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XENOBIOTICS ENHANCE LACCASE ACTIVITY IN ALKALI-TOLERANT γ-PROTEOBACTERIUM JB

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ABSTRACT

Various genotoxic textile dyes, xenobiotics, substrates (10 μM) and agrochemicals (100 μg/ml) were tested for enhancement of alkalophilic laccase activity in γ-proteobacterium JB. Neutral Red, Indigo Carmine, Naphthol Base Bordears and Sulphast Ruby dyes increased the activity by 3.7, 2.7, 2.6 and 2.3 fold respectively. Xenobiotics/substrates like *p*-toluidine, 8-hydroxyquinoline and anthracine increased it by 3.4, 2.8 and 2.3 fold respectively. Atrazine and trycyclozole pesticides enhanced the activity by 1.95 and 1.5 fold respectively.

Keywords: γ-proteobacterium, laccase, xenobiotics, dyes, agrochemicals

INTRODUCTION

Laccases (benzenediol oxygen oxidoreductases, EC 1.10.3.2) are polyphenol oxidases that require O_2 to oxidize phenols, polyphenols, aromatic amines and different non-phenolic substrates by one electron transfer resulting in the formation of reactive radicals (11). They are members of the multicopper protein family that has developed from small sized prokaryotic azurins to eukaryotic ceruloplasmin. Laccases are widely distributed in plants and fungi (4), where their involvement in melanin formation and in a variety of different, and sometimes contradictory, physiological functions like fungal morphogenesis, plant pathogenesis and fungal virulence has been frequently proposed (29). They also occur in prokaryotes e.g. *Azospirillum lipoferum* (12), *Marinomonas mediterranea* (22), *Bacillus subtilis* spore (14) and γ-proteobacterium JB (2,24). Stimulation of laccase activity in fungi with respect to culture medium composition has been investigated by many workers. Filazzola *et al.* (10) reported the influence of xenobiotics on catalytic activity of constitutive forms of laccases. Metal ions and several organic molecules have also been assayed for their ability to enhance activity of inducible form of laccases (25,29). Gallic and ferulic acids were used, mainly because of their structural analogy with lignin model compounds (11). Dyes that affect laccase synthesis include Ethidium Bromide, Malachite Green, Phenol Red and Thymol Blue (7,15). Laccase induction in bacteria has not been studied before, although bacteria offer many advantages over fungi e.g. the faster multiplication rates resulting in early enzyme production. Fungi are usually acidophilic, while bacteria can inhabit acidophilic to alkalophilic environments making their enzymes more stable to pH (2). However, low levels of laccase activity in bacteria are not sufficient for commercial applications.

Our previous work (unpublished) showed that laccase in γ-proteobacterium JB played no role in melanin synthesis or protection from UV or oxidizing agents, though copper and some dyes induced laccase activity (15). This work shows that several xenobiotics induce laccase activity in this bacterium, isolated from industrial effluents, indicating the physiological role of laccase in protection from toxic environmental compounds in response to respiratory stress. The work will be useful for large scale production of laccase for various industrial applications. Laccases from bacteria are not common. Presently, only a few bacteria have been reported with laccase activity (24). This work is the first report on xenobiotics enhancing laccase activity in bacteria.

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MATERIALS AND METHODS

Chemicals

All chemicals used were of more than 90% purity. Textile dyes were obtained from JCT Textile Mill (India). Xenobiotics/substrates were from Sigma (USA) and HiMedia (India). Agrochemicals were commercially available in India. Dyes and other chemicals were soluble in water, dimethyl sulfoxide or 50% ethanol. Proper controls were put up for each solvent or chemical during laccase production or during evaluation of activity of this enzyme.

