Short communication

Induction of Ligninolytic Enzyme Production by *Dichomitus squalens* on Various Types of Immobilization Support

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Abstract

The aim was to produce a mixture of ligninolytic enzymes for decolourisation of synthetic dyes typically present in textile industry wastewaters. White rot fungus *Dichomitus squalens*, capable of producing laccase (Lac) and manganese peroxidase (MnP) was used. A procedure for the enzyme production in stationary cultures was developed and optimized regarding the type of carbon source and addition of nitrogen to N-limited medium. Beech wood, pine wood and straw were used as inducers and immobilization support materials. Beech wood and straw were the best inducers and immobilization supports, while fructose stimulated the enzyme activities better than other sugars. An additional nitrogen source was beneficial to increase the enzyme activities in the culture medium. Higher Lac than MnP activities were determined in all cases. The obtained enzyme mixtures from the culture filtrate were tested to decolourize three structurally different dyes. The highest initial decolourisation rate was obtained with Remazol Brilliant Blue R. The decolourization efficiencies after 10 h were 62% for RBBR, 50% for Copper(II)phthalocyanine and 19% for Reactive Orange 16.

Keywords: Laccase, manganese peroxidase, *Dichomitus squalens,* enzyme production, mycelium immobilization, decolourisation, synthetic dyes

1. Introduction

Synthetic dyes are used extensively in various technologies like textile, leather, paper, food, hair colourings and similar industries.[1] Industrial waste effluents containing dyes produced by such industries often 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 due to their extracellular ligninolytic enzymes such as manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase (Lac). They have been the most intensely studied dye-decolorizing microorganisms in the last two decades.[2–12]

The ligninolytic enzyme production is regulated by various nutrients including nitrogen, carbon as well as metal ions.[13–17] Also, various types of wood with different physico-chemical compositions affect fungal activities including the enzyme production during cultivation of immobilized fungus on a wooden surface.[18,19] This indicates that the dye degradation capability can be regulated by choosing the imobilization support material. In our previous study with *Ceriporiopsis subvermispora* it was shown that various ratios of Lac and MnP activities can be induced by varying carbon and nitrogen concentrations during cultivation on beech wood which was the best wooden support compared to pine and oak wood. [20]

The white-rot fungus *Dichomitus squalens* has been found to produce MnP and Lac, but not lignin peroxidase. Two MnP and two Lac isoenzyme forms were characterized from this white rot basidiomycete.[21–23] Lac production with this fungus can be influenced by various agricultural residues.[24] It was also indicated that beech wood as the immobilization support induced laccase production beter than polyurethane foam.[23] However, only a few papers in the last decade show the capability of *D. squalens* to decolourise synthetic dyes.[23–27]

The aim of this study was to optimize the cultivation conditions to obtain high Lac and/or MnP activities for testing the decolourisation ability of various types of synthetic dyes. In order to find the induction role of natural organic and waste material in the decolourisation process, we investigated the effect of various carbon sources, nitrogen concentration as well as natural lignim containing material like beech wood and straw as the immobilization support and inducing material on the production of Lac and MnP activities during cultivation of *D. squalens* in stationary liquid cultures.

2. Materials and Methods

2. 1. Microorganism and Chemicals

The fungus *Dichomitus squalens* Reid 750 was obtained from the CCBAS culture collection (Institute of Microbiology ASCR, Prague, Czech Republic). The strain was maintained on MEG (2,5 g/L malt extract, 5 g/L glucose, 10 g/L agar) agar slants at 4 °C.

The substrates for the Lac and MnP activity assays were 2,2' – azinobis (3 – ethylbenzthiazolinone – 6 – sulfonate) (ABTS) and 2,6 – dimethoxyphenol (DMP), respectively; both were purchased from Sigma (USA).

2. 2. Culture Conditions

Fungal precultures inoculated with mycellium from agar plates were grown in 50 ml of N–limited mineral medium (NL)[28] in 500 ml Erlenmayer flasks in shaken cultures. After seven days of incubation at 28 °C, the precultures were homogenized using Ultra-Turrax T25 (Janke & Kunkel, IKA-Labortechnik, Germany) at 9000 rpm for 30 seconds under sterile conditions.

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

The immobilized cultures were grown on 50 pieces of 1-cm^3 cubes of pine- (PW) or beech (BW) wood or polyurethane foam (PUF) and 5 g of straw (STR) cut to 1 cm pieces for 30 days.[21] 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 and straw were autoclaved in a volume of 150 ml of the liquid mineral medium[28] 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 culture homogenate.

