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OPTIMISATION OF REMAZOL BRILLIANT BLUE R DYE DECOLOURISATION AND LACCASE ENZYME PRODUCTION BY *Marasmius cladophyllus* USING RESPONSE SURFACE METHODOLOGY

NUR ADILA MURADI[1], AHMAD HUSAINI[1*], AZHAM ZULKHARNAIN[1], HAIRUL AZMAN ROSLAN[1] and TAY MENG GUAN[2]

[1]*Department of Molecular Biotechnology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300, Kota Samarahan, Sarawak, Malaysia*
[2]*Department of Chemistry, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300, Kota Samarahan, Sarawak, Malaysia*
[*]*E-mail: haahmad@unimas.my; Tel:+6082581160; Fax:+6082581155*

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ABSTRACT

The decolourisation of Remazol Brilliant Blue R dye and laccase activity was investigated using pure culture of an endophytic fungus, *Marasmius cladophyllus*. The fungus is found capable of decolourising 99% of the dye after 12 days of incubation in Glucose Minimal (GM) liquid media (pH 5.5) and laccase activity of 285 U/L was recorded. Response surface methodology (RSM) was used to determine and optimise the significant variable(s) in order to obtain the optimum dye decolourisation conditions and laccase production. It was also used to study the interaction effect of the variables on both responses. Box-Behnken Design was used to identify the significant variable(s) whereas the optimisation process was done by using Central Composite Design. It was found that initial dye concentration of 100-300 mg/L, incubation period of 4-20 days and pH of liquid medium of 4-8 significantly influenced the decolourisation of dye and laccase activity. However, only the relationship of the incubation period and pH is significantly affected both the responses. Maximum dye decolourisation of 100% was successfully achieved and the highest laccase activity of 504.53 U/L was recorded after 16 days of incubation period at pH 7 with 259.46 mg/L initial dye concentration.

Key words: Response surface methodology, Remazol Brilliant Blue R, *M. cladophyllus*, decolourisation, laccase

INTRODUCTION

The expanding textile dyeing industry has caused an increase in the volume and complexity of wastewater released to the environment and has created severe water pollution globally (Cervantes & Santos, 2011). During the dyeing processes, there are estimated about 5-50% of unfixed dyes lost in the industrial textile effluent (Maljaei *et al.*, 2009).

Remazol Brilliant Blue R (RBBR) dye is one of the most extensively used dyes in the textile industry. The dye has broadly been used as a model compound in the studies of dye degradation and it is also a derivative of anthracene, which represents an important recalcitrant and toxic organopollutants (Hadibarata *et al.,* 2012). Textile dyes are recalcitrant compounds because they are stable to temperature, microbial attack and light (Rodriguez *et al*., 1999). Thus, the treatment of textile industrial effluent has been one of the most challenging treatments among other industrial effluents (Fu & Viraraghavan, 2001).

Numerous physiochemical systems have been used to treat industrial wastewater (Yeh & Thomas, 1995) but it has many disadvantages (Stolz, 2001) which have resulted in an urge to develop effective biological system to degrade dyes in textile industrial effluent. Lignolytic fungi are known to degrade textile dyes by using laccase enzyme (Stolz, 2001). Due to the aromatic ring structures in the dye that has similar characteristics as the lignin, the white-rot fungi produces extracellular oxidative enzymes such as laccase, lignin peroxidase (LiP)

* To whom correspondence should be addressed.

and manganese peroxidase (MnP) (Hasnul *et al.,* 2015). The non-specific oxidative enzyme system produced makes the white-rot fungi becomes useful for many types of bio-technological applications which includes the dye decolourisation. Since the endophyte *Marasmius cladophyllus* is a member of lignolytic fungi, it is promising that this endophyte has the ability to degrade textile dyes.

Conventionally, optimisation processes have been conducted by changing one independent variable while maintaining other independent variables involved at specific level. However, this single-dimensional method is time consuming, toilsome and unable to analyse interactions among the variables involved (Trupkin *et al.,* 2003). Fortunately, the development of response surface methodology (RSM) has become a powerful tool to rapidly search for main factors from a multivariable system (Özer *et al.,* 2008; Niladevi *et al.,* 2009). It is an experimental method that is used to find the optimum conditions for a system that are involved with many variables by estimating the quadratic effects and linear interaction of the variables. Other than that, RSM is able to predict the model response in small number of experiments (Hafshejani, 2014). Hence, the main objective of this research is to optimise the decolourisation of RBBR dye and laccase production by *M. cladophyllus* UMAS MS8 using the response surface methodology (RSM) approach.

