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FORMULATION OF HIGH LACCASES PRODUCTION MEDIA USING GHEE RESIDUE OR PEPTONE AS AN INDUCER BY *Trametes hirsuta*

RASHEEDA KHANAM*[1] AND DR. R.GYANA PRASUNA[2]

[1]Department of Microbiology, A.Q.J degree & P.G College, Visakhapatnam,
[2]Department of Microbiology, GITAM Institute of science and Technology, Visakhapatnam

RASHEEDA KHANAM
Department of Microbiology, A.Q.J degree & P.G College, Visakhapatnam

*Corresponding author

ABSTRACT

Trametes hirsuta **(GenBank Accession Number: AB429065.1),** a basidiomycete fungus was used for the production of laccases using different carbon sources namely glucose, sawdust, rice bran and wheat bran. Use of organic medium (agricultural/industrial wastes) is aimed to bring down the cost of media. Among these four carbon sources, the best laccase production was obtained in sawdust followed by wheat bran and rice bran. To improve 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) was used. The use of ghee residue in the formulation of the laccases production media by *Trametes hirsuta* has been found to be beneficial in laccases production. The production of laccases in presence of peptone and ghee residue was 85 folds and 7 folds more respectively than in the medium with only glucose. Similarly an increase of 11 folds and 9 folds in laccases production was obtained in the media containing wheat bran with peptone and ghee residue respectively as compared to the controls with only wheat bran. The production was found to be lowered in the medium with saw dust when peptone or ghee residue was added additionally. The production of laccases was found to be lowest in media containing only Glucose (0.44 U/ml). The optimum temperature was found to be 70^0C and pH 5.0 for the laccases activity. The ghee residue and peptone seem to be acting as inducers in the production of laccases.

KEY WORDS

Laccase, Ghee residue, peptone, wheat bran, rice bran, sawdust, *Trametes hirsuta.*

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 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[1].

Laccases are the oldest and most studied enzymatic systems for various industrial applications[2],such as textile dye bleaching, 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].Water from such contaminated sources have 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[4].

Fungal laccases are environment friendly and have wide applications[5]. They help us reduce pollution and the toxicity of the currently used chemicals through their oxidation/reduction mode of action[1]. However the 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[6]. This requires formulation of media which may have cheaper and easily available carbon and nitrogen sources. In this work we have screened three organic wastes namely wheat bran, rice bran and saw dust as a source of carbon for laccases production by the test fungus. Phenolics obtained from plants or other sources have been used as inducers in production of various enzymes[7]. Laccases are able to oxidize several phenolic substrates[6]. In this work we have used ghee residue and peptone as an inducer for laccases 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[8].

Peptone, a product of incomplete fermentative hydrolysis of protein contains peptides, diketopiperazines and amino acids. Peptone was used as the other possible inducer to compare with ghee residue.

MATERIALS AND METHODS

TEST ORGANISM

The test fungus was obtained from Kakatiya University, Warangal, Andhra Pradesh. It was named as Ph1 for convenience. 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) and 20g Agar.

The test fungus in pure form was sent for identification to Xcelris laboratories, Ahmadabad, Gujarat.

Culture in plate:
The fungal culture appears as white mat, at first raised, floccose-cottony to floccose-woolly, after 2-5 weeks becoming more compact, floccose-woolly, floccose-felty, woolly-felty to felty.

Microscopic Features
Hyphal characters. - Advancing zone: hyphae are hyaline, thin-walled, nodose-septate, branched and 1.8-4.3 µm in diameter.
Aerial mycelium: (a) hyphae as in advancing zone; (b) fibre hyphae numerous, with walls thick and refractive, lutina narrow to apparently lacking, mostly unbranched to sparingly branched or some hyphae very closely branched with many short branches (cf. to binding hyphae of fruit-body), 1.5-3.0 µm in diameter. Fruit-body: (a) basidia 4.8 6.0 µm in diameter; (b) basidiospores hyaline, even, cylindric, 4.6 x 1.8-2.0 µm. Submerged mycelium: (a) hyphae as in advancing zone; (b) in old cultures one to two-celled fragments of hyphae present, less common, thin to slightly thick-walled, often swollen and rounded to irregular in shape, appearing like chlamydospores and are 3.0-7.4µmwide[9].

