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Utilization of banana waste for the production of lignolytic and cellulolytic enzymes by solid substrate fermentation using two *Pleurotus* species (*P. ostreatus* and *P. sajor-caju*)

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Abstract

Pleurotus ostreatus and *P. sajor-caju*, were investigated for their ability to produce various lignolytic and cellulolytic enzymes such as laccase, lignin peroxidase, xylanase, endo-1,4-β-D-glucanase (CMCase) and exo-1,4-β-D-glucanase (FP activity) on Banana agricultural waste (leaf biomass and pseudostems) at solid substrate fermentation. The production patterns of these extracellular enzymes were studied during the growth of the organisms for a period of 40 days. Both organisms exhibited similar levels of enzyme activities and pattern of production. Leaf biomass was found to be a more suitable substrate compared to pseudostems for enzyme production. Very low levels of cellulolytic enzyme activities were detected compared to lignin degrading enzymes by both the organisms. Maximum specific activities of enzymes were obtained between 10 and 20 days of culture growth.

Keywords: *Pleurotus ostreatus*; *Pleurotus sajor-caju*; Solid substrate fermentation; Banana waste

1. Introduction

Banana is one the most extensively consumed fruits in the world and represents 40% of world trade in fruits. India is one of the largest producing countries of this fruit, which it is cultivated in 4.796×10^5 ha yielding 16.37×10^6 t of banana [1]. Each hectare of banana crop generates nearly 220 t of plant residual waste that consists mainly of lignocellulose material. Although these materials can be converted to biogas or compost, transportation makes this practice uneconomical [2]. Most of the residual waste produced is discarded by farmers in to nearby rivers, lakes and on roads, which causes a serious environmental concern.

In recent years there has been significant interest in efficient use of agro-industrial residues [3–5]. Several processes have been developed based on these materials as substrates in bioprocess for production of single cell protein, organic acids, ethanol, mushrooms, enzymes and biologically important secondary metabolites [3,6]. Use of these agricultural wastes in bioprocesses may provide alternative substrates and furthermore, helps to solve environmental problems, which are otherwise caused by their disposal.

The main residual wastes of the banana crop are leaves and pseudostems, both containing high levels of lignocellulose [7]. These lignocellulose materials are efficient substrates for white-rot fungi, which produces lignolytic and cellulolytic enzymes that have numerous applications in industrial processes for food, drug, textile and dye use [8–11]. Since the white-rot fungi grows in nature under solid state conditions it can be advantageous to utilize these to develop bioprocesses using agricultural solid waste as substrates [3].

In the present study, two white-rot fungal species *P. ostreatus and P. sajor-caju* were assessed for their ability to produce lignolytic and cellulolytic enzymes such as laccase (EC 1.10.3.2), lignin peroxidase (EC 1.11.1.14),

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xylanase (EC 3.2.1.8), endo-1,4-β-D-glucanase (carboxy methyl cellulase, CMCase EC 3.2.1.4) and exo-1,4-β-D-glucanase (FP activity EC 3.2.1.91) by solid substrate fermentation on banana residual waste. The dynamics of these extracellular enzymes were studied during the growth of these organisms on leaf and pseudostem residual waste.

2. Materials and methods

2.1. Cultures and maintenance

Pleurotus ostreatus and *P. sajor-caju*, were procured from The Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. The cultures were maintained on PDA slants at 25 °C by subculturing every 30-day interval.

2.2. Inoculum development

Inocula for both cultures were produced on boiled wheat grains supplemented with 0.2% calcium carbonate and 1.2% calcium sulphate. Cultures were incubated at 25 °C for 15 days and these grains with mycelium were used as inocula.

2.3. SSF substrate preparation, inoculation and culture conditions

Agriculture wastes of banana plants were collected, dried and divided into pseudostem portion and a leaf portion, the portions were cut in to ~2 cm pieces. Twenty-five grams of each portion was placed in 1000-ml conical flasks and moistened with 75 ml of distilled water. The flasks were autoclaved for 2 h at 121 °C and then inoculated separately with 3-g wheat grain based inocula of *P. ostreatus* and *P. sajor-caju*. The cultures were incubated at 25 °C in a BOD incubator and samples were collected from day 10 and thereafter at 5-day intervals until day 40.

