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Research article

EXPLOITATION OF INDUSTRIAL WASTE GHEE RESIDUE IN LACCASES PRODUCTION BY *Pycnoporus cinnabarinus* strain SYBC-L14

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ABSTRACT: The objective of this research work was on the production of laccases by agricultural and industrial waste management using a Basidiomycetes fungus, *Pycnoporus cinnabarinus*. Agricultural/industrial wastes such as sawdust, Rice bran and wheat bran can bring down the cost of laccases production. The best laccases production medium was analyzed by incorporating the agricultural/industrial wastes in the production medium. The best laccase production was obtained in the medium with wheat bran followed by Rice bran and sawdust. With the aim of enhancement of laccases production, ghee residue (1%W/V), a byproduct of milk industry was added to the above media. For comparison, media with peptone (1% W/V) were used. The use of ghee residue in the formulation of the laccases production media has been found to be beneficial. The production of laccases in presence of peptone and ghee residue was 1.6 times and 4.16 times more respectively than in the medium with only glucose (15.77 U/ml/min). Similarly an increase by 1.2 folds and 1.3 folds in laccase production was obtained in the medium containing wheat bran with peptone and ghee residue respectively when compared to the control i.e. medium containing only wheat bran (126.99 U/ml/min). 88% increase in laccases production in the medium with agricultural (Wheat bran) and industrial (Ghee residue) wastes when compared to the basal medium containing glucose. The optimum temperature was found to be 80^{o}C and pH 5.0 for the laccases activity. The ghee residue and peptone seem to be acting as inducers in laccases production.

Key words: Laccases, Ghee residue, wheat bran, Rice bran, Saw dust, *Pycnoporus cinnabarinus*.

INTRODUCTION

The increase in the use of enzymes in diverse industrial fields has made it necessary to look for the cheap and economic sources for them. Many of such these enzymes are widely distributed in nature; laccases being one of them. Laccases (benzenediol: oxygen oxidoreductases, E.C 1.10.3.2) are multi-copper enzymes belonging to the group of blue oxidases. They are defined as oxidoreductases in Enzyme Commission (EC), which oxidizes diphenol and allied substances and use molecular oxygen as an electron acceptor [13]. Laccases are the oldest and most studied enzymatic systems for various industrial applications such as textile dye bleaching [9], pulp bleaching and bioremediation. In order to improve the color and quality of the Kraft pulp chlorine-based bleaching is adopted followed by discharging of waste waters containing chlorinated aromatics into water bodies [3]. This cytotoxic and cytomutagenic effects on various living organisms ultimately harming human beings too. A similar process is also observed in textile industries using harmful cytotoxic coloring dyes as the effluents are released in water bodies [11]. Fungal laccases have wide applications [6]. They can help us reduce the above, being an environment friendly to overcome the toxicity of the currently used chemicals. However industrial production of laccases by conventional media is costly. Fungi are capable of using a wide variety of organic wastes as a source of carbon and nitrogen [4]. In this work we have screened three organic wastes namely wheat bran, rice bran and saw dust as a source of carbon for laccase production by the test fungus. Phenolics obtained from plants or other sources have been used as inducer in production of various enzymes, [12]. Laccases would be able to oxidize phenolic substrates.

With this view we have used ghee residue as an inducer for laccase production. Ghee residue, a by-product of the dairy industry, was found to contain crude protein content of 25.8% which was found to be suitable for the fungus to produce laccases. The phenolic compounds, defined as the hydroxy derivatives of benzene and its condensed aromatic systems were found to be present in the byproduct of milk industry in the manufacturing of ghee i.e. ghee residue. They were screened by performing the test for phenolic compounds [2].
In order to prove the efficiency of Ghee residue, Peptone, a product of incomplete fermentative hydrolysis of protein containing peptides, diketopiperazines and amino acids, was used as an another possible inducer.

MATERIALS AND METHODS

Test Organism

The laccases producing fungus was obtained from Kakatiya University, Warangal, Andhra Pradesh. It was named as KR for convenience. The above organism was sent for identification to Xcelris laboratories, Ahmadabad, Gujarat.
The fungal culture was maintained in pure form by inoculating it in malt extract medium containing the following composition (g/L), 15g Malt extract powder, 1g Dipotassium hydrogen ortho phosphate, 1g Ammonium chloride, 15 ml Citric acid (1N), 20g Agar.