MATERIALS AND METHODS

Fungal growth medium, culture condition and dye decolourisation experiment

The endophytic fungus used in this study, *Marasmius cladophyllus* UMAS MS8 was successfully isolated from the stem of a healthy flowering Senduduk plant, *Melastoma malabathricum* (Ngieng *et al.,* 2013). Decolourisation of Remazol Brilliant Blue R (RBBR) dye and laccase production by *M. cladophyllus* were screened by growing the *M. cladophyllus* UMAS MS8 in glucose minimal (GM) liquid medium supplemented with Remazol Brilliant Blue R (RBBR) dye based on a method developed by Ngieng *et al* (2013). The glucose minimal (GM) medium contained in (g/L) of the following: K_2HPO_4, 1; $ZnSO_4 \cdot 7H_2O$,0.01; $CuSO_4 \cdot 5H_2O$, 0.05; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$,0.01; KCl, 0.5; glucose, 10 and $NaNO_3$, 3. The pH of the medium was adjusted to pH 5.5 before being autoclaved at 121ºC for 15 minutes. Remazol Brilliant Blue R, RBBR dye was added from a stock solution to a final concentration of 200 mg/L. A 5 mm^2 agar plug from a 7-day old culture of *M. cladophyllus* was used as inoculum. The flask was then incubated in the dark at room temperature. Uninoculated flask with the RBBR dye was used as the control. All of the experiment was prepared in duplicates. The decolourisation of the RBBR dye was observed and monitored for a period of 15 days.

Optimisation of RBBR decolourisation and laccase activity by response surface methodology

The RSM approach was applied to screen for significant variables and optimising the input variables in order to obtain optimised dye decolourisation and laccase activity. Design Expert (Version 7.0.0, Stat-Ease, Inc., Minneapolis, United States) software was used to produce run sheets, analyse data and identify the relationship between input variables and the responses.

Box–Behnken Design was used to screen for significant variable(s) that affecting dye decolourisation and laccase activities. Four independent variables were chosen: incubation period (Factor A, 4-20 days), pH of the media (Factor B, 4-8), initial dye concentration (Factor C, 100-300 mg/L) and number of plug (Factor D, 1-5). The percentage of dye decolourisation and laccase activities were analysed in this experiment.

The significance of the model together with the operating parameters were tested and identified by using Fisher's test for Analysis of Variance (ANOVA). Variables that have confidence level more than 95% ($p>F\leq0.05$) have the significant effect on the responses and was further optimised by using Central Composite Design (CCD).

For statistical analysis, a quadratic polynomial equation by CCD was produced to predict responses as function of independent variables and their interaction. Generally, the response for the quadratic polynomials is described below:

$$Y=\beta_0 + \Sigma\beta ixi + \Sigma\beta iixi_2 + \Sigma\Sigma\beta ijxijxj \qquad (1)$$

where Y is the response (dye decolourisation); β_0 is the intercept coefficient, βi is the linear terms, βii is the squared terms and βij is the interaction terms, and xi and xj are the uncoded independent variables (Ghadge & Raheman, 2006).

Analytical methods

Residual RBBR dye in original and treated sample were measured by observing the absorbance of the dye at 592 nm wavelength using UV-VIS spectrophotometer (UV 7500, Techcomp). Sample obtained was analysed by calculating the decrease in absorbance with the reference of the uninoculated control.

The percentage of RBBR decolourisation was calculated using the following formula:

$$\text{Decolourisation (\%)} = \left(\frac{Ab_0 - Ab_1}{Ab_0}\right) \text{X } 100 \qquad (2)$$

where Ab_0 is absorbance of dye in control flask and Ab_1 is absorbance of dye after decolourisation (Özsoy *et al*., 2005).

Enzyme Assay

Laccase activity was measured at 420 nm (ε_{420} = 36000 M^{-1} cm^{-1}) based on the oxidation of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmol of substrate per minute (Murugesan *et al.,* 2007).

