PRODUCTION OF LIGNINOLYTIC ENZYMES DURING SOLID STATE FERMENTATION OF COFFEE PULP BY SELECTED FUNGI

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ABSTRACT

In our study, *Pleurotus eous* and *Chaetomium globosum* the peak enzyme values (5.53 IU/ml; 2.6 IU/ml respectively) were observed on the 20th day, whereas in *Ganodermalucidum*, the enzyme activity steadily increased till the 30th day (3.6 IU/ml) followed by a steady decline thereafter. In coculture experiments, the maximum laccase activity (8.8 IU/ml) was shown by *Pleurotus flabellatus* + *Pleurotus eous* on the 20th day of biodegradation. The highest peroxidase enzyme activity was observed in coffee pulp colonized by *Phanerochaete chrysosporium* monoculture (3.1 IU/ml) on the 30th day after inoculation followed by *Pleurotus flabellatus* monoculture which showed the maximum 2.83 IU/ml of peroxidase activity on 40th day of biodegradation. *Aspergillus terreus* showed very low peroxidase activity, while peroxidase activity of *Chaetomium globosum* on 30th day (2.3 IU/ml) was on par with the 40th day activity of *Fomes badius.* Significant increase in peroxidase activity over the respective monocultures was observed in all the white rot + white rot dual culture combinations and in *Pleurotus flabellatus* + *Chaetomium globosum* (3.6 IU/ml) dual culture.

Keywords: Ligninolytic enzymes, white rotters, brown rotters, soft rotters, biodegradation.

INTRODUCTION

Coffee pulp is one of the most abundantly available agro-industrial wastes, produced during the pulping operations. Thus, for every 2 tonnes coffee cherries processed, nearly one ton pulp is generated (Adams and Dougan, 1981). Only the coffee bean has a real commercial value. It represents 55.4% of the fruit on dry weight basis and the rest is considered to be the byproducts or residues. At different stages from harvesting to the processing and consumption, several residues *viz.,* coffee pulp or husk, leaves and spent-ground are generated in more than two million tonnes quantity yearly (Pandey *et al.,* 2001). Among these byproducts, coffee pulp is the most important. Operation of the coffee cherries to obtain coffee beans in many coffee-producing areas of the tropics. Undoubtedly, the discovery of "ligninase," a peroxidase in *Phanerochaete chrysosporium* byTien and Kirk, (1983) was a major but the role of other enzymes like oxygenases and oxidases in lignin degradation has not been completely ruled out. Two families of extracellular glycosilated proteins, designated lignin peroxidases (LiPs) and manganese peroxidases (MnPs) are involved in the process of biological degradation (Paszczynski *et al.,* 1986). The degradation of aromatic substances forms an important part of the natural carbon cycle (Evans and Fuchs, 1988). The extracellular enzymes that are involved in the initial steps of lignin and xenobiotic degradation by white rot fungi are lignin peroxidases (LiPs), manganese-dependent peroxidase (MnP), laccase and H_2O_2 -producing oxidase (*e.g*. aryl alcohol oxidase and glyoxal oxidase) (De Jong *et al.,* 1994). The exact role of laccase in lignin degradation is still disputed. However, laccases are able to cleave phenolic lignin model compounds (Higuchi, 1990). Laccases are involved in the biodegradation of lignins, which constitute the main non- carbohydrate component in wood and are among most abundant groups of biopolymers in the biosphere (Eggert*et al.,* 1996).

The ligninolytic enzymes are also capable of *in vitro* oxidation of a variety of aromatic environmental pollutants.

Ligninases have applications in delignification of lignocellulose materials, which can be used as the feedstock for the production of biofuels, paper pulp or as animal feed. They may also be used in pulp bleaching, paper mill waste water detoxification, pollutant degradation or conversion of fungal species differed in their ability to produce peak activities of the different lignocellulolytic enzymes like FP-ase, CMCase, xylanase and β-xylosidase (Azizi *et al*., 1991; Ramamoorthy *et al*., 1999). The lignocellulolytic enzyme activites have been reported in *Aspergillus terreus* and in *Phanerochaete chrysosporium* utilizing corn stover as substrate (Koijam *et al*., 2000), in *Pleurotus sajor- caju* growing on rice straw (Rai and Saxena, 1990), in *Chaetomium globosum* (Lakshmikant, 1990), in a strain of the brown rot fungus *Coniophora puteana* (Schmidhalter and Canevascini, 1992), in *Trichoderma reesei* growing on wheat straw (Kim *et al*., 1985; Maheswari *et al*., 1993), in water hyacinth (Ali *et al*., 1991) inoculated with *Aspergillus terreus* and*Trichoderma reesei,* in *Aspergillus niger* (Ray *et al*., 1993) and in *Aspergillus sp*. (Dina *et al*., 2001).

