Effect of Natural Phenolic and Lignin rich Inducers on the Production of Laccases by *Streptomyces griseus* MTCC 4734

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Abstract

The production of laccase by *Streptomyces griseus* grown in submerged cultures in the presence of different natural phenolic and lignin rich sources was studied in comparison to aromatic inducers which are traditionally used. Among the different inducers studied, *Spirulina* was shown to enhance the laccase production to a greater extent more than the aromatic inducers (190 Ug^{-1} in the presence of *Spirulina* and (132 Ug^{-1}) in the presence of Tween 80). Bajra and grapes also showed a significant rise in laccase activities. The optimum pH and temperature for the partially purified enzyme from this strain were found to be 4.5 and 30^{o}C. The enzyme obtained was stable at high pH and temperatures.

Keywords Laccase, natural inducers, *Spirulina, Streptomyces griseus*

Introduction

Laccases (p- diphenol : oxygen oxidoreductase ; EC 1.10.3.2) belong to the family of blue multicopper oxidases , which catalyze the one electron oxidation of substrates coupled to four electron reduction of oxygen to water (1). These are known to catalyze the oxidation of a wide variety of substrates including phenolic compounds and aromatic amines. They are also involved in lignin degradation and plant pathogenesis.

Laccases have been found in higher plants and numerous fungi. Laccase activities have also been demonstrated in many bacteria and a few species of Streptomyces such as *S.lavendulae* (2), *S.viridosporus* (3), *S.cyaneus* (4) *and S.psammoticus* (5).

Currently, the catalytic properties of laccases are being exploited for a range of biotechnological applications like pulp bleaching, dye decolorization (6) and detoxification of environmental pollutants (7). The ever-increasing demand for this potential enzyme in the industrial sector requires large quantities of this enzyme from microbial sources. Hence, any attempt to increase their production is of considerable interest. The production of laccases can be increased by the addition of inducers which include a variety of aromatic and phenolic inducers.

The purpose of the present study is to check for the effect of different types of natural inducers which include phenolic and lignin rich along with known aromatic inducers.

Materials and Methods

Microorganism and its maintenance

Streptomyces griseus MTCC 4734 used in the present study is an aerobic, filamentous actinomycete purchased from Microbial Type Cell Culture (MTCC), IMTECH, Chandigarh, India. It was isolated from soil (Tripathi, CDRI, Lucknow). The culture was grown and maintained on medium containing (g L^{-1}): glucose – 4.0, yeast extract- 4.0, malt extract – 10.0, $CaCO_3$ – 2.0, agar – 12.0, dis. H_2O – 1L, pH = 7.2 (adjusted with KOH) and temperature 30^{o}C.

Primary screening on solid media

To detect the ability of the organism to produce lignin modifying enzymes, indicator compounds were preferred (8). The traditional screening reagent, tannic acid and synthetic phenolic reagent, guaiacol were added in solid media in the concentrations 0.5% and 0.01% respectively. Guaiacol was added to the media before autoclaving and tannic acid was autoclaved separately before addition to the media.

Cultivations in liquid media

For inoculum preparation, the culture was aseptically transferred into the inocculum media and was allowed to grow for 48hrs at 30^{o}C. Submerged fermentation in 500ml Erlenmeyer flasks was carried out, in the presence of different inducers, under shaking conditions (120 rpm) for 8 days at 30^{o}C.

Effect of inducers

1. Aromatic inducers: Various aromatic compounds such as guaiacol (2-methoxy phenol), pyrogallol (benzene 1, 2, 3 triol), xylidene and tween 80 (9); prepared in 95% ethanol were added at a concentration of 1mM. The inducers were added to the flasks just before inoculation.
2. Natural inducers: (a) Phenolic inducers: Concentrated wet mass of *Spirulina*, whole fruits of green pea, green beans, apple, black grapes and onion bulb (purchased from the local market) were added to the media at a concentration of 8g% before sterilization and autoclaved at 15 lb pressure for 15min.
   (b) Lignin rich inducers: Lignin powder (Hi Media), barley and bajra (Pearl millet) powders were also added to the media at a concentration of 8% before sterilization and autoclaved at 10 lb pressure for 20min.

Enzyme extraction

The contents of the flasks were mixed thoroughly and centrifuged at 10,000rpm for 15min at 4^{o}C.The supernatant was collected and used for the enzyme assay and partial purification.

