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Production of Remazol Brilliant Blue R decolourising oxygenase from the culture filtrate of *Funalia trogii* ATCC 200800

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Abstract

Decolourisation of Remazol Brilliant Blue R, an azo textile dyestuff, by crude filtrate of *Funalia trogii* ATCC 200800 growing in solid-state fermentation (SSF) medium containing wheat bran and soybean hull was studied. Optimum pH and temperature for laccase and horseradish like peroxidase (HRP) production in SSF medium were determined at 5 and 30 °C, respectively. Maximum enzyme synthesis was found in 10 days old cultures. We also found Remazol Brilliant Blue R decolourising enzymatic activity in the culture filtrate of *F. trogii*. The optimum pH and temperature for enzymatic decolourisation were determined at 3.0 and 50 °C, respectively. Both veratryl alcohol and peroxide ions (H_2O_2) accelerated the peroxidase enzyme reactions, whereas decelerated the decolourisation of RBBR with the culture filtrate of *F. trogii*. Sodium azide (NaN_3), cysteine and sodium cyanide (NaCN) inhibited RBBR decolourising activity, laccase and peroxidase activities. When sodium metabisulphite ($Na_2S_2O_5$) was used as an inhibitor, a significant inhibition of laccase and dye decolourising enzyme activities but no peroxidase activity was observed. Initial colourless and later orange bands were obtained by the activity staining process with RBBR and laccase substrate (guaicol), respectively, after separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The molecular mass of this band was estimated as about 65 kDa by SDS-PAGE. Since the reaction was catalysed in the absence of H_2O_2 as co-substrate, it was concluded that this enzyme was a laccase.

Keywords: Decolourisation; Remazol Brilliant Blue R; *Funalia trogii*; Laccase; Solid-state fermentation

1. Introduction

Synthetic dyes are extensively used for dyeing and printing in various industries. Over 7×10^5 t and approximately 10,000 different dyes and pigments are produced annually worldwide, about 10% of which are lost in industrial effluents [1]. These dyes are poorly degradable because of their structures. Treatment of wastewater containing dyes usually involves physical and/or chemical methods. Although these treatment methods are efficient, there is always the possibility of producing highly toxic by products and/or require high levels of energy. Microbial decolourisation has been claimed to be less expensive and less environmentally intrusive alternatives [2]. Ligninolytic fungi have been reported to degrade xenobiotic compounds. Their enzymatic systems that involve enzymes of lignin degradation are capable of transforming polycyclic hydrocarbons [3,4], phenols [5–7], and dyes [8–10]. Many bacteria and fungi are used for the development of biological processes for the treatment of textile effluents [11–13]. Up to now, the majority of studies on biological decolourisation have focused on fungal strains. *Phanerochaete chrysosporium* [14], *Trametes versicolor* [15], *Coriolus versicolor* [16–18], *Cunninghamella polymorpha* [19], *Geotrichum candidum* [20] and *Rhizopus arrhizus* [21] are the major fungal strains used for decolourisation purposes. In addition, it has recently been demonstrated that extracellular enzymes of white rot fungi such as peroxidases (lignin peroxidase (LiP), horseradish like peroxidase (HRP) and manganese peroxidase (MnP)) and phenoloxidase (laccase) can be used to degrade and detoxify polyaromatic hydrocarbons, polychlorinated biphenyls and certain dyes [8–10,22–25]. Although several works

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refer to the LiP of *P. chrysosporium* as being the main decolourising agent, a recent investigation as regard with the degradation of selected phthalocyanine dyes and their degradation products, showed the presence of laccase and MnP [26].

In a recent work by Lorenzo et al. [27] it was shown that it was possible to stimulate the yield of laccase activity of *T. versicolor* by using several agricultural wastes. Enzymes such as lignin peroxidase manganese peroxidase and laccase are all involved in lignin degradation; participation in the decolourisation of dyes [8–10,22,23]. However, few studies have been conducted on anthraquinone dyes, which were xenobiotic chemicals similar to azo dyes but different in structure [2,28].