The extracellular enzyme activities with reference to the lignocellulolytic enzymes of filamentous fungi have been studied using various substrates like poplar wood (Szakacs and Tengerdy, 1997), wheat straw (Mata and Savoie,1998; Savoie and Mata,1999), sago hampas (Kumaran *et al*., 1997), wood or sawdust (Arora and Sandhu, 1987; Buswell *et al* ., 1996) and several synthetic lignin compounds (Sigoillot *et al*., 1999), sugarcane bagasse (Pal *et al*., 1995), paddy straw and rice husk (Saravanan *et al*., 2002). Lignolytic enzyme production using model substrates under SSF with two white rot fungi, *Trametes versicolor* and *Pleurotus ostreatus* was attempted by Gupta *et al*. (1997). The role of laccases in lignin metabolism (Ganisan *et al*., 1998) and their regulation during the development of fruiting bodies were studied in detail by Tan and Wahab (1997). Sinegani *et al*. (1999) studied the production of ligninolytic enzymes by imperfect fungi and yeast induced by culture additives. Laccase production by *Polyporus sp.* was found to be significantly higher than *Aspergillus terreus* and *Phanerochaete chrysosporium* in the liquid culture media supplemented with phenolic derivatives (Sineganiet *al*., 2000). Anne *et al.* (2001) studied the control of laccase overproduction for paper manufacturing and food industries applications in *Pycnoporus cinnabarinus.*

Lignin peroxidase played an important role in delignification of lignocellulosic materials (Kirk and Farrel, 1987) and in degradation of recalcitrant organic pollutants (Hammel, 1987). Production of extracellular lignin peroxidase by *Pleurotus ostreatus, Pleurotusostroformis, Trametesversicolor, Pleurotus sajor-caju*and *Phanerochaete chrysosporium* (Sen *et al*., 2001) and by a brown rot fungus *Polyporus.* A ligninolytic enzyme system showing enhanced decomposition and activity was obtained by cocultivation of *Phanerochaete chrysosporium* and *Pleurotus ostreatus* on combinations of lignocellulosic wastes (Verma and Madamwar, 2001). Mass inoculum development of *Ceriporiopsis subvermispora,* a non-sporulating fungus and a potential lignin degrader had been used in paper industry either individually or with other lignin degrading microorganisms in cocultures (Bajpai *et al*., 2001; Saxena and Vohra, 2001). White rot fungi like *Daedala flavida, Phlebia radiate* and *Phlebia floridensis* were the best producers of lignin peroxidase, manganese peroxidase and laccase in wheat straw (Arora and ChanderRampal, 2000; Verma and Madamwar, 2001). Keeping these aspects in mind, the present investigation was undertaken to study the production of ligninolytic enzymes during solid state fermentation of coffee pulp by selected fungi.

MATERIALS AND METHODS

Substrate:

Coffee pulp, the solid waste of coffee industry, processing the coffee beans by wet processing method was used as the substrate for biodegradation studies.

Organisms:

The selected white rot fungi were *Phanerochaete chrysosporium, Pleurotus eous, Pleurotus flabellatus;* brown rot fungi namely *Ganoderma luciderm* and *Fomes badius* ; Soft rot fungi namely *Chaetomium globosum* and *Aspergillus terreus* are used for biodegradation of coffee pulp. These fungal cultures were maintained on malt extract (2%) agar medium.

Biodegradation Studies:

Biodegradation of coffee pulp was studied in solid state in Erlenmeyer flasks (250ml) using the selected mushroom fungi and their fungal associations. Ten g of coffee pulp containing 60% moisture was taken in individual Erlenmeyer flasks (250ml). The flasks were plugged with cotton and autoclaved at 121°C for 15 min. Single mycelial agar block (8 mm) from seven days-old cultures of the selected fungi was used as inoculum for monoculture experiments. For coculture studies, two agar blocks of the test white rot fungus and its respective fungal association were used as inocula. The conical flasks were incubated at 28 ± 2°C for a period of 40 days in the culture room. At each ten days interval of study, the entire content of each flask was withdrawn, and the enzyme activities *viz*., laccase was based on the oxidation of guiacol (Mahadevan and Sridhar, 1986) and peroxidase were assayed using fresh biodegraded samples. The peroxidase assay was based on the measurement of purpurogallin, a chromogenic compound released from H_2 donor, pyrogallol with H_2O_2 as the substrate due to the activity of peroxidase (Kar and Mishra, 1976). All the experiments were carried out in triplicates and were replicated twice.

