Short communication

Production of Ligninolytic Enzymes by *Ceriporiopsis subvermispora* for Decolourization of Synthetic Dyes

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Dedicated to the memory of professor Vojko Ozim

Abstract

The aim of this research was to produce a mixture of ligninolytic enzymes for decolourization of synthetic dyes, which are usually present in waste waters from textile industry. White rot fungus *Ceriporiopsis subvermispora*, known for the production of laccase (Lac) and manganese peroxidase (MnP) for application in bio-pulping was used for this purpose. Procedure for the enzyme production in stationary cultures was optimized regarding the type of sugar as a carbon source, type of wood as inductor and immobilization support material as well as additional nitrogen supply. By varying the culture conditions, production media with the excess of Lac activity, equivalence of both activities and excess of MnP activity were prepared and further used to decolorize three structurally different dyes. The initial decolourization rate of Remazol Brilliant Blue was more than twice higher at the excess of Lac activities than at the excess of MnP in the medium, while the initial decolourization rate of the dyes Copper(II)phtalocyanine and Reactive Orange 16 was three times higher when the excess of MnP was present in the enzyme mixture compared to the medium with excess of Lac. Results showed that Lac and MnP from *C. subvermispora* can be used for decolourization and that 'tailor made' enzyme mixtures can be produced by varying cultivation conditions.

Keywords: Decolourization, synthetic dyes, *Ceriporiopsis subvermispora*, laccase, manganese peroxidase, culture immobilization on wood

1. Introduction

Industrial waste effluents with dyes from the dyestuff and textile industry represent a serious hazard to the environment. Besides conventional chemical and physical methods for dye degradation, white rot fungi have gained significant importance in this respect. Because of their ligninolytic enzymes like manganese peroxidase (MnP) lignin peroxidase (LiP) and laccases (Lac), they are the most intensively studied dye-decolorizing microorganisms in the last two decades.[1–6]

White-rot fungus *Ceriporiopsis subvermispora* causes degradation of lignin in various types of wood. It is used for wood treatment in bio-pulping process in which wood is pretreated prior to pulping. The paper quality was improved and energy was saved because the lignin degradation was enhanced by the extracellular activities of MnP and Lac of this ligninolytic fungus. Nine types of active isolates were found for this species.[7] Studies of the enzymatic machinery produced by *C. subvermispora* grown under ligninolytic conditions in liquid cultures showed that it produced MnP and laccase.[8] Similarly it was found that up to seven isoenzymes of MnP and four isoenzymes of Lac are produced in liquid cultures of defined composition. These enzymes are also produced when grown on wood chips. [9] High Lac activity was associated with wheat bran in the medium, where two isoenzyme forms were found. No supplemental copper was necessary to increase the enzyme activity.[10] On the other hand, even 11 isoenzyme forms of MnP were isolated from liquid and solid state cultures of this basidiomycete.[11]

The ligninolytic enzyme activity is regulated by various nutrients including nitrogen, carbon as well as metal ions.[12–16] On the other hand it is also indicated in the literature that various types of wood with different physicochemical compositions affect fungal activities including enzyme production during cultivation of immobilized culture on wooden surface.[17,18]

There is no evidence in the available literature, that enzymes produced by *C. subvermispora* were used for the dye decolourization. In our previous biodegradation study of the dye RO16 with immobilized *Irpex lacteus* it was assumed that Lac played important role in dye decolorization.[19] To further investigate the role of laccase in the decolourization process we investigated the following: the effect of various carbon sources, additional nitrogen as well as different types of wood, as immobilization support and inducing material on the production of Lac and MnP activities during cultivation of *C. subvermispora* in stationary liquid cultures. The aim of this research was to optimize the cultivation conditions to obtain high Lac and/or high MnP activities for testing the decolourization ability on various types of synthetic dyes.

2. Materials and Methods

2. 1. Microorganism and Chemicals

The fungus *Ceriporiopsis subvermispora* B 920 was obtained from MZKI culture collection (Natural Institute of Chemistry, Slovenia). The strain was maintained on MEG (5 g/L malt extract, 10 g/L glucose, 20 g/L agar) agar slants at 4 °C.

Substrates for enzyme activity assays, 2,2' – azinobis (3 – ethylbenzthiazolinone – 6 – sulfonate) (ABTS) and 2,6 – dimethoxyphenol (DMP) were purchased from Sigma (USA).