Enzyme assay

The reaction mixture used to determine laccase activity consisted of 66.6mM sodium malonate at pH = 4.5, 1.3mM 2, 6- dimethoxy phenol and 500 µlt of sample. Absorbance changes at 465nm and 30^{o}C were monitored for 5min (Molar absorption coefficient = 10,000 M^{-1} cm^{-1}) (10).
One activity unit was defined as the amount of enzyme that oxidizes 1µmole of the substrate (DMP) per minute.

Partial purification

The supernatant collected after centrifugation was used for fractionated precipitation by ammonium sulphate between 30% to 50% saturation. The precipitate was suspended in 100mM phosphate buffer, pH = 6.8 and centrifuged at 10,000rpm for 15min at 4 ^{o}C; many times (11).

Laccase characterization

The partially purified laccase was used in SDS-PAGE. 20µlt of the sample was mixed with 10µlt sample buffer (containing SDS+ bromophenol blue+ glycerol) and boiled for 5min.This sample along with molecular weight markers was loaded and run on a 12% SDS-PAGE gel. Protein bands were visualized by staining with coommassie brilliant blue R250.

Effect of pH and temperature was observed using the partially purified enzyme.

Total protein estimation

For total protein estimation, the media was centrifuged at 3,000rpm for 15min at 4^{o}C. The supernatant was discarded and the pellet was washed with distilled water and resuspended in 100mM phosphate buffer, pH = 6.8. This was then sonicated for 2min and centrifuged again at 10,000rpm for 15min at 4 ^{o}C. The supernatant was collected and used for protein estimation by Bradford's method.

Results

Primary screening

A reddish brown halo (indicative of positive reaction) was observed around the colonies in the presence of guaiacol. No reliable results were obtained in tannic acid indicator plates.

Effect of inducers

Results implied that *Spirulina* (190 Ug^{-1}) and black grapes (70 Ug^{-1}) enhanced the laccase production more than other polyphenolic inducers. And bajra - Pearl millet (*Pennisetum glaucum*) (63 Ug^{-1}) increased the laccase production among lignin rich inducers. Among aromatic inducers, Tween 80 (132 Ug^{-1}) was found to enhance laccase production by this strain.

The laccase productions by other inducers were found to be less than those mentioned above (table 1).

Table 1: Laccase activities from S. griseus in the presence of different aromatic and natural inducers

Aromatic inducers	**Natural inducers**	
	Phenolic inducers	**Lignin rich inducers**
Tween 80 132 Ug^{-1} Guaiacol 68 Ug^{-1} Pyrogallol 80 Ug^{-1} Xylidene 55 Ug^{-1}	*Spirulina* 190 Ug^{-1} Black grapes 70 Ug^{-1} Green pea (*Pisum sativum*) 42 Ug^{-1} Green Beans 41 Ug^{-1} Apple (*Malus domestica*) 32 Ug^{-1} Onion (*Allium cepa*) 55 Ug^{-1}	Bajra - Pearl millet (*Pennisetum glaucum*) 63 Ug^{-1} Barley *(Hordeum vulgare*) 29 Ug^{-1} Lignin 176 Ug^{-1}

Laccase characterization:

On SDS-PAGE gel, the enzyme showed only a single band corresponding to a molecular weight of 36 KDa.

The optimum pH and temperature for laccase produced by this strain were observed as 4.5 and 30^{o}C respectively though the enzyme was found to be stable at high pH (8.0) and temperatures (80^{o}C). The specific activity after ammonium sulphate precipitation was found to be 0.403 (Umg^{-1}).

Discussion

Laccases exhibit an extraordinary substrate range which is the reason for their attractiveness for many biotechnological applications. Recent work has shown the use of some lignocellulosic wastes for the production of laccases. The utilization of cheap or waste substrates for the production of enzymes is of prime importance since it contributes to the economy of industrial production. Hence this study focuses on the utilization of cheap lignin and phenol sources which are locally available. Polyphenols are found in a wide array of phytochemical-bearing foods which includes legumes, fruits such as apples,

Raspberries, black grapes, cherries, pears and vegetables like broccoli, cabbage, onion, etc. Similarly, *Spirulina* is known to contain phenols in excess. Lignin rich sources include wheat bran, flax seeds, cereals like millet, barley and fruits like kiwi, etc. Consistent with this data the inducers chosen from above induced the production of laccases. Though the enzyme activities obtained were low, these inducers still enhanced the production. Hence, this work shows the possibility of using new and natural sources for the production of laccase.

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