JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2016. Vol. **10**(2), p. 1613-1617

Laccase: Production and Purification by *Pseudomonas aeruginosa*

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(Received: 18 March 2016; accepted: 20 May 2016)

Laccase is belonging to the oxidases of blue multi-copper and will be participating in polymer degradation, monomer cross-linking and aromatic compounds' ring cleavage. This is distributed widely in fungi and higher plants. This is also present in Deuteromycetes, Basidiomycetes and Ascomycetes and also much abundant in white-rot fungi which are lignin-degrading. Also it is used in synthesizing organic substances, having phenols and amines as typical substrates with reaction products as oligomers and dimers which are derived out of coupling from radical reactive intermediates. In recent time, these enzymes are gaining application in fields like paper and pulp, food and textile industry. Also of late it is used in biofuel cells, biosensors design, and also a tool for medical diagnostics and an agent for bioremediation in cleaning up certain explosives and pesticides in soil as well as herbicides. This present work is focusing on screening, the process optimization in achieving maximum production with partial purification in extracellular laccases by Pseudomonas aeruginosa. Purification of laccase through filtrate of bacterium culture is achieved as a greatest action after the utilization of DEAE-Sephadex Affinity chromatography, chromatography (Sephadex) of Gel filtration and precipitation of ammonium sulphate. The experimental results are showing better performance in purification and production of laccase through Pseudomonas Aeruginosa.

Keywords: *Laccase, Pseudomonas aeruginosa,* Enzyme Activity, Optimization, Purification.

In recent times, enzymes are gaining greater importance in the Industries and laccases has been one of them that are being widely seen in nature. Among enzymatic systems[1] the most studied and the oldest will be Laccases. These are enzymes containing 15– 30% of carbohydrate and having mass of molecule as 60–90 kDa. They are copper having (EC 1.10.3.2) oxygen oxidoreductases: 1,4-benzenediol found normally in higher microorganisms and plants. These are oxidases of glycosylated polyphenol containing 4 copper ions in a molecule that are carrying out one electron oxidation in phenolic and related compound, also it can reduce water from oxygen[2,3]. When a laccase oxidizes a substrate, it will lose one electron and forms usually the free radical that might further undergo oxidation or reactions of non-enzymatic kind including polymerization, hydration and disproportionation[4]. These types of enzymes will be polymeric and contains generally one of each type 3, type 2 and the type1subunit or Centre of copper, wherein the type 3 and type 2 remains close together in forming clusters of tri-nuclear copper. However, during latest work in few years the applications of laccase include oxygen cathode development in cells of biofuel, the kraft pulp bio-bleaching, synthetic dyes of decolonization, synthesis of organic, cleaning of laundry, biosensors, bioremediation, immunoassays labelling, drug analysis, wines and

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juices clarification, laccase fungicidal design and preparations for bactericidal. The Laccases have been distributed widely in insects, fungi, higher bacteria and plants. In the plants, the laccases will be found in turnip, cabbages, potatoes, apples, pears, and various other vegetables. Theseare isolated from Deuteromycteous; Ascomyceteous and the Basidiomycetous fungi having more than sixty fungal strains belonging to it[3]. The fungi of white-rot Basidiomycetes can degrade efficiently the lignin available in comparison with Deuteromycetes and Ascomycetes which are oxidizing compounds of phenolic to yield phenoxy radicals along with quinines[5]. The Laccases are playing very important part in pulp and paper industry, food industry, the textile industry, cosmetics, synthetic chemistry, biodegrading phenolic environmental pollutant, removing endocrine disruptors and soil bioremediation[2]. The Laccases could be categorized as two groups depending on their source as either fungal or plant. But however these laccases (diphenol oxidases) were found in insects and bacteria. This enzyme belongs to copper protein containing four ions of metal categorized as three kinds commonly known as T3, T2 and T1. The blue color in the enzyme is due to T1 copper and its absorbance is characteristically about 610nm. The characteristics signal of EPR is generated by T2 copper but detecting it spectromatically is not possible, whereas the diamagnetic T3 copper is bi-nuclear. The main objective of this study is developing a purification procedure for extracellular laccases found in Pseudomonas aeruginosa, and determining individual catalytic and physicochemical properties of the enzymes and studying the property of kinetics of these enzymes.