Mutagenicity of dyes

Mutagenicity of the dyes was determined by Ames (1) and *rec* (8) assays as described previously (3,5). Ames test measures the frequency of reversal of *his*$^{-}$ mutations in *Salmonella typhimurium* to *his*$^{+}$ prototrophy by mutagens/carcinogens. *Rec* assay measures the mutagenic/carcinogenic potential of a chemical by comparing the ability of recombination proficient (*rec*$^{+}$) and deficient (*rec*$^{-}$) strains of *Bacillus subtilis* to repair the DNA damage caused by that chemical.

Organism and culture conditions for enzyme production

γ-Proteobacterium JB was grown in M162 medium (6) at 37ºC, 150 rpm for 72 h after inoculation with 1% (v/v) of 16-18 h pre-inoculum grown in same medium. The culture was centrifuged at 10000 *g*, 4ºC for 10 min and the supernatant was used as extracellular enzyme prepration. Cell pellet was disrupted using a Braun Labsonic sonicator (4-5 bursts of 1 min each at 100 % power). The cell extract was obtained by centrifugation at 13000 *g*, 4ºC for 15 min and used as crude intracellular enzyme preparation. Dry weight of 72 h old culture was measured by centrifuging 20 ml culture of γ-proteobacterium JB at 10000 *g* and drying the pellet at 50ºC till constant weight. Viable cell count (cfu/ml) was measured by standard procedure. All experiments were carried out at least in triplicates. Laccase activities were determined 72 h after the onset of growth and conditions were kept identical for each compound like medium, shaking, pH and temperature.

Enzyme assays

Laccase activity was determined by measuring the oxidation of guaiacol at 465 nm. The reaction mixture contained 2 mM guaiacol and 50 mM phosphate buffer (pH 6.5). One unit was the amount of enzyme that increased the absorbance by 0.001 absorbance units per min at 55ºC. In all cases, initial rate of reaction was measured over 10 min (2). Assays for lignin peroxidase and manganese peroxidase were performed as described by Tien and Kirk (28).

Statistical analyses

Student's *t*-test was performed using SigmaStat® statistical software version 2.03.

RESULTS

The organism used was Gram-negative, non-sporulating, non-hemolytic, singly occurring, short rod. It did not grow on McConkey and cetrimide agar and was oxidase positive but catalase, indole, methyl red, Voges-Proskauer, citrate negative. Growth on glucose was positive with no gas and no acid production. 16S rDNA sequencing of γ-proteobacterium JB was carried out previously in our laboratory (2). A BLASTn algorithm search of GenBank database (htt://www.ncbi.nlm.nih/BLAST) exhibited 98 % identity with the closest match γ-proteobacterium F8 and the present organism was named γ-proteobacterium JB. The bacterium produced laccase but was not found to produce lignin peroxidase and manganese peroxidase enzymes even when tested in 100 times concentrated cell free supernatant and intracellular preparations.

Effect of mutagenic dyes on laccase activity

Laccase activity was enhanced on addition of some dyes in the culture medium (Table 1). Neutral Red, Indigo Carmine, Naphthol Base Bordears and Sulphast Ruby showed 3.7-, 2.7-, 2.6-, and 2.3 fold increase in activity. In the presence of Disperse Blue D_2R, no laccase activity was observed although it enhanced the growth of organism by 1.4 fold. Reactofix Golden Yellow, Navilene Brown and Navilene Orange reduced the enzyme activity by 2.6, 1.7 and 1.3 fold respectively. Navilene Yellow Brown, Fuschin and Crystal Violet increased laccase activity slightly but reduced growth.

Effect of xenobiotics on laccase activity

Ten aromatic xenobiotics were tested for laccase activity (Table 2). *p*-Toluidine induced (3.4 fold) the activity maximally, followed by 8-hydroxyquinoline (2.8 fold), phthalic acid (2.1 fold), Tween-20 (2.0 fold), nicotinic acid (1.7 fold), 2,4,6-trichlorophenol (1.7 fold) and dinitrosalicylic acid (1.48 fold). Veratric acid decreased the enzyme activity by 1.6 fold.