For the polymerisation of phenolic monomers, HRP, LiP and MnP widely used in presence of peroxide ions and various reactor designs were suggested for wastewater treatment [29,30]. In contrast to HRP, laccase uses only dissolved molecular oxygen for catalytic activity [31]. More importantly, laccase is an oxidase with a redox potential of 780 mV and can catalyse the oxidation of organic pollutants by reduction of molecular oxygen straightforwardly to water in the absence of hydrogen peroxide or even other secondary metabolites [32].

Therefore, it is advantageous to replace HRP with laccase since it utilises molecular oxygen instead of peroxide ions, which causes inhibition and deactivation of the enzyme [29,33]. Its availability in a wide variety of natural sources as well as the easy supply of oxygen from the ambient air makes the polyphenoloxidase-based oxidation cheaper than oxidations based on peroxidases.

Two major difficulties in wastewater treatment with LiP were found. First the key enzyme for dye degradation, LiP is released by the fungal cells following a strict secondary metabolism under either carbon and nitrogen limitation. Second, dye degradation by lignin peroxidase consumes a considerable amount of hydrogen peroxide; veratryl alcohol and LiP may not be simultaneously produced in most real effluents [34]. Hatvani and Mecs [35] and Robinson et al. [36] indicated decolourisation of RBBR in the absence of manganese (Mn), which shows that MnP did not play any role in decolourisation of RBBR.

Remazol Brilliant Blue R (RBBR) is an industrially important dye and is being used frequently as starting material in the production of polymeric dyes. RBBR, an anthraquinone derivative, represents an important class of often toxic and recalcitrant organopollutants. It structurally resembles certain polycyclic aromatic hydrocarbons, which are substrates of ligninolytic peroxidases [22]. Therefore we used RBBR for enzymatic decolourisation. In our report, decolourisation of RBBR by crude filtrate of *Funalia trogii* ATCC 200800 produced during solid-state fermentation of wheat bran and soybean as a natural lignocellulosic substrate was investigated. We also determined the enzyme responsible for decolourisation of Remazol Brilliant Blue R in the crude filtrate of *F. trogii*.

2. Materials and methods

2.1. Chemicals

All chemicals used in our study are of analytical grade. Potato dextrose agar, acetic acid, NaOH, KH_2PO_4, K_2HPO_4, NaH_2PO_4, Na_2HPO_4, $CaCl_2{\cdot}H_2O$, $MgSO_4{\cdot}7H_2O$, $NH_4H_2PO_4$, $CuSO_4{\cdot}5H_2O$, yeast extract, $MnSO_4{\cdot}H_2O$, H_2O_2, veratryl alcohol, $Na_2S_2O_5$, L-cysteine, NaCN, NaN_3 and $CaCO_3$ were obtained from Merck, Remazol Brilliant Blue R, folin, serum albumin, guaicol, glucose was obtained from Sigma, $FeSO_4{\cdot}7H_2O$ was obtained from Carlo Erba.

2.2. Microorganism

F. trogii was obtained from Environmental Biotechnology Laboratory of Environmental Engineering Department, University of Mersin, Turkey. The fungus was maintained on Potato Dextrose Agar (PDA, Merck) slants and incubated at 30 °C for 5 days and stored at +4 °C.

2.3. Inoculum preparation

Two square agar slices (1 cm^2 each) punched out from the cultures grown on potato dextrose agar plates were used to inoculate 15 ml stock basal medium, SBM; 5 g/l glucose, 0.005 g $CaCl_2{\cdot}H_2O$, 0.005 g/l $MgSO_4$, 0.5 g/l $NH_4H_2PO_4$, 0.001 g/l $FeSO_4$, 0.02 g/l $CuSO_4$, 0.01 g/l yeast extract, 0.001 g/l $MnSO_4$, in 100 ml Erlenmeyer flasks. pH of these media were adjusted with a buffer solution (0.1 M K_2HPO_4 and KH_2PO_4) to 5.0, 5.5 and 6.0 and incubated at 30 °C under static conditions (Sanyo MIR 152).

