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ORIGINAL RESEARCH ARTICLE

OPTIMIZATION OF LACCASE PRODUCTION FROM POTENTIAL LACCASE PRODUCER ISOLATED FROM MIXED MICROBIAL CULTURE OF DYEING AND TEXTILES EFFLUENT

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Abstract: The laccase enzyme produced by Microorganism was found to be very useful in degradation of synthetic dyes that are commonly found in textile waste, dye industries and lignocelluloseic wastes when used freely or in immobilized form. In the present study for isolation of laccase producing microorganisms sample collected from different forest regions, soil samples near from textile and dye industries and textile waste were used. Out of different bacterial, fungal and actinomycets isolate, one potent Laccase producing fungus namely *RS 3* were selected for further study depending upon its high productivity. Using the gene specific sequencing primers, the purified PCR amplicons was sequenced. The sequences were analyzed using Sequencing Analysis 5.2 software. Blast result and phylogenetic tree analysis clearly indicate that fungal strain *RS3* is *A. nidulans*. For the Laccase assay substrate ABTS has been used. The intense brown color development due to oxidation of guaiacol by laccase can be correlated to its activity often read at 420 nm. The process parameters for enzyme production were optimized viz. Carbon source, Nitrogen source, pH and Time of incubation, it was found that Enzyme activity was maximum in basal medium containing starch (Carbon source), Peptone (Nitrogen source) and at pH 5.5 when incubated for 12h (Time of incubation).

Key Words: *A.Nidulans*, Enzyme Optimization, Fungal Laccase, Textile effluent.

INTRODUCTION

Laccases are one of the important enzyme for industrial applications because extensive studies have shown the potential of fungal phenol oxidases as a biological alternative for chemical oxidative processes e.g. pulp delignification, textile industry, food industry, bioremediation, organic synthesis, pharmaceutical sector and nano-biotechnology [Kunamneni *et al.,* 2008b]. Recently, most of the laccase studied are of fungal origin, especially from white-rot fungi, *Anthracophyllum discolor* [Bustamante *et al.,* 2010], *Pycnoporus sanguineus* [Eugenio *et al.,* 2009], *Trichoderma harzianum* [Sadhasivam *et al.,* 2008] etc.

In mycology, sequences from the ITS region of the nuclear rDNA are commonly used for the identification of fungi [Koljalg *et al.,* 2005; Naumann *et al.,* 2007; Nilsson *et al.,* 2008]. The ITS sequence including both ITS1 and ITS2, which are separated by the conserved short 5.8S rRNA, has been commonly used to infer phylogenetic relationships of closely related species as well as to assess the variability of a population.

Among multiple communities of enzymes, laccases are widely present in the nature and are oldest and most attractive enzymatic system. Laccase (benzenediol: oxygen oxido reductases [EC 1.10.3.2]) belongs to a group of enzymes called blue copper oxidases, capable of oxidizing phenols and aromatic amines by reducing molecular oxygen to water. They are wide spread in nature and have been found in plants, fungi, bacteria and insects.

The objective of this work is to optimize overall process of laccase production, which contributes to develop more effective and economically feasible technology for the treatment of dye industries effluent.

Laccase catalyzed the oxidation of variety of organic compound including phenols, methoxy-substituted phenols, amino phenols, diamines and so on (Morozova *et al.,* 2007). They are exceptionally versatile enzymes and majority of laccases are found in white-rot-causing polypores, geophilous saprophytic fungi, as well as some insect and bacteria. In fungi, laccases function in lignin degradation, pathogenesis, detoxification, and morphogenesis. Therefore, laccases produced from fungi have attracted considerable attention of academicians and researchers. The genus *Trametes*, which belongs to the white-rot fungi, is assumed to be one of the main producers. Among them, *Trametes pubescencs* has been described as promising laccase producer (Galhaup and Haltrich, 2001).

Laccase is so important because it oxidized both the toxic and nontoxic substrates. It plays an important role in food industry, paper and pulp industry, textile industry, synthetic chemistry, cosmetics and soil bioremediation and biodegradation of environmental phenolic pollutant, removal endocrine disruptors (Couto *et al.,* 2006). Along with different application, laccases plays crucial rule in dye de-colorization.

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In this study, laccase-producing fungi were isolated from various samples. In addition, the production of laccase by the potent strains was confirmed in liquid culture. The selected strain was also identified by molecular and phylogenetic analysis.

MATERIALS AND METHODS

Sample Collection

For isolation of laccase producing microorganisms sampling was done from different forest regions, various soil samples near from textile and dye industries were collected. In addition to this effluents were collected comprising textile waste, dye industries and lingo cellulosic wastes. Sampling has been carried out randomly using sterile plastic bags and sterile bottles.

