A Novel Laccase from Basidiomycete *Fomes fomentarius VKPM F-1531*

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Abstract. Laccases are a group of copper-containing phenoloxidases, which activity can stimulated by copper ions. The conditions of obtaining, isolation and purification of extracellular laccase of the basidiomycete *Fomes fomentarius VKPM F-1531* were studied. The optimal concentration of copper in the glucose-peptone nutrient medium and duration of the surface-liquid cultivation for the highest yield of the enzyme were established. The efficient synthesis of fungal laccase by the fungus *F. fomentarius VKPM F-1531* requires the optimal concentration of copper in the nutrient medium of 200 mg/l. The highest enzyme activity was achieved on the 14th day of surface-liquid cultivation on glucose-peptone nutrient medium with optimal $CuSO_4$ supplementation and it was more than 6–6.5 times higher than the one obtained on the conventional glucose-peptone medium. The protein fraction that precipitated in the range from 50% to 70% saturation had a laccase activity for 40 times higher than the initial activity in the culture liquid. The molecular weight of the purified laccase determined by the SDS-PAGE was about 60 kDa.

Keywords: Laccase · *Fomes fomentarius* · Basidiomycete · Copper induction

1 Introduction

Laccases (EC 1.10.3.2) are a group of copper-containing phenoloxidase enzymes capable to catalyze the reduction of an oxygen molecule to two water molecules without formation of hydrogen peroxide. At the same time, a wide range of organic substrates can be oxidized, including lignin and its monomers, as well as inorganic compounds [1–3].

Laccases were found in various types of living organisms, but are the most abundant in xylotrophic basidiomycetes belonging to the group of white rot fungi.The functional role of laccases *in vivo* includes, first of all, the lignin degradation [2], as well as participation in the life cycle of fungi: during the development of the fruitful body, pathogenesis, synthesis of pigments, etc. [3–5].

However, stability (including thermal stability and acid resistance), the ability to work without the activity regeneration stage and the ability to increase activity in the

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presence of redox mediators to indirectly oxidize wide range of organic and inorganic substrates make this enzyme suitable and attractive for industrial applications [1–3]. At the same time, the practical application of laccases are annually expanding. Nowadays they possess a great biotechnological potential and can be used: for biopulping and biobleaching, transformation of colorants in the textile industry; for obtaining antitumor and anti-inflammatory drugs; for creating biochips and biosensors to detecte chemical compounds; for the production of adhesives; for design new lignocellulosic constructions and technical materials based in the woodworking and chemical industries; for wastewater and soil treatment etc. [1–3].

It has been reported that copper ions are widely known inducers for laccase synthesis, and its production is regulated at transcriptional level [5, 6]. There are several studies on laccase synthesis stimulation by optimal copper concentrations in the nutrient media [5–12]. For example, it has been shown that the optimal concentration of copper for the synthesis of laccases for micromycete *Trichoderma harzianum WL1* and the actinomycete *Streptomyces psammoticus* is in the medium is 2 mM; for ascomycete *Paraconiothyrium variabile* - 10 mM [7–9].

Fungus *Fomes fomentarius VKPM F-1531* is an effective producer of extracellular phenol oxidases during the liquid-state fermentation [11]. The current investigation focuses on the studying the effect of various copper concentrations on production and activity of laccases secreted by basidiomycete *Fomes fomentarius*, as well as to obtain a highly active purified laccase and to study a number of its biochemical properties.

2 Materials and Methods

2.1 Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Technique

The strain of the fungus *F. fomentarius* was isolated into pure culture from the fruiting body of *F. fomentarius* (tinder fungus), which has grown on decayed wood samples, and further deposited at All-Russian National Collection of Industrial Microorganisms.

Basidiomycete was cultivated under liquid-state conditions on a modified glucose-peptone medium (HPS) with the media compositions of, g/l: glucose - 20.0; peptone - 3.0; $KH_2PO4*12H_2O$ - 1.0; $K_2HPO_4*2H_2O$ 1.0; $MgSO_4*7H_2O$ 0.25; $CuSO_4*7H_2O$–0.2 2 at a temperature of 22 ± 1 °C for 14 days under static conditions. The initial pH was 6.1 ± 0.1. Each flask was inoculated with 2 agar plugs (1 × 1 cm) cut from the growing 7–9 day old wort agar culture of *F. fomentarius VKPMF-1531*.

The effect of copper ions on the laccase activity of the *Fomes fomentarius VKPM F-1531* was studied using the liquid-state cultivation on glucose-peptone medium (HPS) in the presence of copper ions (CuSO4) in the concentration range 0–300 mg/l in 50 mg/l steps. The solutions of copper sulfate and glucose were added aseptically into the medium after sterilization at the start of the cultivation.

