International Journal of Waste Resources

Aoyama A, Int J Waste Resources 2014, 4:2
DOI: 10.4172/2252-5211.1000146

Research Article IJWR, an open access journal

Newly-Isolated Laccase High Productivity *Streptomyces* Sp. Grown In Cedar Powder as the Sole Carbon Source

Akihisa Aoyama*, Kazuhiro Yamada, Yoshinobu Suzuki, Yuta Kato, Kazuo Nagai and Ryuichiro Kurane

Department of Biological Chemistry, College of Bioscience and Biotechnology, Chubu University, Japan

***Corresponding author:** Akihisa Aoyama, Department of Biological Chemistry, College of Bioscience and Biotechnology, Chubu University, 1200 Matsumoto-cho, Kasugai, Aichi 487-8501, Japan, Tel: +81-568-51-1111(5638); Fax: +81-568-51-4832; E-mail: ap1s2000@gmail.com

Received date: April 18, 2014; **Accepted date:** May 23, 2014; **Published date:** May 30, 2014

Abstract

Microorganisms with greater potential to degrade lignin than well-known white-rot fungi were sought and identified, but the fungi were less frequently employed for slow growth and little enzyme productivity. They were subjected to enriched cultures in order to explore the bacteria instead from 300 soil samples with cedar powder as the sole carbon source were prepared. From these, a culture with actinomycetes which showed the most oxidation activity of 2,6-Dimetoxyphenol (2,6-DMP) known as laccase substrate was selected and labeled as KS1025A strain. Characteristics of the bacteria and behavior of the secreted enzymes were examined. As a result, it was identified as a strain of *Streptomyces* sp. from the 16S rDNA gene sequence homology. The optimum temperature and pH for laccase activity of the secreted enzyme of this strain are 50°C and 4.5, respectively. Since Mn^{2+} was not directly oxidized, it was assumed that it did not contain manganese peroxidase. However, when $MnSO_4$ was added during 2,6-DMP oxidation reaction, activity increased. After 120 hours of culture, 14 U/mL of laccase activity could be achieved by this strain, greatly exceeding known values by white rot fungus, namely 1.8U/mL after approximately 20 days. Furthermore, since reaction could continue without the addition of H_2O_2 during 2,6-DMP oxidation reaction, the culture solution is thought to contain free oxidizing agents. In addition, approximately 50% of 0.05% lignin sulfonic acid was decolorized by this strain in 5 days. The strain or the enzyme produced by it may be utilized for rapid biodegradation of lignin when adding hard (or soft) biomass containing lignin to produce bioethanol.

Keywords: Laccase; Streptomyces; Lignin; Bioethanol; Peroxidase; Lignosulfonate

Introduction

In recent years, effective use of wood-based (or grass-based) plant waste material (wood, bagasse, etc.) for biomass has been studied. For example, cellulose/hemicellulose within the plant material comprises approximately 50% of its dry weight, and by hydrolyzing, sugars such as glucose, etc. can be extracted [1-3]. The obtained sugars can be used to produce bioethanol, etc [4] through fermentation. However, plants generally contain very strong polymer fibers which are formed by bonding cellulose fibers or hemicellulose fibers with lignin to maintain their shape. Although, lignification by lignin is advantageous when these plants are used as construction material; when used as biomass, this is an obstacle. Since various problems arise from the standpoint of the environment and processing costs, such as the need for alkali or acidic degradation or mechanical grinding, the use of plant material is limited [5]. Therefore, reduction of processing costs is an important issue [6,7].

In addition, if lignin degradation is carried out chemically, phenol derivatives are generated, inhibiting the hydrolyzation of cellulose by enzymes [8] and subsequent fermentation of sugars gained by hydrolyzation [9].

On the other hand, plants also serve as important storage sites for organic matter in the biosphere. Some natural microorganisms can degrade and utilize such organic matter as a source of carbon and energy. In particular, white-rot fungi degrades lignin by extracellularly secreting enzymes [10,11] and are considered to play an important role in carbon circulation in the ecosystem. However, enzyme production requires approximately 20 days in culture [12,13] and is difficult to use. Hence, focusing on lignin degradation by extracellular enzymes from fast-growing microorganisms, microorganisms with a high ability to degrade lignin, excluding filamentous fungi, were sought.