Enrichment

Selective enrichment has been carried out in presence of 0.02% guiacol using nutrient broth and PDB under the conditions of optimum temperature of 25°C. Three cycles of enrichment has been carried out by successive transfer of pre-enriched samples into fresh media. The samples from last enrichment were used to isolate laccase producing bacteria and fungi.

Screening

Near about 101 bacterial, 88 fungal and 25 Actinomycets strains were isolated which were further subjected to primary and secondary screening by plate assay to select efficient laccase producer.

Characterization of Screen Fungal strain RS3

The selected fungal strain were characterized by using morphological and molecular characterization methods (18s rDNA)

Laccase assay

ABTS has been reported as efficient substrate for laccase assay. The green color development due to oxidation of ABTS by laccase can be correlated to its activity often read at 420 nm. ABTS (5mM) in sodium acetate buffer (50mM pH 5.0) was used as substrate. One unit of enzyme activity (U) is defined as the amount of enzyme that released 1 µ mole per minute of oxidized product.

Laccase assay was done using 2, 2-diazino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). Assay mixture contains 100µl of culture filtrate, 100µl of ABTS in 800µl of 50mM Sodium acetate buffer. Change in absorbance was monitored at 420nm. Enzyme activity was calculated by using the formula

$$\mathbf{E.A\ (U/ml) = \Delta A \times V / d \times \varepsilon \times v}$$

Where ΔA= change in absorbance per minute at 420 nm
V= Volume of reaction mixture (ml).
v=Volume of Enzyme (ml)
ε=extinction coefficient of ABTS at 420nm (ε mM) =36mM-1cm-1
d= Light path of cuvette (cm).

Optimization of Enzyme Production

100ml of basal media in 250ml flask was inoculated with 5ml inoculums containing 7days old culture of *A. nidulans* & incubated at 25°C at 150 rpm for growth and production of laccase. The process parameters for enzyme production were optimized viz. Carbon source, Nitrogen source, pH and Time of incubation.

Determination of Optimum Carbon source

Different Carbon sources viz, Starch, Glucose, Lactose, Manitol and Sucrose were used at 2% level with basal medium in separate flask, at the time of incubation all other condition was kept constant. The enzyme activity in u/ml was determined after incubation.

Determination of Optimum Nitrogen source

Different Nitrogen source viz; Yeast Extract, Peptone, Ammonium chloride, Ammonium sulfate, and Sodium nitrate were used at 2% level with basal medium contain starch as a carbon source, all other process parameter were kept constant.

Determination of Optimum pH

Basal medium contain starch and peptone with varying pH (4.5-6.5) used for determination of optimum pH. The influence of pH on laccase activity was studied by recording the absorbance of enzyme catalyzed reaction at optimum temperature, using ABTS as substrate dissolved in buffers of different pH (acetate buffer pH 4.5, pH 5, phosphate buffer pH 5.5, pH 6 and pH 6.5) and incubated at 25°C for 15min and absorbance were recorded at 520nm.

Determination of Optimum Time of incubation

To determine optimum time of incubation, Basal medium of pH 5.5 containing starch and peptone as a carbon and nitrogen source respectively were used. The samples was withdrawn after every 48 hours and assayed for presence of laccase activity.

RESULT AND DISCUSSION

Isolation and characterization

The 22 positive fungal laccase producers were screened by observing brown zone around the colony, out of that 6 presume to be potant, 10 showed medium potency and 6 showed weak laccase activity. Out of bacterial isolate 23 showed weak laccase activity.25 Actinomycets were also been isolated but none of

them showed promising enzyme activity. The isolates screened after primary screening were subjected for secondary screening which was based on enzyme activity. As laccase is extracellular enzyme the cell supernatant was used as source of enzyme and laccase activity was determined using ABTS as substrate.

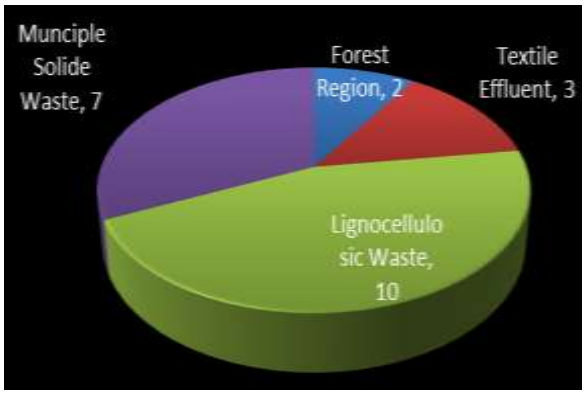

Figure 1: A total of 88 fungal isolates were obtained 22 of which showed positive laccase activity.

