Production and optimization of *Bacillus subtilis* laccase by solid state fermentation of agro-byproducts

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Abstract: Laccases (E. C. 1.10.3.2, p-diphenol: dioxygen oxidoreductase) are a group of multi-copper containing enzymes that catalyze one-electron oxidation of phenolic compounds with concomitant reduction of oxygen to water. Laccases are increasingly being used in food industry for production of cost-effective and healthy foods. To sustain this trend widespread availability of laccase and efficient production systems have to be developed. Laccase producing bacteria were isolated from soil samples collected from different regions of Allahabad. Samples were screened and identified for better laccase producing bacteria on nutrient agar plate containing guaiacol.

Introduction

Laccases (E.C. 1.10.3.2, p-diphenol: dioxygen oxidoreductase) are a group of multi-copper containing enzymes that catalyze one-electron oxidations of phenolic compounds with concomitant reduction of oxygen to water. In structural terms, these enzymes can be either monomeric or multimeric glycoprotein’s, which may exhibit additional heterogeneity because of variable carbohydrate content or differences in copper content. There are many reports on the purification and biochemical characterization of fungal laccases. The key characteristics of laccase are the standard redox potentials of its redox centers. Laccases are currently seen as very interesting enzymes for industrial oxidation reactions, because they are capable of oxidizing a wide variety of substrates. Therefore Laccases find wide commercial applications within food industry, pulp and paper industries, textile industry, synthetic chemistry, cosmetics, and soil. Bioremediation and biodegradation of environmental phenolic pollutants and removal of endocrine disruptors (Cuoto et al., 2006). Laccases are widely distributed in fungi, higher plants, bacteria and insects. More than 60 fungal strains, belonging to various classes such as Ascomycetes, Basidiomycetes and Deuteromycetes, have been demonstrated to produce laccase (Gianfreda et al., 1999). Recently some bacterial laccases have also been characterized from *Azospirillum lipoferum* (Givaudan et al., 1993), *Bacillus subtilis* (Martins et al., 2002), *Streptomyces lavendulae* (Suzuki et al., 2003) and *S. cyaneus* (Arias et al., 2003).

Biological agents such as microorganisms and enzymes have received great attention because of their potential to remove pollutants from environment without harsh side effect. Laccase allows them to oxidize a wide range of compounds, which makes them suitable for biotechnological and environmental applications (Saparrat et al., 2002). However high amounts of the enzyme are needed for these applications as well as to study and understand its properties and roll in lignin biodegradation. In this way researches have been developed in order to increase laccase production by searching for new sources of it (Min et al., 2001) and by screening for inducers like amino acids (Dhawan and Kuhad., 2002) aromatic compounds (Eggert et al., 1996) additional carbon sources and copper (Saparrat et al., 2002) can increase the production of laccase.

The present work was directed towards the characterization of laccase producing bacteria isolated from soil. Attempts were made to optimize production parameters of laccase produced from *Bacillus subtilis*. And attempts were also made to partially purify laccase enzyme.

Material and Methods

Isolation and screening of laccase producing bacteria from soil: Soil samples were collected from different places of Allahabad (U.P), India. These soil samples were serially diluted up to 10-6 and spreaded over the solid nutrient agar plates and incubated for 24 hrs at 37 ± 1°C the colonies grown was screened for laccase activity. Laccase activity was visualized on NA plates containing 0.02% guaiacol (2-methoxyphenol) as a reddish brown zone, due to oxidative polymerization of guaiacol by the action of laccase (Kiiskinen et al., 2002).

Identification of isolates: The isolates were identified on the basis of cultural, morphological and biochemical characters as given in Bergey’s Manual of Systematic Bacteriology (Holt et al., 1984).