In addition, among enzymes secreted extracellularly from white-rot fungi, lignin peroxidase (LiP) [14,15], manganese peroxidase (MnP) [16], and laccase [15,17-19] are known to be associated with lignin degradation. In this study, enzyme activity of newly-isolated actinomycete strains and that of white-rot fungi were comparatively considered.

Materials and Methods

Enriched culture

Two hundred milligrams of soil sample was placed in a microtube and suspended in 300 µL of added sterile saline solution. After standing for one-minute, 50 µL of the supernatant was added to 500 µL of screening medium (composition as below) with cedar powder as the sole carbon source. The medium was covered with a gas-permeable plate seal and cultured for 2 weeks at 150 spm. After 2 weeks, 50 µL of the cultured medium was transferred to a new medium of 500 µL and was further cultured. This process was repeated four times and the medium was subcultured four times.

After subculturing for the fourth time for 1 week, the medium was centrifuged at 4725 g for 10 minutes at room temperature. The supernatant was extracted as an extracellular crude enzyme liquid.

Approximately 300 soil samples were used as enriched cultures.

Screening medium compositions

The following composition was sterilized at 121°C for 15 minutes; 20 g/L cedar powder, 10 g/L $(NH_4)_2SO_4$, 10 g/L $NaNO_3$, 5 g/L KH_2PO_4, 1 g/L K_2HPO_4, 1 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L NaCl, 1 g/L yeast extract, and 0.5 g/L $CaCl_2 \cdot 2H_2O$.

In addition to the above composition, 15 g/L of agar was added to the screening plate medium.

Lignosulfonate (a water soluble lignin derivative [20]) decolorization

For the lignosulfonate medium, 0.5 or 1 g/L of lignosulfonate was added instead of cedar powder. One loop was taken from the PD medium with the preserved strain, inoculated into 5 mL of ISP2 medium (composition g/L: 4 yeast extract, 10 malt extract, 4 glucose), and precultured at 28°C, 150 spm, for 48 hours.

One hundred microliter of preculture liquid was inoculated into 10 mL of lignosulfonate and cultured at 28°C, 150 spm, for 120 hours. Next, the culture was centrifuged for 10 minutes at 12000g, and A480nm was measured for the supernatant, which is the maximum absorption amount in the visible range of lignosulfonate.

Strain separation and preservation

The culture fluid was appropriately diluted, spread on the screening plate medium, and cultured statically at 28°C for up to 2 weeks. A single colony was transferred to the PDA medium as the preserved strain. PDA medium: potato dextrose agar medium (hereinafter referred to as "PDA medium").

Identification of the strain by 16S rDNA gene sequencing

A platinum loop was taken from the colony grown on the PDA medium and extracted by a DNA extraction kit, ultra clean microbial DNA Isolation kit (12224-50, MO BIO Laboratories, Carlsbad, CA, USA), as a template. As primers, 27f (5'-AGAGTTTGATCMTGGCTCAG-3') [21] and 519r (5'-CWATTACCGCGGCKGCTG-3') [22] were used. Sequencing was implemented from the template DNA, and the gene sequence was identified. The homology of gene sequence was searched in DNA Data Bank of Japan (DDBJ) [23]. In addition, the collected sequence data were multiple aligned by ClustalW in DDBJ.

The neighbor joining method was used to reconstruct the phylogenetic tree [24].

Enzyme production

The preserved strain was inoculated into 500 µL of screening medium, and cultured at 30°C, 110 spm for 120 hours, and the medium was centrifuged for 10 minutes at 4,725 g. The supernatant was extracted as the extracellular enzyme.

Measurement method of laccase activity

An enzyme liquid mixture containing 60 µL of enzyme liquid, 10 µL of 20 mM 2,6-dimetoxyphenol (2,6-DMP), 10 µL of 20 mM $MnSO_4$, 120 µL of 100 mM malonic Na buffer fluid (pH 4.5), and 10 µL of 2 mM H_2O_2 was added to a 96-well microplate and allowed to react for 10 minutes at 45°C, after which A469nm was measured [25,26].

Enzyme activity is shown in Units (U). One Unit is the enzyme level at which 1 µmol of substrate oxidizes in 1 minute.

2,6-DMP (ε_{469} = 49,600 $M^{-1}cm^{-1}$) [25]

Optimum pH measurement method

An enzyme liquid mixture containing 60 µL of enzyme liquid, 10 µL of 20 mM 2,6-Dimetoxyphenol (2,6-DMP), 10 µL of 20mM $MnSO_4$, 120 µL of 100 mM sodium malonate buffer (3.0, 3.5, 4.0, 4.5 5.0, 4.5, 5.0, 5.5 and 6.0), and 10 µL of 2mM H_2O_2 was added to a 96-well microplate and allowed to react for 10 minutes at 45°C, after which A469nm was measured.