To study the effect of sugar type on enzyme production, we used N-limited mineral medium (NL) with 1.0 g/L diammonium tartrate and 10 g/L glucose, fructose or saccharose. The effect of nitrogen on enzyme production was studied in NL medium with additional nitrogen concentrations (0.1, 1.0, 2.0, 3.0, 4.0 g/L diammonium tartrate) and 10 g/L glucose or saccharose as the carbon source. Aliquots of the culture liquid were collected every second day to determine the MnP and Lac activities.[20] All experiments were performed in three replicates.

2. 3. Enzyme Assays

Laccase activity was measured by monitoring the oxidation of 5 mM ABTS at A_{420}.[29] MnP activity was measured by monitoring the oxidation of 2,6-dimethoxyphenol at A_{469}.[6,30] One unit of the enzyme activity exhibited the amount of enzyme oxidizing 1 μmol of corresponding substrate per minute. All spectrophotometrical measurements were carried out using Perkin Elmer spectrophotometer, type Lambda 25 (USA).

2. 4. Dyes and Dye Decolourization

Dyes used in this study were: Reactive Orange 16 (RO16; azo dye) λ_{max}= 494 nm, Remazol Brilliant Blue R (RBBR; anthraquinone dye) λ_{max}= 592nm, and Copper(II)phthalocyanine (CuP; phthalocyanine dye) λ_{max}= 694 nm. All dyes were purchased from Sigma (USA).[20]

For the *in vitro* dye decolourisation experiments with a crude culture liquid, a culture filtrate obtained from *D. squalens* cultures with various MnP and Lac activities was used. The reaction mixtures consisted of 100 mM Na-tartrate buffer pH 4.5, 50 mg/L dye and 500 μl of a crude culture liquid in a final volume of 1 ml. Dye decolourisation was measured continuously using Perkin Elmer spectrophotometer, type Lambda 25 (USA).

Dye decolourisation was calculated by comparing the absorbance, measured at the maximum absorbance wavelength for each compound during the decolourisation treatment. Initial decolourisation rates were determined within the first 10 minutes of the decolourisation experiment.[20]

3. Results and Discussion

3. 1. Effect of Culture Conditions on Enzyme Production

The first series of experiments was made on four types of immobilization material (beech wood, pine wood, straw and polyurethane foam) using three carbon sources, glucose, saccharose and fructose at a concentration of 10 g/L of the NL mineral medium with additional 1.0 g/L of diammonium tartrate to estimate the best wooden support and type of sugar for the maximal production of Lac and MnP. As a reference, cultivations in static cultures without immobilization support were carried out.

In most cases, fructose stimulated the enzyme activities better than other sugars. The results with this sugar are shown in Figure 1.

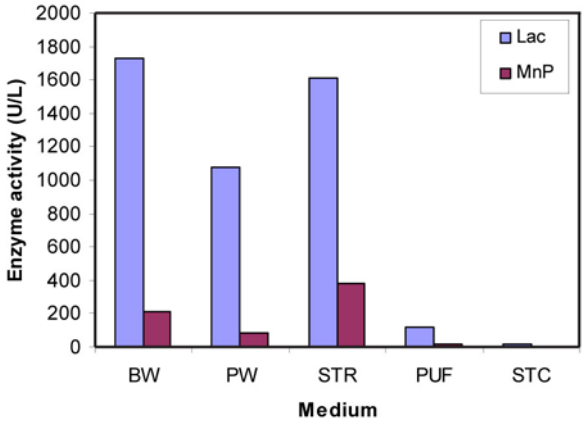

Figure 1. The effect of the type of immobilization support material (BW-beech wood, PW-pine wood, STR-straw, PUF-polyurethane foam, STC-stationary culture without support) on the enzyme production of *D. squalens* grown in the media with fructose.

It can be seen that the lignocellulosic support materials like wood and straw stimulated the enzyme production. Structural cell-wall components like cellulose, hemicelluloses and lignin are dominating available carbon sources for fungi grown on wood and straw. 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.[18,19] Our results confirm that beech wood as well as straw induce Lac activities. Straw also contains cellulose, hemicelluloses and lignin. The composition and contents varies with the type of straw as well as the with the growth location. It contains phenolic compounds, which stimulate laccase production.[31,32] However, straw better induced the production of MnP activities compared to those with beech wood. PUF used as inert support material only slightly increased Lac activity compared to the reference experiments with static cultures and all three carbon sources, where both activities were lower than 10 U/L.

