



Degradation of the phenolic β -ether lignin model dimer and dyes by dye-decolorizing peroxidase from *Bacillus amyloliquefaciens*

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Abstract

Objective The dye-decolorizing peroxidase from *Bacillus amyloliquefaciens*, BaDyP, was identified to be an efficient catalyst for the degradation of phenolic β -ether lignin model dimer guaiacylglycerol- β -guaiaicyl ether (GGE) and dyes.

Results *Efeb* gene encoding BaDyP from *B. amyloliquefaciens* MN-13 consisted of 1257 bp and the open reading frame encoded 418 amino acids. The *efeb* gene was expressed in *Escherichia coli* BL21 and a recombinant BaDyP of 50 kDa was achieved. The BaDyP exhibited activity in oxidizing GGE and decolorizing azo and triphenylmethane dyes. At pH 4.5 and 30 °C the BaDyP not only completely degraded GGE by the cleavage of β -O-4 ether bond and C α -C β bond, and C α oxidation without any oxidative mediator, but also decolorized four synthetic dyes, including congo red, bromine cresol green, eriochrome black T and crystal violet. This was achieved with decolorization rates of 65.7%, 70.62%,

80.06% and 62.09%, respectively, after 72 h of incubation.

Conclusions BaDyP was identified as a bacteria peroxidase with great potential for the degradation of lignin and bioremediation of dye-contamination.

Keywords *Bacillus amyloliquefaciens* · Biodegradation · Dye-decolorizing peroxidase · Dyes · Gas chromatograph mass spectrum · Lignin model dimer

Introduction

It is well known that synthetic dyes from the textile industry and lignin from the paper industry present two major sources of organic matter that are resistant to biological wastewater treatment systems (Sierra-Alvarez and Lettinga 1991; Pasti-Grigsby et al. 1992). Consequently, the question of how to degrade the dyes and lignin in wastewater effectively and simultaneously is critical for the treatment of wastewater pollution.

Heme-containing peroxidases, consistently existing in eukaryotic and prokaryotic cells, catalyze a vast range of oxidation processes, using hydrogen peroxide as an electron acceptor (Van Rantwijk and Sheldon 2000). Some well-known enzymes belonging to the superfamily of heme peroxidases, including the extracellular fungal lignin peroxidases (LiPs),

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manganese peroxidases (MnPs) and versatile peroxidases (VPs), are involved in the process of lignin-degradation and dye-decolorization (Pollegioni et al. 2015). Remarkably, recent studies have indicated that dye-decolorizing peroxidases (DyPs) also exhibit great potential for degrading lignin and a variety of synthetic dyes, which makes them potentially useful for the bioremediation of wastewater (Colpa et al. 2012). DyPs represent a newly discovered superfamily of heme peroxidases that are found in both fungi and bacteria (Kim and Shoda 1999). DyPs are classified into four subfamilies (A, B, C and D) according to their sequence characteristics. Usually, different types of DyPs show different catalytic properties to lignin model compounds (Rahmanpour et al. 2016; Ahmad et al. 2011). To date, about 8318 DyP sequences have been deposited in the InterPro database, and approximately thirty of these enzymes have been isolated and characterized (Yoshida and Sugano 2015). However, there has been little discussion about the degradation of GGE by BaDyP from *Bacillus amyloliquefaciens*.

Bacillus amyloliquefaciens strain MN-13 with the ability to degrade lignin was previously isolated from cattle feces and stored in our lab (Yang et al. 2018). To gain a further insight into the nature of the lignin-degrading biocatalyst in strain MN-13, gene encoding DyP was obtained from it was cloned and heterologously expressed in *Escherichia coli*. Based on the analysis of degradation products of phenolic lignin dimer GGE by purified BaDyP, a hypothetical pathway of BaDyP for GGE degradation was proposed. The decolorization effect of BaDyP on dyes was also investigated in this paper.