SCREENING FOR LACCASES PRODUCTION
Screening for laccase production was done on the 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[10]. Positive cultures were visualized by reddish brown coloring zones in the screening plates since laccases catalyze the oxidative polymerization of Guaiacol to form reddish brown coloring zones in the medium.

GROWTH CURVE:
The fungus was inoculated in malt extract medium in a 250 ml Erlenmeyer flask and was incubated at room temperature. The growth measurement was done by the dry weight of the fungus. The inoculated flask containing the medium was taken out for dry weight estimation at every 2 days time interval. The fungus was filtered using pre weighed filter paper and was dried in hot air oven at 80^0 C till standard weight was obtained.

OPTIMUM pH
The malt extract media was prepared with different pH ranging from pH 3.0-9.0 by using acetate buffer and the inoculated organism was incubated at room temperature for the period of time where the maximum growth was found.

OPTIMUM TEMPERATURE
To estimate the optimum temperature for the fungal growth, the malt extract media was prepared with the optimum pH by using acetate buffer and was incubated for the period of time where the maximum growth was found at temperatures ranging from 0^0 C to 60^0C.

FERMENTATION BY SUBMERGED FERMENTATION TECHNIQUE:
In submerged fermentation (SmF) *Trametes hirsuta (Ph1)* was grown in a continuous liquid phase. The efficiency of the laccases production was assayed with the aid of media designed with optimum concentrations of every component which influences the production.

STANDARDIZATION OF PRODUCTION MEDIUM COMPOSITION:
Laccases production in liquid culture was investigated using glucose, sawdust, rice bran and wheat bran as the carbon source individually.

COMPOSITION OF THE MEDIUM CONTAINED (*in g/L)*
10g Carbon source, 1.00g aspargine, 0.5g Yeast extract, 0.5g K_2HPO_4, 1.00g $MgSO_4.7H_2O$, 0.001g $FeSO_4.7H_2O$ [11].

CARBON SOURCE:
Laccase production time was standardized using composite medium containing glucose and Guaiacol. The optimum time for production of laccase was found to be 15 days. A set of

production media with the appropriate carbon source was inoculated with the *Trametes hirsuta***.** The inoculated medium was incubated for 15 days at 27-30^{0}C in 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 study 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 in the production medium peptone/Ghee residue (1.0%W/V) is added 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 *Trametes hirsuta* and was incubated for 15 days in orbital shaking incubator at 120 rpm at 27-30^{0}C.

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:

Laccase activity in the sample was spectrophotometrically determined by using Guaiacol as a substrate by monitoring the rate of product (dark brown color) formation. In the reaction mixture, the following components were added;

1.0 ml of 2mM Guaiacol, 3.0 ml of 10mM acetate buffer of desired pH range and1.0 ml of crude enzyme filtrate (to be tested)[12].

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

EFFECT OF pH AND TEMPERATURE ON ENZYME ACTIVITY:

For the study of effect of pH and temperature on enzyme activity, the media showing better production of laccases for *Trametes hirsuta* was selected, the fungal mycelium was filtered after 15 days of incubation and the filtrate is used as the crude enzyme source. It was incubated in the range of pH 3.0-9.0 at 27-30^{0}C. The enzyme activity was determined spectrophotometrically later.

The optimum pH for the enzyme activity for the respective cultures was used for incubating the crude enzyme sample at the temperatures ranging from 0^{0}C-90^{0}C.

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,

$$\text{Vol. activity (U/ml)} = \left[\frac{\triangle A/T \times Vt \times \text{dil. Factor} \times 10^6}{E \times Vs}\right]/1000$$

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

STATISTICAL ANALYSIS:

The results obtained were interpreted by using Minitab16 software.

RESULTS AND DISCUSSION

This paper summarizes the important reports on the cheap and best production media 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 Ph1 was reported as *Trametes hirsuta* (GenBank Accession Number: AB429065.1). *Trametes hirsuta* is a plant pathogen. It is found on dead wood of deciduous trees, especially beech wood. It is found all year round and persists due to its leathery nature [13].

