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Full Length Research Paper

Independent behavior of bacterial laccases to inducers and metal ions during production and activity

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Laccase, a blue copper oxidase, is an enzyme that is involved in the oxidation of aromatic compounds which prove otherwise difficult to degrade in the environment. The substrates of laccase are xenobiotics and synthetic dyes. The isolation of bacterial strains was investigated for laccase production and its activity. The medium for production was a high nitrogen medium containing aromatic compounds as inducers, for instance, guaiacol, phenol red and black liquor (from pulp and paper industry) gave higher production. The enzymes of isolated bacteria had the optimum pH and temperature in the range of 3 to 5 and 32 to 37°C, respectively. Furthermore, metal ions had an effect on the laccase enzyme; $MnSO_4$ and $CuSO_4$ showed a significant increase in laccase activity. However, the effects of metal ions on either laccase production or laccase activity were not clear. The laccase production and activity were dependent on species of bacterial strains. The laccase bacteria were identified as *Rhodococcus* sp., *Enterobacter* sp., *Staphylococcus saprophyticus* and *Delftia tsuruhatensis*. The synthetic dyes were determined in the reduction of color using the G32 strain; this strain gave 20 to 65% of dye reduction within three days.

Key words: Bacterial laccases, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), inducers, metal ions, dye reduction.

INTRODUCTION

Laccase, benzenediol oxygen oxidoreductases (EC 1.10.3.2), is a copper blue enzyme that oxidizes aromatic compounds using molecular oxygen as an electron acceptor, resulting in the formation of reactive radicals and water (H_2O) from the reaction. The substrates of this enzyme have various compounds for example, phenolic and non-phenolic compounds.

Some non-phenolic compounds require mediators to catalyze the oxidation of the enzyme (Sharma et al., 2007). Fungal, plant and bacterial laccases have been investigated and their substrates have been classified into five groups: L-tyrosine and monophenol (for example, hydroxyphenylacetic and salicylic acids); *o*-diphenols (for example, guaiacol, catechol and pyrogallol); *p*-diphenol and *p*-aromatic compound (for example, hydroquinone, creso and *p*-phenylenediamine); *m*-diphenol (for example, resorcinol and orcinol) and other substrates (for example,2,2'-azino-bis(3-ethylbenzothiazoline-6 sulphonic acid) (ABTS), syringaldazine) (Faure et al., 1995). These enzymes are used in biotechnology, for instance, in the detoxification of chemicals in wastewater, degradation of lignin in pulp and paper, degradation of inorganic compounds to soil organic matter and the decolorization of dyes in textile (Niladevi and Prema, 2008).

Bacterial laccase was first reported in *Azospirillum lipoferum* (Givaudan et al., 1993); it plays a role in cell pigmentation, oxidation of phenolic compounds (Faure et al., 1994, 1995) and/or electron transport (Alexandre et al., 1999). Bacterial protein sequence studies have indicated that the laccases are represented by high G+C Gram positive bacteria and α-, γ- and ε–proteobacteria (Alexandre and Zhulin, 2000); as have been shown in *Bacillus* sp., *Streptomyces* sp., and a γ proteobacterium (Bains et al., 2003; Sharma et al., 2007). Most bacterial laccases are intracellular enzymes or periplasmic proteins as shown in *A. lipoferum* and *B. subtilis*. Laccase

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like activity has also been found in bacteria, for example, the copper efflux protein CueO from *Escherichia coli* and the copper resistance protein CopA from *Pseudomonas syringae* and *Xanthomonas campestris*. These were due to the dependence of the 2,6-dimethoxyphenol oxidation on Cu^{2+} addition (Solano et al., 2001). The purpose of this study was to isolate bacterial strains that produce laccase enzyme and whether the extracellular enzyme could reduce dyes in a short period of cultivation.

MATERIALS AND METHODS

Most of the chemicals used were purchased from Fluka, whereas alkali lignin and ABTS were from sigma and black liquor was from the pulp and paper industry.