Materials and methods

Strain and plasmids

Bacillus amyloliquefaciens MN-13 (NCBI accession numbers of 16S r RNA gene sequence: KP292553) was preserved on nutrient agar slant at 4 °C. pET30a expression plasmid and *E. coli* BL21 (DE3) were purchased from TransGen Biotech.

Cloning and construction of expression vector

The genomic DNA of strain MN-13 was prepared using a Genomic Extraction Kit. The peroxidase gene

efeb from *B. amyloliquefaciens* MN-13 was amplified using the forward primer: 5'-CGGGATCCATGAGC-GATGAACAAAACAAGGAAAAAC AGATTCA-3' (*Bam*H I bold), and the reverse primer: 5'-GCGAAGCTTTCACGATT CCAGCAGCCTCT-3', (*Hind* III bold), which was designed based on the genome sequence of *B. amyloliquefaciens* DSM7 (NCBI NO. NC_014551). PCR was carried out under the following conditions: an initial denaturing step at 94 °C for 5 min; 30 cycles at 94 °C for 30 s; annealing at 62 °C for 30 s; extension at 72 °C for 1 min 20 s; and a final extension cycle of 72 °C for 10 min. The purified DNA product was cloned into the pET30a.

Expression, purification and pH and temperature ranges of BaDyP

Escherichia coli BL21 (DE3) was transformed with the pET30a-*efeb* and grown in 5 ml of LB medium containing 50 µg/ml Kanamycin at 37 °C overnight. The cells carrying pET30a-*efeb* were incubated in 50 ml culture of LB media with 50 µg/ml Kanamycin at 37 °C until OD_{600 nm} reached 0.6 ~ 0.8, and then induced with 0.8 mM IPTG at 16 °C. After 12 h the cells were collected, disrupted by sonication, and the recombinant BaDyP in the soluble fraction was extracted and purified. The peroxidase activity was determined according to the oxidation of the veratryl alcohol method (Alam et al. 2009).

pH ranges of BaDyP were determined using 50 mM of KCl/HCl buffer for pH 2.0 and 50 mM of acetate buffer for pH 3.0 ~ 6.0 were used at 30 °C. 50 mM of acetate buffer was used for determining temperature ranges.

Degradation of GGE by BaDyP

The reaction system contained 2 ml 0.05 M succinic acid buffer (pH 4.5), 200 µl BaDyP (0.672 U/ml), 100 µl 0.1 mM GGE acetone solution and 200 µl 0.625 M H₂O₂. The reaction was carried out at 30 °C for 10 h, then extracted into 10 ml of ethyl acetate. The organic layer was collected, dried and filtered through a 0.45 µm filter film. The residues were dried and analyzed by GC-MS of their trimethyl silyl (TMS) derivatives, according to the method described by Raj et al. (2007). In the control experiment, BaDyP was replaced with inactive BaDyP heated for 10 min at 100 °C.

Dye-decolorizing assays

Four synthetic dyes, including two azo dyes (congo red and eriochrome black T) and two triphenyl-methane dyes (bromine cresol green and crystal violet) were selected for the decolorization assay. The decolorization assays of dyes were performed at 30 °C for 72 h in a reaction mixture containing BaDyP (0.672 U/ml, 500 µl), 60 mg/l dyes and 50 mM H₂O₂ in 4.5 ml of 0.05 M citrate buffer (pH 4.5). Two control experiments, including degradation of dyes by inactive BaDyP and H₂O₂, and 60 mg/l dyes in citrate buffer, were carried out. The dye degradation was analyzed by ultraviolet spectral scanning (200 nm ~ 800 nm), and expressed as follows:

$$\text{Decolorization ratio (\%)} = \frac{(A_{ck} - A_t)}{A_{ck}} \times 100\%$$

(A_{ck} = absorbance of dye sample treated by inactive BaDyP; A_t = absorbance of dye sample treated by BaDyP).

