Cellular & Molecular Biology
Cell. Mol. Biol. 2014; 60 (5): 59-63
Published online December 24, 2014 (http://www.cellmolbiol.com)
Received on May 25, 2014, Accepted on August 6, 2014.
doi : 10.14715/cmb/2014.60.5.11

Production of cellulolytic enzymes by *Pleurotus* species on lignocellulosic wastes using novel pretreatments

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Abstract

In the present investigation three species of *Pleurotus* i.e. *P. sajor-caju* (P1), *P. florida* (P2) and *P. flabellatus* (P3) along with two lignocellulosic substrates namely paddy straw and wheat straw were selected for evaluation of production of extracellular cellulolytic enzymes. During the cultivation of three species of *Pleurotus* under *in vivo condition*, the two lignocellulosic substrates were treated with plants extracts (aqueous extracts of ashoka leaves (A) and neem oil (B)), hot water (H) and chemicals (C).Among all treatments, neem oil treated substrates supported better enzyme production followed by aqueous extract of ashoka leaves, hot water and chemical treatment. Between the two substrates paddy straw supported better enzyme production than wheat straw. *P. flabellatus* showed maximum activity of exoglucanase, endoglucanase and β-glucosidase followed by *P. florida* and *P. sajor-caju*.

Key words: Lignoellulose, Exo-1,4-β-glucanase; Endo-1,4-β-glucanase; β-glucosidase.

Introduction

Lignocelluloses are the most abundant biomass available on the earth (1). Large amount of these wastes are generated through the activities of agriculture, forests, food and wood processing industries. Biomass in the forms of wastes accumulates every year in large quantities, causing deterioration of the environment and loss of potentially valuable resources. A wide variety of lignocelluloses including wheat straw, paddy straw,sugarcane baggase,wood logs, saw dust, coconut wastes, cotton stalks and various other agro-industrial wastes can be degraded efficiently and effectively by white rot fungi especially *Pleurotus* species commonly known as oyster mushroom. Fungi, which metabolize the lignin portion of complex with the help of lignolytic enzymes, leaving a white rot residue, called as white rot fungi. *Agaricusbisporus* and oyster mushroom (*Pleurotus* spp.) are also called as white rot fungi, having the above character (2, 3).

Pretreatment of lignocellulosic material is necessary for effective bioconversion and biodegradation. The objective of the pretreatment is to render biomass materials more accessible to the action of enzymes or microoganisms (4). The goals of the pretreatments are to remove and separate hemicellulose from cellulose, to disrupt and remove the lignin sheath, to decrease crystallinity of cellulose and to increase the pore size of cellulose to facilitate the penetration of hydrolysis agent (5). It generally involves a combination of mechanical size reduction, alkali swelling, acid hydrolysis, steam and other fiber explosion technique or exposure to supercritical fluids etc. (4).

In view of the harmful effects of chemicals, research efforts have been initiated to exploit the fungicidal potential of botanicals to replace chemicals.Pretreatment of lignocellulosic wastes by plant extracts for sterilization, extracellular enzymatic production and biodegradation can be economical, ecofriendly and novel method.

Materials and methods

The cultures and their maintenance

The pure cultures of *Pleurotus florida, P. flabellatus and P. sajor caju* used in the present investigation were procured from Indian Agricultural Research Institute(IARI), New Delhi. Throughout the study the cultures were maintained on malt extract agar (MEA) medium at 23-25^{0}C and was sub-cultured at the regular interval of three weeks.

Cultivation

Spawn preparation

Spawn is referred to as the vegetative mycelium of the fungus, which is grown on cereal grains i.e. grains of wheat. The preparation of spawn involved soaking of wheat grains in water followed by mixing of buffers, sterilization and inoculation with pure culture of appropriate *Pleurotus* species under aseptic conditions. The spawn was prepared in 500 ml of dextrose bottles or in polypropylene bags. After 3-4 days of inoculation fungal mycelium started spreading on the grains. The mycelium was white net web like in appearance. The bottles or bags were nearly half filled in 10-12 days and in 18-21 days these were completely filled with white mycelial growth.The spawn was prepared at 25^{0}C.

Pretreatment of substrates for cultivation

The selected two lignocellulosic substrates- paddy

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straw (*Oryza sativa*) and wheat straw (*Triticum aestivum*) were used for the cultivation of *P. sajor-caju* (P1), *Pleurotus florida* (P2) and *P. flabellatus* (P3). These substrates were pretreated by (i)Plant extracts i.e. neem oil (*Azadirachta indica*) and ashoka leaves aqueous extract (*Saraca indica)* (ii) hotwater and (iii) chemicals.