RESULTS AND DISCUSSION

Screening of RBBR decolourisation in liquid medium

From the experiment, it was proven that *M. cladophyllus* UMAS MS8 is capable to decolourised RBBR dye in the liquid medium. The blue colour of RBBR dye disappeared after being treated with mycelial plugs of *M. cladophyllus* after day 12.

The highest percentage of dye decolourisation obtained was observed on day 12 which was 98.6%. In addition, the highest laccase enzyme activity was obtained on the day 12 and recorded as 285 U/L. This indicates that the decolourisation of RBBR dye correlates with the production of laccase enzyme (Figure 1).

Screening of significant parameter(s) using Box-Behnken Design

Factors that have a confidence level more than 95% ($p>F\leq0.05$) have a significant effect on the responses and thus chosen for further research. For dye decolourisation, only incubation period was significantly affected the decolourisation (Table 1).

Box–Behnken Design, which is suitable to fit quadratic surface and usually performed well in optimization experiment (Özer *et al.*, 2008) was used to screen for the significant parameter(s) that affecting the biodegradation of dye. In order to analyse the significance of operating parameters on the biodegradation of RBBR, four independent variables were chosen: incubation period, pH of the media, initial dye concentration and number of plug. Total numbers of 29 runs were designed by Design Expert software (Version 7.0.0, Stat-Ease, Inc., Minneapolis, United States) and each run was done in triplicates. The run sheet together with the co-responding responses which were the percentage of decolourisation and laccase enzyme activity was tabulated in Table 1.

The significance of the model, along with the operating parameters was tested and identified by using Fisher's test for Analysis of Variance (ANOVA). From the ANOVA test, the model was validated as significant with F-value of 4.06 for the percentage of decolourisation (Response 1) (Table 2) and 5.28 for laccase enzyme activity (Response 2) (Table 3).

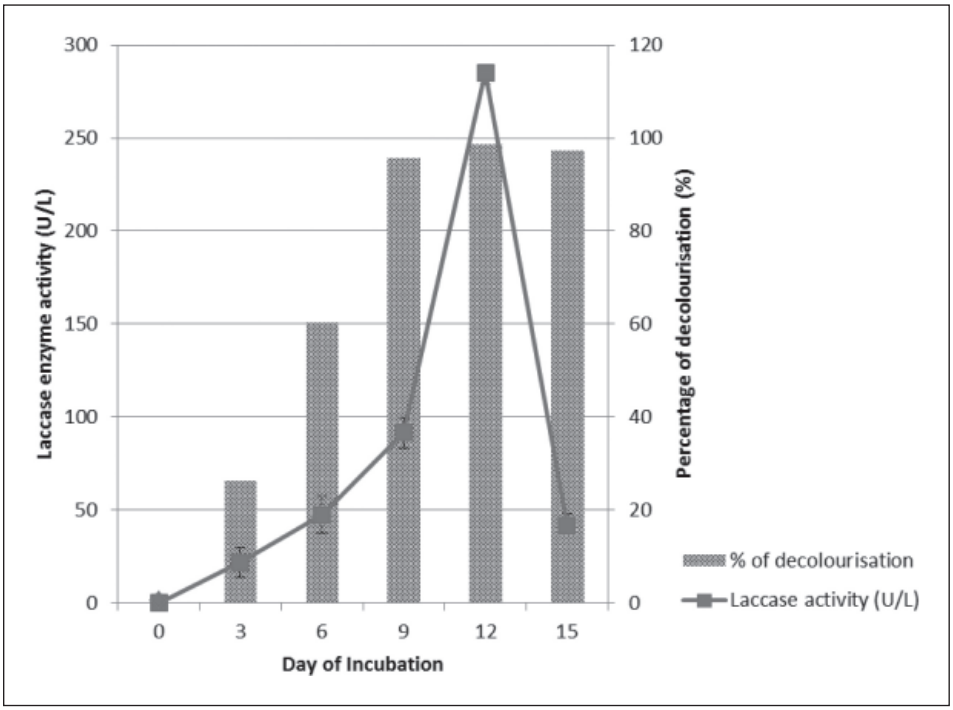

Fig. 1. Laccase enzyme activity and dye decolourisation versus days of incubation period.