Results

Expression of *efeb* gene in *E. coli* Transetta (DE3)

Sequence analysis indicated that the *efeb* gene consisted of 1257 bp and the open reading frame encoded 418 amino acids. The sequence information is available in the NCBI database with Accession No. MH886503. It is 99% homologous to other known *efeb* gene sequences from *Bacillus* sp., such as *Bacillus* sp. 275 (CP019626.1) and *B. amyloliquefaciens* strain LM2303 (CP018152.1). After 12 h of IPTG induction, a high expression level of BaDyP (0.672 U/ml) was achieved. BaDyP was purified as a 50 kDa protein (Fig. 1, lane 4).

Temperature and pH ranges of BaDyP

The optimum pH and temperature of BaDyP were determined using veratryl alcohol as a substrate (Fig. 2). The optimum pH value of BaDyP was pH 4.0 (a in Fig. 2), and the enzyme activity at pH 4.0 decreased significantly with increasing temperature (b in Fig. 2). The highest enzyme activity of BaDyP

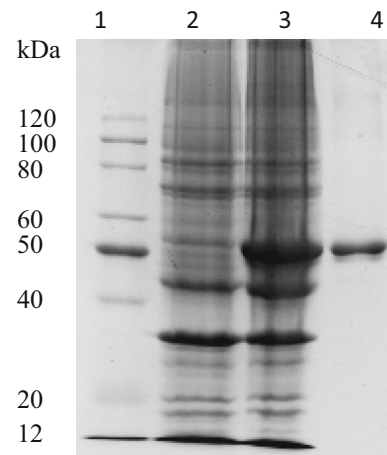


Fig. 1 SDS-PAGE of recombinant BaDyP produced in *E. coli* Transetta (DE3) cells harboring pET 30a-*efeb*. Lane 1: Protein marker; lane 2: Total cellular protein in the *E. coli* without IPTG induction; lane 3: Total cellular protein in the *E. coli* with IPTG induction; lane 4: BaDyP purified by Ni-NTA column (80 mmol/l imidazole)

(0.509 U/ml) was detected at 30 °C, while the enzyme activity of BaDyP was only 0.098 U/ml at 70 °C.

Degradation of GGE by BaDyP

The degradation products of GGE by BaDyP were analyzed by GC-MS and the results were shown in Fig. 3. The signal of GGE was observed at R.T 24.9 min in the total ion chromatograph (TIC) spectrum. When GGE was treated by inactivated BaDyP no other compounds appeared (a in Fig. 3). The TIC spectrum of GGE degradation by BaDyP (b in Fig. 3) showed that the signal of GGE disappeared, and some new signals emerged at R.T. 7.7 min, 10.4 min, 16.2 min, 24.2 min and 25.6 min, respectively, which were considered to be degradation products of GGE and their trimethylsilylation (TMS) derivatives. Comparison of their mass spectra with those stored in the NIST11 library enabled these compounds to be identified as guaiacol, O-trimethylsilyl guaiacol, O, O-bis (trimethylsilyl) vanillyl alcohol, 4-(1,3-dihydro-2-propenyl)- 2-methoxyphenol, bis(trimethylsilyl) ether, and O-trimethylsilyl vanillin, respectively (Supplementary Fig. 1). Among these compounds, 4-(1,3-dihydro-2-propenyl)-2-methoxyphenol was considered to be produced from 1-(4-hydro-3-methoxyphenyl) propanetriol in the process of GGE-degradation products' trimethylsilylation, because there is no