Bacterial cultivation

The samples from soil and wastewater of the pulp and paper industry were diluted and spread on M162 minimal medium (Degryse et al., 1978) with 0.1% guaiacol, incubated at 37°C for three to five days. The putative positive colonies were selected and streak on the same medium to confirm positive activity with guaiacol by the formation of a reddish-brown halo. Each isolated strain was cultured in nutrient broth containing 0.1% guaiacol at 37°C, then; the supernatants were collected by centrifugation and used as a crude enzyme. The crude enzyme was examined for laccase activity. The growth of cell culture was measured by using optical density at 600 nm.

Laccase activity

The activity was measured by monitoring the oxidation of ABTS. The reaction mixture contained 200 µl aliquots of the crude enzyme and 0.2 mM ABTS in 0.1 M sodium acetate buffer (pH 4.5) in a total volume of 1 ml. The reaction was held at 32°C for 10 min; the 0.5 ml of 80% trichloroacetic acid (TCA) was added to stop the reaction. The green formation of oxidized ABTS was determined at A_{420}. The control of reaction was performed using substrate blank (200 µl of nutrient broth instead of crude enzyme) and enzyme blank (without ABTS). The optical density of sample was subtracted from the optical density of both blanks. One unit of enzyme was defined as the amount of enzyme required to oxidize 1 µmol of ABTS per min. The molar extinction coefficient is 36,000 $M^{-1}cm^{-1}$.

Conditions of laccase production

The inducers were added in nutrient broth in order to improve the quantity of laccase production. The crude enzyme from each inducer was examined to determine laccase activity. The inducers used were guaiacol (0.1%), veratryl alcohol (0.1%), black liquor (0.1%), alkali lignin (0.002%), benzoic acid (1 mM), malachite green (10 µM), phenol red (10 µM), ethidium bromide (2.5 µM) and 4-aminobenzoic acid (1 mM).

Detection of temperature and pH on optimum laccase activity

The crude enzyme from a suitable inducer of each strain was incubated with ABTS as a substrate at temperatures of 32, 37, 40 and 45°C to determine the optimum temperature. The optimum pH was determined by incubation of the crude enzyme with ABTS in buffer of pH 3.0 to 9.0. Sodium acetate buffer was used in pH 3.0 to 6.0, whereas sodium phosphate buffer was used in pH 7.0 to 8.0 and Tris-base buffer was prepared for pH 9.0.

Effects of metal on laccase production and activity

The effect of metals was investigated in laccase production by addition of each metal at final concentration of 1 mM in nutrient broth containing suitable inducers. The crude enzyme was examined for laccase activity. Alternatively, the crude enzyme from nutrient broth containing suitable inducers was tested for the effect of metals on activity by incubation with ABTS and 1 mM of each metal. The reaction was held at 32°C for 10 min and then monitored laccase activity.

Dye decolorization

Ten percentage of bacterial inoculums were transferred in screening medium (Chen et al., 2003) containing yeast extract (10 g/L), NaCl (5 g/L) and various dyes (1 g/L); either brilliant blue G, methyl orange or neutral red. The medium was incubated at 37°C with shaking. The color of dyes was determined by spectrophotometer using absorbance at 465, 460 and 550 nm for brilliant blue G, methyl orange and neutral red, respectively. The percentage of dye reduction was calculated.

Identification of bacterial laccases

The deoxyribonucleic acid (DNA) of bacterial strains was extracted by boiling cell suspension for 10 min and then chilled on ice. The suspension was centrifuged at 10,000 rpm for 5 min and the supernatant was kept to use as DNA template. The DNA template was added to 45 µl of DreamTaq polymerase chain reaction (PCR) Master Mixture (Fermentas) using the primer pair of 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTT-GTTACGACTT-3′). The PCR reaction was performed by PTC-200 Peltier Thermal cycler (DNA Engina). The amplification conditions were as follows: 95°C for 3 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 2 min; and final extension at 72°C for 10 min. The PCR product was purified by gel extraction kit (Thermo scientific) and DNA sequencing was performed (1^{st} BASE sequencing Int). The sequence was compared to the NCBI nucleotide database (www.ncbi.nih.gov/blst) and multiple sequence alignment was done using the CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2).