With beech wood, the highest activity of Lac (1733 U/L) was present after 19 days of cultivation while with straw, the highest Lac activities (1609 U/L) occured after 21 days of cultivation. Here the Lac activity with beech wood is about 7% higher. The highest MnP activities were much lower; with beech wood MnP activity (215 U/L) occured after 29 days of cultivation, while about 70% higher MnP activity (378 U/L) occured after 23 days of cultivation. For comparison, the results obtained with an inert material (PUF) showed that the highest Lac activities (160 U/L) were obtained with saccharose and the highest MnP activities (16 U/L) with fructose.

Further, the effect of additional nitrogen in the form of diammonium tartrate added in the NL media with 10 g/L glucose was tested using various immobilization supports and, in parallel, under the reference conditions without any support.The results are shown in Figure 2.

It is seen that the additional nitrogen increases both activities during growth on lignocellulosic material. According to the literature, low levels of nitrogen occur in the wood as well as straw resulting in a high C:N ratio. Decrease in this ratio can be achieved by using an additional nitrogen source, which results in a more intense enzyme production during growth.[26] A particular type of wood as well as straw contains lignin which induces the enzyme activities. This induction effect is even more pronounced in combination with an additional nitrogen source. This is in accordance with the results of this investigation.

For example, Lac activities on straw with 2 g/L of diammonium tartrate increased from 1150 U/L to 1708

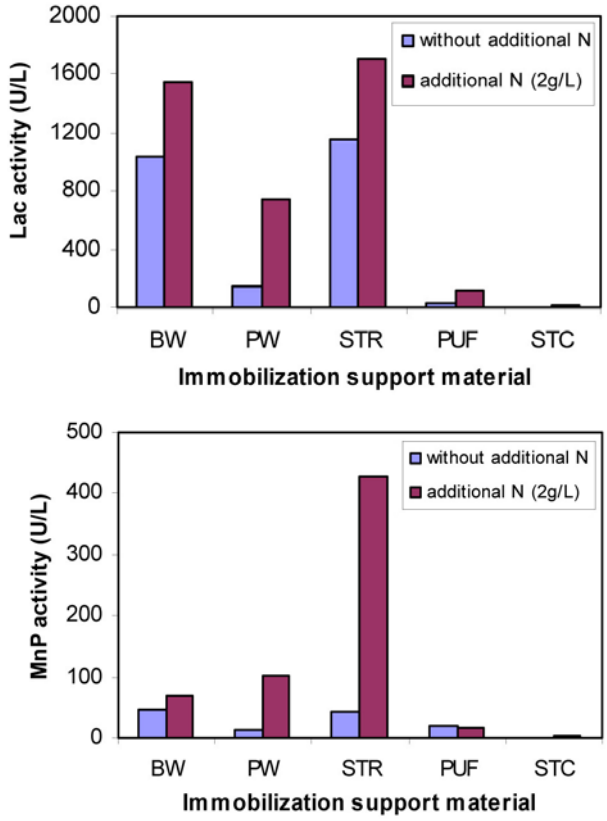

Figure 2. The effect of the additional nitrogen (2 g/L of diammonium tartrate) in the N-limited mineral medium on Lac and MnP production with *D. squalens* grown on various immobilization supports.

U/L and on beech wood from 1031 to 1553 U/L while the MnP activities on straw increased from 42 to 428 U/L and on pine wood from 12 to 103 U/L, all between 21 and 28 days of cultivation. In the cultures with polyurethane foam and in the reference conditions, the effect of additional nitrogen was not significant.

These results confirmed that lignocellulosic support material such as beech wood and straw is necessary to

achieve high Lac activities, which is favorable from the practical point of view. Both Lac and especially MnP activities could even be increased in the media by providing an additional nitrogen source. However, the results show that *Dichomitus squalens* always produces higher Lac activities and that the preparation of excess MnP activities by varying culture conditions that was possible in *C. subvermispora*, [20] could not be attained with *Dichomitus squalens*.