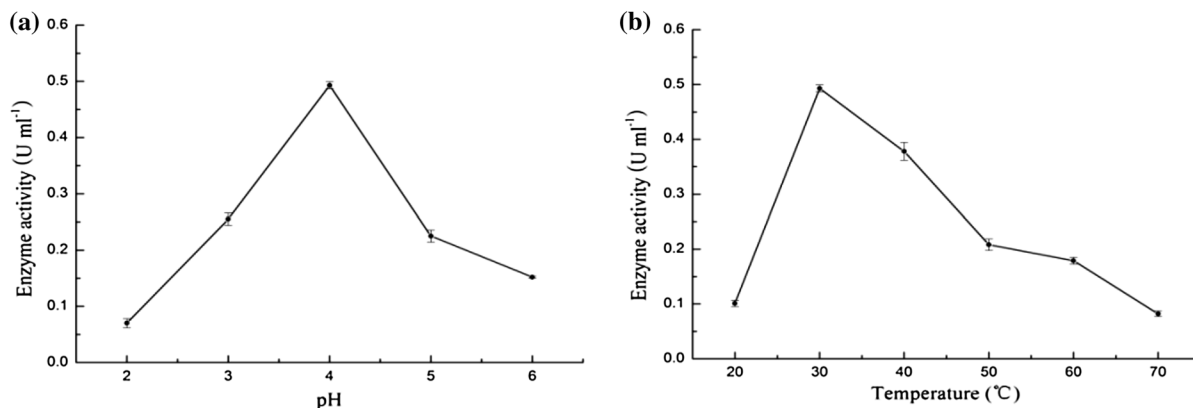


Fig. 2 Temperature and pH ranges of BaDyP

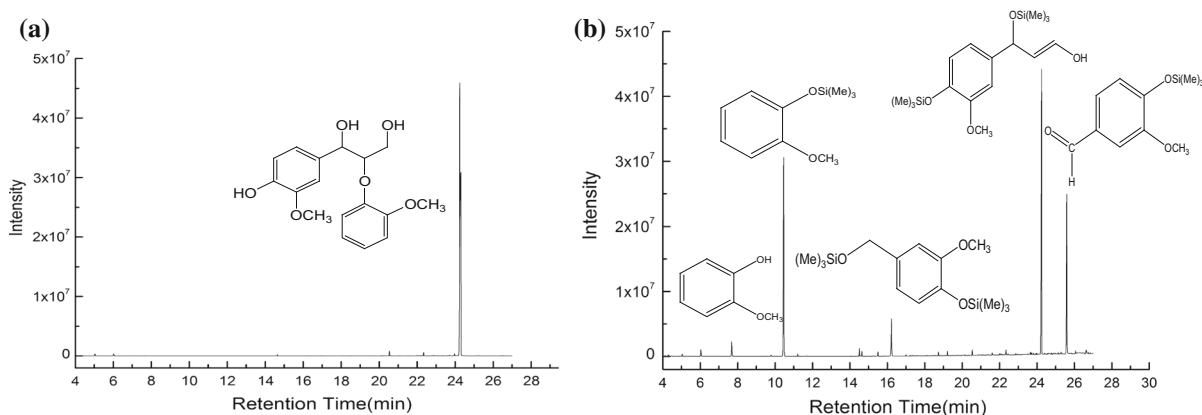


Fig. 3 GC-MS analysis of GGE degradation products by BaDyP. **a** Degradation of GGE by inactive BaDyP; **b** degradation of GGE by BaDyP

evidence to prove that heme-containing peroxidase could catalyze the dehydration of polyhydric alcohols. Also, it could be confirmed that 0.1 mM GGE was completely transformed to guaiacol, vanillyl alcohol, 1-(4-hydroxy-3-methoxyphenyl) propanetriol and vanillin under the conditions of pH 4.5, 30 °C for 10 h, resulting in a degradation rate of 100%.

Decolorization of dyes by BaDyP

After 72 h of exposure to BaDyP and hydrogen peroxide, the solution containing 60 mg/l dye appeared to be almost transparent. The decolorization level of congo red, bromine cresol green, eriochrome black T and crystal violet by BaDyP was 65.7, 70.62, 80.06 and 62.09%, respectively. UV–Visible spectra of the reaction mixtures containing BaDyP and two control solutions (Fig. 4) showed that the absorption

peaks emerging in the visible region for congo red (a in Fig. 4), bromine cresol green (b in Fig. 4) and eriochrome black T (c in Fig. 4) disappeared in the reaction mixtures added with BaDyP, and the maximum peak of crystal violet at 568 nm (d in Fig. 4) declined significantly.