Table 1. Run sheet of Box-Behnken Design and responses obtained

Std	Run	Type	Factor 1 A:pH	Factor 2 B:IP	Factor 3 C:Initial Conc.	Factor 4 D:No. of plug	Response 1 Decolourisation (%)	Response 2 Enzyme Activity (U/L)
26	10	Center	6.00	12.00	200.00	3.00	99.48	233.89
29	15	Center	6.00	12.00	200.00	3.00	99.27	284.58
27	18	Center	6.00	12.00	200.00	3.00	99.10	274.35
28	19	Center	6.00	12.00	200.00	3.00	99.56	240.19
25	26	Center	6.00	12.00	200.00	3.00	100.00	266.25
1	1	IBFact	4.00	4.00	200.00	3.00	64.51	6.33
6	2	IBFact	6.00	12.00	300.00	1.00	100.00	317.92
20	3	IBFact	8.00	12.00	300.00	3.00	100.00	205.83
8	4	IBFact	6.00	12.00	300.00	5.00	100.00	345.83
13	5	IBFact	6.00	4.00	100.00	3.00	55.09	7.58
24	6	IBFact	6.00	20.00	200.00	5.00	100.00	184.58
16	7	IBFact	6.00	20.00	300.00	3.00	99.94	203.75
5	8	IBFact	6.00	12.00	100.00	1.00	100.00	36.25
15	9	IBFact	6.00	4.00	300.00	3.00	95.49	40.08
22	11	IBFact	6.00	20.00	200.00	1.00	98.59	162.92
17	12	IBFact	4.00	12.00	100.00	3.00	100.00	80.83
18	13	IBFact	8.00	12.00	100.00	3.00	100.00	262.5
23	14	IBFact	6.00	4.00	200.00	5.00	86.83	15.75
12	16	IBFact	8.00	12.00	200.00	5.00	100	264.17
19	17	IBFact	4.00	12.00	300.00	3.00	98.53	77.08
10	20	IBFact	8.00	12.00	200.00	1.00	100.00	163.33
3	21	IBFact	4.00	20.00	200.00	3.00	98.01	78.75
2	22	IBFact	8.00	4.00	200.00	3.00	8.21	5.67
4	23	IBFact	8.00	20.00	200.00	3.00	97.39	282.08
7	24	IBFact	6.00	12.00	100.00	5.00	100.00	132.50
21	25	IBFact	6.00	4.00	200.00	1.00	51.64	4.67
11	27	IBFact	4.00	12.00	200.00	5.00	99.28	164.17
9	28	IBFact	4.00	12.00	200.00	1.00	97.97	29.58
14	29	IBFact	6.00	20.00	100.00	3.00	97.26	134.17

Table 2. ANOVA for Response 1 (Dye decolourisation) Surface Quadratic Model

Source	Sum of Square	Degrees of Freedom	Mean Squares	*F*-value	*P*-value	
Model	9660.66	14	690.05	4.06	0.0066	significant
A-pH	231.44	1	231.44	1.36	0.2628	
B-Incubation period	4386.13	1	4386.13	25.80	0.0002	
C-Initial Conc.	144.28	1	144.28	0.85	0.3726	
D-No. of plug	119.76	1	119.76	0.70	0.4154	
AB	775.07	1	775.07	4.56	0.0509	
AC	0.54	1	0.54	3.177E-003	0.9558	
AD	0.43	1	0.43	2.523E-003	0.9606	
BC	355.70	1	355.70	2.09	0.1701	
BD	285.27	1	285.27	1.68	0.2162	
CD	0.000	1	0.000	0.000	1.0000	
A^2	256.17	1	256.17	1.51	0.2399	
B^2	2634.14	1	2634.14	15.49	0.0015	
C^2	104.66	1	104.66	0.62	0.4458	
D^2	41.09	1	41.09	0.24	0.6306	
Residual	2380.49	14	170.04			
Lack of Fit	2380.03	10	238.00	2046.10	< 0.0001	significant
Pure Error	0.47	4	0.12			