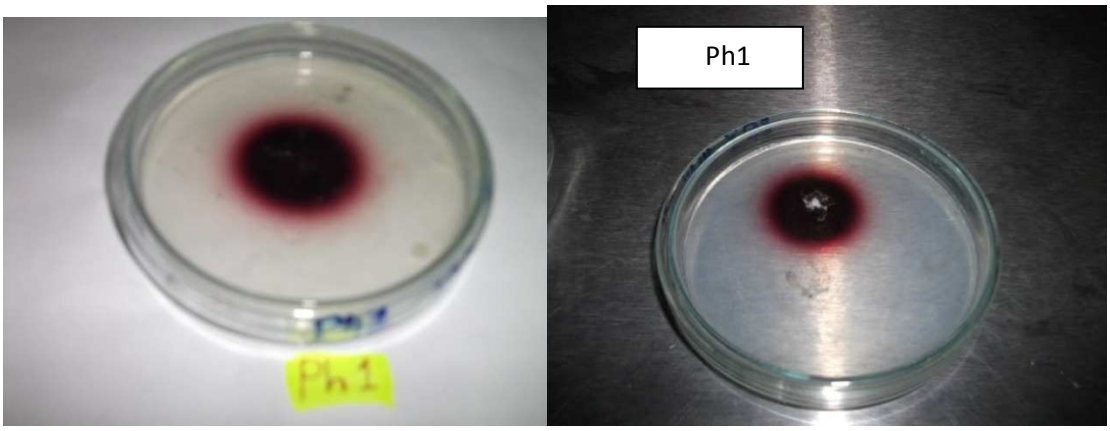
It is Saprobic on the deadwood of hardwoods (very rarely reported on conifer wood); annual; causing a white rot; growing in clusters on logs and stumps; summer and fall; widely distributed across North America.

Cap: Up to 10 cm across and 6 cm deep; semicircular, irregularly bracket-shaped, or kidney-shaped; often fusing laterally with other caps; very densely hairy; often finely, radially furrowed; with concentric zones of texture; zones with gray, whitish, and brownish shades, but usually not contrasting markedly; margin often brownish to brown or blackish. Pore Surface: Whitish, becoming a little brownish, grayish, or yellowish with age; with 3-4 circular to angular pores per mm; tubes with fairly thick walls, to 6 mm deep[14].

Trametes hirsuta is also available in natural samples such as decay wood in various parts of Hosur, Tamil Nadu, India. Samples collected in sterile plastic covers were brought to the laboratory without exposing to the external environment further. The sample was surface sterilized to eliminate surface contaminants, and then the material was used for fungi isolation on the selective agar media. Potato dextrose agar was used for fungi isolation and malt extract medium either broth or solid medium were used further in the study.

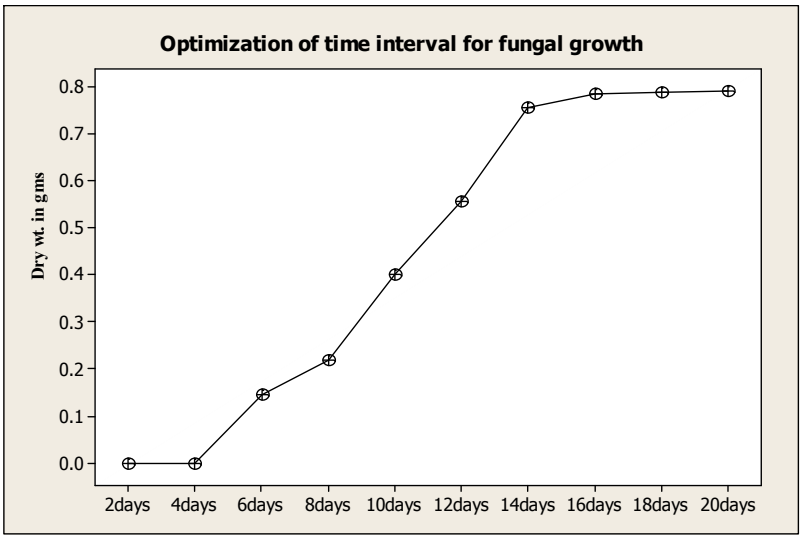
When the test organism was inoculated in the screening media for laccases production using Guaiacol as a substrate it shows positive report by the formation of dark reddish brown zones around the fungal culture.

