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Research Article

FACTORS FOR THE GROWTH, LIGNINOLYTIC ENZYMES AND TARTARIC ACID PRODUCTION OF *GRAMMOTHELE FULIGO*

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ABSTRACT
Grammothele fuligo (Berk. & Broome) Ryvarden is a wood rotting resupinate Agaricomycete growing on the petioles of *Livistona chinensis* (Jacquin) R. Brown. The effect of different media, temperature, pH, days of incubation and carbon sources on its *in vitro* growth and ligninolytic enzymes was evaluated. Optimum growth occurred on Glucose-peptone medium at 24°C, pH-7.0 after 12th day and glucose was found to be the best carbon source for growth. The fungus *in vitro* produced tartaric acid. Manganese peroxidase, Lignin peroxidase and Laccase activities were assayed *in vitro* for 30 days of incubation on glucose peptone medium and on ten carbon sources. Manganese peroxidase activity was the highest expressed activity after 12 days of incubation. The fungus expressed highest manganese peroxidase activity in medium containing xylose as carbon source after 12 days of incubation. It exhibited least activities for lignin peroxidase and laccase activities. Tartaric acid production gradually increased up to 30 days of incubation at 24°C and pH 7.0. Optimum tartaric acid production occurred on xylose and minimum production on lactose. Increased production of tartaric acid by the fungus after optimum days of incubation; attaining maximum biomass; is proposed to be related to the transformation of generative hyphae to the skeletal hyphae.

Key Words: *Grammothele Fuligo, Ligninolytic Enzymes, Tartaric Acid, In Vitro Studies*

INTRODUCTION
Lignin is the most abundant recalcitrant natural aromatic polymer on earth, being only degraded by few micro-organisms (Martinez *et al.,* 2005). Fungi play an important role in degradation and mineralization of lignocellulosic substrates. The fungi absorb nutrients available in the ambient when the molecules are small, and when they are bigger they use their enzymes (Esposito and de Azevedo, 2004). White rot fungi mineralize lignin through a process that was defined as an enzymatic combustion (Kirk and Farrell, 1987). The ability of white rot fungi to mineralize lignin is due to the possession of extracellular ligninolytic enzyme complexes that differ significantly in their composition. These fungi secrete one or more extracellular enzymes essential for lignin mineralization. These include mainly peroxidases (Lignin peroxidase and Manganese peroxidase) and laccases (Ruiz-Duenas and Martinez, 2009).
The ligninolytic enzymes are produced during secondary metabolism under nutrient-limited cultures (Tekere *et al.,* 2001).
Many studies have proved that the nature and composition of culture medium regulate ligninolytic enzymes production by white rot fungi (Zakariashvili and Elisashvili, 1992; Galhaup *et al*., 2002; Songulashvili *et al*., 2007; Abdel-Azeem and Salem, 2012). It has been concluded that the differential enzyme production is highly dependent on conditions or strains used (Tekere *et al*., 2001). Many white rot fungi, with notable exception of *Phanerochaete chrysosporium*, produce an extracellular laccase under commonly adopted ligninolytic conditions (Gold and Alic, 1993).
Ligninolytic enzymes have a potential in several industrial and biotechnological processes (Maciel *et al*., 2010). It is therefore interesting to evaluate these ligninolytic activities in different white rot fungi. In the present study, the influence of culture conditions (basal medium, temperature, pH, days of incubation and carbon sources) on mycelial biomass production (growth) and reproduction has been determined and influence of time period and different carbon sources on ligninolytic enzymes and tartaric acid production

has also been evaluated. The production of tartaric acid by the fungus represents the first report in literature.

MATERIALS AND METHODS

Microorganism

Grammothele fuligo (Berk. & Broome) Ryvarden was isolated from the dead petiole of *Livistona chinensis* (Jacquin) R. Brown from the Botanical Gardens, Panjab University, Chandigarh, (India), on Malt Extract Agar (Malt Extract 20g, Agar agar 20g, distilled water to make 1000ml) by single hyphal tip isolation at 24°C in petri-plates. For all experiments, the fungus was sub-cultured on the same medium. The stock cultures were kept at ±4°C in the refrigerator.