Screening for Laccases Producing Fungi

Screening for laccase producing organisms was done on the screening medium plates containing following composition (g/l):

3.0 peptone, 10.0 glucose, 0.6 KH_2PO_4, 0.001 $ZnSO_4$, 0.4 K_2HPO_4 0.0005 $FeSO_4$, 0.05 $MnSO_4$, 0.5$MgSO_4$, 20.0 agar (pH-6) supplemented with 0.02% Guaiacol [14]. Positive cultures were visualized by reddish brown coloring zones in the screening plates since laccase catalyzes the oxidative polymerization of Guaiacol to form reddish brown coloring zones in the medium.

Characterization of *Pycnoporus cinnabarinus*

Pycnoporus cinnabarinus is a wood-rotting fungus that forms a reddish-orange fruit body with spore-releasing pores on its undersurface. It is a white rot fungus. It breaks down both cellulose and lignin in the cell walls of its host's wood. It is also one of the three species belonging to the genus *Pycnoporus*, first formalized by P. Karst in 1881. It grows in a semi-circular (half a circle) or amorphous-elongated shape, attaching to tree or log exteriors, growing hyphae of varying degrees of branching and wall thickness within a single fruit body and forming pileus that ranges in color from a reddish-apricot color to a deep red-orange. *P. cinnabarinus* lives as a detritivore on hard wood trees, degrading cellulose, hemicellulose, and lignin found in cell wood walls [8].

P. cinnabarinus occurs across the entire northern hemisphere in the North Temperate Zone, extending from Central American & and North African latitudes to mid-Canada and northern Russia, and so can therefore withstand freezing temperatures. However, its growth rate is shown to decline significantly at temperatures above 30^oC and it is not found in tropical zones. The fruit body releases spores in the autumn and spring and can survive over the winter. *P. cinnabarinus*, however, is usually found on the dead hard-wood of deciduous trees and rarely on the dead wood of conifers [10].

Studies on Growth Conditions of Fungal Growth

The fungus was inoculated in malt extract medium and was incubated at room temperature. The growth measurement of the fungus was done by the dry weight estimation at 2 days of time intervals. The fungus was filtered using filter paper and was dried in hot air oven at 80^0 C till standard weight was obtained.

Optimum pH

A similar procedure was followed to estimate the optimum pH for the fungal growth. The malt extract media was prepared with different pH ranging from pH 3.0-9.0 by using acetate buffer, phosphate buffer, Tris HCl buffer and was incubated at room temperature for the period of time where the maximum growth was observed.

Optimum temperature

To estimate the optimum temperature for fungal growth, the malt extract medium was prepared with the optimum pH by using acetate buffer and was incubated for the period of time where the maximum growth was observed at temperatures ranging from 00C to 600C.

Fermentation by Submerged Fermentation Technique

In submerged fermentation (SmF) microorganisms were grown in a continuous liquid phase using *Pycnoporus cinnabarinus* strain SYBC-L14**.** The efficiency of the laccase producing organisms can only be exploited with the aid of media designed with optimum concentrations of every component which influences the production. Research conducted in this regard mainly aimed at picking up proper carbon sources, nitrogen sources and inducers.

Standardization of Medium Composition for Laccases Production

Laccases production in liquid culture was investigated using glucose, sawdust, rice bran and wheat bran as the sole carbon sources.

Composition of the Basal medium (in g/l):

Carbon source-10g, aspargine-1.00g, Yeast extract-0.5g, K2HPO4-0.5g, MgSO4.7H2O-1.00g, FeSO4.7H2O-0.001g [15].

Carbon Source

Laccase production time was standardized using composite medium containing glucose and Guaiacol. Standard time for production of laccase was found to be on the 15th day. A set of production media with the appropriate carbon source was inoculated with the Pycnoporus cinnabarinus strain SYBC-L14. The inoculated medium was incubated for 15 days at 27-300 C in an orbital shaking incubator at 120 rpm. After incubation the fungal culture was filtered using a muslin cloth. The best carbon source for the respective fungal cultures was noted down by using the filtrate as a crude enzyme source and identifying enzyme activity using Guaiacol as a substrate. This investigation was continued with the use of inducers (peptone or ghee residue) in combination with alternative carbon sources like glucose, sawdust and wheat bran in order to increase the production of enzyme.