2.4. Sampling, extraction and analytical methods

Enzymes were extracted from 5 g of sample with 20 ml cold 0.05 M acetate buffer (pH 6.5). The homogenate was filtered through of 200 mesh nylon cloth and the filtrate centrifuged at 6000 × *g* at 4 °C for 20 min. The supernatant was analyzed for activities of laccase [12], lignin peroxidase [13], xylanase [14], carboxy methyl cellulase (CMCase) [14] and filter paper activity (FP activity) [14].

Laccase unit activity was defined as the amount catalyzing 0.1 absorbance change in guaiacol per minute. The amount of the enzyme catalyzing a change of 1.0 absorbance unit in *o*-dianisidine per minute was defined as one activity unit of liginin peroxidase. One activity unit of FP activity, carboxy methyl cellulase (CMCase) and xylanase was expressed as 1 μmole of glucose or xylose equivalents liberated per min, respectively. Specific activities of these enzymes were estimated by determining the total protein contents in the enzyme extract expressed as units per milligram of protein.

The amount of reducing sugars were estimated by a dinitrosalicylic acid method [15] and protein concentrations were estimated by the method of Lowry et al. [16].

3. Results

Banana agricultural waste was divided into leaf biomass and pseudostems. To assess their suitability for the production of lignolytic and cellulolytic enzymes they were cultivated separately. Production of laccase, peroxidase, xylanase, endo glucanase (CMCase) and exo glucanase (FP activity) during degradation of banana biomass by *P. ostreatus* and *P. sajor-caju* was determined and their specific activities presented. The amounts of extracellular proteins released during solid substrate fermentation of banana residual wastes are presented in Fig. 1.

3.1. P. ostreatus on leaf biomass

The patterns of formation of various extracellular enzymes produced by *P. ostreatus* on leaf biomass of banana waste are shown in Fig. 2. It is clear that the specific activities of laccase and peroxidase were increased from day 10 to day 20 and thereafter gradually decreased. The maximum specific activities of these enzymes were 1.7106 and 0.1632 units mg^{-1} on days 20 and 15 for laccase and lignin peroxidase, respectively. In the case of xylanase, the maximum specific activity

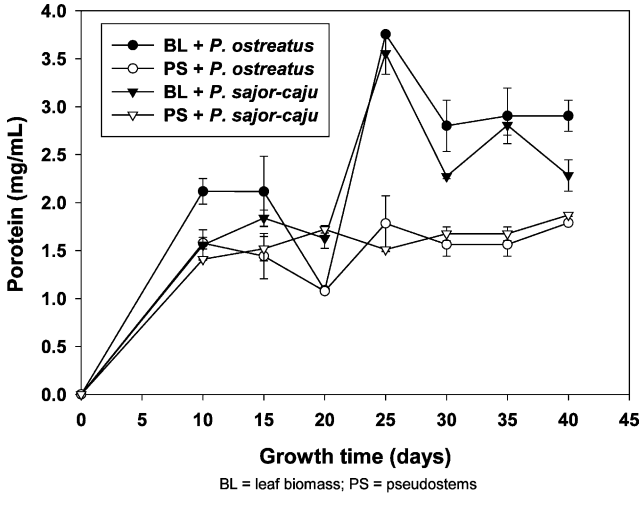

Fig. 1. Extracellular protein contents during the growth of *Pleurotus* species on banana waste.

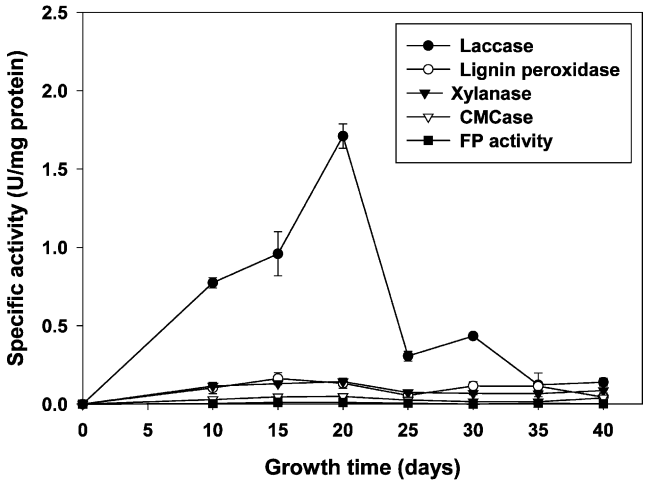

Fig. 2. Production patterns of lignolytic and cellulolytic enzymes on leaf biomass of banana waste by *P. ostreatus*.