RESULTS AND DISCUSSION

Coffee pulp is one of the most abundantly available agro industrial wastes produced, during the pulping operation of the coffee cherries to obtain coffee beans in many coffee- producing areas of the tropics (Roussos *et al*., 1995).

Fig. 1: Laccase activity (IU/ml) of selected white rot, brown rot and soft rot during solid state biodegradation of coffe pulp.

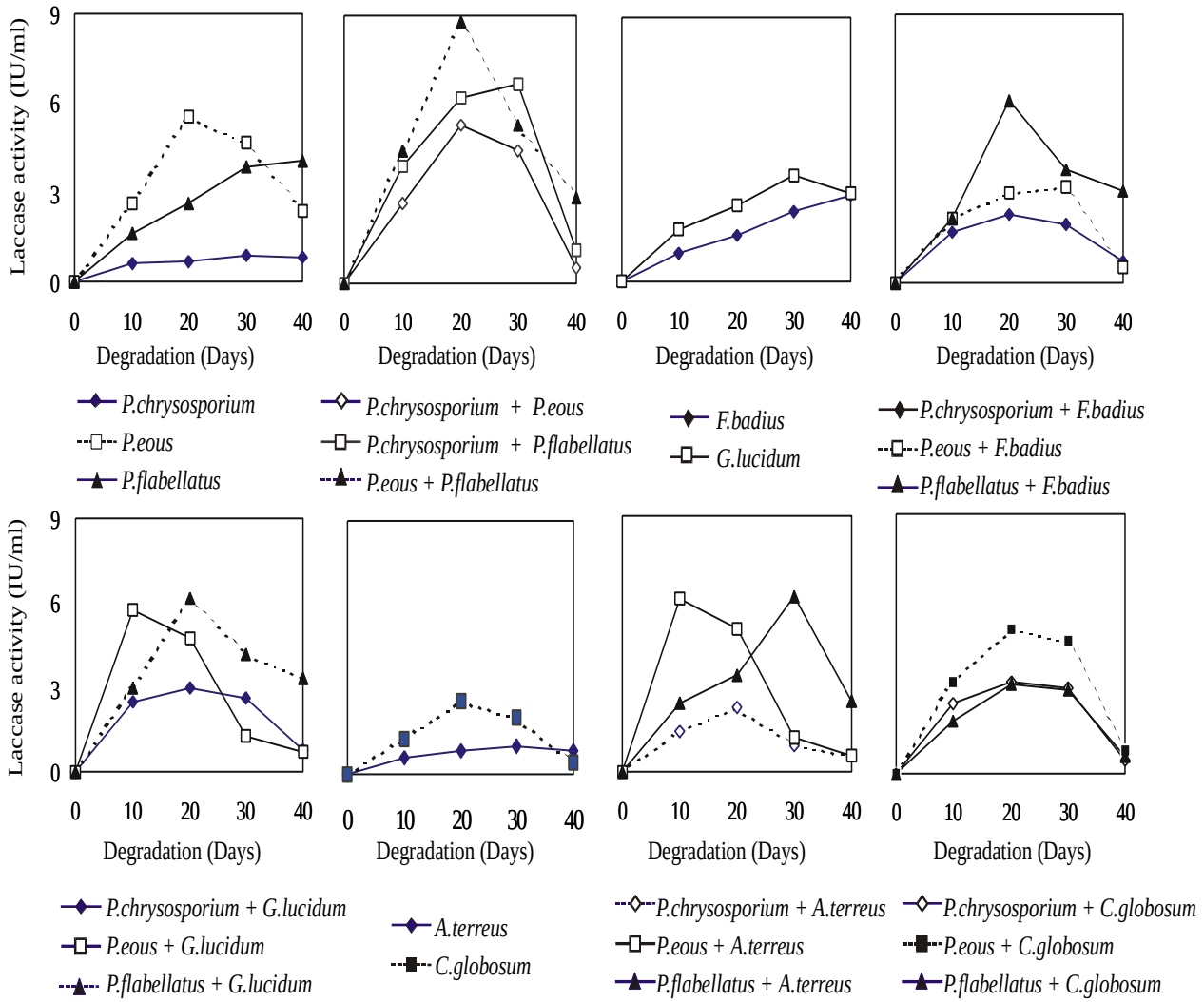
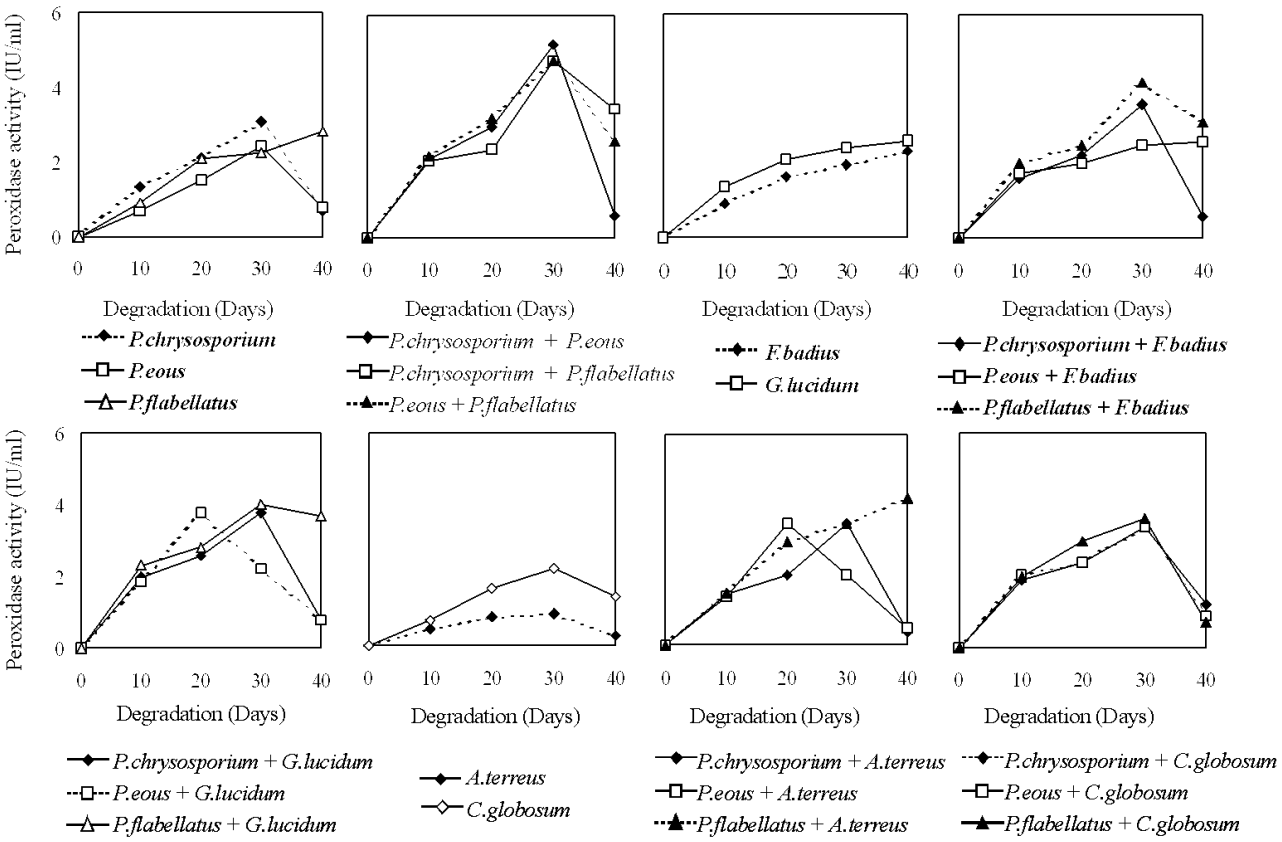