Nitrogen Source

In addition to yeast extract, peptone/Ghee residue (1.0% w/v) was added in the production medium as an inducer. The production medium with alternative carbon source (Glucose, sawdust and wheat bran, Rice bran) each with inducer either peptone or Ghee residue was inoculated with P. cinnabarinus strain and was incubated for 15 days in orbital shaking incubator at 120 rpm at 27-300C (Fig.1)

After incubation the fungal culture was filtered using a muslin cloth. The best carbon source with the best inducer was noted down by using the filtrate as a crude enzyme source and identifying enzyme activity using Guaiacol as a substrate.

Assay of Laccases

Using Guaiacol, Laccase activity in the sample was determined spectrophotometrically by monitoring the rate of product (dark brown color) formation.

The reaction mixture consists of 1.0 ml 0.2% Guaiacol prepared, 3.0 ml of acetate buffer (pH 5.0) and 1.0 ml of enzyme (to be tested) [1].

The kinetic reaction was spectrophotometrically recorded at 465 nm [3] for 3 Minutes with 1 min of time interval at 30°C, as an increase in absorbance. The blank contained all the assay constituents except the active enzyme, buffer or heat inactivated enzyme was used in its place.

Fig1: Incubation of *P. cinnabarinus* in the production media at 120rpm for 15 days

Effect of pH and Temperature on Enzyme Activity

The pH optima of laccases are highly dependable on the substrate. When using Guaiacol as substrate the pH optima are more acidic and are found in the range 3.0-5.0. For the study of effect of pH and temperature on enzyme activity, the media showing better production of laccases for *P. cinnabarinus* was selected, the fungal mycelium was filtered after 15 days of incubation and the filtrate was used as the crude enzyme source .It is incubated in the range of pH 3.0-9.0 at 27-30^{0}C and enzyme activity was determined spectrophotometrically. The better pH for the enzyme activity was used for incubating the crude enzyme sample at the temperatures ranging from 0^0 C- 90^{0}C to determine the enzyme activity spectrophotometrically. Therefore the better pH and temperature can be applied to find the highest enzyme activity.

Effect of Substrate Concentration on Enzyme Activity

The crude enzyme (1ml) obtained from the cheap and best media was incubated with the increasing concentrations (0.1%-0.5%) of the substrate (Guaiacol) at the optimum temperature in optimum pH buffer (sodium acetate buffer) and its activity was noted spectrophotometrically at 465nm.

Calculation of Enzyme Activity (U/ml)

The enzyme activity was calculated by using the formula,

$$\text{Vol. activity (U/ml)} = \frac{[\,\Delta A/T \times Vt \times \text{dil. Factor} \times 106\,]/1000}{\varepsilon \times Vs}$$

ΔA=Increase in Absorbance at 465 nm
T= Time of observation
Vt = Final vol. of reaction mixture= 5.00
Vs = Sample volume= 1.00
E Guaiacol = extinction coefficient of the product= 27.75

Statistical Analysis

The data presented in results were interpreted statistically using Minitab 16.

RESULTS AND DISCUSSION

This paper summarizes the important reports on the cheap and best production medium for the production of industrially most important laccases by submerged fermentation method and optimization of pH and temperature for its activity.

As per the reports sent by xcelris laboratory, Ahmadabad, the fungus KR was reported as *P. cinnabarinus strain* SYBC-L14 (GenBank Accession Number: HQ891298.1). When the test organism was inoculated in the screening media for laccases production using Guaiacol as a substrate it shows positive report by the formation of dark reddish brown zones around the fungal culture (Fig.2).

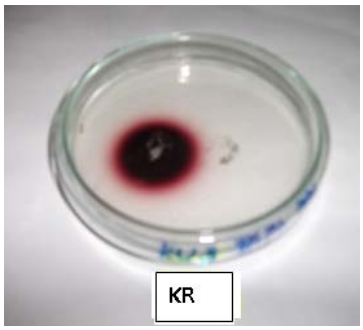

Fig2: Positive report for the screening test of *P.cinnabarinus* (KR)

The fungus *P. cinnabarinus* shows the maximum growth in 14 days of incubation at room temperature in malt extract medium (pH6.8) Fig.3.