Related study of laccase

Sources and mechanism of laccase

The Laccase is found generally in fungi and higher plants but in a recent study it is found in certain bacteria like Marinomonas mediterranea S.lavendulae, and S.cyaneus[6–8]. The laccases will appear more in the fungi, than higher plants. The Basidiomycetes like Phanerochaete chrysosporium, Lenzites, betulina and Theiophora terrestris[9], and white-rot fungi[10, 11] like Trametes versicolour[14], Pleurotus ostreatus[13], and Phlebia radiate[12] will also be producing laccase. Many species of Trichoderma like T. longibrachiatum[16] and T. harzianum, T. atroviride[15] are sources for laccases. Laccase taken from Monocillium indicum has been characterized as first laccase showing peroxidase activity as taken from Ascomycetes. Pycnoporus cinnabarinus is producing laccase in the form of ligninolytic enzyme, whereas Pycnoporus sanguineus is producing laccase in the form of phenol oxidase. The laccase in plants is playing a role for lignifications while in fungi its role is in implicating sporulation, delignification, and formation of fruiting body, plant pathogenesis and pigment production[19, 20]. The catalysis of laccase is occurring because one oxygen is getting reduced into water that is accompanied with oxidation of single electron having broad range of compounds in aromatic category that includes aromatic amines[9], monophenols substituted with methoxy and polyphenol[10]. Laccases is containing four atoms of copper termed as Cu T1 (where this reducing substrate is binding) and cluster of T3/ T2 trinuclear copper (electron transferred from copper of type I to the copper of type II and copper of type III trinuclear cluster and also oxygen reduction into water in trinuclear cluster)[3]. The four ions of copper are categorized as (T3) Type 3, (T2) Type 2 and (T1) Type 1. The three types could be distinguished with the use of Electronic Paramagnetic Resonance (EPR) and UV/visible spectroscopy.

Properties of laccase enzyme

The Laccases are usually tetrameric glycoprotein, monomeric and dimeric. Glycosylation is playing an essential role in susceptibility of proteolytic degradation, secretion, copper retention and thermal stability. When purified, laccase enzymes could demonstrate considerable amount of heterogeneity. The composition of glycoprotein and Glycosylation content varies as the medium composition is growing.

Applications of laccase

The Laccase is essential because it can oxidize both the nontoxic and toxic substrates. It is used in industry of food processing, textile industry, chemical industry, industry of wood processing, and pharmaceutical industries. This enzyme will be very specific, sustainable ecologically and can be proficient catalyst.

Proposed work

Overview

The Laccase is polyphenol oxidase containing copper which can act on broad ranges of substrates. This present work is focusing on optimization of process parameters and screening, to achieve maximum purification and production of extracellular-laccases through Pseudomonas aeruginosa.

Laccase production

The production of Laccase through Pseudomonas aeruginosa is confirmed with use of a media of Nutrient agar containing guaiacol 0.01% as an indicator compound. The reddish brown color zone is observed around colonies after the incubation @ 32ºC for a period of 6 days.

Optimization of culture conditions for enzyme production

This is done through varying conditions of culture that is effecting laccase production and determining optimum conditions in the laccase production from the Pseudomonas aeruginosa. The Production medium will be used in analysis.

Effect of temperature

To determine temperature's effect on the laccase production, production medium is inoculated in one ml culture Pseudomonas aeruginosa while incubating at the temperatures viz. 41, 33, 53 and 22ºC for a period of 24 - 48 h in pH 8. The Laccase assay is measured @ 520 nm.

Effect of pH

Effect of the pH on the laccase production is carried out through incubating a flask having 100 ml media of production inoculated in one ml culture Pseudomonas aeruginosa in different pH 9, 12, 7, and 5 for a period of 24 48 h in optimized temperatures. The Laccase assay is measured @ 530 nm.

Laccase activity

Highest activity of laccase is observed in (0.0392 U/ml) 41ºC when slight decrease of enzyme activity is observed in (0.0389 U/ml) 52ºC. This present study will be contrasting with another study which was done by Ding *et al*., (2012); wherein influence of the temperature on laccase activity is studied and then reported as 60°C is optimal temperature aiding laccase activity. Whereas Adejoye and Fasidi (2009) have reported that the maximum of laccase activities is at 28°C (51.5 ± 2.21 U/ml). The incubation temperature is playing a very essential role in metabolic activities in microorganisms. This optimal temperature in laccase is differing greatly from the one strain to another. The decrease and increase in temperature is leading to gradual decrease of protein products. In this present investigation there has been some gradual increase of enzyme activities ranging from 41° to 22 C and @ 52°C enzyme activity starts decreasing.

Production and partial purification laccase

For production of laccase, 12millilitre Nutrient broth is inoculated in loopful culture Pseudomonas aeruginosa for incubating in 32ºC @ 120 rpm. This culture is served as the seed culture for inoculating further after 24 h. A 100ml sterilized production media is prepared containing the carbon at optimized 2.5% and the nitrogen source having optimized pH in accordance tocomposition as stated by Unyaayar *et al*.,(2005) and sterilized cum inoculated in seed culture of 6% (v/v) and then incubated in optimized temperature with interval of optimized time. This redissolved protein is applied to column of Sephadex G-100, which is equilibrated with the buffer. This column is subsequently washed in equilibration buffer of