2. 2. Culture Conditions of *Ceriporiopsis subvermispora*

Fungal precultures inoculated from agar plates were grown in 50 ml of N-limited mineral medium[20] in 500 ml Erlenmayer flasks. After seven days of incubation the precultures were homogenized using Ultra-Turrax T25 (Janke & Kunkel, IKA-Labortechnik, Germany) at 9000 rpm for 30 seconds under sterile conditions. Homogenate was filtered using sterile gauze with of 1–2 mm^2 pores (Tosama, Slovenia) and used as fungal inoculum. All incubations were carried out at 28 °C.

For experiments in stationary liquid cultures 5% (v/v) of the filtered culture homogenate was used to inoculate 100 ml of fresh mineral medium[20] in 250 ml Erlenmeyer flasks. At the selected times aliquots of culture liquid were collected for the determination of enzyme activities.

Immobilized cultures were grown on 1 cm^3 pine (PW), beech (BW), oak (OW) wood and polyurethane (PUF) cubes for 30 days. In order to prepare the solid support for fungal colonization, PUF cubes were washed three times with hot distilled water to remove all foreign matter and air dried. The cubes were autoclaved in a volume of 150 ml of the liquid mineral medium[20] in 250 ml Erlenmeyer flasks. After autoclaving, 50 ml of the medium was removed and the rest of it was inoculated with 5% (v/v) of the filtered culture homogenate.

To study the effect of sugar type, we used high-nitrogen mineral medium (5,4 mM diammonium tartrate) with additional 10 g/L glucose, fructose or saccharose. Various additional nitrogen (N) concentrations (0.1, 1.0, 2.0, 3.0, 4.0 g/L diammonium tartrate) were prepared in mineral medium with 10 g/L glucose or saccharose as carbon source. Aliquots of culture liquid were collected every two days to determine the MnP and Lac activities and to evaluate the effect of culture conditions on enzyme production. All experiments were performed in three replicates.

2. 4. Enzyme Assays

Laccase activity was measured by monitoring the oxidation of 5 mM ABTS at A_{420}.[21] Mn-dependent and Mn-independent peroxidase (MnP) activity was measured by monitoring the oxidation of 2,6-dimethoxyphenol at A_{469}.[5,22] One unit of enzyme activity was defined as an amount of the enzyme oxidizing 1 μmol of substrate per min. All spectrophotometrical measurements were carried out using Perkin Elmer spectrophotometer, type Lambda 25 (USA).

2. 5. Dyes and Dye Decolourization

Dye decolourization was calculated by comparing the absorbance, measured at the maximum absorbance wavelength for each compound, during the decolourization treatment. Dyes used in this study were: Reactive Orange 16 (RO16; an azo dye) λ_{max} = 494 nm, Remazol Brilliant Blue R (RBBR; an anthraquinonic dye) λ_{max} = 592nm, and Copper(II)phthalocyanine (CuP; a phthalocyanine dye) λ_{max} = 694 nm. All dyes were purchased from Sigma (USA).

For *in vitro* dye decolourization by the crude culture liquid, a culture filtrate obtained from *C. subvermispora* cultures with various MnP and Lac activities was used. The reaction mixtures consisted of 100 mM Na-tartrat buffer pH 4.5, 50 mg/L dye and 100 μl of crude culture liquid in a final volume of 1 ml. Dye decolourization was measured continuously using Perkin Elmer spectrophotometer, type Lambda 25 (USA). Initial decolourization rates were determined during the first 60 minutes of the decolourization experiment.

3. Results and Discussion

3. 1. Effect of Culture Conditions on Enzyme Production

The first series of experiments was done with stationary liquid cultures using three different kinds of sugar, saccharose, glucose and fructose as a carbon source in the

mineral medium[20] to estimate the maximal production of Lac and MnP. It was found that the highest activity of Lac was present in the medium with saccharose (257 U/L) after 28 days of cultivation, while MnP activities were low (19 U/L) using this sugar. The maximal MnP activity occured in the medium with glucose (97 U/L) after 16 days of cultivation where the Lac activity was only 17 U/L. Additional nitrogen source (0,1–4 g/L of diammonium tartrate) in the media with glucose did not cause any special increase or decrease in either MnP or Lac activity. Maximum activities occurred mainly after four weeks of cultivation, which is in agreement with results obtained by other researchers.[23]