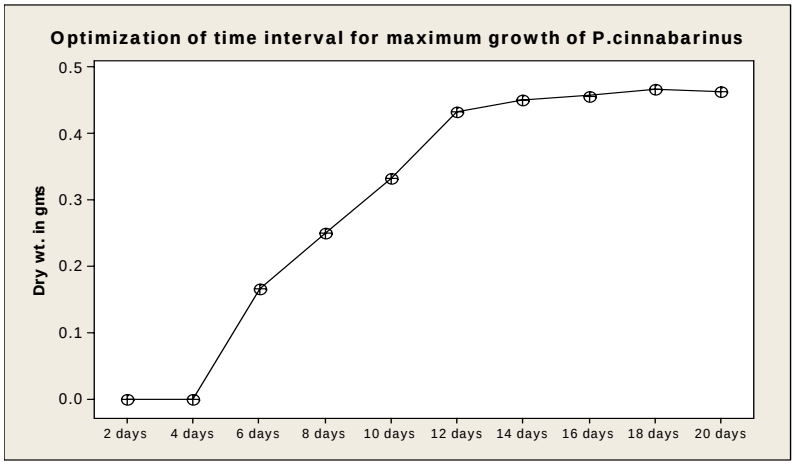

Fig.3: Optimization of time interval for the fungal growth

When the organism was inoculated and incubated in the malt extract medium of different pH ranging form 3.0-9.0 at room temperature for 14 days, the maximum growth was observed in the medium with pH3.0. (Fig.4).

Among the formulated laccases production media using agro waste (wheat bran, rice bran), industrial waste (sawdust) as a carbon source, higher laccase production was obtained in the medium with wheat bran followed by sawdust, glucose and rice bran (Table 1.). Further addition of peptone or ghee residue as an inducer lead to higher production of laccases. 140.78U/ml in the medium with Wheat bran and ghee residue/WBG followed by wheat bran with peptone /WBP (113.45U/ml) and Sawdust with ghee residue/SDG (90.35U/ml) (Fig.6). Lowest production of laccases was found in the medium with only Rice bran (0.94U/ml). 88% increase in laccases production in the medium with agricultural (Wheat bran) and industrial (Ghee residue) wastes when compared to the basal medium containing glucose.

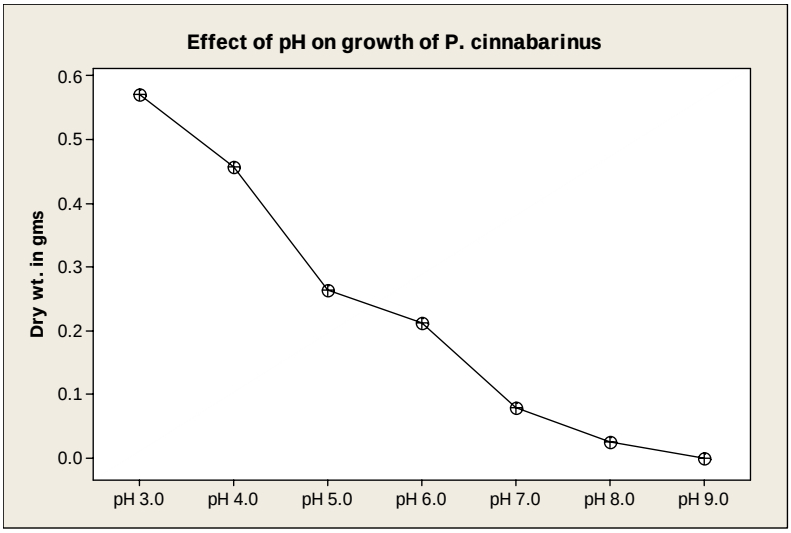

Fig.4: pH optimization for the fungal growth

Similarly, the maximum growth was observed at temperature 30^0C when the fungus was inoculated and incubated in the malt extract medium (pH3.0) at different temperatures ranging from 0-60^0C for14 days (Fig.5).