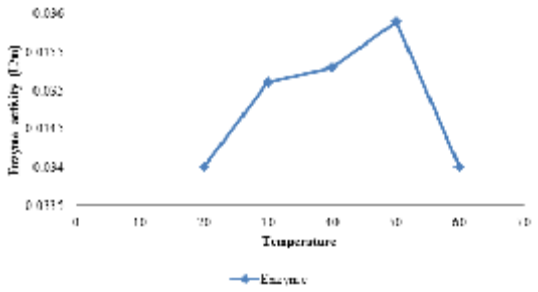

Fig. 1. Effect of temperature on laccase activity

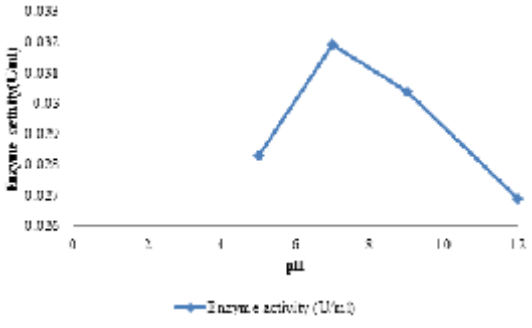

Fig. 2. Effect of ph on laccase activity

790 ml, and these enzyme fractions are eluted with the gradient of linear concentration of 0.6 M NaCl in same buffer. The fractions having laccase activity are collected, subjected to dialysis using membrane and then loaded onto A-50 DEAESephadex. This column is elated and equilibrated with a buffer at the flow rate 0.8 ml/ min. The fractions having laccase activities are concentrated after collection.

Determination of molecular mass

The method of Osborn and Weber is used to check homogeneity in enzyme preparation through SDS-PAGE. This separating gel is acrylamide 13% in 0.385 mol/L Tris·HClhaving buffer of pH 8.9 and the stacking gel is acrylamide 6% in the 0.067 mol/L Tris·HClhaving buffer 6.9. A weight marker of molecules is attained from the Bangalore Genei Pvt. Ltd. (India Bangalore) Gel and is run at the constant current of 22 mA.

Characterization of laccase

Optimal pH and temperature

The optimal pH of laccase enzyme activities were determined using the 12 ¼mol·L-1 ABTSas substrate in the buffer of 52 mmolL-1 sodium acetate at various pH (5.2 to 2.2) and the 12 ¼mol·L-1 ABTS in the buffer of 53 mmol·L-1 sodium phosphate at the various pH (10.00 to 6.0). The pH effect on enzyme activities and the stability is measured after the incubation at the different pH in 30 °C. The optimal temperatures for the laccase activities were determined at various temperatures (80 °C, 70 °C, 60 °C, 50 °C, 40 °C, 30 °C, 20 °C) by incubation of purified laccase in 10 ¼mol·L-1 ABTS with a buffer of 50 mmol·L-1 sodium acetate having (pH 4.5) for 2 hours stable heat. The stability heat in purified laccase is determined by the incubation of purified enzyme over 15 minute periods at various temperatures from 70 °C to 40 °C. After the incubation, enzyme activity is decided by the use of ABTS in 0.05 mol·L-1 acetate buffer.

RESULTS AND DISCUSSION

Effect of temperature on laccase activity

The Effect of the temperature on the laccase activity Highest activity of laccase is observed in (0.0359 U/ml) 50ºC (Fig:1). This present study will be contrasting with study done by Ding *et al*., (2012) wherein influence of the temperature on the laccase activities were reported after a study at 60°C as optimum temperature in laccase activity. The incubation temperature is playing an essential role in metabolic activities in microorganisms. Optimum temperature in laccase is differing greatly from the one strain to another. The decrease and increase of temperature will lead to gradual decrease of protein products. And in this present investigation there is gradual increase of enzyme activity ranging from 40 -20°C and @ 60°C enzyme activity is found decreasing.

Purification of laccase

Purified laccase is determined using SDS PAGE which is monomeric protein. This single band which is corresponding to relative 45 kDa and the marker of standard protein is showing it as the single polypeptide-protein. The suggestion by many of the reports is that molecular weight in protein is ranging from 80 – 30 kDa.

Effect of pH on laccase activity

A highest activity of laccase is observed in (0.0319 U/ml) pH 7, while the lowest activity of laccase is observed in (0.0269 U/ml) pH 12 (Fig:2). This present study will be contrasting with study done by Ding *et al*., (2012) wherein the highest activity of laccase is reported in pH 3. This maximum release in laccase activity is at 0.25 U/ml of Ganoderma sp. as reported by Sivakumar *et al*., (2010) in pH 6.0.

CONCLUSION

The Laccases are versatile enzymes that can act as catalysts in oxidation reactions that are coupled in reduction of four-electron to molecular\ oxygen of water. They constitute multicopper enzymes that are broadly distributed in the fungi and higher plants. They tend to detoxify and decolorize the effluents of industries and help the treatment of wastewater. They can act both on the nonphenolic and phenolic compounds of lignin-related and also environmental pollutants that are highly recalcitrant which helps researchers in placing them in different biotechnological applications. This is effectively used at pulp and paper industries, the xenobiotic degradation, bioremediation, textile industries and acting as a biosensor. In this research the laccase is purified and produced through pseudomonas aeruginosa. The Laccase from the Pseudomonas aeruginosa is

purified after extraction and has shown stability in pH 8 along with temperature at 50°C for a period of 2.5 hour. This enzyme which is produced is having a wide working range of pH and has shown even better thermo-stability. It is observed lower the value of Km and the higher of Vmax value is indicating the enzyme is having higher affinity for its substrate as well as higher catalytic activities as compared to other reported laccase.

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