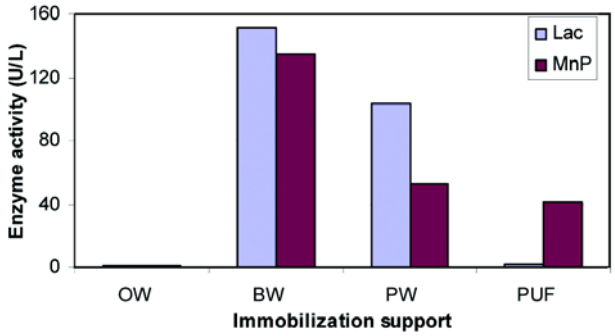
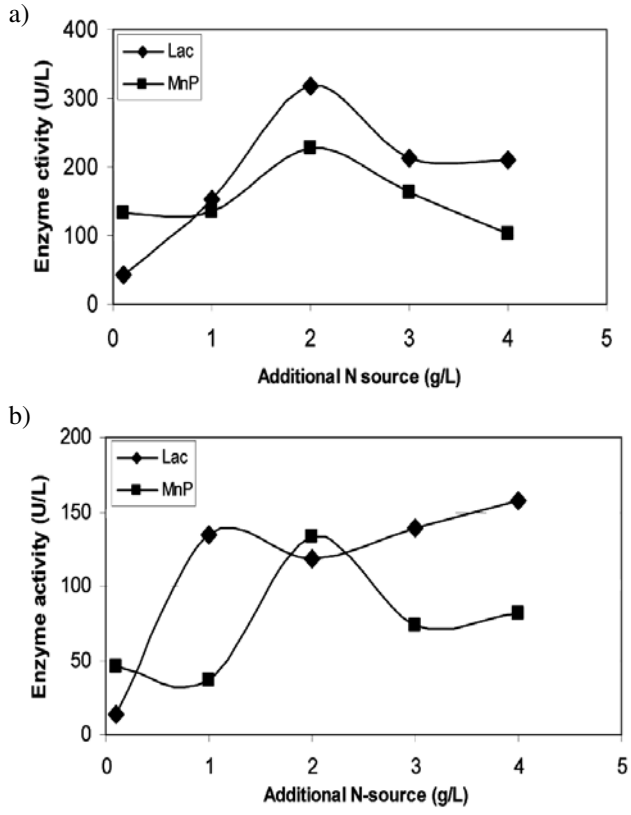


Figure 1. The highest ligninolytic enzyme activities (expressed in U/L) obtained by *C. subvermispora* immobilized on oak wood (OW), beech wood (BW), pine wood (PW) cubes and polyurethane foam (PUF) using mineral medium with glucose.

Glucose was chosen as a C-source in further experiments. The investigation continued by testing various inducing and support materials in the form of cubes such as different types of wood and polyurethane. The results are presented in Figure 1 which show that the fungus grown on PUF cubes produces low enzyme activities (2 U/L Lac and 41 U/L of MnP) while activities are higher in the presence of wood. Structural cell-wall components like cellulose, hemicelluloses and lignin form the dominant available carbon sources for fungi grown on wood. In addition, wood contains small amounts of soluble sugars, lipids, peptides and starch as well as minerals and a wide range of extractives and volatiles. All these compounds vary with various tree species and therefore affect the fungal growth and enzyme production in different ways.[18] For example, oak is very resistant to rottenness because it contains tannin that can suppress fungal growth and also enzyme production. Pine wood contains various resins which are a sort of natural protection against rot. Presented results show that, overall, beech wood stimulated the most production of Lac as well as MnP since 152 U/L of Lac after 21 day and 135 U/L of MnP activities after 14 day of cultivation were achieved. However, some activities were obtained also on pine wood: 104 U/L of Lac after 11 day and 53 U/L of MnP activities after 7 day of cultivation. Activities below 1 U/L were detected on oak wood.

Beech wood and pine wood were selected for further experiments. The effect of additional nitrogen in the form of diammonium tartrate and 10 g/L glucose as the source of sugar was investigated. The results are shown in Figures 2a and 2b. In the range of nitrogen concentrations investigated here, Lac activities increased with additional nitrogen on both, beech wood and pine wood. However, beech wood gave better results; maximal Lac activity (318 U/L) occurred on this support after 28 days when nitrogen concentration was 2,0 g/L. These activities were between 118 and 156 U/L after 25 and 30 days of cultivation on pine wood when the additional nitrogen concentration was in the range 1–4 g/L. Production of MnP activities also depended on the type of wood as well as additional nitrogen. Better results were also obtained with 2,0 g/L of diammonium tartrate where the maximal MnP activity on beech wood achieved 227 U/L while on pine wood it was 133 U/L on the 30th day of cultivation. According to the literature data, very low levels of nitrogen occur in the wood resulting in a high C:N ratio. Decrease in this ratio can be achieved by additional nitrogen source and consequently more intense fungal activity during wood decom-



Figures 2a and 2b: The highest ligninolytic enzyme activities (expressed in U/L) obtained by *C. subvermispora* grown in mineral medium on beech wood (a) and pine wood (b) cubes depending on the amount of additional nitrogen source.

position.[18] This is in accordance with the results of this investigation, that a particular type of wood contains some unique component which induces particular enzyme activity. This induction effect is even more pronounced in combination with additional nitrogen source. Furthermore, the ratio between Lac and MnP activities can be controlled by adjusting nitrogen concentration as well as type of wooden support.