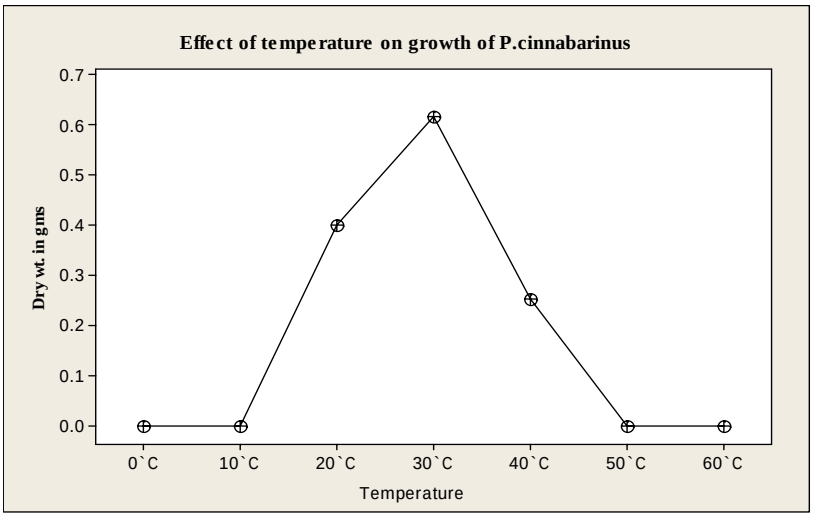

Fig.5: temperature optimization for the fungal growth

The carbon source in the medium plays an important role in ligninolytic enzyme production. Lignolytic systems are activated during the secondary metabolic phase of fungi and are triggered by nitrogen depletion. The effect of different type of nitrogen source was analyzed, which influences the laccase production significantly. Further the optimum temperature and pH for enzyme activity produced in the cheap and best media i.e., wheat bran with ghee residue (WBG) was found to be 80^0C (Fig.7) and 5.0 (Fig.8) respectively. Since laccases oxidize phenolic compounds (Pcs), they may also induce its production. Phenolic compounds are a diverse group of chemicals (over 8000 currently known; L. Bravo, 1998), produced as secondary metabolites by most plants, as natural deterrents to grazing animals. Pcs get incorporated into milk and milk products. Pcs are found in considerable amounts in ruminant milk (mg/Kg) [5] Thus, it was found that pcs are present in the ghee residue [2].

Table 1: Optimization of carbon source for laccases production

Carbon source	Nitrogen source	Fungal mass (gm)	Production of laccases(U/ml)
Glucose	Yeast extract	0.18 ± 0.04	15.77±0.12
Sawdust	Yeast extract	0.79± 0.01	118.24±0.16
Wheat bran	Yeast extract	0.41 ± 0.10	126.99±0.11
Rice bran	Yeast extract	0.80± 0.05	0.94±0.08