2.4. Preparation of crude enzyme extract

SSF medium containing 90% wheat bran 10% soybean hull were humidified with SBM (50% v/w) and autoclaved for 60 min. Pre-cultures were inoculated into SSF medium and inoculated for 5, 10 and 15 days in order to determine the incubation period for maximum enzyme production. At the end of these incubation periods cultures were taken out of the flasks. The harvested cultures were dehumidified at 40 °C (Electromag 6040BP incubator) for 24 h. After dehumidification cultures were dissolved at pH 6.0 potassium–phosphate buffer (0.1 g culture/ml) and centrifuged (Hettich micro 22R) at 5000 rpm for 15 min. Then the supernatant was filtered (Whatman No. 1). The filtrate (crude enzyme solution) was used for enzymatic analysis, protein determination and decolourisation studies.

2.5. Enzyme assays

Laccase and horseradish peroxidase activities were determined spectrophotometrically, monitoring the oxidation of guaicol as described previously [37]. One unit of activity was defined as the amount of enzyme causing an absorbance

change of 0.1 in 1 h at pH 6.0 and 30 °C. Lignin peroxidase activity was estimated spectrophotometrically by the method of Sik and Unyayar (1998), following oxidation of veratryl alcohol to veratryl aldehyde [37]. Protein concentration was determined by the Lowry method [38].

2.6. Enzymatic decolourisation

Decolourisation of RBBR, with culture filtrates was determined spectrophotometrically (Shimadzu A-160) by measuring the decrease in A_{609} [10,12]. In this study the influence of pH, temperature, dye concentration, and culture filtrate concentration on enzymatic colour removal were investigated. The 150 μl culture filtrate was used in all decolourisation studies unless indicated. Initial decolourisation rate (d*C*/d*t*, mg/l s) of RBBR is a slope of graphic, which shows the decrease in dye concentration in time. The enzyme responsible from the decolourisation of RBBR was named as RBBR oxygenase.

2.7. pH influence

Enzymatic decolourisation was monitored in a medium containing 20 mg/l RBBR, at different pH values (2.5, 3.0, 3.5, 4.0, 4.5 and 5.0) at 30 °C. Medium pH was adjusted by sodium acetate buffer.

2.8. Temperature influence

Different (20, 30, 40, 50, 60, 70 and 80 °C) reaction temperatures were used for the determination of maximum initial decolourisation rate. These reactions were conducted at pH 3.0 in the presence of 80 mg/l RBBR.

2.9. Dye concentration

The influence of dye concentration on enzymatic colour removal was investigated by using different dye concentrations (70, 80, 90, 100 and 110 mg/l) at 30 °C and pH 3.0.

2.10. Enzyme concentration

Culture filtrate was used as an enzyme source. Different amounts of culture filtrates (50, 150, 300, 350, 400 and 500 μl) were used to assess the significance of culture filtrate in colour removal. Reactions were conducted at 30 °C and pH 3.0 in the presence of 30 mg/l RBBR.

2.11. Influence of enzyme inhibitors

Four different inhibitors ($Na_2S_2O_5$, L-cysteine, NaCN and NaN_3) were used for inhibition of laccase, HRP and RBBR oxygenase. The final concentration of the inhibitors were, 40, 240 and 1000 μM for sodium metabisulphite ($Na_2S_2O_5$); 20, 40, 60, 80, 100, 120, 200 and 300 μM for cysteine; 40, 240 and 1000 μM for sodium cyanide (NaCN); 1, 2.5, 5, 7.5, 10, 15, 20 and 1000 μM for sodium azide (NaN_3).