Optimum temperature measurement method

An enzyme liquid mixture containing 60 µL of enzyme liquid, 10 µL of 20 mM 2,6-Dimetoxyphenol(2,6-DMP), 10 µL of 20 mM $MnSO_4$, 120 µL of 100 mM sodium malonate buffer (pH 4.5) was added to a 96-well microplate and pre-heated 10 minutes, and 10 µL of 2 mM H_2O_2 was added to a 96-well microplate and was allowed to react for 10 minutes various temperatures (30, 40, 50 and 60°C), after which A469nm was measured immediately.

Measurement method of lignin peroxidase activity

An assay was carried out at pH 3.0 using 0.1 M sodium tartrate buffer and 0.01 mM veratryl alcohol as the substrate. Reaction was initiated by adding 4 mM H_2O_2 to 100 mM sodium lactate buffer pH 4.5, 0.1 mM H_2O_2, and 0.5 ml culture supernatant and increased absorbance at 310nm could be observed [27].

Measurement method of Manganese (II) peroxidase activity

Manganese (II) peroxidase activity was identified by observing the formation of a Mn^{3+}-malonate complex at 275 nm [28]. The reaction mixture contained 0.5 ml of 10 mM Mn^{2+} in 100 mM sodium maloate buffer pH 4.5, 0.1 mM H_2O_2 and 0.5 ml culture supernatant.

Enzyme protein concentration measurement

Measurement of enzyme protein concentration was carried out by the Bradford method [29], using the Bradford Protein Assay kit (Bio-rad Laboratories, Atlanta, GA, USA) with BSA as the control.

Enzyme concentration

The enzyme was concentrated to 10 times using a centrifugal ultrafiltration filter, Vivaspin 20-3K (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

SDS-PAGE and Zymogram method

SDS-PAGE and Zymogram analysis was conducted with 8% acrylamide gel using a modified method of Díaz et al. [13]. The protein of the gel was embedded and denatured by SDS for 10 minutes at 45°C in the presence of a reducing agent.

After electrophoresis at 20 mA, the gel was placed in a cleaning solution (0.1% Triton X and 100 mM sodium acetate buffer, pH5.0). The cleaning solution was replaced after 15 minutes. Cleaning was

carried out three times. The gel was transferred to a substrate solution (18 mM 2,6-DMP, 0.2 mM H_2O_2, 100 mM Acetate Na Buffer pH 5.0), allowed to react for 10 minutes at 45°C to color, and was then scanned. Next, the gel was placed to deionized water which was replaced after 15 minutes. The gel was cleaned three times with deionized water, stained by the CBB staining fluid, Gelcode Blue Stain Reagent (Thermo Fisher Scientific, Waltham, MA, USA), and then scanned.

Results

Screening

Three hundred types of soil samples were individually added to a screening medium to form an enriched culture. As a result, a high level of 2,6-DMP oxidation activity could be observed by enzymes in the enriched cultured with a soil sample collected from Aichi, Japan. Laccase activity of the sample could progress without the addition of H_2O_2.

Isolation of the KS1025A strain

The active culture fluid was appropriately diluted and spread on a screening plate medium, and an actinomycete-like colony could be subsequently obtained. This strain was designated as KS1025A. After it was preserved, the enzyme was measured, and a high level of laccase activity as illustrated in Figure 1 could be observed. The enzyme of this strain was active even without the addition of $MnSO_4$ and H_2O_2, but activity increased with such addition. The assay of the Mn^{3+}-malonic acid complex indicated oxidation activity from Mn^{2+} to Mn^{3+} (as manganase peroxidase activity). However, although veratryl alcohol oxidation (as lignin peroxidase activity) was examined, no activity was observed (data not shown). Hence, Laccase is thought to be the main enzyme group.