Procedure

The effects of basal media, temperature, pH, days of incubation and carbon source on growth and reproduction of *G. fuligo* were observed in still cultures grown in 100 ml Erlenmeyer flask containing 25 ml of the basal medium (autoclaved at 15 psi pressure for 15-20 mins). Each flask was inoculated with an agar plug of 10 mm diameter cut from the margin of a 4-day old culture with a mycelial inoculum equivalent to 2.5 mg dry weight. Three replicates were kept for each parameter.

Determination of mycelial dry weight and final pH: At the end of each experiment, the mycelia were harvested through pre-weighed Whatman filter paper No. 1 and dried at 45°C in a hot air oven and their dry weights were measured using an electronic balance (Sartorius Analytical BL 210S). The final pH of the culture filtrate of the individual replicate was checked over Digital pH Meter 813.

Basal Media

The fungus was grown in Glucose-peptone medium which was found to be the best medium for growth.

Temperature

In the experiment on the effect of temperature, the fungus was incubated at 16, 20, 24, 28 and 32°C, in basal medium (Glucose-peptone) with a pH of 5 (selected arbitrarily) for 10 days of incubation.

H-ion concentrations:

In the experiment on the effect of pH, the pH levels of the medium were adjusted to 3.0-9.0 with a difference of unit pH. The pH of each aliquot was adjusted to a separate unit value aseptically with sterile 1N-HCl and 1N-KOH and checked over Digital pH Meter 813. The flasks were incubated for 10 days at 24°C.

Days of Incubation

The effect of days of incubation on growth, enzyme production and organic acid production (tartaric acid) was studied for 30 days, at 24°C and at pH-7.0 (found optimum).

Carbon Source

The effects of the carbon sources (fructose, glucose, lactose, maltose, pectin, raffinose, sorbose, starch, sucrose and xylose) on *G. fuligo* mycelial biomass production, reproduction, ligninolytic enzyme production and tartaric acid production were evaluated at 24°C, pH-7.0 and after 12-days of incubation. The glucose of the glucose-peptone medium was substituted singly by each of the carbon compounds so as to provide 0.333g/l of carbon - a substituent of glucose (10g/l) in the basal medium.

Qualitative Screening for Ligninolytic Enzyme

Lignin modifying enzyme (LME) assay (Archibald, 1992): Azure-B agar clearance method was used to detect the activity of lignin modifying enzyme. The discoloration of blue colored medium was recorded which indicate the activity of lignin modifying enzymes.

Quantitative Assays for Lignin Peroxidase, Manganese Peroxidase and Laccase Activity

The culture filtrate was assayed for enzymatic activity. All enzymes were assayed spectrophotometrically in duplicate, using a SHIMADZU UV spectrophotometer 1800. Lignin peroxidase (LiP) activity was determined using the method of Atalla *et al*. (2010). Manganese (II) peroxidase (MnP) activity was measured following the method whereby guaiacol was used as a substrate (Atalla *et al*., 2010). Laccase activity was determined using guaiacol as a substrate (Coll *et al*., 1993).

Detection of Organic Acid

The detection of organic acid was done by TLC (Stahl, 1969). The solvent used was 96% ethanol, water, 25% ammonia (25:3:4). The acid in the test culture was detected by comparing its R_f value with the standard reference organic acid.

Quantitative Assay for Tartaric Acid Determination

The quantitative determination of tartaric acid from the culture filtrate was done using pyridine-acetic anhydride as coloring agent, using a SHIMADZU UV spectrophotometer 1800 at 420 nm (Marier and Boulet, 1958).

Statistical Analyses

All the experiments were performed in triplicates. The means of three replicate values for all data in the experiments obtained were tested in a one way ANOVA at P=0.05 using PASW Statistics 18 software and Tukey's test was used to evaluate differences between treatments.