2.12. Gel electrophoresis and staining

SDS-PAGE, to determine the molecular weight of the partially purified enzyme, was performed with a 10% polyacrylamide containing 0.1% sodium dodecyl sulphate according to the method of Laemmli [39]. The protein bands were visualised by Commassie blue G-250 (Merck) and compared with standard molecular weight markers: β-galactosidase, 118.0 kDa; bovine serum albumin 79.0 kDa; ovalbumin 47.0 kDa; carbonic anhydrase 33.0 kDa; β-lactoglobulin 25.0 kDa; lysozyme 19.5 kDa (MBI Fermentas). Relative mobilities of the standard markers versus common logarithms of their molecular masses were plotted. With the linear regression output, the molecular masses of the proteins in the crude filtrate were estimated.

2.13. Activity staining

For the activity staining of the decolourising activity, the SDS was removed by washing the gel at room temperature in solutions containing 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), isopropanol 40% for 1 h and 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2) for 1 h, respectively. The renaturation of the enzyme proteins was carried out by placing the gel overnight in a solution containing 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), 5 mM β-mercaptoethanol and 1 mM EDTA at 4 °C. The gel was then transferred onto a glass plate, sealed in film, and incubated at 37 °C for 4–5 h [40]. The gel was stained with Remazol Brilliant Blue R and guaicol. Any clear and coloured zones indicated the presence of the decolourising activity of the enzyme.

3. Results and discussion

In this study, effects of pH, temperature and incubation period on enzyme production in SSF medium were investigated. We used wheat bran and soybean hull as SSF medium for enzyme production from *F. trogii*. Enzyme (laccase, HRP, LiP) production in the media of different pH values on 5th, 10th and 15th days of incubation period(s) at different temperatures were determined. It was seen that all of these parameters affect the enzyme production and maximum enzyme synthesis by *F. trogii* in SSF medium was observed at pH 5.0, 30 °C and on 10th day of incubation period (Figs. 1 and 2).

4. Dye decolourisation by crude enzyme

For the decolourisation of dye, HRP LiP and MnP were widely used in the presence of peroxide ions [8–10,22,23]. The major enzymes involved in dye decolourisation by *F.*

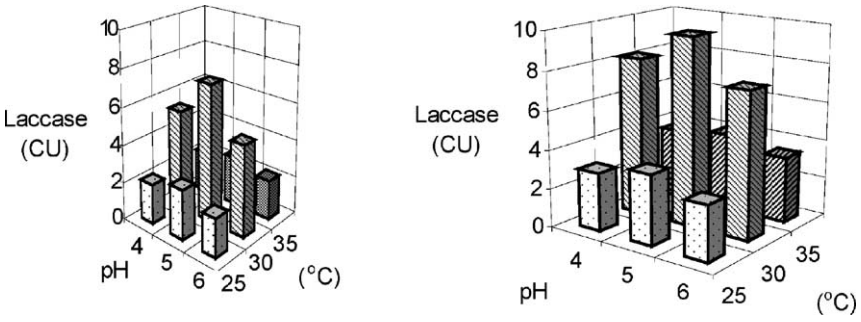

Fig. 1. Activity of laccase in 5 and 10 days old *F. trogii* culture.

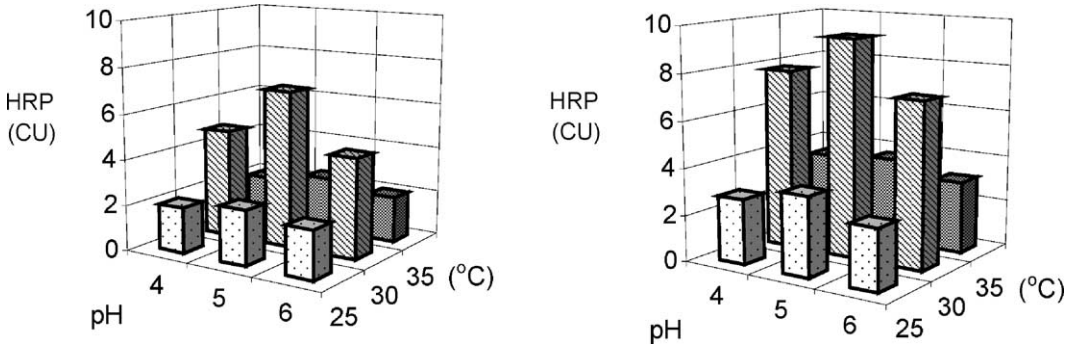

Fig. 2. Activity of HRP in 5 and 10 days old *F. trogii* culture.