Figure 1
Positive report for the screening test byTrametes hirsuta (Ph1)



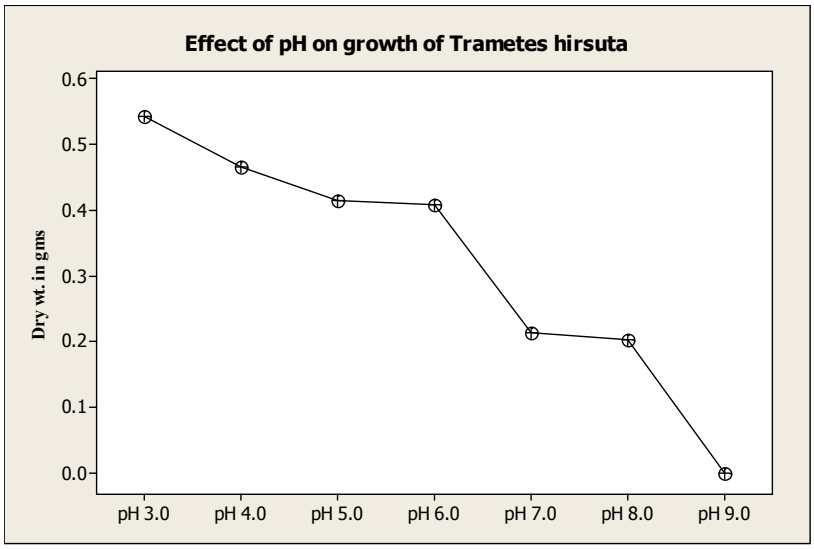
GROWTH: The maximum growth was seen in 12-14 days of incubation at room temperature in malt extract medium (pH6.8).

Graph 1
Optimization of time interval for the fungal growth



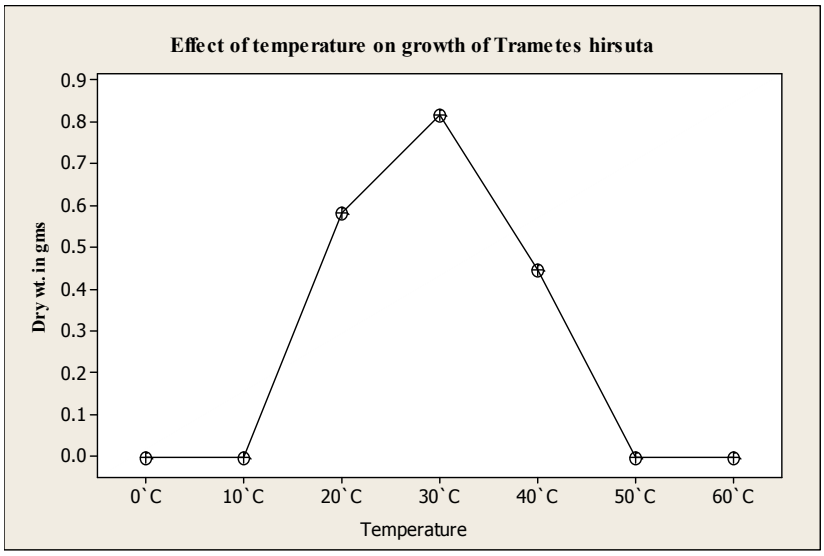
The maximum growth was observed in the medium with pH3.0 when the organism was inoculated and incubated in the malt extract medium with pH ranging form 3.0-9.0 at room temperature for 14 days.

