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Biotransformation of phenolics with laccase containing bacterial spores

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Abstract Treatment of effluents containing phenols such as textile dyes with fungal laccases is usually limited to the acid to neutral pH range and moderate temperatures. Here we demonstrate for the first time that spore-bound laccases which are stable at high temperatures and pH values can be used for phenolic dye decolorisation. Laccase containing spores from *Bacillus* SF were immobilized on alumina pellets. Both immobilized and free spores were able to completely decolorize the common textile dyes Mordant Black 9, Mordant Brown 96/Mordant Brown 15, and Acid Blue 74 within 90 min of incubation time and decolorized solutions were successfully used in re-dyeing.

Keywords *Bacillus* · Spores · Biodegradation of phenolics · Effluent treatment

Introduction

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) have found several applications in enzyme remediation. Laccases as well as immobilized laccases have been used for the treatment of phenolic effluents, PAHs and PCBs. The enzymes render phenolic compounds less toxic via degradation or polymerization reactions and/or cross-coupling of pollutant phenols with naturally occurring phenols (Bohmer et al. 1998; D'Annibale et al. 2000; Ullah et al. 2000). Besides microbial processes (Fischer-Colbrie et al. 2005), laccases have also a potential for the decolorization of textile dyes (Kandelbauer and Guebitz 2005).

Laccases catalyze the removal of a hydrogen atom from the hydroxyl group of ortho and para-substituted monophenolic and poly-phenolic substrates and from aromatic amines by one-electron abstraction, to form free radicals, capable of undergoing further depolymerization, repolymerization, demethylation or quinone formation.

For dye decolorization, the laccases from a number of fungi such as *Sclerotium rolfsii*, *Trametes modesta*, *T. hirsuta*, *T. versicolor*, *Pleurotus pulmonarius* among others have been investigated (Kandelbauer and Guebitz 2005). However, the potential of bacterial laccases for this purpose has not been assessed so far. In contrast to fungal laccases which are known for many years, laccase activity in bacteria has only been described in 1993 for *Azospirillum lipoferum* and has been characterized later in more detail (Diamantidis et al. 2000). Interestingly, the spore coat protein CotA of *Bacillus subtilis* was identified as laccase (Hullo et al. 2001). Since spores serve microorganisms to survive drastic conditions, spore coat enzymes might also withstand high temperatures or extreme pH values which would be advantageous for industrial applications.

It has previously been shown that residual enzyme after enzymatic decolorization of dyeing effluents can restrict the reuse of water (Abadulla et al. 2000). Enzyme protein can adsorb to fabrics and thus lead to unwanted color differences in dyeing with enzymatically decolorized effluents. Therefore, for continuous applications in dye decolorization and also in other applications immobilization of laccases is required and several successful examples have been reported (Abadulla et al. 2000; Kandelbauer et al. 2004).

In a previous study higher decolorization rates were reported with an immobilized laccase from the white-rot fungus *Trametes modesta* when compared to the free enzyme. Despite the promising efficiency of dye decolorization in general, no decolorization activity at all was observed at pH values higher than pH 7 (Kandelbauer et al. 2004). This limits the industrial potential since many dyeing processes (e.g., cotton) are performed in the alkaline pH range. Alternatively, spore laccases which are also active in the alkaline pH range could be used for dye decolorization without liberation from spores allowing simple immobilization of

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spores or application in membrane reactors. In this study, we report for the first time the use of a spore laccase from *Bacillus* SF for dye decolorization.

Experimental

Cultivation of *Bacillus* SF

Bacillus SF was isolated as described previously (Gudelj et al. 2001). Cultures were grown on Caso-agar (Merck) plates pH adjusted with NaOH to 8.5 for 2 days at 55°C and thereafter stored at 4°C. The medium for liquid cultivation of *Bacillus* SF consisted of two different solutions which were combined after autoclaving. Solution A (g l^{-1}): 3.5 KH_2PO_4 , 4.98 $\text{NaHPO}_4 \cdot 2\text{H}_2\text{O}$, 2.5 $(\text{NH}_4)_2\text{SO}_4$, 10 yeast extract, 10 peptone from casein L, 60 ml 1 M NaOH, Solution B (g l^{-1}): 4.5 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ l^{-1} , 100 ml iron citrate (8.9 g l^{-1}), 25 ml trace element solution SL6 l^{-1} , 10 ml 1 M NaOH l^{-1} . The medium for the induction of sporulation of *Bacillus* SF consisted of (g l^{-1}) 8 nutrient broth (Merck), 1 KCl, 0.25 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and after autoclaving (121°C, 30 min) sterile 1 ml l^{-1} of the following solutions was added 1 M $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.01 M MnCl_2 , 0.001 M FeSO_4 , and 0.5 ml of 1 M NaOH solution. The 1 l baffled Erlenmeyer flasks containing 250 ml of the above medium were inoculated with *Bacillus* SF actively growing on agar plates and incubated at 55°C on a rotary shaker (130 rpm) for 3 days and then 5 ml l^{-1} were transferred to the sporulation medium.