3. 2. Dye Decolourization

On the basis of the above results, we prepared three different culture media for incubated the fungus to achieve the production of three combinations of Lac and MnP activities. The combinations are presented by the enzyme activity ratio to express the excess of Lac activities, equivalence of both activities and excess of MnP activities. Three structurally different dyes were used to test the decolourization activity of prepared enzyme mixtures. The results of enzyme performance expressed as initial rates of decolourization are shown in Table 1.

Table 1. Initial velocity (expressed in mg/Lmin) of decolourization for anthraquinonic dye RBBR, phthalocyanine dye CuP and azo dye RO16 with crude enzyme mixture produced by *C. subvermispora* immobilized on beech wood cubes.

medium type	enzyme activity ratio	initial velocity of decolourization (mg/L min)		
	Lac/MnP	RBBR	CuP	RO16
BWG, N 2g/L	4,5	0,225	0,122	0,003
BWS, N 2g/L	1	0,100	0,148	0,001
BWS, N 1g/L	0,6	0,099	0,360	0,010

BWG: beech wood in mineral Kirk's medium with additional glucose as sugar source and additional nitrogen (N) source.
BWS: beech wood in mineral Kirk's medium with additional saccharose as sugar source and additional nitrogen source.

The ability of white rot-fungi to decolorize synthetic dyes has been well documented.[5,6,12, 24–28] To our knowledge, this is the first report on the decolourization activity of the enzymes produced by *C. subvermispora.* Results presented here show that the decolourization rate of RBBR more than doubled with the excess of Lac activities than with the excess of MnP activities in the media. This is in agreement with the results of Šušla et al,[24] who explained that laccases are able to decolourize RBBR due to the fungal ability to also degrade PCBs. Champagne and Ramsay[25] also found that the RBBR decolourization increased with increasing Lac concentration. CuP is in general more difficult to degrade than the two other dyes.[24] However, an involvement of MnP in decolourization process has been observed, when lower laccase activity was compensated by other enzymes. This can be seen from Table 1, where the decolourization rate of CuP as well as RO16 is three times higher when the excess of MnP is present in the enzyme mixture compared to the medium with the excess of Lac. It was observed that MnP played an important role in decolourization of various azo dyes.[26,27] Such Lac and MnP enzyme cooperation in liquid cultures has been already suggested.[25,28]

4. Conclusion

The mixture of ligninolytic enzymes produced by the white rot fungus *C. subvermispora*, already successfully applied in bio-pulping process, could be also used for the decolourization of synthetic dyes. A simple process to produce enzyme mixtures with various ratio of Lac/MnP is proposed, using appropriate cultivation conditions like carbon source, additional nitrogen as well as type of wood as immobilization carrier. This way 'tailor made' enzyme mixtures with excess of laccase or excess of manganese peroxidase can be produced for application in decolourization technology of specific synthetic dyes.

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Povzetek

Raziskave so potekale z namenom pripraviti mešanico ligninoliticnih encimov za razbarvanje sintetičnih barvil, ki so običajno prisotna v odpadnih vodah iz tekstilne industrije. Za proizvodnjo encimov je bila uporabljena gliva bele trohnobe *Ceriporiopsis subvermispora,* ki je znana po sintezi lakaz in mangan peroksidaz za uporabo v industriji celuloze. Postopek za proizvodnjo encimov v stacionarnih kulturah je bil optimiran glede na vrsto sladkorja kot vira ogljika, vrsto lesa kot induktorja in nosilca za imobilizacijo kulture ter dodanega vira dušika. Na osnovi različnih pogojev gojenja so bili pripravljeni mediji s prebitkom lakaz, prebitkom mangan peroksidaz ter enakostjo obeh aktivnosti. Ti so bili v nadaljevanju raziskav uporabljeni za razbarvanje strukturno različnih organskih barvil. Začetna hitrost razbarvanja RBBR je bila dvakrat visja v mediju s prebitkom lakaz kot v mediju s prebitkom mangan peroksidaz, medtem ko je začetna hitrost razbarvanja CuP in RO16 bila tri krat večja v mediju s prebitkom mangan peroksidaz kot v mediju s prebitkom lakaz. Rezultati kažejo, da se encimi glive *C. Subvermispora* lahko uporabljajo za razbarvanje in da se lahko s izbranimi pogoji gojenja proizvede encimske mešanice »po meri«.