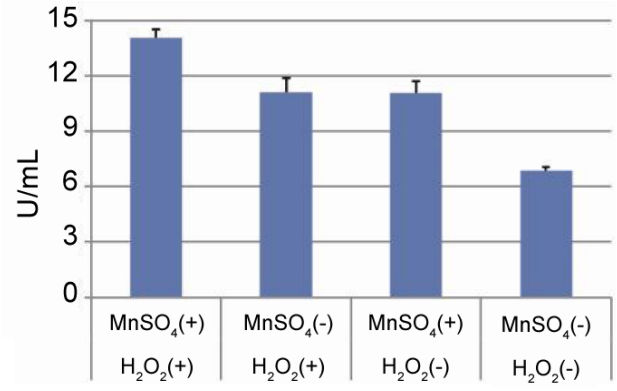

Figure 1: Laccase activity by KS1025A extracellular enzymes, Laccase activity in a pH4.5 sodium malonate buffer of KS1025A culture supernatant is shown. For H_2O_2(+), 0.2mM H_2O_2 was added. The bars indicate the standard deviations of three samples.

After culturing the KS1025A strain in a PDA medium at 28°C for 7 days, a wrinkled, white to grey colony formed in 7 days. Mycelium was verified by microscope observation, but sporulation was not observed after a month of culture.

By identifying the 16S rDNA gene sequence of this strain, homology with *Streptomyces atratus* was determined to be 97.39% and a phylogenetic relationship could be shown (Figure 2).

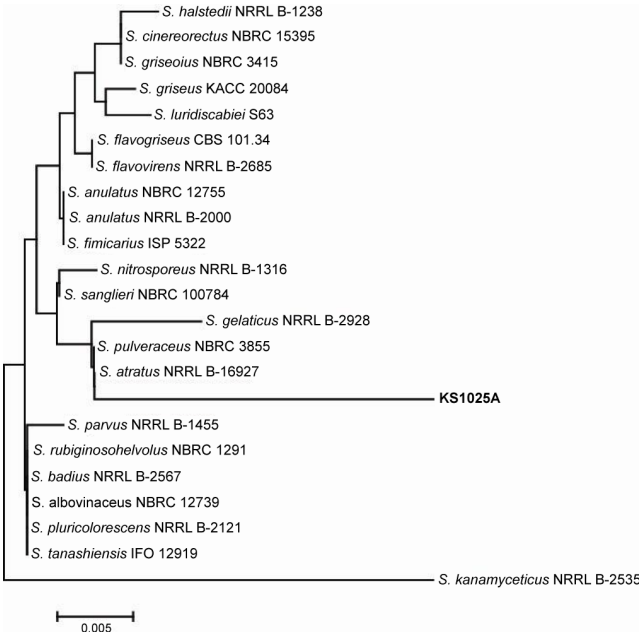

Figure 2: Phylogenic relationships of the isolated strains and some related species based on 16S rRNA gene-sequences. The neighbor joining method was used to generate branching pattern. The bar indicates 0.05 nucleotide substitutions per site. The sequence data were cited from DDBJ.

Enzymological properties of enzymes produced by KS1025A

The optimum temperature and pH for laccase activity are 50°C and pH 4.5 respectively, as shown in Figure 3.

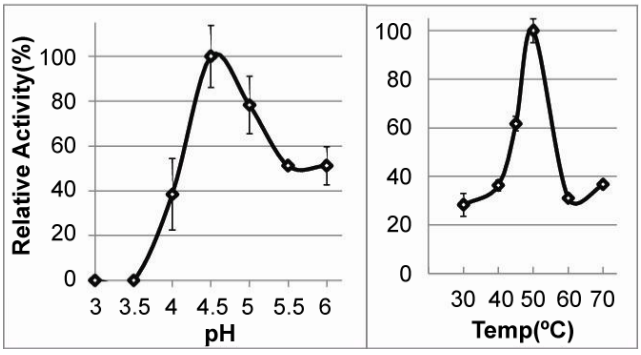

Figure 3: The optimum temperature and pH for laccase activity of KS1025A strain enzyme. Left: The relative activity when 2,6-DMP is oxidized in malonic acid buffer at various pHs, where the optimum pH is centered. Right: The relative activity when 2,6-DMP is oxidized in sodium malonate buffer at pH4.5 at various temperatures, where the optimum temperature is centered. The bars indicate the standard deviations of the two samples.

The zymogram image is shown in Figure 4A, where 2,6-DMP oxide turned yellow. From the culture supernatant of KS1025, two large bands could be verified approximately between MW>175 kDa and MW 90 kDa. The CBB stained image is shown in Figure 4B. A large band could be seen at approximately 42 kDa for the KS1025A enzyme, but a band could barely be seen in the area where a strong reaction could be verified by Zymogram. From these observations, KS1025A derived Laccase is thought to be an enzyme with highly specific activity.