RESULTS

Isolation of bacterial laccases

The bacterial strains were isolated from soil samples and waste water of the pulp and paper industry by determining their ability to use guaiacol as an energy source in minimal medium at 37°C. There were four strains selected, coded G1, G2, G32 and G44, which were potential strains giving laccase activity in minimal medium. All strains were investigated for growth and laccase activity in nutrient broth containing guaiacol, as a nitrogen source is needed for laccase production. After 48 h, growth still increased continuously while the laccase activities showed the highest activity at 48 h and

Table 1. Suitable inducers of laccase production for each bacterial strain.

Isolated strain	Inducer* (concentration)	Laccase activity (mU/L)	
		24 hours	48 hours
G1	Guaiacol (0.1%)	33	56
	Veratryl alcohol (0.1%)	51	43
G2	Guaiacol (0.1%)	45	66
	Phenol red (10µM)	67	79
G32	Guaiacol (0.1%)	53	66
	Black liquor (0.1%)	61	80
G44	Guaiacol (0.1%)	55	38
	Ethidium bromide (2.5µM)	11	77

*Nine inducers were investigated in nutrient broth for all strains (data not shown).

higher than in minimal medium (data not shown). Hence, the nutrient broth was used for laccase production rather than minimal medium.

Inducers of laccase production

Laccase has broader range of substrates which can induce enzyme production in microorganisms. We have determined the capacity of inducers in nine compounds to be suitable for laccase production in each strain. Guaiacol was able to induce laccase in all strains and was used as a comparative inducer. The suitable inducers resulting in the highest activity in four isolates are shown in Table 1. Guaiacol still remained a good inducer for G1 strain. Some dyes at low concentration could trigger laccase production, as shown in G2 and G44 strains using phenol red and ethidium bromide, respectively. The G32 used black liquor, which contains lignin from pulp and paper bleaching, to induce laccase activity. These results show that laccase activity of different strains could be triggered with different substrates. The inducer may be involved in mechanisms of enzymatic secretion or catabolic metabolism.

Property of crude enzymes in different temperature and pH

The crude enzyme was investigated to determine the optimum temperature and pH for laccase activity by incubation with ABTS substrate. The optimum temperature was 37°C for G1 and G44 strains by giving laccase activity at 75 and 56 mU/L, respectively (Figure 1a). The G32 strain had optimum temperature at 32°C. Only the G2 strain showed higher laccase activity at high temperature (45°C). The optimum pH was determined using a range of pH (3.0 to 9.0) for laccase activity at a temperature of 32°C. All strains gave laccase activity in acid conditions (Figure 1b). The optimum pH of laccase activity was 3.0 in almost all strains except the G2 strain which had an optimum pH of 5.0. The enzyme did not work well in alkaline conditions (pH 7.0 to 9.0; data not shown). For ABTS substrate, this was similar to most laccases which were active at acid pH. The oxidation of ABTS is not involved in protons; thus the mechanism is involved in the hydroxide (OH^-) inhibition of laccases (Xu, 1997).

Effect of metals on laccase production and activity

It has been reported that metal ions stimulated laccase formation when added to actively growing culture of *Trametes pubescens* (Galhaup et al., 2002). Copper (Cu^{2+}), has been shown to be effective in increasing laccase production in many microorganisms at low concentration. To compare with various metal ions, Cu^{2+} was added in medium at concentration of 0.1 and 1 mM, while the other ions were added at 1mM. The manganese (Mn^{2+}) ion was the most effective in stimulating laccase production in the G1 strains by increasing the activity 4.8 fold (Figure 2). Cu^{2+} (0.1 mM) and Ca^{2+} also activated laccase production particularly in the G32 strain. Moreover, the cadmium (Cd^{2+}) did not affect growth of the G32 strain and slightly stimulated laccase production. In parallel experiments, the effects of these metal ions were tested in laccase activity. Most laccase enzymes showed higher activity with the addition of Mn^{2+}, calcium (Ca^{2+}), lithium (Li^+) and Cu^{2+}, particularly for the G1 and G44 strains (Figure 2). The Cd^{2+} ion had the most negative effect on enzyme activity. However, the laccase enzymes of the G32 and G44 strains showed activities towards cadmium concentration. This implies that the metal ions have an effect on enzyme activity more than on enzyme production. The laccase enzymes of bacterial strains

