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Isolation of laccase enzyme producing bacteria and optimization of its production parameters to obtain maximum yield

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Abstract : *Enzymes are among the most beneficial products obtained for human needs through microbial sources and they have come up as a sustainable alternative to the use of harsh chemicals in industries. Laccase, a multicopper oxidoreductase, able to catalyse oxidation of phenolic and other toxic compounds, finds a potent substrate in the lignocellulosic agro-industrial residues. For the purpose of study, soil containing dumped saw dust were collected from two small scale saw mills located in Rajendra Nagar and Pirmuhani areas of Patna respectively. Bacterial population from these samples were isolated and screened for laccase activity on a guaiacol supplemented medium. Results indicated five strains to exhibit a positive response. However only one among them (Lac4) was found to show a visibly significant activity. On biochemical analysis, the strain was identified to be of Bacillus species. The enzyme production was carried out and assayed under normal conditions by spectrophotometric analysis. For optimization of production conditions, three parameters were kept under observation, i.e.; Incubation time and temperature, pH and different carbon sources. Study revealed that the maximum enzyme activity was obtained at 37ºC after 24 hours of incubation if the pH is kept at 6 and Dextrose (1%) supplemented as carbon source. The methods used were practical for a microbiology laboratory that chooses to perform enzyme assay by spectrophotometric analysis and assures a significant rate of accuracy in the result.*

Keywords: *Enzyme activity, Guaiacol, Laccase.*

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Introduction :

Enzymes are among the most important product obtained for human needs through microbial sources. These biocatalysts are utilized in a number of environmental and biotechnological applications.

Laccase (Benzendiol:oxygen oxidoreductases) is a multicopper oxidoreductase, able to catalyze the one electron oxidation of a wide array of substrates, such as phenols, aromatic amines, and other toxic aromatic compounds, with simultaneous reduction of oxygen to water (Xu et al 1996).The molecule is a glycoprotein, containing four copper atoms classified into three types, referred as Type 1 (T1), Type 2 (T2) and Type 3 (T3).The atoms are distributed in one mononuclear (T1) center and one trinuclear (T2 - T3) center. T1 copper is responsible for the characteristic blue colour and also acts like a primary electron acceptor during oxidation of substrates (Ducros et al 1998). The T2/T3 trinuclear site is where the reduction of molecular oxygen takes place by accepting electrons from T1 site. Next electrons are transferred to the two electron acceptor (Thurston 1994).

The lignocellulosic agro-industrial residues have great potential to act as substrate for producing high titres of laccase. Small sized sawmill residues such as sawings and saw dust in many part of the world are simply dumped or burned. The dumped shedding remain for prolonged period in saw mills and may provide a substrate for microbial proliferation, that are potent source of laccase extraction. This very property has been utilized in this study.

Laccase has been applied for numerous processes such as bioremediation of industrial effluents (i.e. Degradation of colored products and woods), Wine clarification, which specially removes toxic phenols like epicatechin, ferulic acids, oxidation of organic pollutants (Collins et al 1996), and development of biosensor (Kulys and Vidziunaite 2003) to improve food sensory parameters and also used as a biofuel cells (Palmore and Kim 1999).

High amount of enzyme is needed for these applications, as well as to study and understand its properties and role in lignin biodegradation This has impelled the search for new sources (Min et al 2001) and inducers like amino acids (Dhawan and Kuhad 2002), additional carbon sources, various pH and temperature exposures, etc.

Materials and Methods :

The study was conducted at the Patna Women's College Laboratory, Department of Industrial Microbiology, Patna.

Sampling :

Saw dust containing soil samples were collected from two saw mills located in different places in Patna. The saw mills were located in Rajendra Nagar and Pirmuhani. Two samples were collected from different areas in each mill. The serial dilution of the soil sample was prepared upto 10^{-6} dilution in normal saline. 0.1 ml of diluted sample was plated on solidified Nutrient Agar medium (supplemented with guaiacol). The plates were incubated for 24 hours at 37±1 ºC in an incubator and the colonies grown were observed.