All the isolated and sub cultured fungal colonies were inoculated on petri plates containing SDA, amended with 1% 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). Development of dark green to purple color around the colonies indicates the presence of laccase being produced by the fungus. The organism which showed rapid growth was selected for further study. Out of 6 potent Laccase producing fungus one namely *RS 3* were selected for further study depending upon productivity.

Figure 2: Primary Screening

Characterization of Screen Fungal isolate

Fungal genomic DNA isolation: Fungal genomic DNA was isolated using geneO-spin Microbial DNA isolation kit (geneOmbio technologies, Pune; India).

Figure 3: Secondary Screening

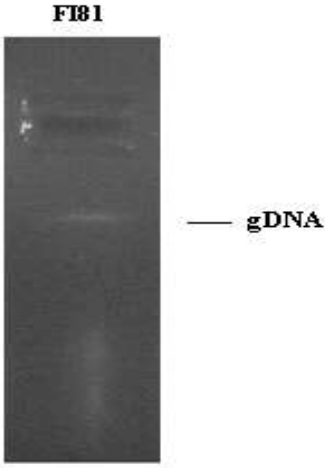

Figure 4: 1% (W/V) Agarose Gel electrophoresis: Lane 1: FI84 gDNA

PCR

Fungal ITS region was amplified using standard PCR reaction. The primer pair ITS1 and ITS4 was used in a PCR reaction with an annealing temperature of 54°C. After amplification, products were purified by using a geneO-spin PCR product Purification kit (geneOmbio technologies, Pune; India) and were directly sequenced using an ABI PRISM BigDye Terminator V3.1 kit (Applied Biosystems, USA).

Agarose gel electrophoresis of PCR products for confirmation of PCR amplification

After PCR is completed, the PCR products were checked on 2% Agarose by Agarose Gel Electrophoresis and amplicon size was compared using reference Ladder. 2% agarose gel spiked with Ethidium bromide at a final concentration of 0.5µg/ml was prepared using Agarose (LE, Analytical Grade, Promega Corp., Madison, WI 53711 USA) in 0.5X TBE buffer. 5.0 µl of PCR product was mixed with 1 µl of 6X Gel tracking dye. 5µl of gScale 100-1000bp size standard was loaded in one lane for confirmation of size of the amplicon using reference ladder. The DNA molecules were resolved at 5V/cm until the tracking dye is 2/3 distance

away from the lane within the gel. Bands were detected under a UV Trans illuminator. Gel images were recorded using BIO-RAD GelDocXR gel documentation system. The PCR product of size 650bp was generated through this reaction.

Figure5: 2% (W/V) Agarose Gel electrophoresis: Lane M: DNA marker; 200-1000bp; well No. 2- Negative Test Control; Well No3- FI84 PCR product

DNA sequencing

Using the gene specific sequencing primers (its1 and its4) and abibigdye® terminatorv3.1 cycle sequencing reaction kit (applied biosystems, USA), the purified PCR amplicons was sequenced. The sequences were analyzed using Sequencing Analysis 5.2 software. BLAST analysis was performed at Blast N site at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). DNA sequencing was performed using one of the PCR primers. Blast Result and phylogenetic tree analysis clearly indicate that fungal strain *RS3* is *A. nidulans*

Classification

Scientific classification
Domain: Eukarya
Kingdom: Fungi
Phylum: Ascomycota
Class: Eurotiomycetes
Order: Eurotiales
Family: Trichocomaceae
Genus: Aspergillus
Species: *A. nidulans*

Optimization of Laccase Production

Determination of Optimum Carbon source: It was found that Enzyme activity was maximum in basal medium containing starch (0.623 u/ml) followed by sucrose, Manitol, Glucose (0.451, 0.392,0.167U/ml respectively). Presence of Lactose as carbon source can significantly decreases Laccase production (0.004u/ml).