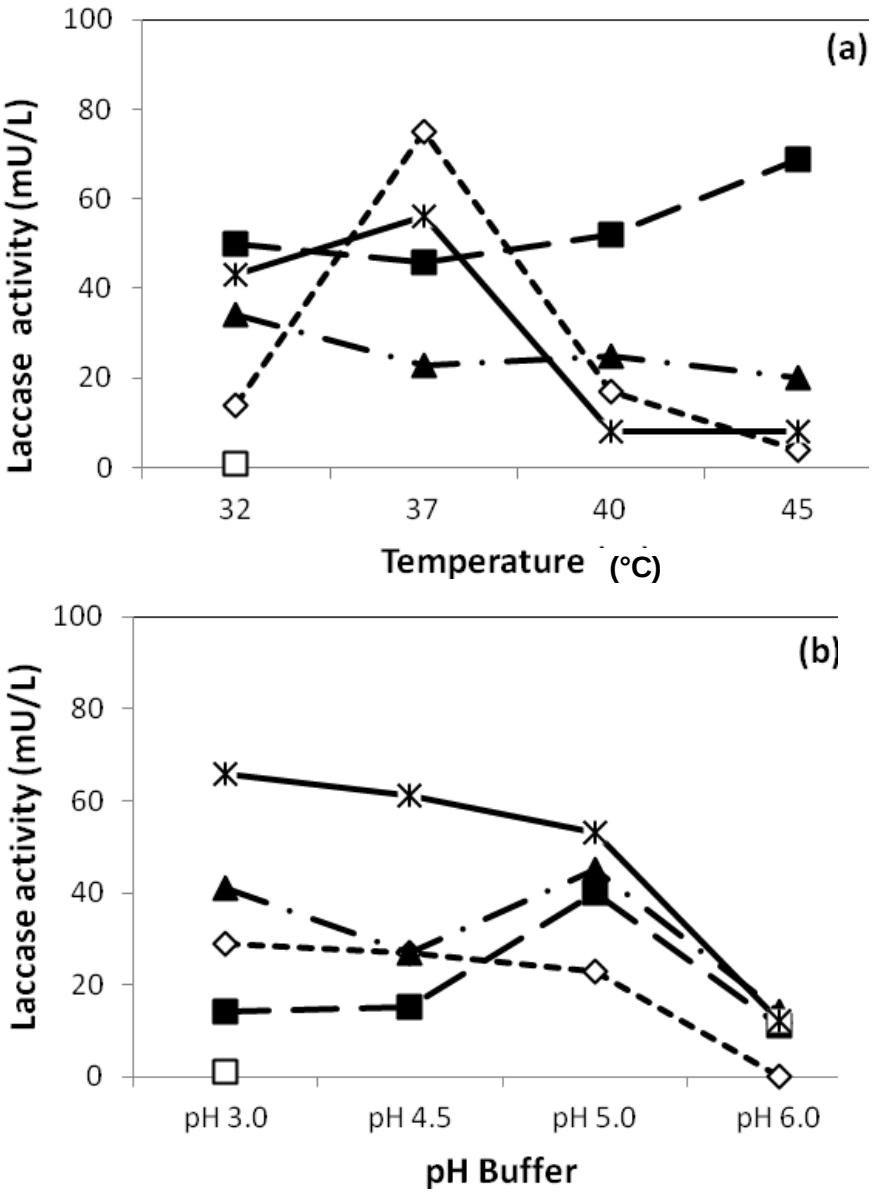

Figure 1. Properties of bacterial crude enzyme. (a) Optimum temperature of laccase; (b) Optimum pH of laccase. Symbols of each strain showed; ---◇---, G1; —■—, G2; —▲ -, G32; —✳—, G44.

resistant to various metal ions; may have potential in the environment for wastewater treatment.