Each value is the average of three replicates and ± indicates standard deviation

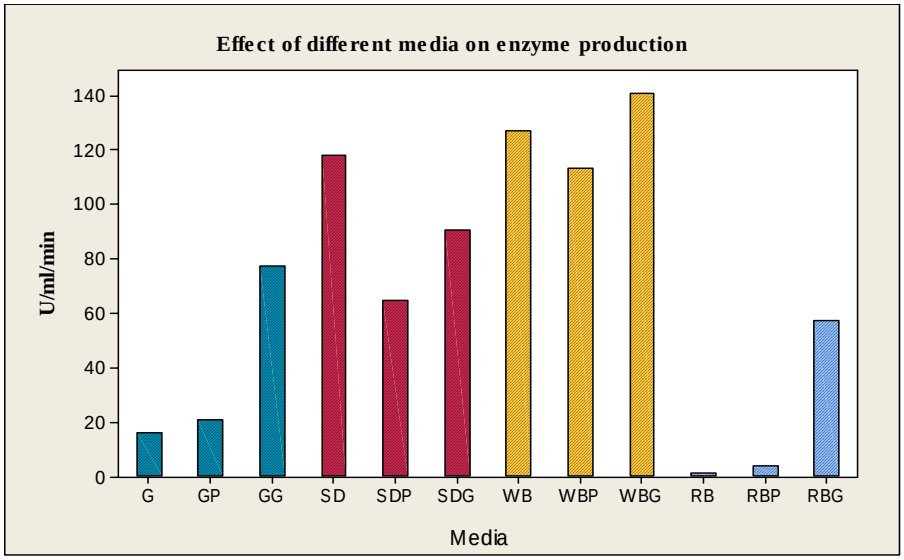

Fig.6: Optimization of media for laccases production

GHEE RESIDUE

Ghee residue is obtained as a by-product of ghee manufacturing industry and is produced in large quantity (about 91000 tones per annum) in India. During its manufacture the solids not fat (SNF) present in cream or butter appears as small particles known as ghee-residue. It has been obtained after molten ghee has been either strained out with muslin cloth or separated by continuous centrifugal clarifiers. The yield of ghee-residue varies with the method of preparation of ghee. This is due to the variation in the non-fatty serum constituents of the different raw materials used for the preparation of ghee. The average yield of ghee-residue is Maximum in direct creamery (DC) method (12%) followed by about 3.7%yield in creamery butter (CB) and desi butter (DB) method. Keeping quality of all types of Ghee residue clarified at 120°C is 3months. Its shelf life can further be increased to more than 4months by pressing it in cake form. Physical attributes: Ghee residue is moist brownish sediment. On average, particle diameter of ghee residue is about 115 µ and density is 1.14 g/cm^3 **[***vedyadhara.ignou.ac.in/wiki/images/* BPVI-16-Block_4-Unit-14.pd. Unit 14 *Buttermilk* and *Ghee Residue*].Soluble nitrogen content of ghee-residue prepared from cream or creamery butter decreases with heating time. This decrease is due to the denaturation of the proteins. Main sugars in ghee-residue prepared at 120^{o}C are lactose, galactose and glucose. As the period of heating is increased, the lactose content of ghee-residue decreases with a corresponding increase in galactose and glucose content. Ghee-residue is a rich source of natural antioxidants and its antioxidant properties are due to its constituents affected by various technological parameters. The overall antioxidant properties are due to both lipid and non-lipid constituents [*vedyadhara.ignou.ac.in/wiki/images/* BPVI-16-Block_4-Unit-14.pd.. Unit 14 *Buttermilk* and *Ghee Residue*].

Since laccases oxidize phenolic compounds (Pcs), they may also induce its production. Phenolic compounds are a diverse group of chemicals (over 8000 currently known) [7], produced as secondary metabolites by most plants, as natural deterrents to grazing animals.

Pcs get incorporated into milk and milk products. Pcs are found in considerable amounts in ruminant milk (mg/Kg) and it was also found to be present in Gee residue [5]. Ghee residue, by virtue of its chemical composition, nutritional quality, physical characteristics, bulk of production and long shelf life permitting its collection and centralized handling has great potential and is more amenable to exploit its utilization. Ghee residue can be utilized in a number of products like chocolate burfi, samosa filling, chapatis etc. However, most dairy plants in India have not been utilizing ghee residue profitably except for fat extraction. Most of the ghee residue goes to waste *[vedyadhara.ignou.ac.in/wiki/images/BPVI-16-Block_4-Unit-14.pd.*Unit14 *Buttermilk* and *Ghee Residue*].
In order to utilize whole ghee residue commercially it can be used as an important component in the production medium for laccases production to put it in the market.