trogii are also LiP, HRP and laccase. These three enzymes were assayed and MnP, LiP were not detected for *F. trogii* in SSF medium. Hatvani and Mecs [35] showed that laccase production was less sensitive increasing the nitrogen (N) concentrations and relatively high laccase and horseradish like peroxidase activities were measured at high N concentrations, at which point the production of MnP and the degradation of poly-R-478 were inhibited completely. As we used a nitrogen (N) rich medium we did not expect the production of MnP. As in the case of xenobiotics, synthetic dyes are much more degradable by LiP than by MnP [34]. In addition to this, they indicated decolourisation of RBBR in the absence of manganese (Mn), which shows that MnP did not play any role in decolourisation of RBBR [35,36].

Decolourisation activity was observed upon addition of the enzyme. No LiP activity was detected in crude enzyme solution. Our results are correlated with the results of Zhen and Yu [41] as they indicated the prohibition of the LiP by the fungal cells in the presence of carbonaceous compounds and nitrogen nutrients in industrial effluents. Because of the presence of wheat bran as a high carbon source and the presence of soybean as a high nitrogen source, production of LiP was prohibited in our SSF medium.

4.1. Influence of temperature

Maximum initial degradation rate of RBBR was determined at 50 °C (Fig. 3). It was reported that when laccases from various fungi were used for colour removal, the best decolourisation was obtained at temperatures between 50 and 60 °C [42–46]. Our results are in agreement with this, suggesting that colour decolourisation might be due to the laccase activity.

In another report peroxidase enzyme purified from *Geotririchum candidium* was used for colour degradation of RBBR and optimal temperature for colour decolourisation was estimated. It was shown that the activity decreases at temperatures above 30 °C, e.g., 90% decrease at 50 °C [47]. Peroxidase enzyme has been reported to lose activity at high temperatures such as 50 °C [48,49].

4.2. Influence of pH

We found that *F. trogii* RBBR decolourising activity was maximal at pH 3.0 (Fig. 4). According to other reports there

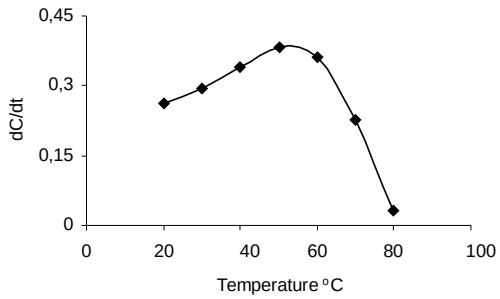

Fig. 3. Influence of temperature on enzymatic decolourisation of RBBR. The 80 mg/l RBBR, 150 μl culture filtrate at 1 min reaction at pH 3.0.

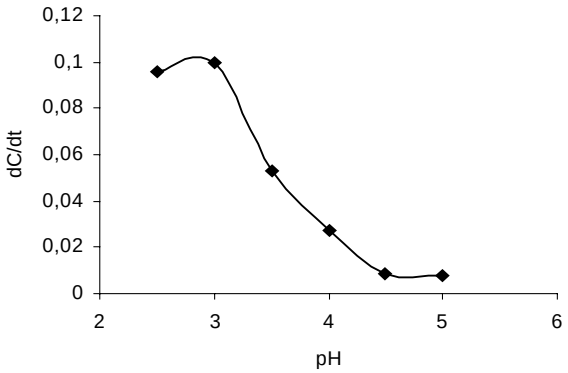

Fig. 4. Influence of pH on enzymatic decolourisation of RBBR. The 20 mg/l RBBR, 150 μl crude enzyme, 1 min reaction at 30 °C.

were three enzymes, laccase, lignin peroxidase and manganese peroxidase, which were active at pH 3.0 and could decolourise dyes [45,50].