These results indicate that the chromophore structures in these dyes were destroyed by BaDyP, and colorless degradation products were produced. Meanwhile, there were some new absorption peaks appearing in the wavelength range from 250 nm to 280 nm in each UV–Visible spectrum of reaction mixture treated with BaDyP. As those peaks were consistent with the B belt in the absorption spectra of aromatic compounds, the presence of those peaks was concluded to be the result of mono-aromatic ring molecule production by the degradation of the dyes. Also, it needs to be noted that the UV–Visible spectra of solutions

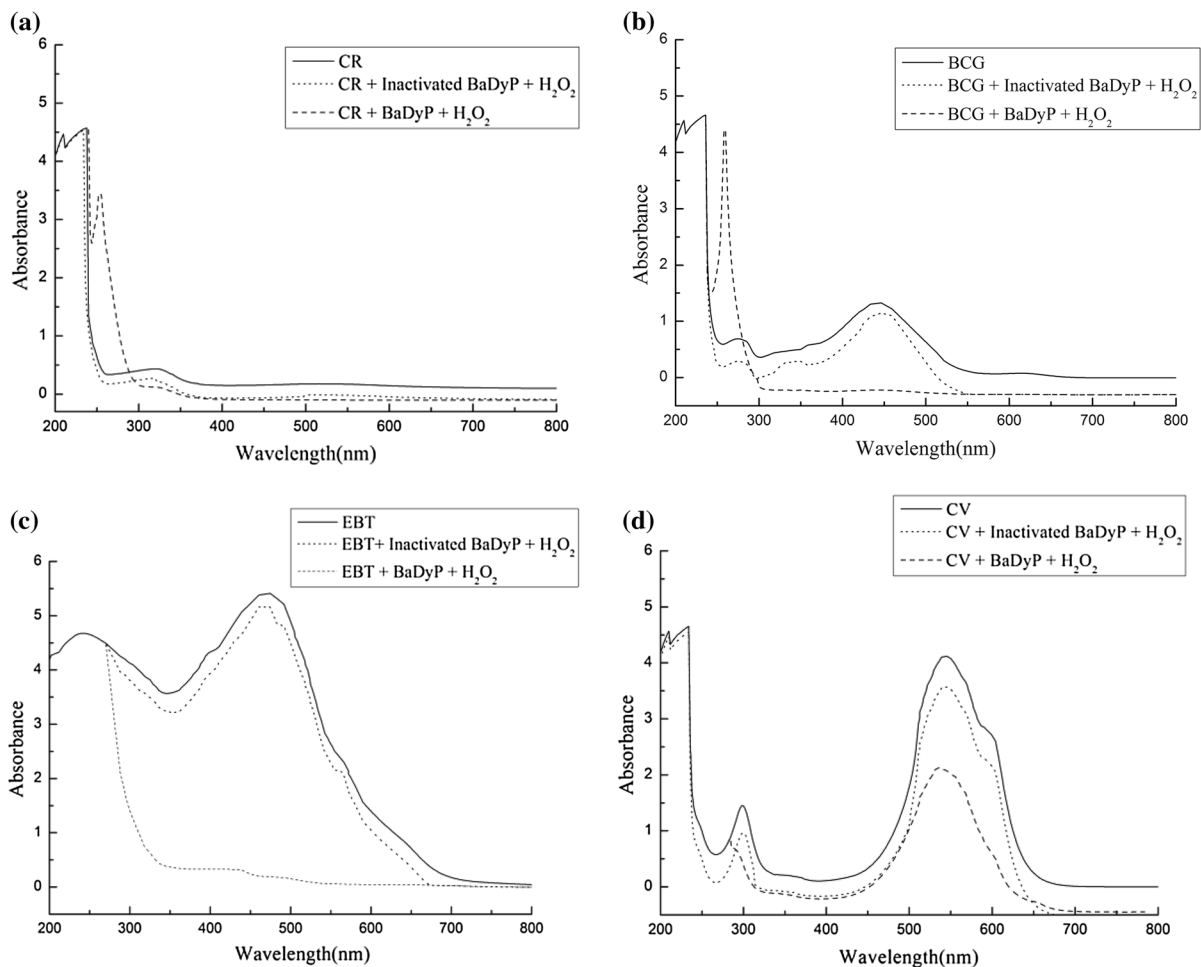


Fig. 4 UV/Vis absorbance spectra of congo red (a), bromine cresol green (b), eriochrome black T (c) and crystal violet (d) in 50 mM Sodium citrate buffer (pH 4.5) treated with recombinant BaDyP and H₂O₂