Immobilization of the spores

Alumina pellets (Sigma) were silanized twice at 50°C for 24 h in a 3% (v/v) solution of aminopropyl triethoxy silane (Sigma) in acetone. The silanized pellets were washed with distilled water and immersed in 2.5% (v/v) aqueous glutardialdehyde solution (Sigma) for 2 h at 20°C. Thereafter, the pellets were incubated with 60 mg spores l^{-1} for 5 h at 20°C. The immobilized spore pellets were washed with buffer (pH 8.5) and kept refrigerated until further use. Effective laccase activity of solution was determined before and after immobilization.

Laccase activity

Before its usage in decolorization, the effective activities of the laccase and laccase activity of spore dispersions were determined using the oxidation of 2,6-dimethoxyphenol (DMP). The reaction mixture contained 50 mM sodium malonate (pH 4.5), 1 mM DMP, 1 mM MnSO_4 , and 700 μl sample in a total of 1 ml. The formation of 2,2',6,6'-dimethoxydiphenquinone at 30°C was followed spectrophotometrically at 468 nm and apparent laccase activity was calculated from the molar extinction coefficient $\epsilon = 49.6\text{ mM}^{-1}\text{ cm}^{-1}$.

Isolation of the laccase from the spores

Culture (40 ml) were harvested through centrifugation at $9000 \times g$ for 30 min. The supernatant was withdrawn and the pellet was washed twice through resuspension in 10 ml of deionized water and centrifugation ($9000 \times g$, 15 min). After a final centrifugation at $16000 \times g$ for 20 min cells (spores) were resuspended in an equal volume of a solution containing 0.1 M NaOH, 8.0 M urea, 1% sodium-dodecylsulfate. Cells were incubated for 15 min at 25°C and subsequently centrifuged at $16000 \times g$ for 20 min. The supernatant was filtrated through 0.22 μm membrane filter (Osmonics Inc., Germany) at 2 bar positive pressure. Sodiumdodecylsulfate (SDS) was removed from the crude enzyme preparation through precipitation with an equal amount of 0.5 M potassium acetate buffer pH 5 as potassium dodecylsulfate (PDS). After incubation for 15 min at 25°C PDS precipitate was removed through centrifugation at $16000 \times g$ for 20 min and filtration through 0.22 μm membrane filter. Urea was removed through centrifugation with vivaspin (Satorius AG, Germany) centrifugal concentrators molecular cutoff 5 kDa and exchanging the buffer with 0.2 M N,N,N-tris(hydroxymethyl) aminomethane hydrochloride buffer pH 8.5.

Temperature and pH-stabilities

The thermal stability of the laccase was examined by preincubation in 10 mM phosphate buffer pH 8.0 of free and immobilized spores at various temperatures and measurement of remaining activity with the assay described above. The pH-stabilities were determined similarly incubating free and immobilized spores at 25°C in different buffers ranging from pH 5.0 to 9.5.

Dye decolorization experiments

For decolorization experiments, the commercial textile dyes Diamond Black PV 200 (CI Mordant Black 9 (DBPV 200)), Diamond Fast Brown (CI Mordant Brown 96/CI Mordant Brown 15 (DFB)) kindly donated from DyeStar and Indigo Carmine (IC) (Acros) were used. Aqueous solutions containing 50 mg l^{-1} dye treated with 5 g l^{-1} spores or 7 g l^{-1} immobilized spores at 60°C for 90 min. Samples were withdrawn every 5 min and analyzed by UV/Vis spectroscopy using a U-2001 Hitachi spectrophotometer in the spectral range of 240–800 nm with a scan speed of 1200 nm min^{-1} . To account for the effects of temperature and pH, controls were performed without spores while the typical laccase inhibitor NaF (1 mM) was added to account for possible adsorption effects.