R^2=0.8023, R^2_{adj}=0.6046, adequate precision= 8.213

Table 3. ANOVA for Response 2 (laccase enzyme activity) Surface Quadratic Model

Source	Sum of Square	Degrees of Freedom	Mean Squares	*F*-value	*P*-value	
Model	2.817E+0.005	14	20120.12	5.28	0.0018	significant
A-pH	46480.83	1	46480.83	12.20	0.0036	
B-Incubation period	77790.37	1	77790.37	20.42	0.0005	
C-Initial Conc.	24000.33	1	24000.33	6.30	0.0250	
D-No. of plug	12826.90	1	12826.90	3.37	0.0878	
AB	10402.98	1	10402.98	2.73	0.1207	
AC	700.13	1	700.13	0.18	0.6747	
AD	284.77	1	284.77	0.075	0.7885	
BC	343.73	1	343.73	0.090	0.7683	
BD	27.98	1	27.98	7.346E-003	0.9329	
CD	1167.59	1	1167.59	0.31	0.5886	
A^2	24122.50	1	24122.50	6.33	0.0247	
B^2	97705.95	1	97705.95	25.65	0.0002	
C^2	7056.37	1	7056.37	1.85	0.1950	
D^2	8319.81	1	8319.81	2.18	0.1616	
Residual	53331.30	14	3809.38			
Lack of Fit	51408.08	10	5140.81	10.69	0.0177	significant
Pure Error	1923.22	4	480.81			

R^2= 0.8408, R^2_{adj}= 0.6816, adequate precision= 6.432

Factors that have confidence level more than 95% ($p>F\leq0.05$) have the significant effect on the responses and will be chosen for further research. For dye decolourisation response, only incubation period is significantly affecting the percentage of dye decolourisation (Table 2). This is may be due to most of the decolourisation in the experiment achieved more than 90% decolourisation, which indicates there is no significant difference between dye decolourisation.

For the second response, which is the enzyme activity of laccase, incubation period with *F*-value of 0.0005 has the most significant effect on response 2. This significance effect is also given by pH of media and initial dye concentration with *F*-value of 0.0036 and 0.0250 respectively (Table 3).

Number of plugs however, gave no significant influence to production of enzymes with F-value of 0.0878. This indicates that the number of plugs added into the media gave no significant effect to both responses and hence, this factor was eliminated in further studies.

Therefore, the three variables that had significant effect on laccase activity which are incubation period, pH and initial dye concentration were further selected in the optimization experiments. Number of plugs that had no significant effect on both responses and hence, it was eliminated from the optimisation studies.

Optimisation experiment by using Central Composite Design (CCD)

The three significant factors identified by using Box-Behnken Design were used in optimising the dye decolourisation and enzyme yield by using Central Composite Design (CCD). A total of 20 experiments with different combinations of incubation period (Factor A, 4-20 days), pH (Factor B, 4-8) and initial dye concentration (Factor C, 100-300 mg/L) were performed in triplicates. The regression of data was analysed to test the sufficiency of the quadratic model and thus, the following second-order polynomial equation (in coded units) were derived (Eqn. 3 & 4):

$$Y\text{ (Decolourisation)} = 100.01 + 2.29A + 1.47B - 0.36C - 1.83AB - 0.38AC - 0.53BC - 1.33A^2 - 0.75B^2 - 0.65C^2 \quad (3)$$

$$Y\text{ (Enzyme Activity)} = 318.66 + 96.98A + 90.63B + 27.08C + 77.48AB + 21.51AC + 1.61BC - 59.16A^2 - 53.54B^2 - 28.49C^2 \quad (4)$$

where *Y* represents the response, A: the coded value of factor incubation period, B: the coded value of factor pH and C: the coded value of initial dye concentration factor.

The behaviour of dye decolourisation with respect to the differences in days of incubation and pH is shown in Figure 2. The interaction of these two parameters gave significant positive effect on the decolourisation of RBBR. Dye decolourisation was found to increase with the increase of pH in the incubation period ranges from day 4 to day 16. After day 16, the dye decolourisation declined when the pH was above 6. The interaction of incubation period and pH indicated that incubation period had profound effect on the decolourisation of RBBR as compared to the effect of pH towards the dye decolourisation alone.

The decrease in the decolourisation of RBBR dye after day 16 when pH is above 6 might be due to the age of *M. cladophyllus*. After day 16, the growth rate of the fungus was reduced and it was unable to survive in environment that contained high pH value. Hence, less percentage of decolourisation achieved after day 16 when pH was beyond 6. Besides that, the plot also indicates that *M. cladophyllus* performed better decolourisation of dye in acidic condition where it was able to completely decolourise the dye in pH ranges from 4 to 6 up to 20 days of incubation.