Graph.2
Effect of pH on fungal growth



Similarly the maximum growth was observed at temperature 30^{0}C when the fungus was inoculated and incubated in the malt extract medium (pH3.0) at different temperatures ranging from 0-60^{0}C for14 days (Graph.3)

Graph.3
Effect of Temperature on fungal growth



LACCASES PRODUCTION

Among the production media for laccases, formulated by using agro waste (wheat bran, rice bran), industrial waste (sawdust) as a carbon source, higher laccase production was obtained in the medium with sawdust followed by wheat bran, rice bran and glucose (table: 1). Even though the production is more with SD solely (Table.1) its combination with either peptone or ghee residue is not better than WBP and WBG (Graph: 4). Further addition of peptone or ghee

residue as an inducer leads to higher production of laccases in Wheat bran with peptone/WBP (112.81U/ml) followed by wheat bran with ghee residue/WBG (91.19U/ml) and Rice bran with ghee residue /RBG (59.15 U/ml). The Lowest production of laccases was found in the medium with only glucose (0.44U/ml).The Comparative study on laccases production on different production media with carbon sources showed highest production with Saw dust (SD).

Table: 1

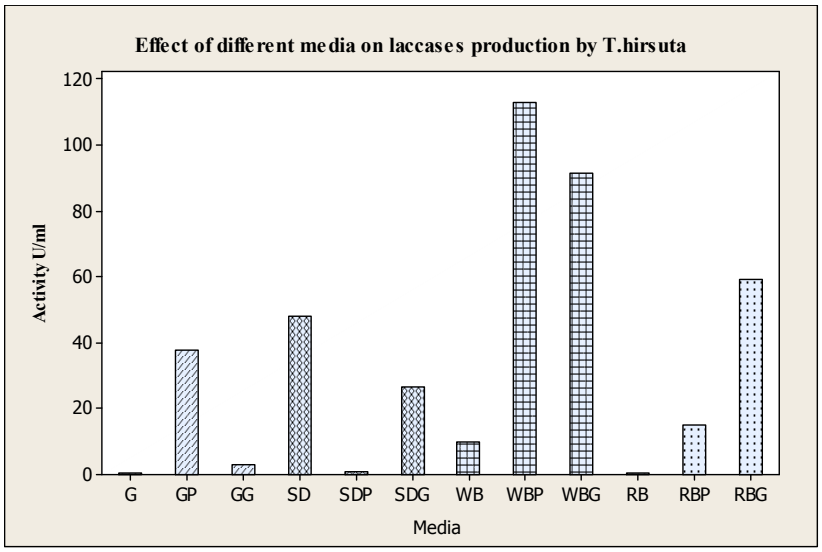
comparative study on media with different carbon sources for laccases production by Trametes hirsuta

MEDIA WITH DIFFERENT CARBON SOURCE	NITROGEN SOURCE	INDUCER	PRODUCTION OF LACCASES (U/ml)
Glucose(G)	Yeast extract	-	0.44
Saw dust(SD)	Yeast extract	-	48.03
Wheat bran(WB)	Yeast extract	-	9.87
Rice bran(RB)	Yeast extract	-	0.60

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 types of nitrogen sources was analyzed, which influence the laccase production significantly.

Graph: 4

Effects of carbon sources and inducers on production of laccases by Trametes hirsuta