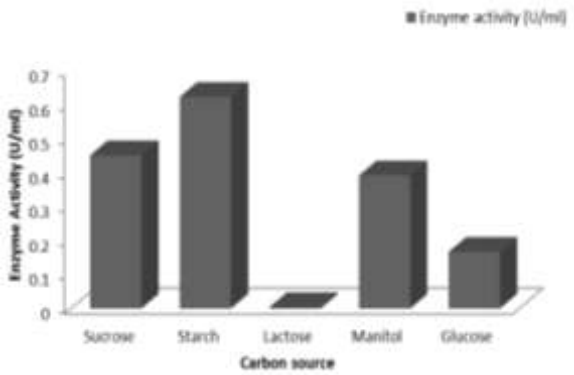

Figure 6: Enzyme Activity (U/ml) in basal medium containg different Carbon source

Determination of Optimum Nitrogen source: Isolate *A. nidulans* produce maximum amount of laccase in presence of Peptone (0.734 u/ml). It was found that all other nitrogen sources (Yeast Extract, Ammonium chloride, Ammonium sulfate, and Sodium nitrate) cannot play significantly role in laccase production (Enzyme activities 0.103, 0.108, 0.039 and 0.254 u/ml respectively).

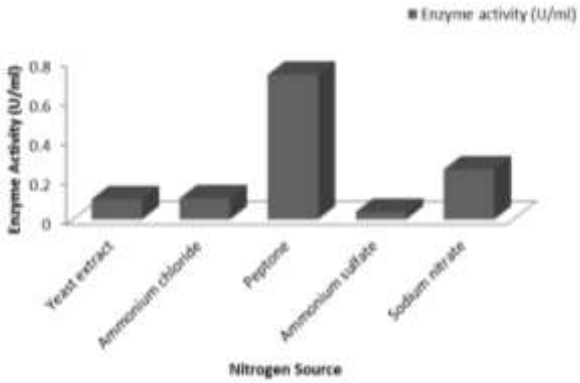

Figure 7: Enzyme Activity (U/ml) in basal medium containg different Nitrogen source

Determination of Optimum pH: Five levels of pH were studied by maintaining all other parameter constant at 30◦C. The maximum production of laccase and biomass were attained at pH 5.5. The enzyme activity of laccase decreased with increasing pH. Maximum activity of laccase was found in basal medium of pH 5.5. (0.1u/ml), followed by 5, 6, 4.5 and 6.5 pH (0.043, 0.036, 0.013, 0.005 u/ml respectively).

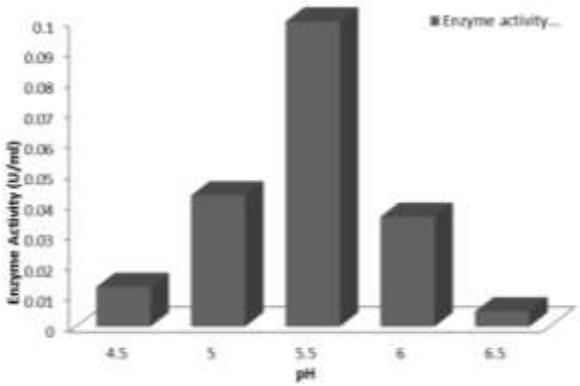

Figure 8: Enzyme Activity (U/ml) in basal medium of different pH

Determination of Optimum Time of incubation: There is no significant change found in enzyme activity at day 2, 4 and 6 (0.014, 0.014, 0.015 u/ml respectively). But laccase production constantly increased from day 8 to day 12. Maximum activity was found on 12th day of incubation (0. 527 u/ml).

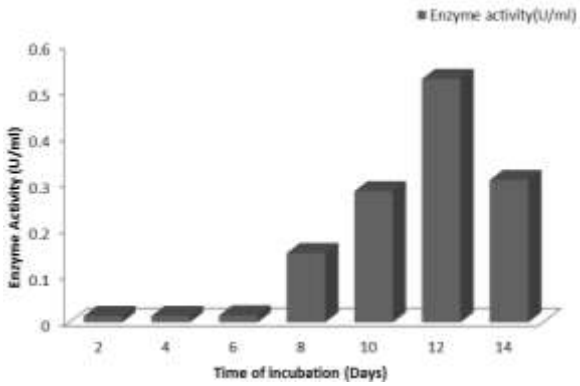

Figure 9: Enzyme Activity (U/ml) in basal medium incubated for different time interval

DISCUSSION

Out of several bacterial, Fungal and Actinomycets isolates, one potent fungal strain were selected for further study which shows maximum laccase activity than that of the other isolates. The selected strain was identified by 18S rDNA sequence (*A. nidulans*) and obtained accession number KP131596. One Factor-at-a-time method was used to determine the effect of different carbon source, Nitrogen source, pH and Time of incubation on laccase production. The different process parameter optimization suggests that the laccase production increased in the presence of Starch as a carbon source and peptone as a nitrogen source when incubated for 12 days. Enzyme production enhanced when the pH of basal medium was 5.5. Small change in pH can significantly affect enzyme production so pH of the medium is one of the important factors, which plays a major role in the production of laccase and must be maintain carefully.

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