Figure 3 shows three-dimensional and contour plot of interactive effect of pH and incubation period towards laccase production. With the increase of incubation period and pH, laccase activity is also increased. The maximum laccase activity obtained from this experiment was 504.53 U/L after 16 days of incubation, at pH 7.19 with initial dye concentration of 259.46 mg/L. This result is almost similar to a study by Chowdhury *et al* (2014), where they found that the optimum pH for laccase activity produced by *Pleurotus fossulatus* was pH 7. Furthermore, Dhakar and Pandey (2013) discovered the optimum laccase activity produced by fungus *Trametes hirsuta* was at pH range from 5.5 to 7.5, where it can be assumed that laccase enzyme from *M. cladophyllus* resemble laccase produced by *T. hirsuta* in term of its optimal pH.

Laccase activity was found decrease at pH 8 where the enzyme activity detected was 307.57 U/L. The difference in pH alters the protein structure of fungus and a decrease in enzyme activity above its optimum pH conceivably because of the instability of enzyme or the enzyme is no longer active in such high pH (Battestin & Macedo, 2007). The effect of pH on enzyme activity is determined by the essence of the amino acids at the active site, which undergo conformational changes triggered by the ionisation of amino acid. Hence, enzymes are highly sensitive to pH changes and they function well in a very limited range, with a known optimum pH (Sabu *et al.,* 2005).

Verification Experiment

In order to validate the results obtained from the statistical analysis of CCD, a verification experiment was conducted under the optimal experimental conditions obtained from RSM (Table 4). The optimal parameters were: incubation period, 16.35 days; pH, 7.16 and initial dye concentration, 204.04 mg/L.

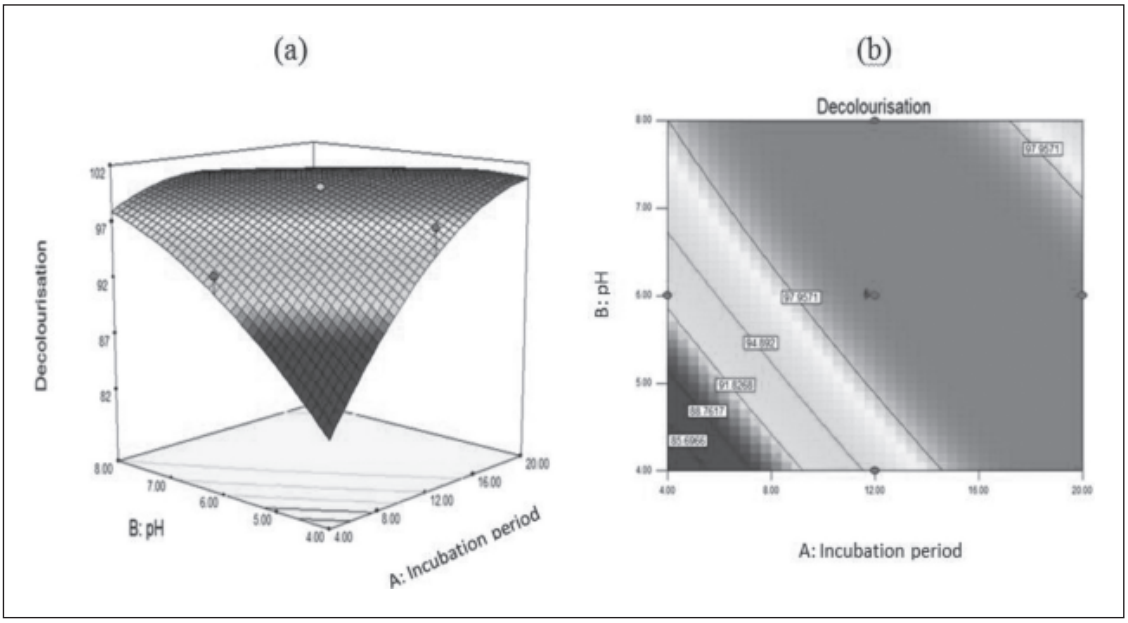

Fig 2. (a) Response surface for dye decolourisation (%) by *M. cladophyllus*. (b) The three-dimensional plot (a) and contour plot (b) showed the influence of incubation period (day) and pH on dye decolourisation. The dots on both plots indicate the design point of CCD.