Substrates and its pretreatment: In the present study, the different substrates such as Orange baggase, Banana peels, Paper cuttings and Apple peels were used. Substrates were sliced, spreaded on

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trays and oven dried at 70 ±2°C for 24 hrs. The dried slices were
grounded and sieved through standard mesh sieves to obtain
particles ranging in size from 200-2400 μm, and stored in polyethylene
bags at room temperature (30±2°C) until use.
**Production medium and conditions for control:** The bacterial
strain was grown in 250 ml Erlenmeyer flasks containing each of
various substrates (Orange baggase, Banana peels, Paper cuttings
and Apple peels) and moistened with mineral salt solution [g-1:
K₂HPO₄ 2.75; KH₂PO₄ 2.25; (NH₄)₂SO₄ 1; MgCl₂.6H₂O 0.1;
NaCl 0.2; FeCl₂.6H₂O 0.02; CaCl₂ 0.01; pH 7.0]. It had an initial
moisture content of 75% and was autoclaved at 121°C for 60 min,
cooled to about 30°C and inoculated with an inoculum of 3 ml. The
contents of the flasks were mixed thoroughly to ensure uniform
distribution of the inoculum and were incubated at 35±1°C for 5
days. The enzyme was extracted and assayed (Krishna and
Chandrasekaran, 1999).

**Enzyme extraction:** The enzyme from fermented substrate medium
was extracted with 0.01 M phosphate buffer (pH 7.0) applying a
substrate: buffer ratio of 1:10 employing a simple contact method
(Krishna and Chandrasekaran, 1996). The enzyme extract was
centrifuged at 8000 g for 20 min at 4°C. The clear supernatant
obtained was used for the purification process.

**Enzyme assay:** Laccase activity was determined by measuring
the oxidation of guaiacol at 530 nm. Enzyme activity was measured
in U/ml which is defined as the amount of enzyme catalyzing the
production of one micromole of colored product per min per ml.
(Jhadav et al., 2009).

**Optimization of production parameters:** The various process
parameters that influence the enzyme production during SSF were
optimized over a wide range. Process parameters such as pH,
incubation temperature, incubation period, inoculum size, moisture
content, copper concentrations, were optimized for maximum enzyme
production in triplicates.

**Results and discussion**

**Isolation:** One laccase-producing bacterial strain was isolated
from soil samples collected. This strain showed a reddish brown
zone around its colonies on the screening agar medium containing
0.02% guaiacol. The selected strain was identified as *Bacillus subtilis*
(isolated from saw mill soil) by its cultural, morphological and
biochemical characteristics.

**Selection of best substrate:** In the present study potato peel,
apple peel, orange bagasse and paper cuttings were used as
substrate. Among all the substrates potato peel gave the maximum
laccase activity (1.2463 U/ml) on fourth day (96 hrs) of incubation.
According to the results obtained in the present work, food wastes,
especially potato peelings, have an enormous potential as supports
for laccase production. The result was supported by Rosales et al.
(2002) when apple peel used as substrate, laccase production
reached a maximum value of 3148 UI on the 5th day of cultivation.
With orange peelings as a substrate, laccase production reached a
maximum value of 3437 UI on the 6th day. When *T. hirsuta*
was grown on potato peelings, laccase activity peaked on the 8th
day (5372 UI/). This value of potato peelings was much higher than that
found in the other substrates (Fig. 2).

**Optimization of production parameters of bacterial laccase
production- Incubation time:** The laccase activity was studied
for the incubation period from 0 to 120 h. It was observed that the
maximum enzyme production from the bacterium was attained after
96 h of incubation period at 35°C. An increase in the enzyme
production from 0 h towards 96 h was observed. After 96 h of
incubation a decrease in the trend of enzyme activity towards 120 h
was observed (Fig.3). The present study found contradictory against
Singh et al. (2009) who observed amongst the 30 experimental
runs, maximum laccase production was 7.4 × 10⁵ rkat/L after 60 h
of incubation time. The major obstacle in the production of laccase is
incubation period because most of the laccase exploitable sources
are fungus (Cueto and Herrera, 2006).

**Effect of temperature:** The laccase activity for different
temperature ranging from 20-50°C was determined. The maximum
laccase production was observed at temperature 35°C± 1°C, i.e.
1.2463 U/ml. An increase in enzyme production was observed from
temperature 20-35°C. On further increasing the temperature
the laccase activity decreased gradually (Fig.4). The result was
supported by Diamantidis et al. (1999) where the optimum
temperature found was 35°C for significant production of laccase
from *Azospirillum lipoferum*.