Biochemical Characterization and Identification:

The colony that gave positive response on Nutrient Agar medium (supplemented with guaiacol) was identified on the basis of morphological characterization and biochemical analysis : Gram's staining, IMViC test, Carbohydrate fermentation test, Catalase test and Hydrogen Sulphide test.

Enzyme production and extraction

Laccase Production:

For laccase production, 100 ml Nutrient broth media was prepared. A loopfull culture of selected bacterial strain was inoculated, using a sterile loop and incubated for 24 hours on shaker incubator at 32±1ºC at 120 rpm. This culture served as the seed culture after 24 hours for further inoculation.

A 100 ml sterile production media was prepared according to the composition given by Unyayar *et al* (2005) sterilized and inoculated with 5 % seed culture (v/v) and was left for 48 hours at 37±1ºC.

After incubation, the cell culture was centrifuged at 10,000 rpm at 4 ºC for 10 minutes. The clear supernatant obtained was used as crude enzyme.

Laccase Assay:

The crude enzyme obtained after centrifugation was assayed for laccase activity according to the method described by Unyayar *et al* (2005).

In this method 2 mM guaiacol (1 ml) and 0.1 M phosphate buffer of pH 6.0 (3.9 ml) was incubated with culture filtrate (0.1 ml) for 1 hour at 30±1 ºC, after which absorbance was read at 465 nm.

One unit of laccase activity (Calorimetric Unit: CU) is defined as the amount of enzyme that causes an increase in absorbance of 0.1 / hour.

Optimization of production medium

The media adopted to study the optimization of growth parameter on laccase production aimed at evaluating the effect of a single parameter at a time and later manifesting it as standard concentration before optimizing the next parameter. For each step laccase activity was assayed to know the optimal yield.

Effect of temperature and incubation time:

To investigate optimum temperature for laccase production 50 ml of broth media was inoculated with 5% seed inoculum of the test organism and incubated at different temperatures (32,37 and 42ºC). The samples were withdrawn at different time intervals (24, 48 and 72 h) and the laccase activity was assayed.

Effect of pH:

Optimum pH condition for maximum laccase production was ascertained by inoculating the broth media having different pH (5.0, 6.0, 7.0, and 8.0) with the organism and subjecting it to incubation at 37ºC. After incubation, the laccase activity was assayed using standard assay method.

Effect of different carbon sources:

Different broth media was inoculated with the test organism supplemented with the additional carbon source such as dextrose, lactose, and sucrose at 1% (w/v) level incubated under the above optimized condition. After incubation, the samples were withdrawn and assayed for laccase activity.

Production of laccase with optimized media:

The production of laccase was determined with optimized parameters on production media. All optimized component was introduced into the production media and laccase activity was detected by laccase assay.

Results and discussion :

Bacterial population obtained on nutrient plates were enumerated. The bacterial count from Sample 1 and Sample 2 was found to be 7×10^4 cfu/g and 10×10^4 cfu/g respectively, while from Sample 3 and Sample 4, it was 12×10^4 cfu/g and 8×10^4 cfu/g respectively.

Table 1. Enumeration of bacterial isolates from Soil sample

Sample 1	Hanuman Timbers	7×10^4
Sample 2	Hanuman Timbers	10×10^4
Sample 3	Vishwakarma Timbers	12×10^4
Sample 4	Vishwakarma Timbers	8×10^4

Among all the isolates obtained, a total of 5 strains were found to exhibit laccase activity. Only 1 out of the selected 5 strains showed maximum activity on screening while the rest 4 showed minimal response.

Table 2. Strains showing positive laccase activity

Isolates	Laccase Activity
Lac 1	+
Lac 2	+
Lac 3	+
Lac 4	+ +
Lac 5	+

+= Positive

The strain 4 showed maximum reddish brown colouration on Guaiacol supplemented medium. Similar results were seen in earlier studies (Unyayar et al 2005).