was obtained (0.1435 units mg^{-1}) on day 20 and thereafter gradually decreased with the growth time (0.0863 units mg^{-1} on day 40). The levels of CMCase for endoglucanase and FP activity for exoglucanase were low throughout the culture period.

3.2. *P. ostreatus* on pseudostem biomass

The specific activities of different extracellular enzymes produced by *P. ostreatus* on pseudostem biomass are shown in Fig. 3. Maximum specific activity of laccase was observed on day 10 (0.4722 units mg^{-1}) followed by a gradual decrease until day 40. Lignin peroxidase specific activity was 0.1537 units mg^{-1} on day 20 and decreased levels of activity could be observed during subsequent period of degradation. The xylanase specific activity was 0.0408 units mg^{-1} on day 20 and thereafter gradually decreased. Very low levels of CMCase and FP activity activities were detected throughout the culture period.

3.3. *P. sajor-caju* on leaf biomass

The specific activities of the extracellular enzymes produced by *P. sajor-caju* during the degradation of banana leaf biomass are presented in Fig. 4. The highest specific activity of laccase was observed on day 10 of the culture (1.6669 units mg^{-1} protein), followed by a gradual decrease. The specific activity of lignin peroxidase increased from day 10 (0.0824 units mg^{-1}) to day 20 (0.4719 units mg^{-1}), and thereafter only low levels could be detected. Xylanase specific activity was 0.1411 units mg^{-1} on day 10 and 0.1174 units mg^{-1} on day 40. Only low levels of CMCase and FP activity could be detected.

3.4. *P. sajor-caju* on pseudostem biomass

Increase in specific activity of laccase from day 10 (0.1122 units mg^{-1}) to day 20 (0.7415 units mg^{-1}), then gradual decrease in its activity on day 25 (0.3745 units mg^{-1}) and day 30 (0.3145 units mg^{-1}) was observed. Lignin peroxidase specific activity was 0.1097 units mg^{-1} on day 10 day which increased on day 20 (0.4865 units mg^{-1}), and then declined on day 40 (0.0089 units mg^{-1}). Xylanase specific activity was 0.0237 units mg^{-1} on day 10 day and 0.0862 units mg^{-1} on day 40. Only very low levels of CMCase and FP activity could be detected (Fig. 5).

4. Discussion

The potential of agricultural residual wastes of banana leaf biomass and banana pseudostems for use

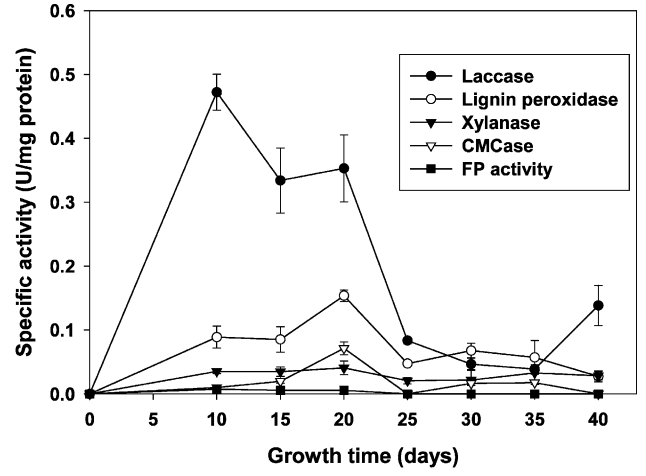

Fig. 3. Production patterns of lignolytic and cellulolytic enzymes on pseudostems of banana waste by *P. ostreatus*.