Effect of substrates on laccase activity

Anthracine increased the enzyme activity maximally by 2.3 fold (Table 3). Tyrosine, *p*-phenylenediamine and ferulic acid increased by 1.5, 1.2 and 1.1 fold respectively.

Effect of agrochemicals on laccase activity

Among the five agrochemicals tested (Table 4), atrazine and trycyclozole pesticides enhanced the enzyme activity by 1.95 and 1.5 fold respectively. Tebuconazole reduced the activity by 1.3 fold.

DISCUSSION

The results show that xenobiotics of environmental interest and natural products can increase the laccase activity in γ-

Table 1. Effect of dyes on laccase activity of γ-proteobacterium JB 72 h cultures.

Dye (10 µM)	Functional/Structural group/ CI No.	Mutagenicity of Dyes Ames test	*rec* assay	Growth (g dry wt l^{-1}/ Log cfu ml^{-1})	Laccase activity (U mg^{-1} dry wt)
None				0.25/9.2	61.6
Indigo Carmine (W)	NA / Anthraquinone / 73015	-	-	0.085/8.9	170.5
Naphthol Base Borders (D)	Naphthol / Anthraquinone / NA	+F	+	0.12/8.9	163.6
Sulphast Ruby (D)	Sulphur / Anthraquinone / NA	-	+S9	0.11/8.9	142.7
Dispers Blue D2R (D)	Disperse / Anthraquinone / NA	+F/B (S9)	+S9	0.35/9.3	N.D
Navilene Yellow Brown (D)	Disperse / Anthraquinone / NA	+F/B	+S9	0.095/8.9	81.6
Navinon Brown (D)	Vat / Anthraquinone / 69015	+F		0.245/9.2	71
Sulphast Green (D)	Sulphur / Anthraquinone / NA	-	+S9	0.25/9.2	62
Sulphur Niritaryl Green (D)	Sulphur / Anthraquinone / NA	-	+S9	0.3/9.2	59.6
Reactofix Golden Yellow (W)	Reactive / Disazo / NA	+F	+S9	0.34/9.3	23.4
Malacryl Red (W)	Basic/ Disazo / NA	-	+	0.25/9.1	58.4
Navilene Brown (D)	Disperse / Monoazo / NA	+F/B (S9)	+S9	0.25/9.1	36
Navilene Orange (D)	Basic / Monoazo / NA	+F/B	+	0.25/9.1	44.4
Neutral Red (W)	Basic / Heterocyclic /50040	+	-	0.25/9.1	227
Fuschin (W)	Acidic / Triphenylmethane / 42685	+	-	0.11/8.9	81
Crystal Violet (W)	Basic / Triphenylmethane / 42555	+	+	0.175/9.1	78

W: water soluble D: Dimethyl sulfoxide soluble
F: inducing frameshift mutations B: inducing base-pair substitutions
CI NO: color Index no. S9: mix (16)
NA: Not available, ND: Not detectable. Student's t-test revealed that increase and reduction in laccase activity was significant ($p<0.001$) for all dyes except $p=0.004$ and 0.29 for Navinon Brown and Sulphast Green, respectively when compared with non-treated controls.

Table 2. Effect of xenobiotics on laccase activity of γ-proteobacterium JB 72 h cultures.

Xenobiotics (10 µM)	Growth (g dry wt l^{-1}/ Log cfu ml^{-1})	Laccase activity (U mg^{-1} dry wt)
None 0.25/9.1	61.6	
2,4,6-Trichlorophenol (E)	0.09/8.9	106.1
Aniline 0.11/8.9	70.1	
Nicotinic acid (D)	0.123/8.9	106.3
Phthalic acid (E)	0.15/9.0	133
Dinitrosalicylic acid (W)	0.27/9.1	91.4
8-Hydroxyquinoline (E)	0.13/9.0	175
p-Toluidine (E)	0.1/8.9	213
Tween-200.25/9.1	124.8	
Veratric acid (E)	0.3/9.2	38.6
Xylidine (w)0.1/8.9	84	

E: 50% ethanol soluble, D: Dimethylsulfoxide soluble, W: Water soluble Student's t-test revealed that increase and reduction in laccase activity was significant ($p<0.001$) for all xenobiotics except aniline ($p=0.004$).