containing dyes and H₂O₂ were not shown in Fig. 4, because these spectra were exactly the same as those of the control solutions (i.e., those with inactive BaDyP and H₂O₂).

Discussion

The BaDyP from *B. amyloliquefaciens* MN-13 was characterized as a bacterial peroxidase capable of degrading phenolic lignin dimer GGE and four synthetic dyes. Based on the degradation products of GGE, including guaiacol, vanillyl alcohol, γ -hydroxy coniferyl alcohol and vanillin (identified by GC-MS), it was concluded that cleavage of the β -O-4 ether bond and the C α -C β bond, as well as C α oxidation, were the

main pathways for lignin-degradation by BaDyP. As is reported by Ahmad et al. (2011), DyP from *R. jostii* RHA1 showed Mn²⁺-dependent oxidative activity and could decompose GGE to vanillin and guaiacol in the presence of MnCl₂. In our study, the BaDyP oxidized GGE to vanillin and guaiacol without any oxidative mediator, such as Mn²⁺. The BsDyP from *Bacillus subtilis* KCTC2023 reported by Min et al. (2015) could oxidize even more recalcitrant nonphenolic VGE (β -O-4 model compound for lignin) to guaiacol and veratryl aldehyde in the absence of an oxidative mediator. This means that the oxidation of GGE and VGE in the absence of oxidative mediators could be considered as a typical characteristic of DyP from *Bacillus* sp.

It is worth mentioning that vanillyl alcohol has not been reported to appear in the process of GGE-degradation by DyPs and other heme-containing peroxidases involved in lignin-degradation until now. This indicates that the presence of vanillyl alcohol might be proof of the $C\alpha$ - $C\beta$ fission in the alkyl side chain occurring before $C\alpha$ -oxidation during the process of GGE-degradation by BaDyP. Obviously, this lignin-degradation pathway is different from that of other typical heme-containing peroxidase for lignin-degradation. For example, MnPs catalyze $C\alpha$ -oxidation of GGE to form α -oxo-GGE, which first occurs in the whole degradation pathway of GGE (Kapich et al. 2005). And it is also distinct from the degradation pathway of LiPs for GGE (Yokota et al. 1990). The hypothetical pathway for BaDyP's GGE-degradation was proposed in Fig. 5. In the proposed pathway, as a typical phenolic lignin β -O-4 model compound, GGE was first cleaved to guaiacol and 1-(4-hydroxy-3-methoxyphenyl) propanetriol. $C\alpha$ - $C\beta$ fission of the alkyl side chain then occurred in 1-(4-hydroxy-3-methoxyphenyl) propanetriol, to produce vanillyl

alcohol. The final product, vanillin, was undoubtedly considered to be produced by the $C\alpha$ oxidation of vanillyl alcohol.

Generally, guaiacol was not detected in the degradation process of GGE or VGE mediated by DyPs, because it is easily converted to the guaiacol-oligomer in the presence of several DyPs (Ahmad et al. 2011). However, no guaiacol-oligomer was found in the process of GGE-degradation mediated by BaDyP. To date, several studies have shown that the radical sites of DyPs formed by the reaction with H_2O_2 depend on pH value. That means different radical transfer pathways of DyPs were formed at different pH, thus resulting in different catalytic activity of DyPs, different substrate and even the same substrate producing different oxidation products at different pHs (Uchida et al. 2015). We conclude that the phenomenon of no guaiacol-oligomer produced in this study may be caused by the pH of the reaction conditions. And at pH 4.5, azo dyes and triphenylmethane dyes could be well decolorized and phenol lignin model dimer GGE could also be efficiently

