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

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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 MnCl₂ was not directly oxidized, it was assumed that it did not contain manganese peroxidase. However, when MnSO₄ 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 H₂O₂ 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 (NH₄)₂SO₄, 10 g/L NaNO₃, 5 g/L KH₂PO₄, 1 g/L K₂HPO₄, 1 g/L MgSO₄·7H₂O, 1 g/L NaCl, 1 g/L yeast extract, and 0.5 g/L CaCl₂·2H₂O.

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 24 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 the supernatant was extracted as the extracellular enzyme.

**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'-AGACTACGGGCGGAGGC-3') [22] were used. Sequencing was implemented from the template DNA, and the gene sequence was multiple aligned by ClustalW in DDBJ. In addition, the collected sequence data were not available 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 MnSO₄·H₂O, 120 μL of 100 mM malonic Na buffer fluid (pH 4.5), and 10 μL of 2 mM H₂O₂ 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 (ε₄₆₀ = 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 MnSO₄·H₂O, 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 2mM H₂O₂ 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 20mM MnSO₄·H₂O, 120 μL of 100 mM sodium malonate buffer (pH 4.5) was added to a 96-well microplate, and pre-heated 10 minutes at 45°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 H₂O₂ to 100 mM sodium lactate buffer pH 4.5, 0.1 mM H₂O₂, and 0.5% 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 Mn³⁺-malonate complex at 275 nm [28]. The reaction mixture contained 0.5 ml of 10 mM Mn²⁺ in 100 mM sodium malonate buffer pH 4.5, 0.1 mM H₂O₂, and 0.5 ml culture supernatant and increased absorbance at 310nm could be observed [27].

**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 Diaz 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$_2$O$_2$, 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$_2$O$_2$.

**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$_2$O$_2$, 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.

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).

**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.
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.

**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 sangleri* (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 thezymogram 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 anserina* [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 *Streptomyces* 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 microorganisms, 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 H2O2. By adding lignosulfate to the medium of the strain, incubation of the A480 nm was decreased about 50%.

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