Appl. Biochem. Biotechnol.* 158, 416-431.

Galai, S., Limam, F., and Marzouki, M. N. (2010). "Decolorization of an industrial effluent by free and immobilized cells of *Stenotrophomonas maltophilia* AAP56. Implementation of efficient down flow column reactor," *World Journal of Microbiology and Biotechnology* 26(8), 1341-1347.

Gursharan, S., Naveen, A., Mona, B., Neena, C., and Prince, S. (2008). "Biobleaching of wheat straw-rich soda pulp with alkalophilic laccase from γ-*proteobacterium* JB: Optimization of process parameters using response surface methodology," *Bioresource Technology* 99, 7472-7479.

Hullo, M. F., Moszer, I., Danchin, A., and Martin-Verstraete, I. (2001). "CotA of *Bacillus subtilis* is a copper-dependent laccase," *J. Bacteriol*.183, 5426-5430.

Kim, C., Lorenz, W. W., Hoopes, T., and Dean, J. F. D. (2001). "Oxidation of siderophores by the muticopper oxidase encoded by the *Escherichia coli* yac K gene," *J. Bacteriol*. 183, 4866-4875.

Klonowska, A., Gaudin, C., Fournel, A., Asso, M., Le Petit, J., Giorgi, M., and Tron, T. (2002). "Characterization of a low redox potential laccase from the basidiomycete C30," *Eur. J. Biochem.* 269, 6119-6125.

Klonowska, A., Le Petit, J., and Tron, T. (2001). "Enhancement of minor laccases production in the basidiomycete *Marasmius quercophilus* C30," *FEMS Microbiology Letter* 200, 25-30.

Lee, Y., Hendson, M., Panopoulos, N. J., and Schroth, M. N. (1994). "Molecular cloning, chromosomal mapping, and sequence analysis of copper resistance genes from *Xanthomonas campestris* pv. *juglandis*: homology with small copper proteins and multicopper oxidases," *J. Bacteriol.* 176, 173-188.

London, J. W., Shaw, L. M., Theodorsen, L., and Stromme, J. H. (1982). "Application of response surface methodology to the assay of gamma-glutamyltransferase," *Clin. Chem.* 28(5), 1140-1143.

Lucas, M. S., Amaral, C., Sampaio, A., Peres, J. A., and Dias, A. A. (2006). "Biodegradation of the diazo dye Reactive Black 5 by a wild isolate of *Candida oleophila*," *Enzyme and Microbial Technology* 39, 51-55.

Mabrouk, M. E. M., and Yusef, H. H. (2008). "Decolorization of Fast Red by *Bacillus Subtilis* HM," *Journal of Applied Sciences Research* 4(3), 262-269.

Maier, J., Kandelbauer, A., Erlacher, A., Cavaco-Paulo, A., and Gübitz, G. M. (2004). "A new alkali-thermostable azoreductase from *Bacillus* sp. strain SF," *Appl and Environm Microbiol* 70(2), 837-844.

Mayer, A. M. (1987). "Polyphenol oxidases in plants-recent progress," *Phytochemistry* 26, 11-20.

Mayer, A. M., and Staples, R. C. (2002). "Laccase: New functions for an old enzyme," *Phytochemistry* 60, 551-565.

McMahon, A. M., Doyle, E. M., Brooks, S., and O'Connor, K. E. (2007). "Biochemical characterisation of the coexisting tyrosinase and laccase in the soil bacterium *Pseudomonas putida* F6," *Enzyme and Microbial Technology* 40, 1435-1441.

Murugesan, K., Dhamija, A., Nam, I. H., Kim, Y. M., and Chang, Y. S. (2007). "Decolourization of Reactive Black 5 by laccase: Optimization by response surface methodology," *Dyes and Pigments* 75, 176-184.

Niladevi, K. N., and Prema, P. (2008). "Effect of inducers and process parameters on laccase production by *Streptomyces psammoticus* and its application in dye decolourization," *Bioresource Technology* 99, 4583-4589.

Pereira, L., Coelhoa, A. V., Viegas, C. A., Correia dos Santos, M. M., Robalo, M. P., and Martins, L. O. (2009). "Enzymatic biotransformation of the azo dye Sudan Orange G with bacterial CotA-laccase," *Journal of Biotechnology* 139, 68-77.

Riva, S. (2006). "Laccases: Blue enzymes for green chemistry," *TRENDS in Biotechnology* 24(5), 219-226.

Rodríguez Couto, S., and Toca Herrera, J. L. (2006). "Industrial and biotechnological applications of laccases: A review," *Biotechnology Advances* 24, 500-513.

Ruijssenaars, H. J., and Hartmans, S. (2004). "A cloned *Bacillus halodurans* multicopper oxidase exhibiting alkaline laccase activity," *Appl Microbiol Biotechnol* 65, 177-182.

Sanchez-Amat, A., Lucas-Eilo, P., Fernandez, E., Garcia-Borron, J. C., and Solano, F. (2001). "Molecular cloning and functional characterization of a unique multipotent polyphenol oxidase from *Marinomonas mediterranea*," *Biochim. Biophys Acta* 1547, 104-116.

Sanchez-Amat, A., and Solano, F. (1997). "A pluripotent polyphenol oxidase from the melanogenic marine *Altermonas sp.* shares catalytic capabilities of tyrosinases and laccases," *Biochem. Biophys. Res. Commun.* 240, 787-792.

Singh, G., Ahuja, N., Sharma, P., and Capalash, N. (2009). "Response surface methodology for the optimized production of an alkalophilic laccase from γ-*Proteobacterium* JB," *BioResources* 4(2), 544-553.

Singh, G., Capalash, N., Goel, R., and Sharma, P. (2007). "A pH-stable laccase from alkali-tolerant γ-*Proteobacterium* JB: Purification, characterization and indigo carmine degradation," *Enzyme and Microbial Technology* 41, 794-799.

Solano, F., Lucas-Elio, P., Lopez-Serrano, D., Fernandez, E., and Sanchez-Amat, A. (2001). "Dimethoxyphenol oxidase activity of different microbial blue multicopper proteins," *FEMS Microbiology Letters* 204, 175-181.

Yang, Q., Yediler, A., Yang, M., and Kettrup, A. (2005). "Decolorization of an azo dye, Reactive Black 5 and MnP production by yeast isolate: *Debaryomyces polymorphus*," *Biochemical Engineering Journal* 24, 249-253.

Yu, J., Wang, X., and Yue, P. L. (2001). "Optimal decolorization and kinetic modeling of synthetic dyes by *Pseudomonas* strains," *Water Research* 35, 3579-3586.

Article submitted: June 21, 2011; Peer review completed: December 8, 2011; Revised version received and accepted: December 20, 2011; Published: January 6, 2012.