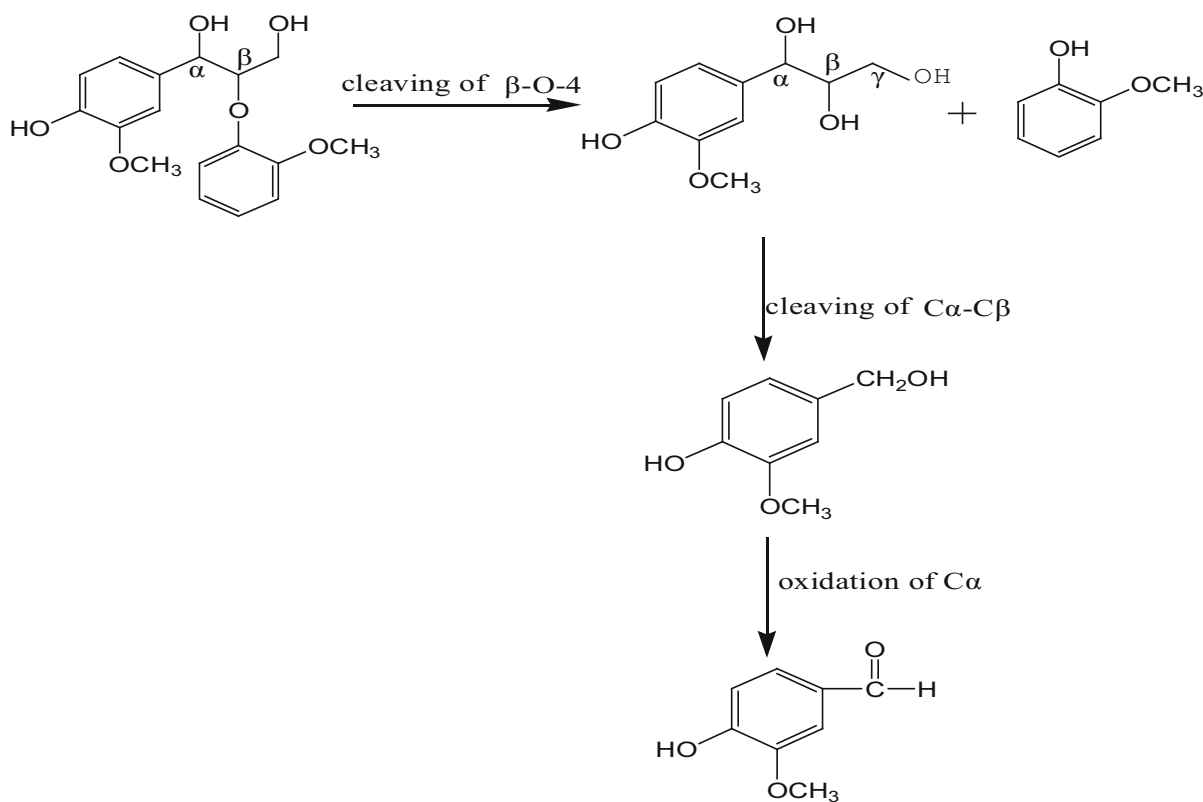


Fig. 5 Decomposition of guaiacylglycerol- β -guaiacyl ether (GGE) to guaiacol and Vanillin

degraded to guaiacol, vanillyl alcohol and vanillin by BaDyP from *B. amyloliquefaciens* MN-13.

Compared to other bacterial peroxidases previously reported, the BaDyP investigated in this study exhibited high oxidative activity in the degradation of lignin dimer and dye-decolorizing. Furthermore, BaDyP showed some distinct characters from other DyPs in the degradation of GGE. To our knowledge, this is the first study presenting a pathway for BaDyP's GGE-degradation by the cleavage of the β -O-4 ether bond and the C α -C β bond, and C α oxidation without any oxidative mediator. These results provide a valuable new reagent for the efficient biocatalytic pretreatment of lignin and synthetic dyes in wastewater.