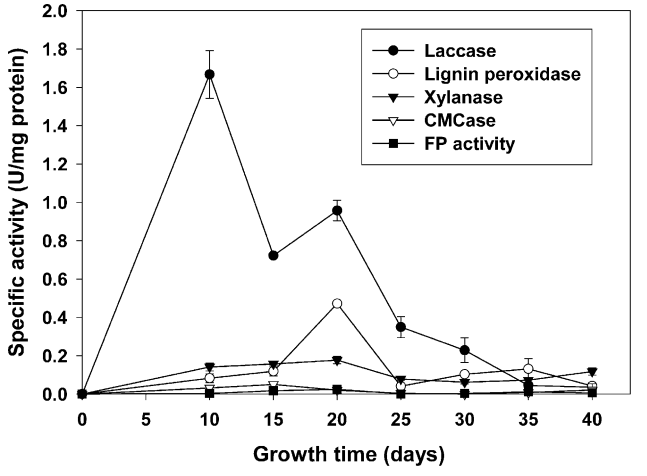

Fig. 4. Production patterns of lignolytic and cellulolytic enzymes on leaf biomass of banana waste by *P. sajor-caju*.

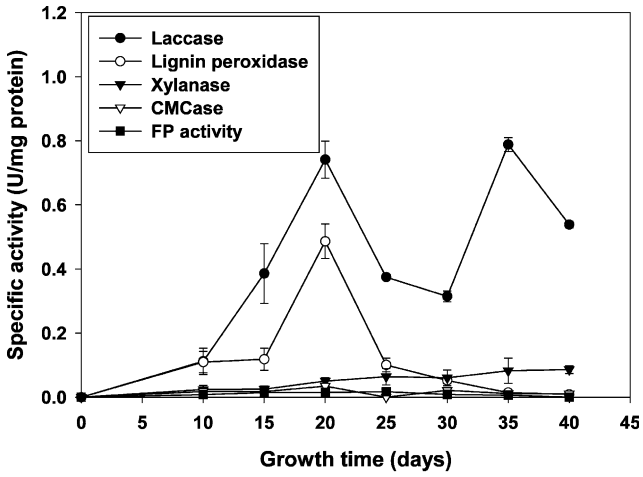

Fig. 5. Production patterns of lignolytic and cellulolytic enzymes on pseudostems of banana waste by *P. sajor-caju*.

as substrates for the production of lignolytic and cellulolytic enzymes by *P. ostreatus* and *P. sajor-caju* was assessed under solid-state conditions. The extracts of extracellular proteins released by organisms during their growth on banana biomass waste were screened for laccase, lignin peroxidase, xylanase, and CMCase and FP activity activities. The amount of extracellular protein produced on leaf biomass by both organisms was almost two times higher than that of pseudostems. It is known that cultivation conditions influences culture growth and activities of enzyme [17]. In the present study, production of various lignocellulolytic enzymes by *P. ostreatus* and *P. sajor-caju* on leaf biomass was much higher than that of pseudostem biomass, which may be due to more surface area in leaf biomass favoring the culture growth. Recently, Venelampi et al. [18] reported that the arrangement of cellulose fibres could affect the biodegradability of various paper products.

In the present investigation laccase activity was high in relation to cellulases and xylanase during the initial stage of degradation. Laccase is a lignin modifying extracellular oxidoreductase whose enzyme activity appears to be regulated in association with morphogenesis and often with the development of fruiting bodies [19,20]. Its activity increases during mycelia growth and high activity coincided with maximum mycelia growth on the substrates by both organisms. Increase in laccase activity during the vegetative phase up to the appearance of fruiting bodies has been reported in *Schizophyllum commune* [21], *Agaricus bisporus* [22], *L. edodes* [23] and *Coprinus congregatus* [24].

Laccase could be used as a morphogenetic indicator, its rapid drop indicates that maximal mycelium growth was achieved. In the present study laccase activities of *P. ostreatus* and *P. sajor-caju* began to decrease before day 20 on leaf and pseudostem biomass. These two *Pleurotus* species therefore take less than 20 days for establishment of mycelium on banana substrate and it can be used as an ideal spawn run period during cultivation of *P. ostreatus* and *P. sajor-caju* on banana waste. Similar yields of laccase and lignin peroxidase were obtained in SSF system using a culture of *P. chrysosporium* [25].