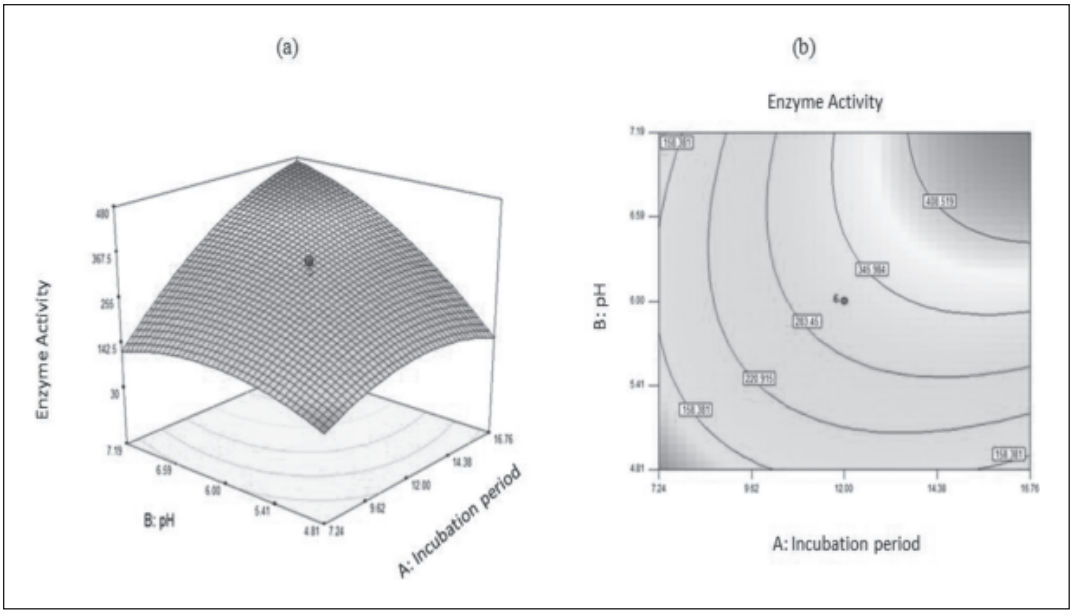

Fig. 3 (a) Response surface for laccase production (U/min) by *M. cladophyllus*. (b) The three-dimensional plot and contour plot showing the influence of incubation period (day) and pH on production of laccase. The production of laccase on different days of incubation is influenced by pH. The dots on both plots indicate the design point of CCD.

Table 4. Result of validation experiment conducted at optimum parameters

Incubation Period (Day)	pH	Initial Conc. (mg/L)	Predicted Decolourisation (%)	Actual Decolourisation (%)	Predicted Enzyme Activity (U/L)	Actual Enzyme Activity (U/L)	Desirability
16.35	7.16	204.04	100	100	467.79	355.00	0.962

The predicted decolourisation value of 100% was successfully achieved in this verification experiment. However, as for laccase enzyme activity, the actual value obtained was 355.00 U/L with standard deviation of 79.75 from the predicted value, 467.79 U/L. The experimental result did not meet the predicted value probably because of the exhaustion of RBBR dye as the enzyme substrate in the medium. There is also a likelihood that *M. cladophyllus* has completely biodegrade the RBBR dye in the media and laccase is no longer being produced after all the substrate was converted into product. These results obtained have confirmed that RSM can predict the decolourisation removal of RBBR dye but not for enzyme activity. Nonetheless, the application of RSM reduces the resources and time taken to complete this study. Finally, the laccase enzyme produced by *M. cladophyllus* and the microorganism itself can be utilised to treat textile effluent in the industrial areas.

CONCLUSION

In this work, the RBBR dye decolourisation and laccase production by *M. cladophyllus,* UMAS MS8 was successfully performed and optimised. The endophytic fungus is capable of degrading RBBR dye. Due to these findings, the endophytic fungus, *M. cladophyllus* has the potential to be exploited for the mycoremediation or enzymatic bio-degradation of the textile effluent. For those small medium enterprises (SMEs), this will definitely give them an option to opt for the bioremediation technology employing *M. cladophyllus* as the bioremediation agent that will be more cost effective compared to high cost water treatment facilities in treating dyed waste water generated.

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