Table 3. Effect of substrates on laccase activity of γ-proteobacterium JB 72 h cultures.

Substrates (10 µM)	Growth (g dry wt l^{-1}/ Log cfu ml^{-1})	Laccase activity (U mg^{-1} dry wt)
None	0.21/9.1	82
Anthracine (D)	0.15/9.0	190
Tyrosine (W)	0.25/9.1	125.3
Ferulic acid (E)	0.23/9.1	93
p-Phenylenediamine (W)	0.3/9.2	103

E: 50% ethanol soluble, D: Dimethyl sulfoxide soluble, W: water soluble. Student's t-test revealed that increase in laccase activity was significant ($p<0.001$) for all substrates except $p=0.003$ for ferulic acid.

proteobacterium JB. Ethidium Bromide is the only dye reported to increase laccase activity in fungi and bacteria (7,15). This prompted us to study the effect of various mutagenic dyes on laccase activity at subinhibitory concentrations. Among the dyes tested, one heterocyclic and many anthraquinone dyes

enhanced laccase activity significantly. Dispers Blue D_2R acted differently. Though it belongs structurally to anthraquinone group but reduced laccase activity to an undetectable level. In Indigo Carmine and Neutral Red, an amine group is common on the benzene rings. Increase in activity may be due to degradation/transformation products of these dyes formed during incubation for 72 h, as laccase from this organism degraded Indigo Carmine to isatin and anthranilic acid (26). The impact of genotoxicity of these dyes on organism is not clearly understood. Enhanced activity of laccase in γ-proteobacterium JB could possibly be due to the respiratory stress induced in the cells (19). Xenobiotics may increase laccase activity by increasing the level of gene expression without affecting laccase isozyme composition. Veratric acid, an inducer in various fungi (18), could not increase laccase activity in γ-proteobacterium JB. *p*-Toluidine, phthalic acid and 2,4,6-trichlorophenol enhanced laccase activity, but decreased microbial growth. These one ring compounds have different substituted groups. Phthalic acid has two carboxylic groups; 2,4,6-trichlorophenol has three chloro groups, while toluidine contained one amino and one methyl group at para position (Fig. 1). Increase in activity does not seem to be caused by specific structure/ substituent group. Xiao *et al.* (29) reported that compounds with different chemical structures affect composition of laccase isozymes in *Trametes* sp. AH28-2.