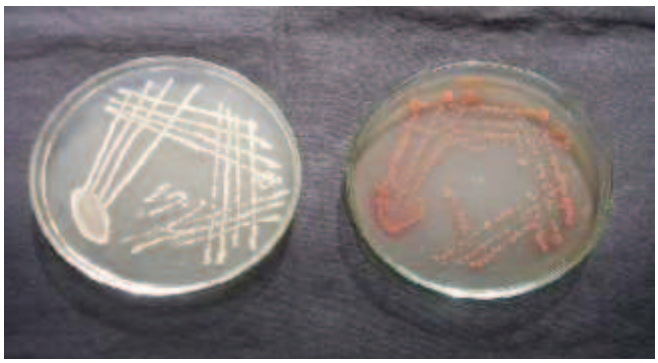

Fig 1. Result of screening - Bacterial isolate growing on (a) Nutrient agar (b) Guaiacol agar

The study of morphological and biochemical characteristics showed the following results:

Table 3. Identification of the potential isolate (Lac4) on the basis of orphological and biochemical characteristics

TESTS	RESULTS
COLONY CHARACTERISTICS:	
1. Configuration	Round
2. Margin	Wavy
3. Elevation	Convex
4. Surface	Entire
5. Density	Opaque
6. Gram's reaction	+
7. Shape	Rod
8. Motility	+
BIOCHEMICAL TEST :	
1. Indole	–
2. Methyl red	W
3. Voges Proskaeur	+
4. Citrate	–
5. Catalase	$A^{\cdot}G^{-}$
6. Hydrogen sulphide	$A^{\cdot}G^{-}$
7. Carbohydrate fermentation	$A^{\cdot}G^{-}$
(a) Lactose	
(b) Dextrose	
(c) Sucrose	

+ = Positive, – = Negative, W=Weak, A=Acid, G=Gas

Enzyme Production and Assay:

The Enzyme activity under normal condition was found to be 0.07 U/ml.

Optimization of production parameters:

After optimization of production parameters, following activity was recorded.

Effect of incubation time and temperature on production of laccase:

Of all the conditions studied, maximum Enzyme activity of 0.07 U/ml was observed after 24 hours of incubation at 37ºC. The result was supported by Diamantidis *et al* (1999) where the optimum temperature found was 37ºC for

significant production of laccase from *Azospirullum lipoferum.*

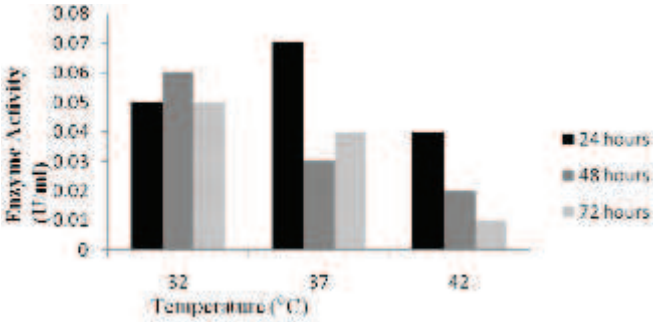

Fig 1. Effect of temperature and time on production of laccase

Effect of pH on production of laccase:

A significant increase in laccase activity was observed at pH 6, suggesting the influence of pH on enzyme production. The value was found to be 1.28 U/ml.

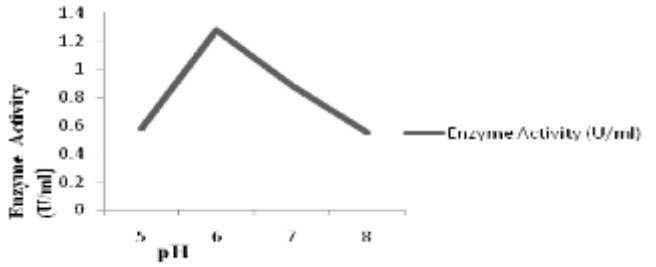

Fig 2. Effect of pH on production of laccase

Effect of different carbon sources on production of laccase:

Dextrose (1%) was found to play a significant role in increasing the enzyme production as compared to other sugars taken for study. Maximum activity of 0.28 U/ml was found in Dextrose supplemented medium.