RESULTS AND DISCUSSION

Results

Qualitative Enzyme Screening: *Grammothele fuligo* exhibited positive lignin modifying enzyme activity. The production of purple color under and around the fungal colony was considered as a positive reaction resulting from decolorization of Azure B.

Organic Acid Production (TLC results): The study of comparative R_f of unknown organic acid in the culture filtrate with the standard reference organic acids shown in Table 1 revealed the production of tartaric acid in the culture filtrate.

Table 1: The R_f value of the unknown organic acid in the culture filtrate of *G. fuligo* in comparison with the selected reference organic acids, from chromatograms run concurrently

Sample	R_f value
Citric acid	0.15
Fumaric acid	0.6
Succinic acid	0.51
Tartaric acid	0.33
Culture filtrate	0.33

Effect of Liquid Basal Media

In the present study, Glucose-peptone medium was found to be an optimum medium for the growth of *G. fuligo*. The growth rate was significantly greater on this medium than in other basal media in the preliminary experiments. The final pH of the Glucose peptone medium changed from 5.0 to 4.3. It did not reproduce and formed vegetative hyphae which were thin-walled, profusely branched with clamp connections, sparsely vacuolated and septate at long intervals.

Effect of Temperature

The fungus showed optimum growth rate at 24°C. The final pH of the basal medium changed from 5.0 to 4.3 at 20°C, 24°C, 28°C & 32°C. Thin-walled, profusely branched hyphae with clamp connections and sparsely vacuolated were formed at 16°C & 20°C and brown colored hyphae (skeletal hyphae/dendrohyphidia) developed at 24°C, 28°C & 32°C which were short and thick-walled.

Effect of Hydrogen-ion Concentration

The fungus produced optimum growth at pH 7.0. The final pH of the basal medium changed significantly with the mycelial growth from 7.0 to 4.6. The fungus produced hyaline to slightly brown colored mycelia at pH 3.0-5.0 and pH 7.0-9.0, however, along with white mycelia dark brown colored mycelia also

developed at pH 6. Hyphae were thin-walled, profusely branched with clamp connections, sparsely vacuolated and short. Thick-walled, branched and light brown hyphae (skeletal hyphae/dendrohyphidia) developed at pH 3, 4, 5, 7, 8 & 9 whereas at pH 6 these hyphae are dark brown in color.

Effect of Days of Incubation

Maximum growth rate was obtained after 12 days of incubation thereafter, it declined (Figure 1). The final H-ion concentration of the basal medium changed significantly from pH 7 to pH 6.2 up to optimum days of incubation thereafter, it decreased gradually to 4.9. It showed gradual increase in the production of LiP up to 30 days of incubation, MnP up to 12 days of incubation after which it decreased gradually and Laccase up to 26 days of incubation afterward which it decreased (Figure 2). Out of the three enzymes screened it showed maximum activity for MnP. There was a gradual increase in *in vitro* tartaric acid production up to 30 days of incubation (Figure 1).