**Effect of pH:** The laccase activity for different pH ranging from 4-
10 was determined. The maximum laccase production was observed
at pH 8, i.e. 1.6528 U/ml. An increase in the enzyme production was
observed from pH 4-8. After pH 8 a gradual decrease in the
trend of enzyme activity towards pH 10 was also observed (Fig.5).
Wang et al. (2010) found that the strain Bacillus sp. WD23 exhibiting
laccase activity could grow at optimum pH 7.0. Bianco et al. (1999)
found laccase isolated from *Bacillus sp*. and *Streptomyces
virdosporus* active at alkaline pH.

**Effect of inoculum size:** The laccase activity for different inoculum
size ranging from 0.5ml to 3ml was determined. The maximum
laccase production was observed at inoculum size of 1.5ml, i.e.
3.1157 U/ml. An increase in enzyme production was observed from
0.5ml to 1.5ml of inoculum size. On further increasing the inoculum
size till 3ml, the laccase activity decreased gradually (Fig.6). Sabu
et al. (2005) found that inoculum plays a significant role in enzyme
production in solid state fermentation. A lower level of inoculum may
not be sufficient to initiate the growth, whereas a higher level of
inoculum may cause competitive inhibition.

**Effect of copper concentration:** Copper act as a cofactor for
laccases. The laccase activity for different copper concentration
ranging from 100 to 500μg was determined. The maximum laccase
production was observed at copper concentration of 200μg, i.e.
1.8398 U/ml. An increase in enzyme production was observed
from 100 μg to 200μg of copper concentration. On further increasing
the copper concentration till 500μg, the laccase activity decreased
gradually (Fig.7). Ohsumi et al. (1998) stated that this may be due
to the toxicity of heavy metals at high concentration which may
cause death by interacting with nucleic acid and enzyme site. It was
observed that when 200μg/ml copper sulphate was added to the
medium, 0.5935 U/ml increase in the production of laccases was
achieved. This was contrary to the result of D’souza et al. (2006)
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Fig. 1: *Bacillus subtilis* on guaiacol media

Fig. 2: Enzyme activity of different substrate

Fig. 3: Production of laccase from *Bacillus subtilis* at different incubation period

Fig. 4: Production of laccase from *Bacillus subtilis* at different temperature

Fig. 5: Production of laccase from *Bacillus subtilis* at different pH

Fig. 6: Production of laccase from *Bacillus subtilis* at different inoculum size

Fig. 7: Production of laccase from *Bacillus subtilis* at different copper concentration

Fig. 8: Production of laccase from *Bacillus subtilis* at different moisture level
who demonstrated that an addition of 2 mM copper sulphate to the medium nearly 100 fold increases in laccase production was achieved.

Effect of moisture level: The laccase activity for different moisture level ranging from 45 % to 85% was determined. The maximum laccase production was observed at moisture level of 75% i.e. 1.2463 U/ml. An increase in enzyme production was observed from 45% to 75% of moisture level. On further increasing the moisture level till 85%, the laccase activity decreased gradually (Fig. 8). De Souza et al. (2006) found similar results for laccase production after five days cultivation of cultures of P. pulmonarius CCB-19 at 75% initial moisture content. Yadav and Tripathi (1991) found during the growth of Trametes versicolor on wheat straw, 55% as the optimum moisture content.

In this study Bacillus subtilis found to be the best laccase producing bacteria and was used for optimizing the best conditions for the laccase production. According to the results highest laccase enzyme was produced on potato peel used as substrate in solid state fermentation (SSF). Production parameters were optimized and the optimum conditions were found to be at incubation temperature 35°C, incubation period 96 h, pH 8, copper concentration 200 ìg, moisture content 75% and inoculum size 1.5ml. It can be further optimized at large scale level (food, feed, textile, pulp and paper industries etc.). The results indicated the suitability of cheaper and abundantly available potato peels waste as solid substrate for large-scale production of laccase in an SSF system, thereby minimizing the high costs when other substrates, and chemicals, are used for laccase production. Maximum utilization of this waste can also contribute to efficient solid-waste management, where continuous accumulation of agricultural wastes poses serious environmental problems.

References