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Supporting Information Supplementary Figure 1—Identification of GGE-degradation products by comparison of mass spectra in NIST.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

References

- Ahmad M, Roberts JN, Hardiman EM, Singh R, Eltis LD, Bugg TD (2011) Identification of DypB from *Rhodococcus jostii* RHA1 as a lignin peroxidase. *Biochemistry* 50:5096–5107
- Alam MZ, Mansor MF, Jala KCA (2009) Optimization of lignin peroxidase production and stability by *Phanerochaete chrysosporium* using sewage-treatment-plant sludge as substrate in a stirred-tank bioreactor. *J Ind Microbiol Biot* 36:757–764
- Colpa DI, Fraaije MW, Bloois EV (2012) DyP-type peroxidases: a promising and versatile class of enzymes. *Enz Eng* 1:2
- Kapich AN, Steffen KT, Hofrichter M, Hatakka A (2005) Involvement of lipid peroxidation in the degradation of a non-phenolic lignin model compound by manganese peroxidase of the litter-decomposing fungus *Stropharia coronilla*. *Biochem Biophys Res Commun* 330:371–377
- Kim SJ, Shoda M (1999) Purification and characterization of a novel peroxidase from *Geotrichum candidum* Dec 1 involved in decolorization of dyes. *Appl Environ Microbiol* 65:1029–1035
- Min K, Gong G, Han MW, Kim Y, Um Y (2015) A dye-decolorizing peroxidase from *Bacillus subtilis* exhibiting substrate-dependent optimum temperature for dyes and β -ether lignin Dimer. *Scientific Reports* 5:8245
- Pasti-Grigsby MB, Paszczynski A, Goszczynski S, Crawford DL, Crawford RL (1992) Influence of aromatic substitution patterns on azo dye degradability by *Streptomyces* spp and *Phanerochaete chrysosporium*. *Appl Environ Microb* 11:3605–3613
- Pollegioni L, Tonin F, Rosini E (2015) Lignin-degrading enzymes. *FEBS J* 282:1190–1213
- Rahmanpour R, Rea D, Jamshidi S, Fülöp V, Bugg TD (2016) Structure of Thermobifida fusca DyP-type peroxidase and activity towards Kraft lignin and lignin model compounds. *Arch Biochem Biophys* 594:54–60
- Raj A, Krishna Reddy MM, Chandra R (2007) Identification of low molecular weight aromatic compounds by gas chromatography–mass spectrometry (GC–MS) from kraft lignin degradation by three *Bacillus* sp. *Int Biodeter Biodegr* 59:292–296
- Sierra-Alvarez R, Lettinga G (1991) The methanogenic toxicity of wastewater lignins and lignin related compounds. *Chem Tech Biotechnol* 50:443–455
- Uchida A, Sasaki M, Tanaka Y, Ishimori K (2015) A Dye-decolorizing peroxidase from *Vibrio cholerae*. *Biochemistry* 54:6610–6621
- Van Rantwijk F, Sheldon RA (2000) Selective oxygen transfer catalysed by heme peroxidases: synthetic and mechanistic aspects. *Curr Opin Biotechnol* 11:554–564
- Yang J, Gao MY, Li M, Li ZZ, Li H, Li HY (2018) *Bacillus amyloliquefaciens* CotA degradation of the lignin model compound guaiacylglycerol- β -guaiacyl ether. *Lett Appl Microbiol* 67:5
- Yokota S, Umezawa T, Higuchi T (1990) Degradation of phenolic β -1 lignin substructure model dimer by *phanerochaete chrysosporium* and its lignin peroxidase. *Holzforschung* 44:271–276
- Yoshida T, Sugano Y (2015) A structural and functional perspective of DyP-type peroxidase family. *Arch Biochem Biophys* 574:49–55

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