The fungus was cultivated under static conditions without aeration and stirring; temperature during the first three days was 30 ± 1 °C, the next day - 22 ± 1 °C, without lighting (in the dark) for 18 days.

2.2 Enzyme Assay and Protein Content Determination

Laccase activity (U/ml) was determined on a PE-5400 UV spectrophotometer according to the oxidation rate of 10 mM pyrocatechol (ε 740 mM/cm^{-1}) in 0.1 M Na-acetate buffer pH 5.4 at a temperature of 22 ± 1° C and wavelength 410 nm. The unit of activity was determined as the amount of the enzyme catalyzing the oxidation of 1 μmol of the substrate for 1 min. Protein concentration was determined by the Bradford method using bovine serum albumin as a standard.

2.3 Isolation and Purification of the Extracellular Fungal Laccase

Fungal mycelium was removed and 14-day culture liquid of *F. fomentarius* F-1531 obtained by centrifugation for 60 min at 6000 g in a Hettich Universal 320R centrifuge (Germany) was subjected to a two-step ammonium sulfate fractionation.

At first, to remove ballast proteins, ammonium sulfate was added to the supernatant up to 50% saturation and kept at +4 °C overnight, than it was centrifuged for 30 min at 12000 g and the precipitate has been taken away. In the supernatant, the concentration of ammonium sulfate was increased to 70%; after centrifugation at 12000 rpm for 20 min, the precipitate was collected. The resulting fraction was dissolved in a small volume of 0.1 M Na-acetate buffer, pH 5.4, and was dialyzed against 0.02 M Na-acetate buffer. Protein concentration and laccase activity were analyzed in all fractions.

2.4 Molecular Weight Analysis Using Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) Technique

Denaturing electrophoresis was carried out on a 12% polyacrylamide gel according to the method of Laemmli on a Mini-PROTEAN device (BioRad, USA). The mixture of marker proteins Precision Plus Protein ™ All Blue Prestained Protein Standards (BioRad, USA) was used as molecular weight standards. Proteins were visualized by staining with Coomassie (brilliant blue R-250) and gel has been photographed.

The data presented are the average of the results of three parallel experiments carried out at least in three chemical replicates. For evaluation of the results we used the standard error method using Microsoft Office Excel 2010.

3 Results

3.1 Influence of Copper Ions on Laccase Activity of *F. fomentarius* F-1531

As a result of studying the dynamics of the laccase activity of *F. fomentarius F-1531* Culture liquid, which has been obtained in the presence of copper ions in the concentration range of 0–300 mg/l on a liquid HPS medium, the maximum activity was reached on day 14 in the presence of Cu^{2+} at a concentration of 200 mg/l (Fig. 1).

The laccase activity in the culture liquid on the HPS medium with $CuSO_4$ (200 mg/L) was in 6 times higher than the activity on a medium without copper sulfate (Fig. 2).

As a result, the optimal concentration of copper ions to stimulate the biosynthesis of laccase by the fungus *F. fomentarius F-1531* was its content in the HPS nutrient medium of 200 mg/l. In this case, the maximum level of laccase activity (14–17 U/ml) was reached on 14 day.

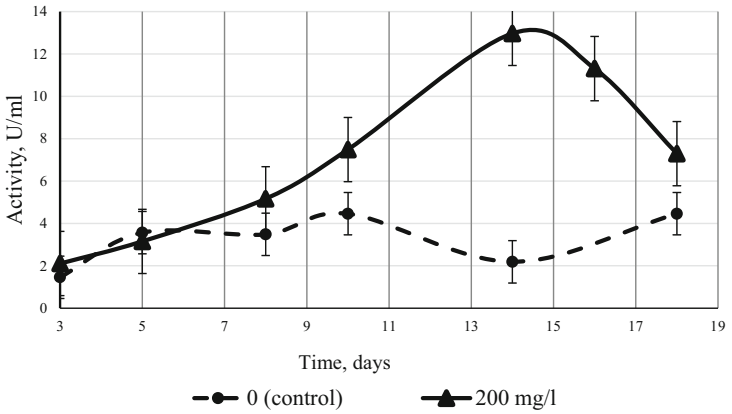

Fig. 1. Time-course of laccase production by *F. fomentarius F-1531* in a liquid HPS medium for 18 days: in the presence of $CuSO_4$ (200 mg/l) and without CuSO4 (control).