Previous studies have shown that laccase was a potential enzyme for bioremediation, particularly in wastewater treatment of some dyes (Peralta-Zamora et al., 2003). The remaining dyes of wastewater from Industries became problems to environment as pollutants. We conducted a preliminary study to investigate the decolorization of synthetic dyes by G32 strain which had black liquor as an inducer. Ten percentages of inoculums were grown in nutrient broth with black liquor for 48 h and transferred into screening medium containing each dye. The remaining dyes were observed using spectrophotometer and then calculation of decolorization was performed. The brilliant blue G showed the reduction of dye at the beginning (30%) and gave the highest decolorization of up to 65% at 72 h (Figure 3). Methyl orange and neutral red gave only 10% decolorization for 48 h; increasing decolorization at 72 h was 20 and 45% for methyl orange and neutral red, respectively. The pH of cultures was kept at pH 6.5 to 7.0 (data not shown). Thus, our results show that the G32 strain activity decreases dye color; and that different types of dyes may increase enzyme oxidation.

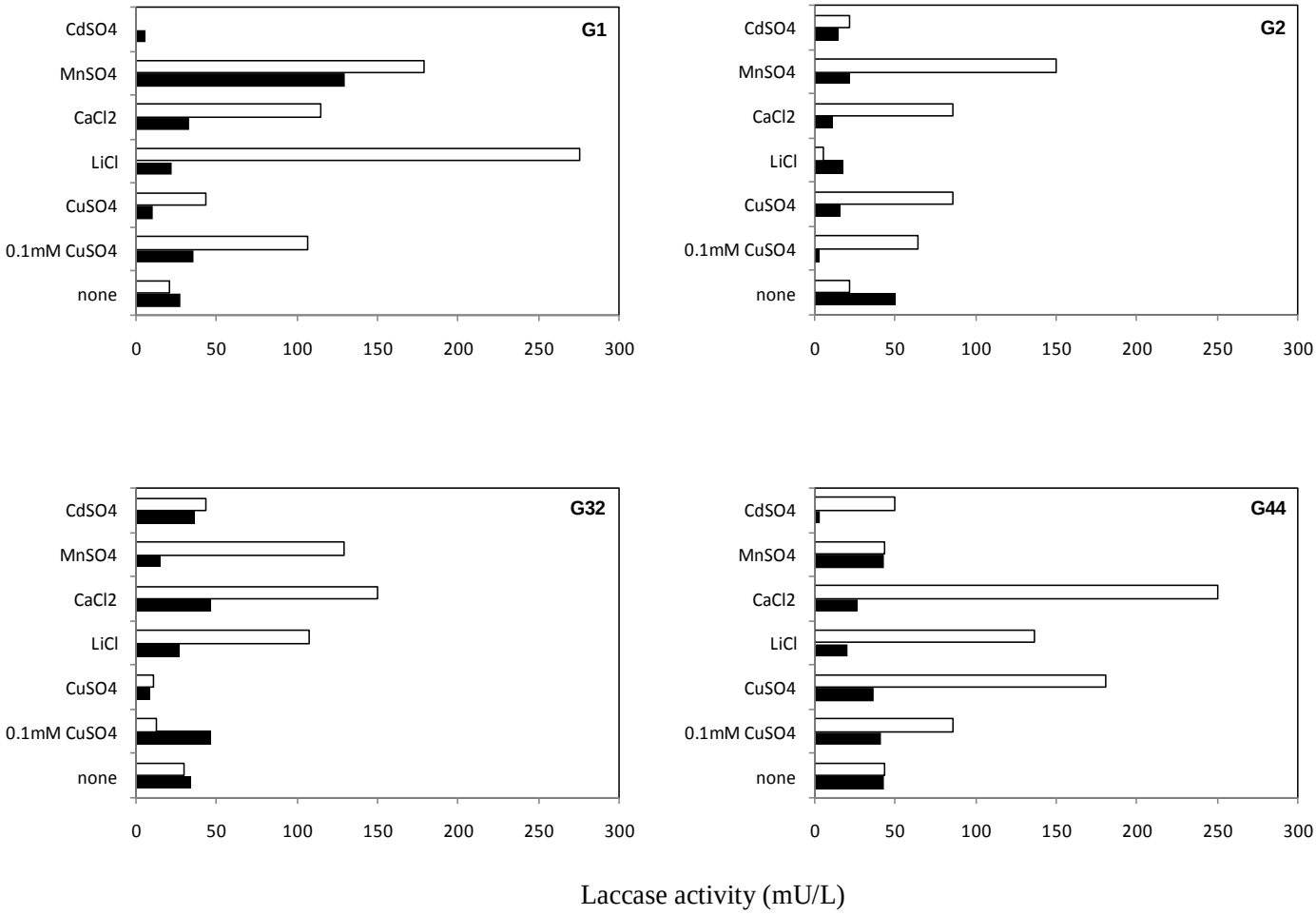

Figure 2. Effect of metal ions on laccase production (dark bars) and on laccase activity (white bars) of bacterial strains.

Identification of bacterial laccases

Analysis of 16S recombinant deoxyribonucleic acid (rDNA) sequences showed that the strain G1, G2, G32 and G44 belonged to *Rhodococcus* sp., *Enterobacter* sp., *Staphylococcus saprophyticus* and *Delftia tsuruhatensis*, respectively, with 99% identity. *Rhodobacter capsulatus* contained putative laccase