4.3. Dye concentration

Different RBBR concentrations (70, 80, 90, 100 and 110 mg/l) were used to determine the effect of dye concentration on enzymatic decolourisation of RBBR. Dye decolourisation rate was improved by increasing RBBR concentrations; however, concentrations above 100 mg/l were inhibitory (Fig. 5).

Young and Yu [1] investigated the effect of dye concentration on the initial decolourisation rate of lignin peroxidase. Although the dye and the enzyme were different they obtained similar results. Various concentrations of 8 dyes exhibited different inhibition effects (Reactive Black-5; 10 mg/l, Reactive Black-15; 50 mg/l, Acid Orange-74; 100 mg/l) [1].

4.4. Enzyme dosage

The decolourisation rate of RBBR increased proportionally with the amount of crude enzyme used up to a dosage of 350 μl enzyme/min (Fig. 6).

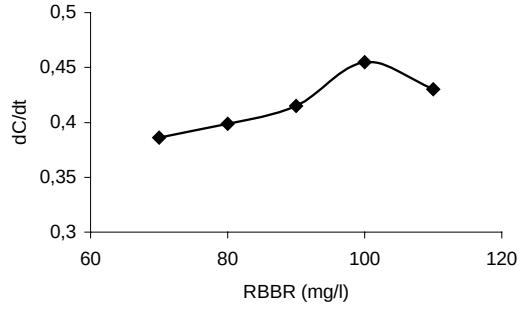

Fig. 5. Influence of dye concentration on decolourisation rate of RBBR, pH 3, 150 μl enzyme, 1 min reaction at 30 °C.

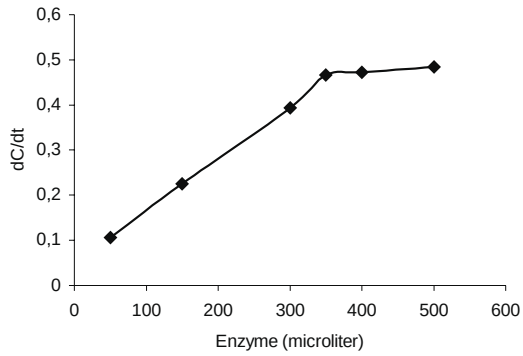

Fig. 6. Influence of enzyme dosage on RBBR decolourisation; pH 3.0, 30 mg/l RBBR, 1 min reaction at 30 °C.

4.5. Influence of H_2O_2, VA dose and inhibitors

LiP and HRP require the presence of H_2O_2 and/or veratryl alcohol [1,8]. In this study various H_2O_2 concentrations were used to determine the influence of H_2O_2 in RBBR decolourisation. Fig. 7 shows that initial colour decolourisation rate decreases with addition of H_2O_2. Therefore the effect of H_2O_2 is negative.

Vyas and Molitoris [22] used the extracellular ligninolytic enzyme of *Pleurotus ostreatus* for decolourisation of RBBR and showed that H_2O_2 was needed to initiate the enzyme activity. Similarly, Young and Yu [1] in their study of decolourisation of dye materials used LiP and they could only initiate enzyme activity in the reaction by adding peroxide ions and VA to the medium. Evidently, the results obtained by Young and Yu [1], and Vyas and Molitoris [22] differed significantly with our findings. In our present study of decolourisation of RBBR with extracellular ligninolytic enzymes of *F. trogii* we have established that effect of H_2O_2 ions were not required to start enzyme activity as mentioned above. Decolourisation of RBBR with culture filtrate of *F. trogii* did not require H_2O_2. Hence, we concluded that a different enzyme or enzymes were involved in our decolourisation process. Furthermore, we have also observed that using peroxide ions and veratryl alcohol caused a significant drop in the initial activity of enzyme/enzymes responsible for decolourisation. Therefore the effects of H_2O_2 and veratryl alcohol were negative. Two groups of ligninolytic enzymes (laccase and peroxidase) are accepted to play a significant role in enzymatic dye decolourisation [2,22,48,51–53]. Laccase, which decolourises RBBR, does not need any peroxide ions (H_2O_2).