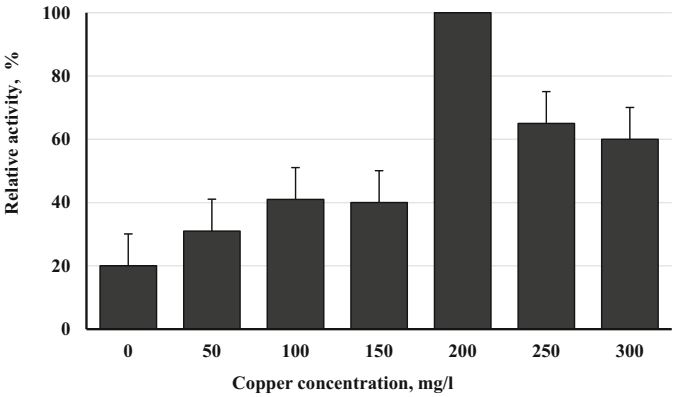

Fig. 2. Profile of phenoloxidase activity in 14-day culture liquid of fungus *F. fomentarius F-1531* in the presence of various concentrations of $CuSO_4$ in culture medium

3.2 Purification *F. fomentarius F-1531* Laccase at the Optimum Concentration of Copper in the Nutrient Medium

Isolation and purification of laccase from 14-day copper supplemented culture broth (200 mg/l Cu^{2+}) of the fungus *F. fomentarius F-1531* was carried out by the ammonium sulfate precipitation method.

As a result of SDS-PAGE electrophoresis and following staining gel by Coomassie R-250, in the purified sample was detected a major laccase protein with a mobility corresponding to a molecular weight of about 60 kDa (Fig. 3).

The protein fraction that precipitated in the range from 50% to 70% saturation had a laccase activity of up to 560 U/ml (for pyrocatechol), which is about for 40 times higher than the initial activity in the culture liquid.

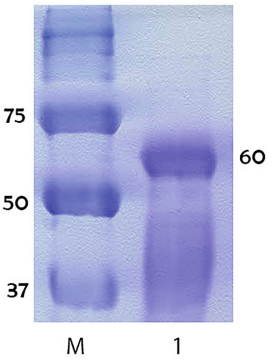

Fig. 3. Electrophoregram of purified enzymes from *F. fomentarius F-1531*: M - protein standards (mass, kDa); 1 - purified enzymes

4 Discussion

To increase the yield of oxidases, including laccases, during the microbial growth, using various inducers, which promote effective biosynthesis are processes that has been reported [1–4]. Copper ions in culture media can serve as inducers, including for the xylotrophic basidiomycete *Fomes fomentarius* [13].

The study of oxidase activity during the *F. fomentarius* liquid-state fermentation with various copper concentrations (0–300 mg/l) for 18 days was found out the time-course of oxidase production and indicated that the maximum laccase activity was reached with $CuSO_4$ (200 mg/l) at 14 day.

The efficiency of oxidase enzymes biosynthesis in the presence of copper was in 6–6.5 times higher than in a medium without copper, as shown on Figs. 1 and 2. This phenomen indicates stimulation of copper-containing oxidase enzymes synthesis in culture broth of fungus *F. fomentarius* [10].

The enzymes were isolated from cultural liquid and purified by ammonium sulfate precipitation. The investigation of salting out the enzymes by the stepwise addition of ammonium sulfate between 0–70% saturation (with the interval of saturation 10–15%) showed that the fraction precipitated between 50–70% of saturation has the highest laccase activity.

The molecular weight of the purified laccase determined by the SDS-PAGE was about 60 kDa which corresponds with the range of 50–90 kDa for the majority of the fungal laccases according to the data from previous studies conducted by other authors [1–4, 13–15]. For example, the purified laccase of the fungus *Fomes fomentarius* obtained under the conditions of solid-state fermentation had a molecular weight of 51 kDa [13].

The combination of the results of gel electrophoresis, an absence of tyrosinase activity, inhibition of the enzymatic activity by EDTA and the capability to oxidize a wide range of phenol derivatives indicates on the laccase nature of the isolated enzyme with high probability [13, 15].

5 Conclusions

Thus, the efficient synthesis of fungal laccase by the fungus *F. fomentarius VKPM F-1531* requires the optimal concentration of copper in the nutrient medium of 200 mg/l. The highest enzyme activity was achieved on the 14^{th} day of surface-liquid cultivation on HPS medium with optimal $CuSO_4$ supplementation and it was more than 6–6.5 times higher than the one obtained on the conventional glucose-peptone medium.

Using the ammonium sulfate fractionation method made it possible to concentrate the enzyme with molecular weight of 60 kDa and laccase activity being 40 times higher than in the original cultural liquid.

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