protein (Alexandre and Zhulin, 2000); *Staphylococcus* sp. (K18D3) also had laccase which was identified as laccase like multicopper oxidase (LMCO) (Kellner et al., 2008). Therefore, the phylogenetic tree was constructed to compare relationship of bacterial strains that produced laccases (Figure 4). The G2 strain was closely related to the bacterial laccases/ laccase-like group while the others were the same group with putative laccase proteins.

DISCUSSION

Laccase enzymes produced from different microorganisms show different properties. They are unique in both culture and environment conditions. For a screening medium, we used a minimal medium containing guaiacol as a common inducer. The growth of bacteria and the enzyme activity were not as high as nitrogen source which is important for laccase production; therefore nutrient broth was used for enzyme production, containing beef extract and peptone as nitrogen sources. The four bacterial strains that we detected (G1, G2, G32 and G44) were responded to different aromatic compounds as determined by higher activity. The addition of metal ions in culture improved laccase activity. In the ascomycete *Podospora anserine*, laccase messenger ribonucleic acid (mRNA) was produced in response to copper and aromatic compounds as a protective mechanism against oxidative stress (Fernandez-Larrea and Stahl, 1996). Bacterial laccase productions may be involved in the activation of gene transcription in presence of metal ions as well as metal responsive elements in *P. anserine*. Additionally, the xenobiotic response elements and antioxidant response elements have been suggested to be involved in the induction