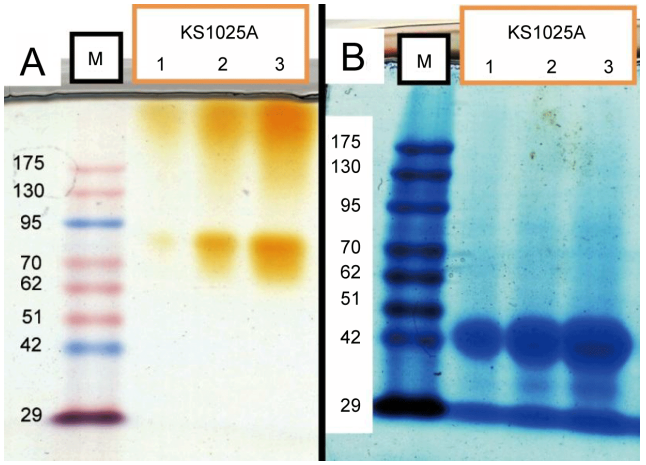

Figure 4: Zymogram image and CBB stained image of KS1025A secreted enzyme. Quantity of migration protein. KS1025A; Lane 1:1.25 μg, lane 2:2.5 μg, lane 3:5 μg. A) Zymogram image: After electrophorosis of protein in an 8% acrylamide gel, bands indicating activity with 18mM 2,6-DMP as a substrate were verified. B) CBB-stained image: After the verification of bands indicating activity by the Zymogram, the same gel was CBB-stained to verify a band for protein.

Lignosulfonate de-colorization

De-colorization rates of lignosulfonate at A480nm by KS1025A in 5 days were 52% for 0.05% lignosulfonate, and 22% for 0.1% lignosulfonate.

Discussion

In this study, actinomycete KS1025A with strong lignin-degradation was newly isolated and identified as *Streptomyces* sp., using a universal primer for PCR amplification and DNA-sequencing. The universal primer mentioned above contained V1-4 regions which could be used to determine the specific sequences of the microorganism [30]. In the phylogenetic tree, the isolated actinomycete was slightly distant to its most homologenic microorganism, *Streptomyces atratus* (97.39%). Thereby we named the identified microbe as *Streptomyces* sp. KS1025A.

Crude enzymes derived from KS1025A do not show manganese peroxidase or lignin peroxidase activity. Therefore, laccase activity is considered to be the main cause of the lignin degradation ability of KS1025A. There are no reports concerning laccase or peroxidase activity in *Streptomyces atratus* (97.39%), *Streptomyces pulveraceus* (97.38%) or *Streptomyces sanglieri* (97.38%).

Optimal pH and temperature of the crude enzyme(s) derived from KS1025A are 4.5 and 50°C, respectively. Acidic pH (4-5) has been reported to be optimal for most laccases, and pH 7-8 for others [31]. In the zymogram analysis, two types of laccases derived from KS1025A could be observed; low molecular weight laccases approximately 90 kDa and others greater than 175 kDa. However, most fungal laccases reported thus far are monomeric proteins with molecular weights between 50 and 80 kDa [17,32,33]. With the exception of the following, laccases from *Agaricus bisporus* [34] and *Trametes villosa* [35] are comprised of two subunits, and those derived from *Podospora anserine* [36] are comprised of four subunits. In addition, the molecular weight of laccases derived from *Streptomyces coelicolor* [31] and *Streptomyces ipomoea* [37] are approximately 32-33 kDa in monomeric form, and exist as dimers in their natural form with molecular weights of 67-69 kDa. The molecular weight of laccase derived from KS1025A is heavier than the above mentioned *Streptomyceses* laccase. Therefore, laccases from KS1025A may be comprised of more than two subunits, which may have been caused by the mild denaturing conditions for SDS-PAGE and zymogram analysis. This analysis was carried out at 45°C for 10 minutes to preserve enzyme activity, although SDS and reducing agents were added. Hence, subunits of laccases may not have completely separated.