Fig. 2: Peroxidase activity (IU/ml) of selected white rot, brown rot and soft rot during solid state biodegradation of coffee pulp.



Laccase

Laccase activity continued to increase gradually to reach the maximum titre on the final day of experiment in *P.flabellatus* (4.08 IU/ml) monoculture. In *P.eous* and *C.globosum* the peak enzyme titre values (5.53 IU/ml; 2.6 IU/ml respectively) were observed on the 20th day, whereas in *G.lucidum*, the enzyme activity steadily increased till the 30th day (3.6 IU/ml) followed by a steady decline thereafter (Fig 1). Similar reports were also made by Geetha and Sivaprakasam (1998) who observed that the laccase activity was higher in the intial stages, which gradually decreased thereafter in sugarcane bagasse – paddy straw mixture inoculated with *Pleurotus spp*.

Yesilada *et al*. (1998) associated that high biodegradation potentials with high laccase activities and high biomass production in *Coriolus versicolor* and *Funalia trogii*. *P. flabellatus, P.eous* and *G.lucidum* monocultures seemed to share these properties. *Chaetomium globosum* among the soft rot fungi was earlier shown to have significant laccase activity (Rai *et al.,* 1993). Our results on quantitative and plate assays of laccase for *C.globosum* corroborated with this finding.

Significant laccase activities have been reported for many white rot fungi (Hatakka, 1994; Vikineswary *et al.,* 2001) and brown rot fungi like *Polyporus sp* (Safari Sinegani *et al.,* 2001). But laccase was not found to be produced by *P.chrysosporium*(Hatakka, 1994). Deuteromycetes like *A.terreus* were also observed to be poor degraders of lignin by Kamal *et al.* (2000). We similarly found that laccase activity was very low throughout the experimental period in the white rot fungus *P.chrysosporium* and the soft rot fungus *A.terreus.* These results were supported by their respective laccase plate assays.

Laccase acts as a developmental marker, the level of which is maintained until the culture begins to fruit and after which the level falls (Tan and Wahab, 1997). In reports on fruiting cultures, the decline in activity of laccase when fruiting bodies develop corresponds with an increase in cellulase activity, which has remained at a low level during the vegetative phase (Wood and Goodenough, 1977).

Results of *P.eous* culture producing fruiting bodies on coffee pulp are in accordance with these reports. *P. flabellatus* which does not fruit on coffee pulp seemed to be inhibited by its caffeine and tannin contents.

In coculture experiments, the maximum laccase activity (8.8 IU/ml) was shown by *P. flabellatus* + *P.eous* on the 20th day of biodegradation. In contrast, the peak activity was observed on the 10th day in *P. eous* + *G.lucidum* (5.74 IU/ml) and *P.eous+ A. terreus* (6.1 IU/ml) cocultures, whereas in *P .flabellatus + A. terreus*, laccase activity continued to increase till the 30th day (6.2 IU/ml) and then decreased (Fig 1).Earlier peak in laccase activity in these cocultures signalled the advancement of reproductive phase. These results were substantiated by the visual observations on their respective SSF flasks. Similar findings were reported by Kerem *et al.* (1992), Singh *et al*. (1994), Asiegbu *et al*. (1996) and by Savoie and Mata (1999) in SSF studies using cocultures of white rot fungi and mushrooms, their weed fungi or bacteria respectively.

3.2. Peroxidases

The highest peroxidase enzyme activity was observed in coffee pulp colonized by *P.chrysosporium* monoculture (3.1 IU/ml) on the 30th day after inoculation followed by *P.flabellatus* monoculture which showed the maximum 2.83 IU/ml of peroxidase activity on 40th day of biodegradation (Fig 2).

Phanerochaete chrysosporium, the most studied wood decaying fungus reportedly elaborated both lignin peroxidase (Tien and Kirk, 1983) and manganese peroxidase (Wariishi *et al*., 1991). Similarly, the peroxidase activity of several species of *Pleurotus* was well documented (Hader *et al*., 2001). Observations similar to our study have been made by Mata and Savoie (1998) who reported that the MnP activity of *L.edodes* reached higher values between 28 and 42 days.

Aspergillus terreus showed very low peroxidase activity, while peroxidase activity of *Chaetomium globosum* on 30th day (2.3 IU/ml) was on par with the 40th day activity of *Fomes badius.* Though lignin mineralization brought about by deuteromycetes was observed to be very limited (Hammed and Prema, 2001; Hatakka, 2001), phenol oxidase production by *Chaetomium* species has been reported by Chefetz *et al* (1998).

Significant increase in peroxidase activity over the respective monocultures was observed in all the white rot + white rot dual culture combinations and in *P.flabellatus* + *C.globosum* (3.6 IU/ml) dual culture. In certain associations *viz*., *P.eous + G.lucidum* (3.8 IU/ml), *P.eous + A.terreus* (3.5 IU/ml) peak peroxidase activity was observed earlier, while in other associations the enzyme activity peaked on 30th day as in the monocultures (Fig.2). Synergy due to colonization of different white rot fungi had beeen found (Verma and Madamwar, 2001) to result in increased production of ligninases in various lignocellulosic wastes. Asiegbu *et al. (*1996) opined that novel combinations of lignin attacking enzymes could be achieved in cocultures and mixed cultures.

From the above results it can be concluded that the different fungal associations can be grouped not only on the basis of their ability to degrade coffee pulp but also on the extent of synergy, physiological compatiblity and endurance during their colonization of coffee pulp.

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