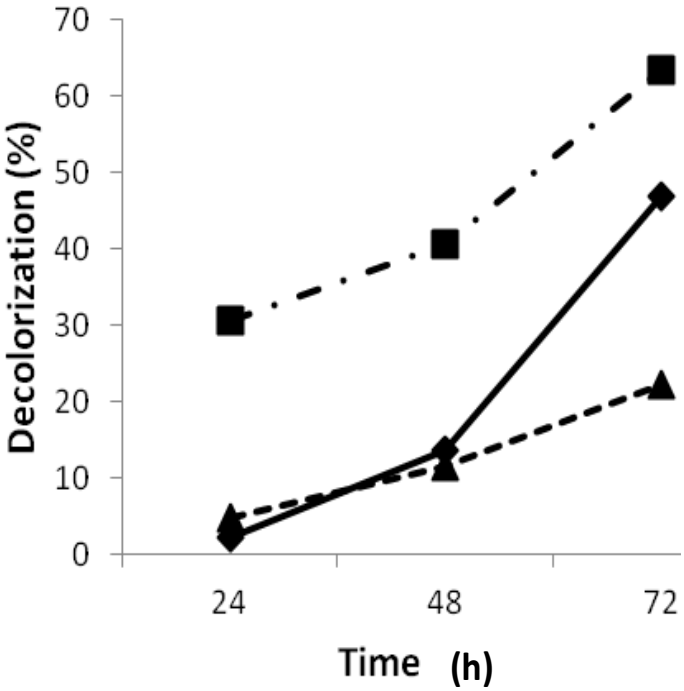

Figure 3. Decolorization of the G32 strain. The strain was grown in laccase production and transferred in medium containing brilliant blue G (■), methyl orange (▲) and neutral red (♦). The cultures were incubated at 37°C with shaking.

of fungal laccases by aromatic compounds (Soden and Dobson, 2001).

The conditions of laccase activity are necessary for the efficiency of enzymes and their applications. The laccase of the bacteria we examined had optimum temperatures in mild conditions (32 to 45°C), unlike fungal laccases which have high activities at high temperatures (40 to 70°C) and had good activities in acidic condition similar to fungal laccases (Baldrian, 2006). The *Streptomyces ipomoea* encoded SilA laccase which had activity of laccase at alkaline pH; however, this strain was able to oxidize ABTS and ferrocyanide only at acid pH (Molina-Guijarro et al., 2009). The catalytic activity of laccases depends on the redox potential difference between laccases and substrates and the OH^- inhibition. The ABTS oxidation is not involved in protons; therefore, the redox potential is independent of pH (Xu, 1997). The specific substrate of each bacterial strain should be investigated to improve laccase activity. This may be a reason for various substrates depending bacterial strains.

Heavy metals normally found in the environment, might affect the stability or activity of enzymes. The fungus, *Trametes hirsute*laccase was stable in many metals at low concentration (1 mM) for seven days (Couto et al., 2005). Higher laccase activity *T. versicolor* showed in the addition of Mn^{2+} and Cu^{2+} (lower than 1 mM) and was inhibited by Cd^{2+} (Lorenzo et al., 2005). In our study, the bacterial laccases were found to be resistant to various metals under supplements of culture conditions. The activity of these enzymes was stimulated in the presence of Mn^{2+}, Cu^{2+}, Ca^{2+} and Li^{+}. The metal ions might affect the active sites of laccase formation (laccases are isoenzymes that oligomerize to form multimeric complexes). They require a molecule, such as copper, manganese, for the catalytic reaction of the oxidation of various phenolic compounds. With respect to practical application, the G32 strain could be used to reduce the color of many dyes. Lignolytic enzymes are involved in decolorization reactions which include laccase and manganese peroxidase. Manganese peroxidase was also detected at low activity in these bacterial strains (data not shown). The property of the bacteria and their enzymes may be useful in bioremediation.

Bacterial LMCOs are receiving more attention with regards to their application in biotechnology (Kellner et al., 2008) as they produce enzymes in short periods of cultivation. Our results show the production of laccases by bacterial strains which had independent conditions. The properties of laccases depend on type of bacteria as they may produce laccase in multiple isoenzymes. We have shown that metal ions assist with induction and/or secretion of enzyme into culture medium and the resistance of enzyme to metal ions. These properties are suitable for the biodegradation and decolorization of metals which contaminate the environment. Moreover, raw naturally occurring materials, such as rice bran, or