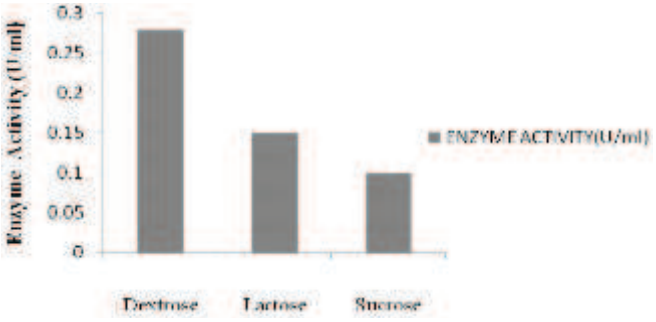

Fig 3. Effect of Carbon sources on enzyme production

Production of laccase with optimized media:

In the final optimized media a remarkable increase in laccase activity was observed. The Enzyme activity was found to be 0.56 U/ml.

Conclusion :

In the present study, laccase producing bacteria was isolated from soil sample collected from saw mills and on the basis of cultural, morphological and biochemical testing the strain Lac 4 was identified to be a *Bacillus spp*.

Various parameters such as temperature, incubation time, pH, Carbon sources, were optimized for maximum production of laccase by the isolate *Lac 4.*

The study revealed that the maximum production of laccase was obtained at 37 ºC after 24 hours at pH 6.0. Further Dextrose (1 %) served as a best carbon source for increasing the production of laccase.

Lac 4 was isolated from the saw dust containing soil sample taken from Hanuman Timber. These samples from saw mills are a rich constituent of lignin. This clearly showed that the lignin containing soil is the source of laccase producing bacteria.

Earlier laccase was mostly obtained from fungal sources. The obstacle was greater probability of contamination as well as longer incubation time. Therefore, production of laccase from bacteria could serve as a more suitable source for bioremediation, waste water treatment, wine clarifications and other industrial and biotechnological applications. The potential isolate Lac 4 could be used as an industrially exploitable source because it can grow at normal room temperature and has shorter incubation time.

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References :

Collins P J, Kotterman M J J, Field J A and Dobson A D W (1996). Oxidation of anthracence and benzo[a]pyrene by laccase from *Trametes versicolor*. *Appl. Environ. Microbiol.* 62: 4 – 7.

Dhawan S. and Kuhad R C (2002). Effect of amino acids and vitamins on laccase production by the bird's nest fungus *Cyathus bulleri*. *Bioresour. Technol*. 84: 35 - 38.

Diamantidis G, Effosse A., Potier P. and Bally R. (1999). Purification and characterization of first bacterial laccase in the rhizospheric bacterium *Azospirillum lipoferum*. *Soil Biol. Biochem*. 32: 919-927.

Ducros V, Brzozowski A M, Wilson K S, Brown S H and Davies G J (1998). Crystal structure of type 2 copper depleted laccase from *Coprinus cinereus* at 2.2 A° resolution. *Nat. Struct. Biol*. 5: 310-316.

Kulys J and Vidziunaite R (2003). Amperometric biosensors based on recombinant laccase for phenols determination. *Biosens. Bioelectron*. 18: 319 – 25.

Min K L, Kim Y H, Kim Y W, Jung H S and Hah Y C (2001). Characterization of a Novel Laccase Produced by the Wood-Rotting Fungus *Phellinus ribis*. *Arch. Biochem. Biophys.* 392: 279 - 286.

Palmore G T R and Kim H H (1999). Electro-enzymatic reduction of dioxygen to water in the cathode compartment of a biofuel cell. *J. Electro. Chem.* 565: 110 – 116.

Thurston C F (1994). The structure and function of fungal laccases. *Mirobiol.*140:19 - 26.

Unyayar A, Mazmanci A M, Atacag H, Erkurt A E and Coral G (2005). A.Drimaren blue X3LR dye decolorizing enzyme from *Funalia trogii*: one step isolation and identification. *Enzy. Microbiol. Technol*. 36:10-16.

Xu F, Shin W, Brown S H, Wahleithner J, Sundaram U M and Solomon E I (1996). A study of a series of recombinant fungal laccase and bilrubin oxidase that exhibit significant differences in redox potential, substrate specificity, and stability. *Biochem. Biophys. Acta.* 1292:303-311.