3. 2. Dye Decolourisation

The ability of white rot-fungi to decolorize synthetic dyes has been well documented. [2–11, 20, 23, 26–27] Three structurally different dyes were used to test the decolourisation activity of the enzyme mixture obtained during the cultivation on beech wood. The results are shown in Figure 3.

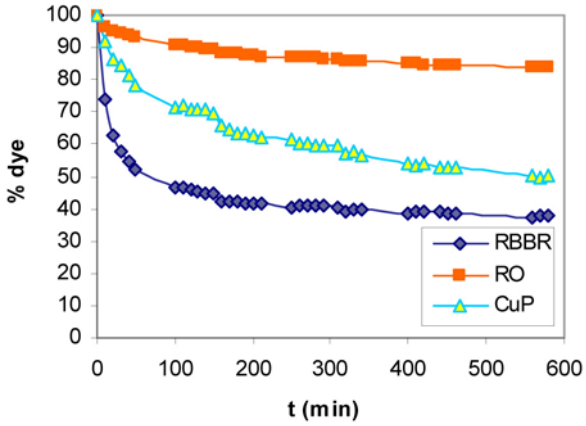

Figure 3. Decolourisation of three different dyes: Reactive Orange 16 (RO16) Remazol Brilliant Blue R (RBBR) and Copper(II) phthalocyanine (CuP) with enzyme mixtures obtained on beech wood (Lac 1550 U/L) and MnP (70 U/L)

The results show that the highest initial decolurization rate was obtained with RBBR and the lowest with RO16. The values are 12,9 mg/L min, 4,1 mg/L min and 1,7 mg/L min for RBBR, CuP and RO16, respectively. The decolourization efficiency after 10 hours for RBBR was 62%. This is an easily degradable dye and is often used for docolourization experiments to test the enzyme docolourization properties. In our investigation, the docolourization of RBBR was the most efficient in the presence of Lac and MnP activities. This is consistent with other investigators[23, 26, 27] who have demonstrated that laccase isoenzymes were able to decolourise RBBR, the important role of MnP as well as cooperation between Lac and MnP during the decolourization process. The decolourization efficiency of CuP was 50%. Presumably the cooperation of Lac and MnP is present here.[23, 33] The decolourization efficiency of RO16 was 19%. Azo dyes are not easily degradable. In our previous study of RO16 decolourization with immobilized *Irpex lacteus*[8] it was assumed that the successful decolourization of RO 16 was due to the mycellium associated laccase. Under such conditions probably some intracellular mediators exist which were not present in the crude culture liquid used in this investigation. Šušla et al [23] also found low decolourization efficiency of RO 16 in culture filtrate of *D. squalens* with relatively high Lac activity (816 U/L), which is consistent with our results.

4. Conclusion

Production of extracellular Lac and MnP activities by *D. squalens* immobilized on lignocellulosic material such as beech wood and straw is highly induced compared to the immobilization on inert support like PUF which would be favorable from the practical point of view. Additional nitrogen in the media even increases relatively high Lac activities on these two supports and furthermore increases MnP activities as well, especially on straw. Considering the degradation of corresponding substrate for each activity determination based on unit of substrate per unit time and conditions investigated here, higher Lac activities are produced compared to MnP activities. Both enzymes as well as their synergistic action, play important roles during the decolourization of specific dyes. The mixture of produced extracellular ligninolytic enzymes could be used for the decolourization process of the tested anthraquinone and phthalocyanine dye.

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Povzetek

Namen raziskav je bil pridobiti mešanico encimov za razbarvanje sintetičnih tekstilnih barvil z glivo bele trohnobe *Dichomitus squalens,* ki proizvaja lakaze in mangan peroksidaze. Postopek sinteze barvil je bil razvit in optimiran glede na vir ogljika in dodatek dušika v gojišče ter ob prisotnosti smrekovega ter bukovega lesa in slame kot nosilca in induktorja. Bukov les in slama sta bila najboljša induktorja, fruktoza najboljši vir ogljika, dodani dušik pa je ugodno vplival na razvoj aktivnosti. Ob upoštevanju specifičnega substrata za določanje posamezne aktivnosti je bila v vseh primerih najvišja dosežena lakazna aktivnost. Največja začetna hitrost razbarvanja je bila dosežena pri barvilu remazol modro R (RBBR). Učinek razbarvanja po 10 urah je bil 62 % za RBBR, 50 % za bakrov(II)ftalocianin (CuP) in 19 % za reaktivno oranžno 16 (RO16).