Dyeing experiments

Bleached cotton fabrics (10×10 cm) were dyed with either CI Reactive Orange 70 or CI Reactive Blue in liquors containing enzymatically decolorized dyes and 20 g l^{-1}

Na_2CO_3 and $60 \text{ g l}^{-1} \text{ Na}_2\text{SO}_4$. An Ahiba Spectradye dyeing apparatus (Datacolor International, Luzern, Switzerland) was used at a liquor to good ratio of 20:1 (40 rpm, step 1: temperature was raised from 20 to 60°C in 20 min; step 2: 60°C, 60 min). Dye load was either low (0.5%) or high (3%) with respect to the dry mass of the fabric. Dyed fabrics were washed at the same liquor ratio with the non-ionic detergent Hostopal CV (1 g l^{-1}) for 30 min at 90°C to remove the unfixed dye. Diode array spectrums (TIDAS instrument from J and M, Aalen, Germany) of dyes both in standard dye baths and in dye baths containing enzymatically decolorized dyes were recorded.

Color differences of the dyed fabrics were determined using a reflectance measuring apparatus (Spectraflash 600 from Datacolor, Luzern, Switzerland) according to the CIELAB color difference concept at standard illuminant D65 (LAV/Spec. Excl., d/8, D65/10°) with a color tolerance interval of 1 CIELAB unit. Color deviation was also quantified by calculating the K/S values from reflectance measurements on the dyed fabrics.

Results and discussion

Laccase activity

The activity of the *Bacillus* SF spore bound laccase was dependent on the age of the spores. Maximum activity was achieved after 9 days of growth. Thereafter, activity remained constant within the observed time of 14 days. Laccase activities of spores were 0.64 U mg^{-1} dry weight of spores. The effective laccase activity of the immobilized spores was 0.48 U mg^{-1} (based on the amount of spores immobilized) indicating that immobilization partially inactivated the laccase and/or restricted the access of the substrate to the enzyme.

Several procedures for the isolation of the laccase from spores were compared (data not shown) and only a drastic treatment led to release of active laccase (6%). Most likely this low activity recovery is due to incomplete re-naturation of the enzyme.

Temperature stabilities

The temperature stability (half life times $t_{1/2}$) of the free *Bacillus* SF laccase liberated from spores was only 5 min at 60°C and pH 8.0. In contrast, the observed half life times of laccase activity of *Bacillus* SF spores revealed high temperature stabilities of 66 h at 60°C and pH 8.0. These values are about six and 20 times higher than stabilities of fungal laccases reported previously for this temperature and optimum pH values of the individual enzymes (11.5 h, Abadulla et al. 2000 and 3 h, D'Annibale et al. 2000). At 80°C the half life was still 30 min. Immobilization of the *Bacillus* SF spores onto alumina pellets slightly increased the half life times from 66 to 80 h at 60°C. Similarly, increased temperature stabilities have been reported previously for fungal laccases. (Abadulla et al. 2000; D'Annibale et al. 2000).

pH stabilities

The pH half life time of the free *Bacillus* SF laccase was 48 h at pH 8.5. However, the laccase activity of *Bacillus* SF spores showed higher stabilities over a broad pH range. Within a pH range from 5.0 to 7.0 the half life was more than 120 h compared to 13 h previously measured for a laccase from *Trametes hirsuta* (Abadulla et al. 2000). In contrast to fungal laccases, laccase activity of *Bacillus* SF spores showed a very high stability at alkaline pH values ($t_{1/2}=67 \text{ h}$ at pH 9.5). These observed half life times indicate a high potential for industrial processes like dye decolorization, detoxification, and transformation of phenolic and other compounds where high temperatures and pH values are common. Previously, an intracellular catalase-peroxide from this *Bacillus* SF has been described which also showed high stabilities at alkaline pH and high temperatures (Gudelj et al. 2001).

Dye decolorization

Based on the high stability of laccase activity of *Bacillus* SF spores compared to the liberated enzyme, only spores and immobilized spores were investigated for dye decolorization. The spore laccases as well as the spores immobilized on inorganic carrier material were used for the decolorization of the common textile dyes DBPV 200 (CI Mordant Black 9), DFB (CI Mordant Brown 96/CI Mordant Brown 15) and IC (CI Acid Blue 74) in order to demonstrate their potential for the treatment of process waters contaminated with excess dyestuff. Both types of spore preparations proved to be very efficient in the complete removal of color within less than 90 min of incubation time.