Aromatic compounds which increase laccase activity are very often toxic to fungal growth and metabolism, and it has been proposed that one of the possible functions of fungal laccase is the polymerization of toxic aromatic compounds. Therefore, laccase may function as a defence mechanism against oxidative stress. Fernandez-Larrea and Stahl (9) reported that oxidative stress, caused by aromatic compounds in *Podospora anserina*, typically was accompanied by the induction of laccase mRNA. Solden and Dobson (27) found that xenobiotic response elements (XREs) are present in the region upstream from promoter of *lac*4 in *Pleurotus sajor-caju,* which are regulated by aromatic compounds.The XRE has a consensus sequence TNGCGTG, which is a *cis*-acting element sufficient to increase transcription of genes in eukaryotes by aromatic compounds (21). Cloning of the gene encoding laccase from γ-proteobacterium JB, especially promoter region, may help elucidate the mechanism of regulation of laccase activity by aromatic compounds. 8-Hydroxyquinoline is a known reducing agent but its role in increased laccase activity is not understood. On the other side, Tween-20 is a non toxic polysorbate used as a surfactant and is a known solubilizing agent of membrane proteins. It may not induce laccase, but may remove the bound laccase from cell membranes of γ-proteobacterium JB into broth. Third group of tested chemicals included various substrates, where anthracine showed maximum activity while ferulic acid did not, though it is known to increase laccase activity in fungi (13). Tested substrates were not oxidized by laccase (except *p*-phenylenediamine), may be due to the high redox potential (E ½) of these compounds. The increased laccase activity in fungi by a number of low molecular weight phenolic compounds has been demonstrated at the physiological level (20,23). This work is the first attempt dealing with enhancement of bacterial laccase activity using these substrates. Among the tested agrochemicals, only atrazine and trycyclozole increased laccase activity (Table 4). Mougin *et al.* (17) also reported stimulating effect of atrazine on laccase activity by *T. versicolor*. In order to investigate whether the increase in laccase activity by xenobiotic compounds resulted in synthesis of new laccase isozymes, native polyacrylamide gel electrophoresis (PAGE) was carried out. Protein bands exhibiting laccase activity were stained red with guaiacol (2 mM) in 50 mM phosphate buffer, pH 6.5. Native PAGE analysis of γ-proteobacterium JB laccase, in presence or absence of xenobiotics, revealed only one band in all cases (data not shown), which suggests that the effect on laccase activity was not due to new isozymes expression. Some conclusions can be drawn from our study. Additional efforts must be devoted to the optimisation of culture conditions to enhance the sensitivity of bacterial response to the treatment

Table 4. Effect of agrochemicals on laccase activity of γ-proteobacterium JB 72 h cultures.

Agrochemicals (100 µg/ml)	Growth (g dry wt l^{-1}) / Log cfu ml^{-1})	Laccase activity (U mg^{-1} dry wt)
None	0.11/9.0	108.6
Atrazine (D)	0.09/8.9	211
Carbendazine (D)	0.1/8.9	121
Trycyclozole (D)	0.1/8.9	160
Hexaconazole (D)	0.13/9.2	102
Tebuconazole (D)	0.11/9.1	81.8

D: Dimethyl sulfoxide soluble. Student's t-test revealed that increase and reduction in laccase activity was significant ($p<0.001$) for all agrochemicals except carbendazine ($p = <0.002$).

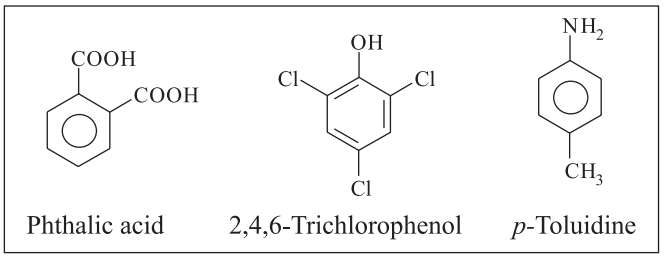

Figure 1. Laccase activity was enhanced by aromatic compounds of different structures.

with xenobiotics. In such conditions, laccase could be used as biomarker to assess environmental contamination. Laccase producing fungi have also been reported to be useful tools for xenobiotics removal in liquid effluents as well as in soil bioremediation (25).

RESUMO

Xenobióticos aumentam a atividade de lacase em γ-Proteobacterium JB alcali-tolerante

Vários corantes têxteis genotóxicos, xenobióticos, substratos (10mM) e agroquímicos (100mM/mL) foram testados quanto ao aumento da atividade de lacase em g-Proteobacterium JB. Os corantes Neutral Red, Indigo Carmine, Naphtol Base Bordears e Sulphast Ruby aumentaram a atividade em 3,7, 2,7, 2,6 e 2,3 vezes, respectivamente. Xenobióticos/substratos como *p*-toluidina, 8-hidroxiquinolina e antracina aumentaram a atividade em 3,4, 2,8 e 2,3 vezes, respectivamente. Atrazina e pesticidas triciclozol aumentaram a atividade em 1,95 e 1,5 vezes, respectivamente.

Palavras-chave: γ-Proteobacterium, lacase, xenobióticos, corantes, agroquímicos.

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