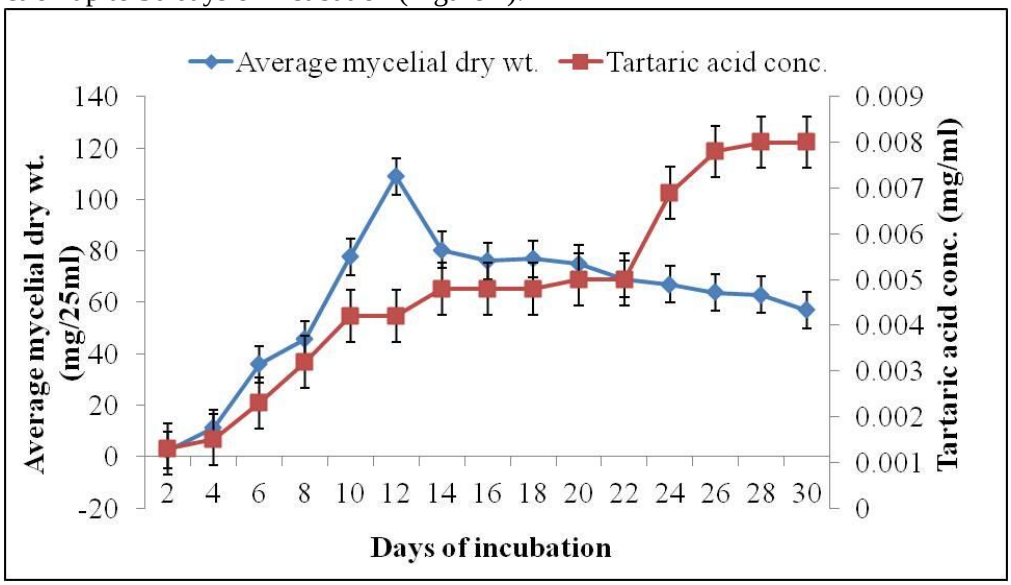

Figure 1: Growth and tartaric acid production of *G. fuligo* on Glucose-peptone medium with different days of incubation at optimum temperature (24°C) and pH (7.0)

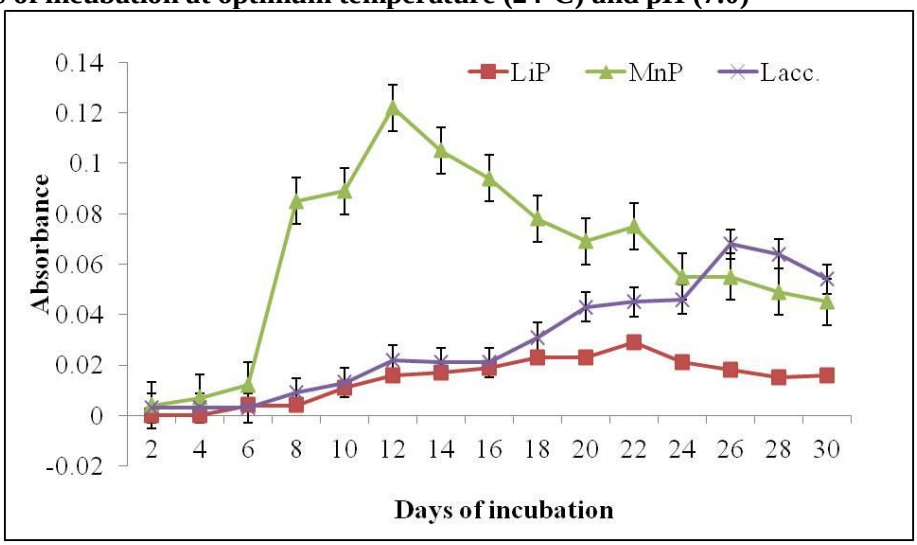

Figure 2: Lignin peroxidase, Manganese peroxidase and Laccase activity of *G. fuligo* with different days of incubation at optimum temperature (24°C) and pH (7.0)

Mycelial growth was in the form of superficial as well as submerged, incomplete, white mycelial mat up to 6 days after that it formed complete mycelial mat with brown colored mycelia which developed after 9 days of incubation. Thin-walled, sparsely vacuolated and profusely branched hyphae with clamp connections developed up to 8 days of incubation and thick-walled, branched, brown colored hyphae (skeletal hyphae/dendrohyphidia) started developing after 9 days of incubation and their development continued up to 30 days of incubation.

Effect of Carbon Source

The maximum average mycelial dry weight was obtained with glucose followed by raffinose, fructose, sucrose, xylose, lactose, maltose, sorbose, starch and poor growth was obtained with pectin (Figure 3). The pH of the medium shifted toward acidity with all selected carbon source. It did not reproduce with any carbon source. It showed maximum activity of LiP in medium containing lactose as carbon source followed by raffinose, sorbose, glucose and maltose; poor activity in xylose, fructose and pectin & nil activity in starch and sucrose (Figure 4). Maximum MnP activity was observed in xylose followed by glucose, sorbose, control, lactose, sucrose, fructose, maltose, starch, pectin and raffinose (Figure 4). Maximum Laccase activity was observed in medium containing xylose followed by control, maltose, lactose, glucose, raffinose, starch, sucrose, sorbose and fructose & activity was nil in pectin containing medium (Figure 4).