Decolorization rates depended upon dye structure. DBPV 200, DFB, and IC were almost completely decolorized after 35, 50, and 65 min, respectively (Fig. 1). A similar trend was found earlier when an immobilized laccase from the white-rot fungus *Trametes modesta* was used to treat the same dyes (Kandelbauer et al. 2004). Although the optimum decolorization rates were generally higher with the immobilized fungal laccase, they were strongly dependent on acidic conditions and no decolorization activity at all was observed at pH values higher than pH 7. In contrast, when using the bacterial spore preparations, decolorization was achieved at pH 9. Furthermore, in previous experiments with fungal laccases, the optimum temperature for dye decolorization was never above 40°C whereas in the present experiments the best results were found at 60°C. No significant decolorization was seen when the laccase inhibitor NaF was present in the incubation mixtures indicating that adsorption did not contribute to decolorization.

Our results indicate access of substrates to the outer spore coat protein of *Bacillus* sp. displaying laccase activity does not seem to be sterically limited. This is in agreement with previous data for immobilized fungal laccases. Consequently, in different general decolorization rates of structurally different dyes are rather due to presence of different electron withdrawing of electron donating substituents on

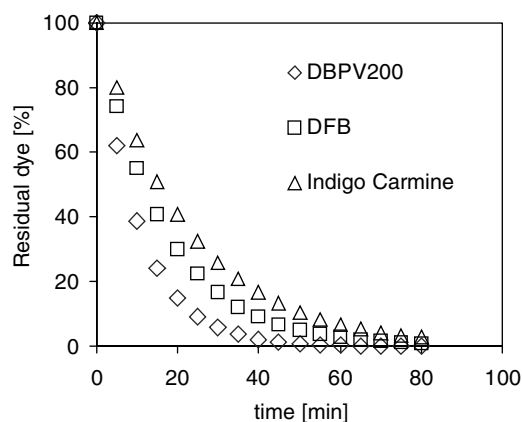


Fig. 1 Decolorization of textile dyes with immobilized spore-bound laccase from *Bacillus* SF

the aromatic rings than due to sterical effects (Kandelbauer et al. 2004). In the next section it will be shown that the spore-treated dye solutions can be re-used for dyeing operations.

Cotton dyeing

Re-using the laccase-treated effluent in the dyeing process we have found previously that enzyme protein obviously strongly interfered with the dyeing process while promising results were obtained with the immobilized laccases (Abadulla et al. 2000). This was indicated by dE^* values of the dyed fabrics below 1.1, which is acceptable to the industry. Shifts in dye absorption maximum in solution could be caused by binding of auxochromes to the dye molecules. Alternatively, degradation products and/or protein could cause aggregation of dye molecules preventing the dye uptake to the fabric, which would cause larger color fail. Thus, immobilization of enzymes seems to be strictly required in order to allow reuse of enzyme-treated effluents.

Decolorized solutions of the three commercial dyes DBPV 200, DFB, and IC were used for the dyeing of bleached cotton with reactive dyes. Results are shown in Fig. 2. Even with low dye concentrations, no significant color differences between fabrics dyed in baths prepared with water or enzyme-treated dye solutions were seen. Only in the case of enzyme-treated solutions of IC high dye concentrations were necessary in a second dyeing step to reach acceptable dE^* differences of the dyed fabrics below 1.1.

Conclusion

In this study we have shown for the first time that spores from *Bacillus* SF with laccase activity can be used for continuous decolorization of dyes when immobilized on alumina carriers. Despite some loss of laccase activity due to the immobilization procedure both pH and temperature stabilities of laccase activity of the immobilized spores were higher than those of the free spores. Dye solutions treated with spore laccase can be used for the preparation of new dyeing baths leading to acceptable color differences of around 1 dE.

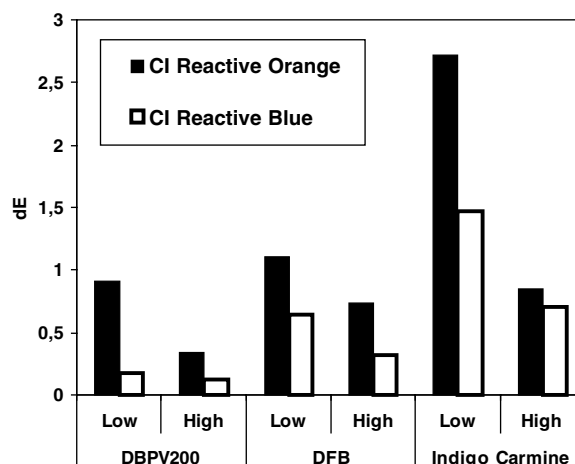


Fig. 2 Color difference (dE) between cotton fabrics dyed with Reactive Orange 70 or Reactive blue 214 (high and low concentration) either in water or decolorized dye solutions. Dye solutions containing the textiles dyes DBPV 200, DFB, and IC were decolorized with immobilized spore laccase from *Bacillus* SF

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