We investigated the effects of inhibitors, in order to confirm if the enzyme involved was laccase in fact. The inhibition of decolourising activity of RBBR was achieved by using different concentrations of sodium azide, cysteine and sodium cyanide. Laccase purified from *Trametes versicolor* has shown different inhibition responses to different substrates. Bollag and Leonowicz [54], for example, showed that with 10 μM NaN_3, the inhibition of laccase activity was 90% using ABTS as substrate. On the other hand, Johannes

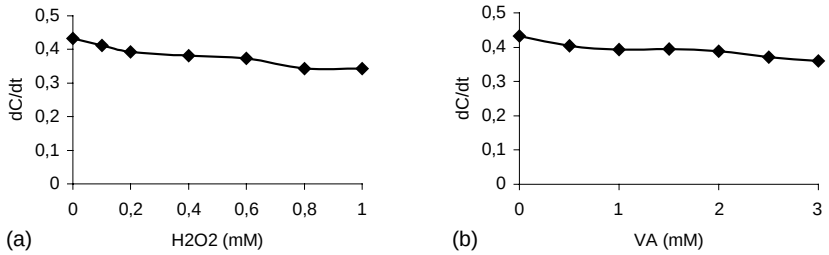

Fig. 7. (a) Influence of H_2O_2 dosage on RBBR decolourisation; pH 3, 1 mM VA, 150 μl enzyme, 80 mg/l RBBR, 1 min reaction at 30 °C; (b) influence of VA dose on RBBR decolourisation; pH 3, 0.2 mM H_2O_2, 150 μl enzyme, 80 mg/l RBBR, 1 min reaction at 30 °C.

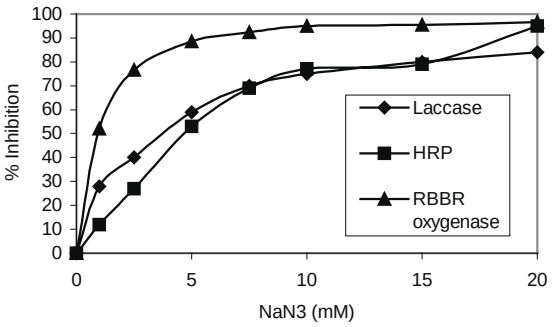

Fig. 8. Influence of NaN_3 for dye degradation, 80 mg/l RBBR, 150 μl, 5 min reaction at 30 °C.

and Majcherczyk [4] reported with the using same concentration of NaN_3, an inhibition of 55% was achieved using syringaldazine as the substrate.

This clearly demonstrates that the inhibition factors of enzymes are dependent on both substrate used and pH of the reaction. In our studies with inhibitors, we achieved a 50% inhibition with guaicol as a substrate (pH 6), and dye material (RBBR, pH 3) using 5 and 1 μM NaN_3, respectively (Fig. 8). Cysteine and sodium cyanide are known to inhibit both laccase and peroxidase enzymes. The RBBR dye decolourising enzyme was inhibited by $Na_2S_2O_5$ and NaN_3, which are known to inhibit enzymes of oxido-reductase group [22,55]. Both laccase and the dye decolourising enzyme (RBBR oxidase) were inhibited by $Na_2S_2O_5$, whereas peroxidase was not inhibited (Table 1). These results emphasised that the main responsible enzyme in RBBR dye decolourising reaction is laccase.