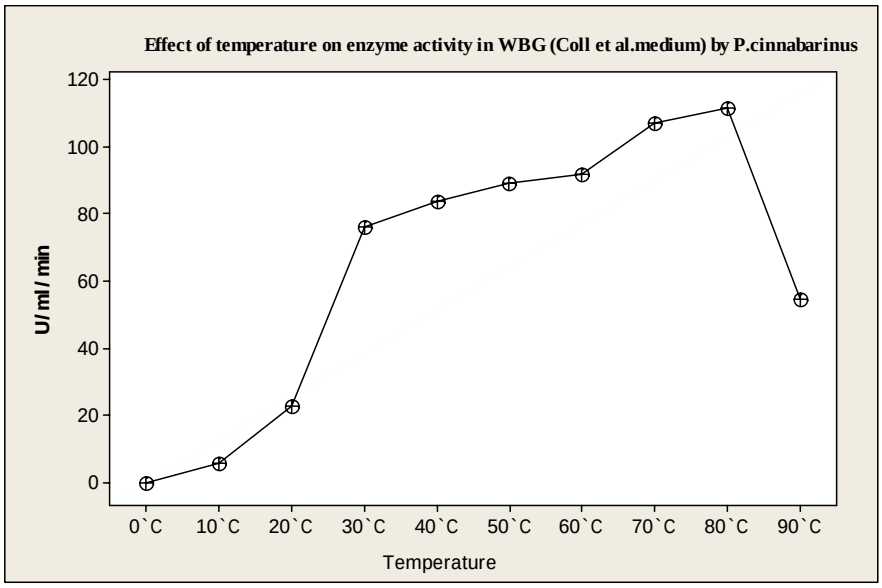

Fig.7: Optimization of temperature for enzyme activity

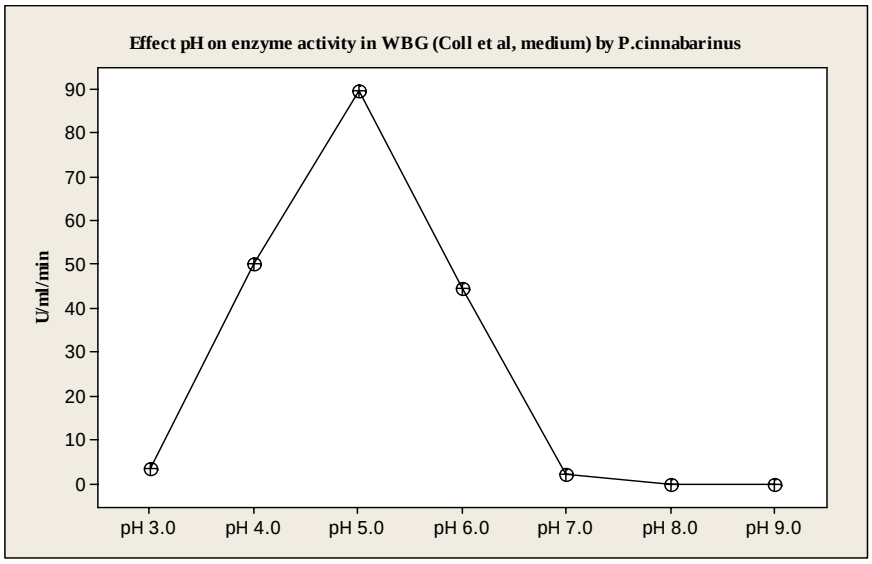

Fig.8: Optimization of pH for enzyme activity

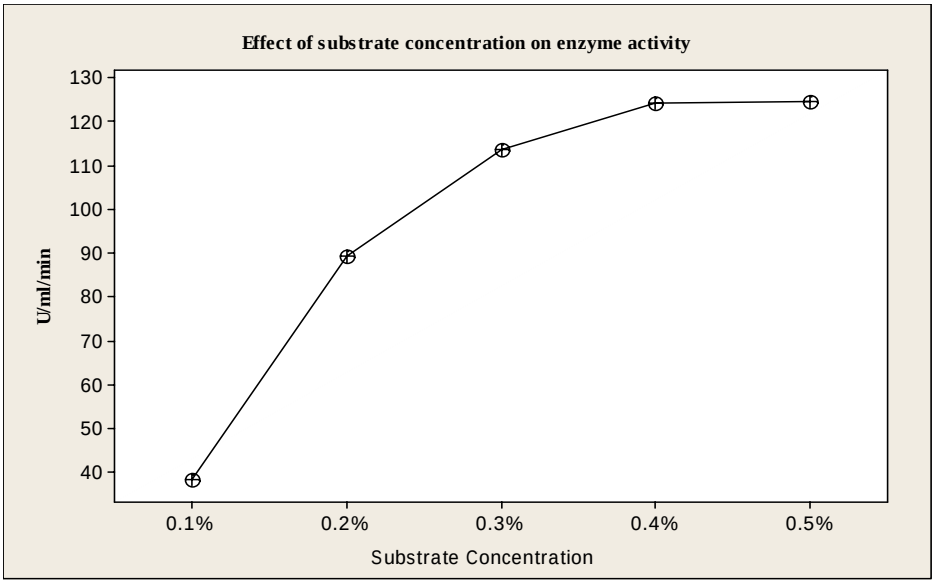

Fig.9: Optimization of substrate concentration for enzyme activity

As the concentration of the substrate (Guaiacol) increases, the units of enzyme activity also increases (Fig.9).

Because of the versatility of potential substrates, laccases are highly interesting as novel biocatalysts in various industrial processes. One of the limitations to the large-scale application of the enzyme is the lack of capacity to produce large volumes of highly active enzyme. Thus, efforts have been made in order to achieve cheap overproduction of laccase by using the fungus *P. cinnabarinus***.** To obtain more robust, active and less expensive enzymes, this organism is under the process of strain improvement capable of producing high concentrations and then optimization of conditions for laccase production.

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