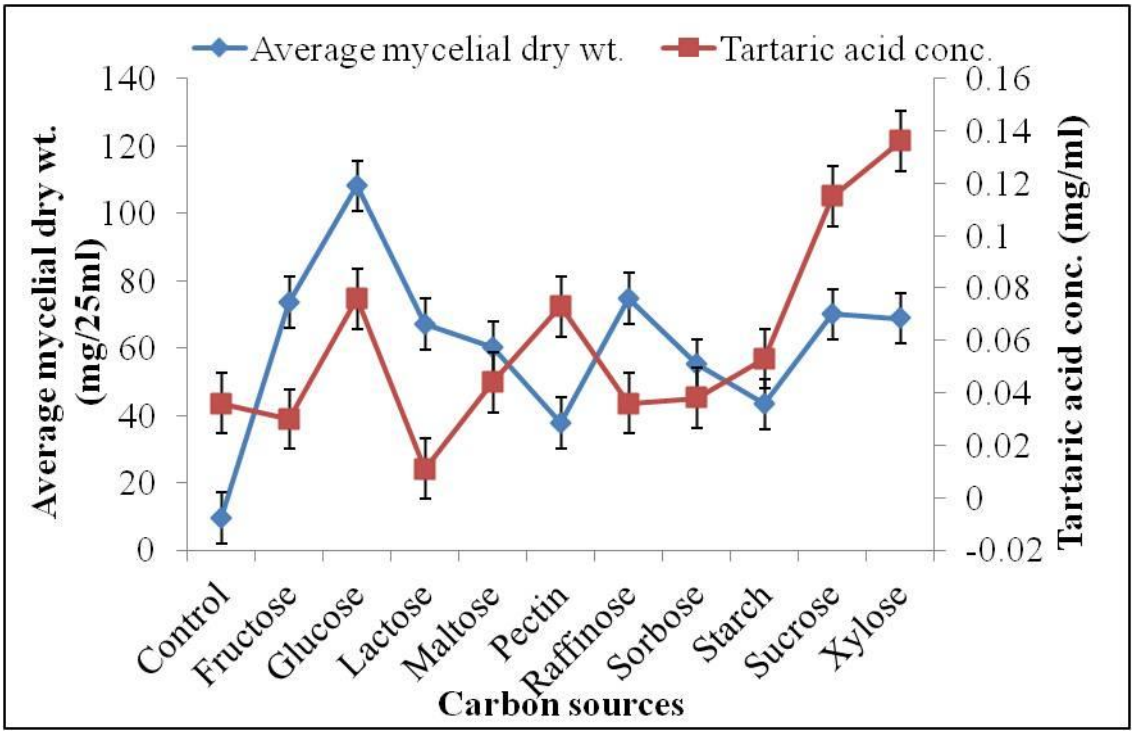

Figure 3: Growth and tartaric acid production of *G. fuligo* on selected basal medium with different carbon compounds at optimum temperature (24°C), pH (7.0) and days (12) of incubation

Tartaric acid production was found to be maximum with xylose followed by sucrose and glucose however, there was minimum production with fructose and lactose (Figure 3). Mycelial growth was in the form of white superficial as well as submerged, incomplete mycelial mat in control and sorbose. It formed mycelial patches in lactose with white and brown colored mycelia. Mycelial growth was in the form of superficial as well as submerged, complete, white mycelial mat with brown colored mycelia in fructose, glucose, maltose, pectin, raffinose, sucrose and xylose whereas only white mycelia formed in starch.

Hyphae were thin-walled, profusely branched with clamp connections and narrow in control and sucrose. These hyphae were broader in fructose, glucose, lactose, maltose, pectin, raffinose, sorbose, starch and xylose. Brown colored hyphae (skeletal hyphae/dendrohyphidia) were short, thick-walled and branched in fructose, glucose, lactose, maltose, pectin, raffinose, sucrose and xylose.