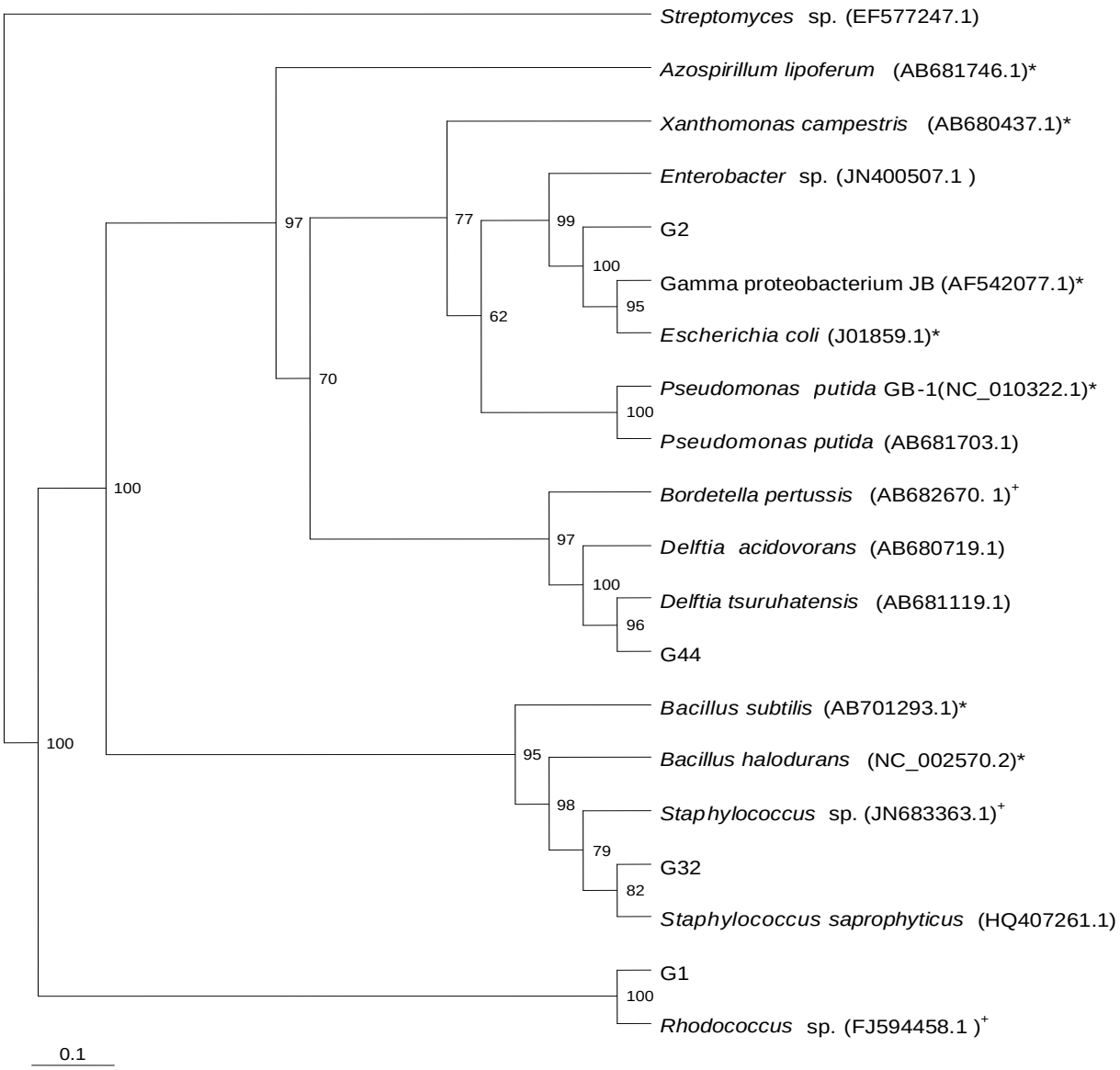

Figure 4. Phylogenetic tree of bacteria produced laccase/laccase-like enzyme. Accession number is given in blanket; Symbol (*) and (+) represent laccases producing bacteria and putative bacterial laccase strains, respectively (Kellner et al., 2008; Sharma et al., 2007; Alexandre and Zhulin, 2000). Each number on a branch indicates the bootstrap confidence values; the scale bar indicates distance of nucleotide substitutions per site.

saw dust, should be investigated to determine whether they produce laccase, as they could affect production and/or activity of all laccase isoenzymes.

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