The present study reveals that banana waste can be used as an alternative substrate to other agricultural/agro-industrial wastes, wheat brawn/straw, saw dust and bagasse, which are already in use for the production of lignino and cellulolytic enzyme production [26]. The yields of ligninolytic enzymes largely varied in different strains of *P. ostreatus* and *P. pulmonarius* when they were cultivated on wheat straw under different culture conditions [27,28]. The maximum production of laccase reported on rubber tree sawdust by *P. sajor-caju* was 27.4 units mg^{-1} protein [29]. In the present study, maximum production of laccase obtained on leaf biomass was 1.7106 and 1.6669 units mg^{-1} protein by *P. ostreatus* and *P. sajor-caju*, respectively. The yields of the enzymes are too low to make a commercially viable process. However, the yields can be improved by optimizing the culture conditions and adopting different culture techniques such as strain improvement, addition of inducers or trace elements etc [28,30,31]. Laccase obtained can be used for detoxification of various pollutants and in the treatment of industrial wastewater [10,32,33]. Lignin peroxidase can be used for bioremediation of pentochlorophenols and other toxic chlorinated compounds [34,11]. Cultivation of *Pleurotus* on banana waste can lead to in process optimization, simplification and cost reduction for production of these enzymes.

P. ostreatus and *P. sajor-caju* cultivation on biomass of leaf and pseudostem resulted into very low levels of CMCase and filter paper activities. Similar levels of cellulolytic activities have been reported in *Pleurotus* spp. during growth on rice straw; no activity was however reported for filter paper and very low activity levels of CMCase were observed [35]. Buswell et al. [36] also reported that no cellulolytic enzyme activity was detected in the culture supernatants of *P. sajor-caju* and *Lentinula edodes* grown on crystalline cellulose and during their cultivation on sawdust. In the present study among cellulases, lowest activities were recorded for FP activity compared to CMCase. The low FP activity limits the rate at which white-rot fungi degrade native cellulose. Its action is necessary for the degradation of highly ordered (crystalline) forms of cellulose where it acts synergistically with CMCase active enzymes [37,38]. This pattern of degradation permits utilization of lignin without loss of cellulose from the lignocellulosic material by some white fungi [39]. Growth of *P. ostreatus* and *P. sajor-caju* on banana waste mostly utilizes lignin contents, supported by greater percentage reduction of

lignin contents (52% reduction on banana leaves; 23% in pseudostem) when compared to cellulose contents (4% reduction in banana leaves; 1.5% in pseudostem) [7]. The lignocellulosic material free from lignin is a good source of animal feed.

In a global context with a switch to biotechnology based bioprocessing, it becomes essential that the paper industry in India should opt for enzyme treatment as an alternative to chlorine bleaching [40]. Xylanase produced on banana waste by *P. ostreatus* and *P. sajor-caju* is low but can be a good source of pulp bleaching enzyme. Application of xylanase with other bleaching agents such as oxygen and hydrogen peroxide in the pulp industry has been extensively investigated and projections of a totally chlorine free pulp technology suggested [41].

Establishing the ideal batch time period for SSF is essential to improve the digestibility of banana waste as animal feed [42]. *Pleurotus* spp have been used for the treatment of environmental pollutants (xenobiotics such as polycyclic aromatic hydrocarbons, polychlorinated biphenols, dioxine, etc), for treatment of wood to produce biological pulps, bio-bleaching of pulps; biotransformation of Pencillin V and G into 6-aminopenicillanic acid [43–45]. The present study envisages development of similar processes through *Pleurotus* cultivation on banana waste.

These results give an insight into the dynamics of extracellular enzyme formation during degradation of banana waste by *P. ostreatus* and *P. sajor-caju*. Protocols for production of various lignolytic and cellulolytic enzymes by *P. ostreatus* and *P. sajor-caju* on Banana waste has been found to be cheaper due to the easy availability of banana residual waste. Present environmental problems due to accumulation of banana plant residual waste also can be eradicated apart from the production of industrially important enzymes.

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