Laccase activity and enzyme productivity of this strain was compared with white-rot fungus. *Pleurotus ostreatus* [13,38-41] is known as a powerful lignin-degrading enzyme producer. When laccase activity of *Pleurotus ostreatus* is compared, a maximum of 1.8 U/mL of enzyme is produced in 21 days of culture, provided that copper is added as a Laccase catalyst [41]. Meanwhile, KS1025A strain produced a maximum of 14 U/ml of 2,6-DMP oxidative enzyme in 5 days of culture. From this, culture time can be reduced by 1/4 if KS1025A is used for enzyme production, and enzyme yield per day would increase 33 times. Possible applications of this strain for lignin degradation include wood-based biomass process, rapid decomposition of lignin colored material, and the industrial production of laccase. To the best of our knowledge, this is the first report on isolating laccase high-productive Streptomyces sp. Application of this strain is potential for de-lignin in wood-based biomass and rapid decomposition of lignin colored substance. Moreover, laccase has wider applications for degrading environmental pollutants such as bisphenol A; endocrine-disrupting chemical.

Conclusions

In this study we have screened high de-lignin enzyme producing microorgasms, and *Streptomyces* sp. KS1025A strain was isolated. The strain produces laccase in a short period and at high activity than previously reported white rot fungi. Remarkably, the laccase from the KS1025 strain was not-needed in addition of oxidation agent such as H_2O_2. By adding lignosulfate to the medium of the strain, incubation of the A480 nm was decreased about 50%.

References

1. Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 83: 1-11.
2. Reddy N, Yang Y (2005) Biofibers from agricultural byproducts for industrial applications. Trends Biotechnol 23: 22-27.
3. Kumagai S, Yamada N, Sakaki T, Hayashi N (2007) Characteristics of Hydrothermal Decomposition and Saccharification of Various Lignocellulosic Biomass and Enzymatic Saccharification of the Obtained Hydrothermal-Residue. J Jpn Inst Energy 86: 712-717.