Table 1
Inhibition of laccase peroxidase and RBBR decolourising activity by various inhibitors

Inhibitor	Concentration (μM)	Inhibition (%)		
		HRP	Laccase	RBBR oxygenase
Cysteine	20	34	19	32
Cysteine	40	46	45	49
Cysteine	60	64	63	61
NaCN	40	53	40	38
NaCN	240	86	82	72
NaCN	1000	95	94	92
$Na_2S_2O_5$	240	15	55	64
$Na_2S_2O_5$	1000	20	95	90

5. Gel electrophoresis

The molecular weight of the enzyme was determined by SDS-PAGE with the molecular weight marker. In order to determine the molecular weight of the decolourising enzyme band, the activity staining was done with Remazol Brilliant Blue R after the gel was renatured. After sufficient staining (30 min), a single non-coloured zone appeared on the band, which was calculated to be about 65 kDa. After staining with guaicol, this non-coloured zone turned into orange-colour (Fig. 9). Our results showed that only one enzyme was responsible for decolourisation of RBBR. We conclude that this decolourising enzyme is laccase.

Hublik and Schinner [46] determined the molecular mass of laccase purified from *Pleorotus ostreatusc* 66.8 kDa. Höfer and Schlosser [56] purified the laccase enzyme of *T. versicolor* having a single band with a molecular mass of approximately 68 kDa. The degradation of the diazo dye Chicago Sky Blue 6B by a purified laccase from *Pycnoporus cinnabarinus* showed a band having a molecular size of 63 kDa determined by SDS-PAGE [23]. These results are in accordance with our findings.

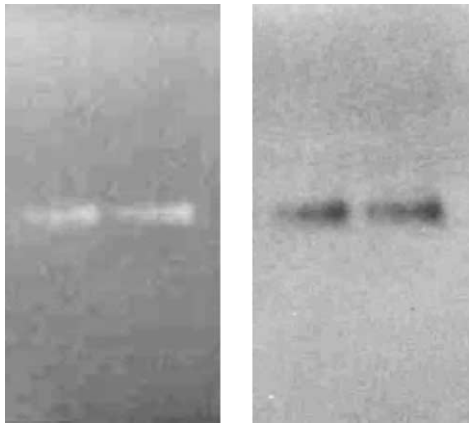

Fig. 9. In order to determine the molecular weight of the decolourising enzyme band, the activity staining was done with Remazol Brilliant Blue R after the gel was renatured. After staining with guaicol, this non-coloured zone turned into orange-colour.

6. Conclusion

Remazol Brilliant Blue R decolourising enzyme was synthesised by *F. trogii* during solid-state fermentation of wheat bran and soybean waste. Decolourisation was seen in the absence of peroxide ions (H_2O_2). Inhibition of decolourisation activity by oxido-reductase inhibitors, NaN_3 and $Na_2S_2O_5$, indicated that Remazol Brilliant Blue R decolourising enzyme was an oxido-reductase group enzyme. These results indicate that RBBR oxygenase is a laccase.

By simply adding a new step to SDS-PAGE, to be more specific, instead of staining gel with normal Commassie blue, we used RBBR for staining operation and achieved a clear zone. Later by treating this colourless zone with guaicol, known as a substrate and indicator of laccase we managed to turn the colourless zone to orange. This was due to oxidation of guaicol by laccase, producing quinones, giving orange-colour. These findings conclusively proved that the decolourising enzyme for RBBR was laccase. Yet another interesting outcome is that the laccase from *F. trogii*, did not require any mediator such as HBT during the decolourisation process, which could have important implications for future industrial usage of this enzyme. These data have a great importance for industrial applications, as it makes possible using this crude filtrate without purification and by this way could reduce cost of enzymatic-based decolourisation process.

As a result, this property of the enzyme makes it as an excellent fungal decolourising enzyme for biotechnological aims. Further kinetics study for RBBR and other dyes are also in progress.

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