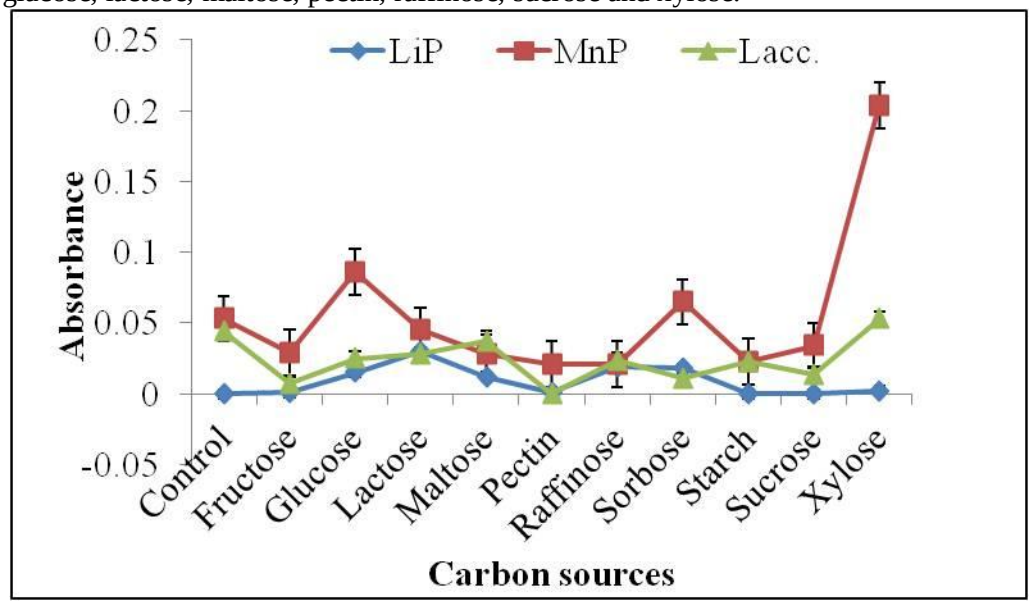

Figure 4: Lignin peroxidase, Manganese peroxidase and Laccase activity of *G. fuligo* in different carbon compounds at optimum temperature (24°C), pH (7.0) and days (12) of incubation

Discussion

The optimum temperature for the growth of this isolate is 24°C and optimum pH is 7.0. It gives optimum mycelial growth rate (109.0mg/25ml) after 12 days of incubation. However, it does not reproduce *in vitro*. MnP activity increases and reaches its highest value (A_{465nm} -0.122) on 12th day of incubation, thereafter, it decreases whereas the highest values of LiP and Laccase activities are obtained after 22 (A_{310nm} -0.029) and 26 (A_{440nm} -0.068) days of incubation respectively. MnP (A_{465nm} -0.204) and Laccase (A_{440nm} -0.053) enzymes activities are found to be high in medium containing xylose as carbon source whereas LiP (A_{310nm} -0.030) activity is high with lactose. The enzyme activities obtained show that the MnP enzyme is predominant in this isolate as compared to LiP and Laccase enzymes. The tartaric acid production started from the 2nd day of incubation and there was a gradual increase in the production up to 30 days. It was also noticed that there was gradual increase in the acidity of the culture filtrate with the increase in the formation of brown colored skeletal hyphae/dendrohyphidia in the culture up to 30 days of incubation. Tartaric acid production was found to be maximum when xylose was used as carbon source followed by sucrose and glucose; however; there was minimum production when culture medium was supplemented with fructose and lactose as carbon sources. The increased tartaric acid production, lignin peroxidase and laccase activity after optimum days of incubation (12 days) is probably correlated to the transformation of generative hyphae to brown colored skeletal hyphae/dendrohyphidia which develop from the generative hyphae consuming their contents. The role of secondary hyphae (skeletal hyphae/dendrohyphidia) in organic acid biosynthesis and enzyme activity is not known. Studies are in progress in this direction.

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