4. Sluiter JB, Ruiz RO, Scarlata CJ, Sluiter AD, Templeton DW (2010) Compositional analysis of lignocellulosic feedstocks. 1. Review and description of methods. J Agric Food Chem 58: 9043-9053.
5. Aoyama A, Kurane R, Nagai K (2013) Penicillium sp. strain that efficiently adsorbs lignosulfonate in the presence of sulfate ion. J Biosci Bioeng 115: 279-283.
6. Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, et al. (2006) The path forward for biofuels and biomaterials. Science 311: 484-489.
7. Sticklen MB (2008) Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol. Nat Rev Genet 9: 433-443.
8. Ximenes E, Kim Y, Mosier N, Dien B, Ladisch M (2010) Inhibition of cellulases by phenols. Enzyme Microb Technol 46: 170-176.
9. Klinke HB, Thomsen AB, Ahring BK (2004) Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl Microbiol Biotechnol 66: 10-26.
10. Leonowicz A, Matuszewska A, Luterek J, Ziegenhagen D, WojtaÅ›-Wasilewska M, et al. (1999) Biodegradation of lignin by white rot fungi. Fungal Genet Biol 27: 175-185.
11. Higuchi T (2004) Microbial degradation of lignin: role of lignin peroxidase manganese peroxidase and laccase. Proc Jpn Acad Ser B Phys Biol Sci 80: 204-214.
12. Baldrian P, Gabriel J (2002) Copper and cadmium increase laccase activity in Pleurotus ostreatus. FEMS Microbiol Lett 206: 69-74.
13. Díaz R, Sánchez C, Bibbins-Martínez MD, Díaz-Godínez G (2011) Effect of medium pH on laccase zymogram patterns produced by Pleurotus ostreatus in submerged fermentation. Afr J Microbiol Res 5: 2720-2723.
14. Tien M, Kirk TK (1988) Lignin Peroxidase of Phanerochaete chrysosporium. Methods Enzymol 161: 238-249.
15. Higuchi T (1990) Lignin biochemistry: Biosynthesis and biodegradation. Wood Sci Technol, 24: 23-63.
16. Paice MG, Reid ID, Bourbonnais R, Archibald FS, Jurasek L (1993) Manganese Peroxidase, Produced by Trametes versicolor during Pulp Bleaching, Demethylates and Delignifies Kraft Pulp. Appl Environ Microbiol 59: 260-265.
17. Thurston C (1994) The structure and function of fungal laccases. Microbiology 140: 19-26.
18. Iimura Y, Katayama Y, Kawai S, Morohoshi N (1995) Degradation and Solubilization of 13C-, 14C-side Chain Labeled Synthetic Lignin (Dehydrogenative Polymerizate) by Laccase III of Coriolus versicolor. Biosci Biotechnol Biochem 59: 903-905.
19. Eggert C, Temp U, Eriksson KE (1997) Laccase is essential for lignin degradation by the white-rot fungus Pycnoporus cinnabarinus. FEBS Lett 407: 89-92.
20. Eggert C, Temp U, Eriksson KE (1996) The ligninolytic system of the white rot fungus Pycnoporus cinnabarinus: purification and characterization of the laccase. Appl Environ Microbiol 62: 1151-1158.
21. DeLong EF (1992) Archaea in coastal marine environments. Proc Natl Acad Sci U S A 89: 5685-5689.
22. Lane DJ (1991) 16S/23S rRNA sequencing. Nucleic acid techniques in bacterial systematics. Chichester United Kingdom. John Wiley & Sons 115–175.
23. Kaminuma E, Mashima J, Kodama Y, Gojobori T, Ogasawara O, et al. (2010) DDBJ launches a new archive database with analytical tools for next-generation sequence data. Nucleic Acids Res 38: D33-38.
24. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406-425.
25. Wariishi H, Valli K, Gold MH (1992) Manganese(II) oxidation by manganese peroxidase from the basidiomycete Phanerochaete chrysosporium. Kinetic mechanism and role of chelators. J Biol Chem 267: 23688-23695.
26. Mester T, de Jong E, Field JA (1995) Manganese regulation of veratryl alcohol in white rot fungi and its indirect effect on lignin peroxidase. Appl Environ Microbiol 61: 1881-1887.
27. Tien M, Kirk TK (1984) Lignin-degrading enzyme from Phanerochaete chrysosporium: Purification characterization and catalytic properties of a unique H2O2-requiring oxygenase. Proc Natl Acad Sci USA 81: 2280-2284.
28. Harazono K, Kondo R, Sakai K (1996) Bleaching of Hardwood Kraft Pulp with Manganese Peroxidase from Phanerochaete sordida YK-624 without Addition of MnSO(inf4). Appl Environ Microbiol 62: 913-917.
29. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.
30. Ezaki T, Ohkusu K (2006) Identification of Intestinal Flora: DNA Probe and Primers. Journal of intestinal microbiology 20: 245–258.
31. Machczynski MC, Vijgenboom E, Samyn B, Canters GW (2004) Characterization of SLAC: a small laccase from Streptomyces coelicolor with unprecedented activity. Protein Sci 13: 2388-2397.
32. Bollag JM, Leonowicz A (1984) Comparative studies of extracellular fungal laccases. Appl Environ Microbiol 48: 849-854.
33. Yaropolov A, Skorobogatko OV, Vartanov SS, Varfolom-eyev SD (1994) Laccase-properties, catalytic mechanism, and applicability. Appl Biochem Biotechnol 49: 257–280.
34. Wood DA (1980) Production, purification and properties of extracellular laccase of Agaricus bisporus. J Gen Microbiol 117: 327–338.
35. Yaver DS, Xu F, Golightly EJ, Brown KM, Brown SH, et al. (1996) Purification, characterization, molecular cloning, and expression of two laccase genes from the white rot basidiomycete Trametes villosa. Appl Environ Microbiol 62: 834-841.
36. Durrens P (1981) The phenoloxidases of the ascomycete Podospora anserina: the three forms of the major laccase activity. Arch Microbiol 130: 121–124.
37. Molina-Guijarro JM, Pérez J, Muñoz-Dorado J, Guillén F, Moya R, et al. (2009) Detoxification of azo dyes by a novel pH-versatile, salt-resistant laccase from Streptomyces ipomoea. Int Microbiol 12: 13-21.
38. Giardina P, Aurilia V, Cannio R, Marzullo L, Amoresano A, et al. (1996) The gene, protein and glycan structures of laccase from Pleurotus ostreatus. Eur J Biochem 235: 508-515.
39. Giardina P, Palmieri G, Scaloni A, Fontanella B, Faraco V, et al. (1999) Protein and gene structure of a blue laccase from Pleurotus ostreatus1. Biochem J 341 : 655-663.
40. Palmieri G, Giardina P, Bianco C, Scaloni A, Capasso A, et al. (1997) A novel white laccase from Pleurotus ostreatus. J Biol Chem 272: 31301-31307.
41. Abo-State MAM, Khatab O, Abo-EL AN, Mahmoud B (2011) Factors affecting laccase production by Pleurotus ostreatus and Pleurotus sajor-caju. World Appl Sci J 14: 1607-1619.