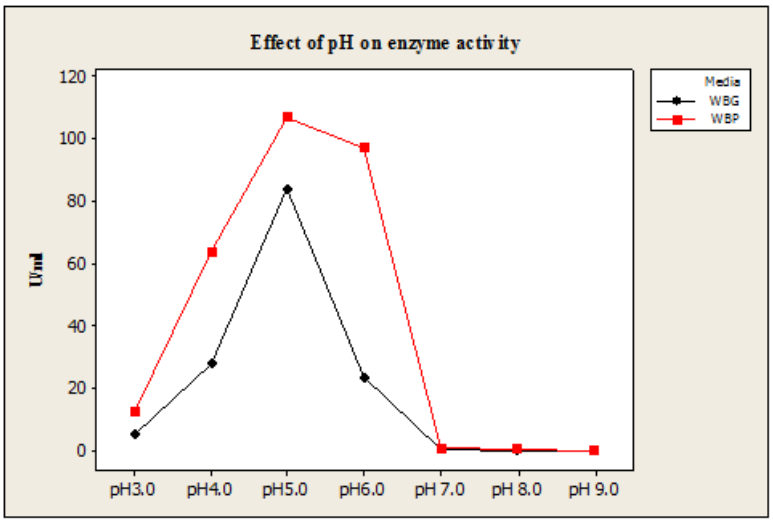


G=Glucose, GP= Glucose and peptone, GG= Glucose and ghee residue, SD= Saw dust, SDP= Saw dust and peptone, SDG= Saw dust and ghee residue, WB= Wheat bran, WBP= Wheat bran and peptone, WBG= Wheat bran ghee residue, RB=Rice bran, RBP= Rice bran and peptone, RBG= Rice bran ghee residue

Further the optimum pH and temperature for the activity of the enzyme produced in the cheap and best media i.e., wheat bran with peptone and wheat bran with ghee residue (Graph: 5) was found to be 5.0 and 70-80^{0}C (graph: 6) 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; 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)[15].Thus, it was found that pcs are present in the ghee residue[8]. As the concentration of the substrate (Guaiacol) increases, the units of enzyme activity increases (graph: 7).

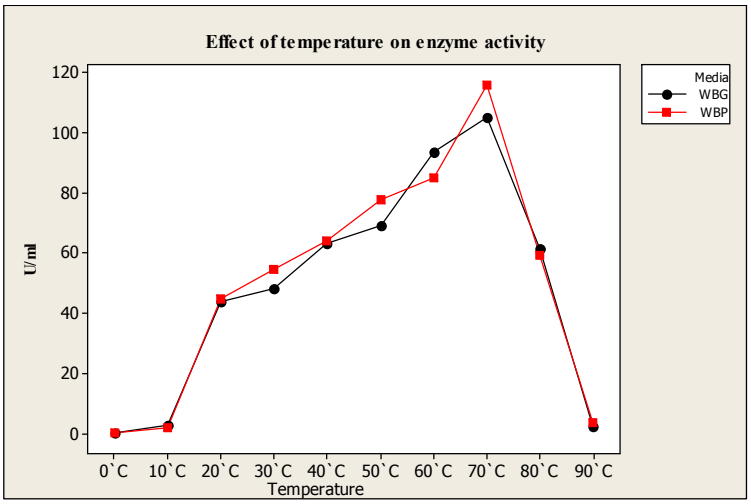
Graph: 5

Optimization of pH for laccases activity in the medium WBP and WBG

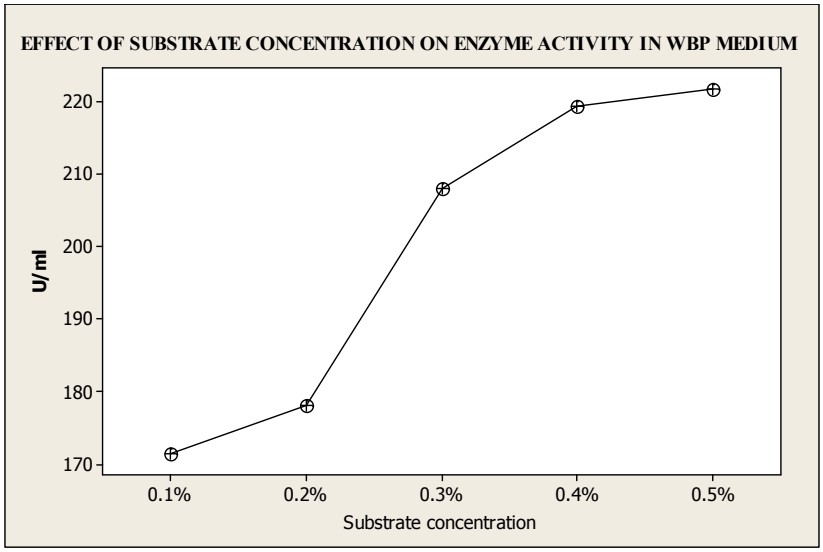


Graph 6

Optimization of temperature for enzyme activity in Medium WBP and WBG with optimum pH5.0



Graph.7
Effect of substrate concentration on enzyme activity



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 *Trametes hirsuta*. 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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