Plant extract treatment

During present investigation, the lignocellulosic wastes were treated with two plant extracts i.e. aqueous extract of ashoka (*Saracaindica*) leaves and neem (*Azadirachtaindica*) oil. 20 ml of these extracts were mixed in 1litre of water at the time of soaking of substrates. After 24 hours of treatments excess water was drained out. The substrates were evenly spread on the clean platform for 30 minutes to further remove free water. These aqueous plant extracts treated substrates were ready for spawning.

Hot water and chemical treatment

In this treatment, hot water and chemicals were used for sterilization of substrates. The substrates were completely dipped in water (50 litres for every 10 kg dry chopped substrates in a drum). The substrates were allowed to stay in water for 20 hours.After that excessive water was drained out. After draining, the substrates were again completely dipped in hot water (temperature 70-80^{0}C) for an hour. For chemical treatment, substrates were added 50 ppm each of nuvan and bavistin and the substrates were allowed to stay in water for 24 hours. This was followed by draining out of water and substrates were evenly spread on platform till the cooling of substrates. These hot water and chemically treated substrates were ready for spawning.

Spawning

Spawning is the process of mixing spawn in the sterilized substrate. 3% wet weight basis spawn grain was mixed with the substrate and filled into polypropylene bags. The mouth of each bag was tied with rubber band and 12 holes of about 1cm diameter were made, two at each corner at the base, four each on the broader area and one each on the narrow, rectangular side to drain out extra water and for proper aeration. 60 bags of each of the treatments were filled and kept in mushroom house on the iron racks on the bricks.

Sample collection and extraction of extracellular enzyme

After every five days interval three bags for each treatment were removed for enzyme assay. The contents of a set of three bags were mixed uniformly. Ten gram sample was homogenized in 100 ml of 50 mM sodium acetate buffer (pH 5.0) for enzyme assay. Homogenized samples were filtered through Whatman No.1 filter paper and filtrate was used for enzymatic studies.

CelluloyticEnzyme assay

Exoglucanase (FPase),endoglucanase (CMCase) and β-glucosidase were assayed by the method prescribed by Mandels *et al*., (6) and modified by Sandhu and Kalra (7).

Exo-1,4-β-glucanase (EC 3.2.1.91) and Endo-1,4-β-glucanase (EC 3.2.1.4)

Substrates used for exoglucanase (FPase, EC 3.2.1.91) and endoglucanase (CMCase, EC 3.2.1.4) were Whatman filter paper no.1 and carboxymethyl cellulose, respectively, prepared in 0.1 M acetate buffer separately. 0.5ml of crude enzyme extract was inoculated with 8 disc of Whatmann filter paper no.1.0.5ml of 0.1M acetate buffer (pH 5.0) also added in it. All solutions were taken in triplicate. Solutions were kept in the water-bath at 45^{0}C for 6 hrs and then 1 ml of alkaline CuSO4 was added in each test tube and again kept in boiling water-bath at 100^{0}C for 20 minutes. Then solutions were taken out of water-bath and 1ml of arsenomolybdate solution was mixed in each test tube. Final volume was made up 10 ml of each test tube with the distilled water. After cooling for 30 min at room temperature, absorbance was read at 540 nm by UV-visible spectrophotometer (Elico SL 164).

β-Glucosidase (EC 3.2.1.21)

0.5 ml of crude culture filtrate was added with 1 ml of PNPG in 0.1 M acetate buffer (pH 5) and incubated at 45^{0}C for one hour. The reaction was terminated by adding 1.5 ml of 10% Na_2CO_3. The yellow coloured p-nitrophenol liberated and determined by measuring absorbance at 425 nm. P-nitrophenol was used as standard. Reaction mixture containing boiled dialised culture filtrate was used as blank.

Results

Exoglucanase (EC 3.2.1.91) or FPase activity

In vivo exoglucanase activities of *Pleurotus* species on differently treated substrates is given in figure 1 and 2. Figure 1 shows that the exoglucanase activity of *P. flabellatus* was maximum among all the three species of *Pleurotus*, on plant extract treated substrates. Low activity of the enzyme by all the species of *Pleurotus* was seen on the 5th day of incubation. The enzyme showed gradual increase in the activity on the 10th day and reached at peak on 25th day.Maximum exoglucanase activity by *P. flabellatus* on 25th day of cultivation was observed as 11.48 and 10.59 unit/ml/hr on ashoka leaves treated paddy straw and wheat straw and 11.92 and 11.70 unit/ml/hr on neem oil treated paddy straw and wheat straw, respectively. In figure 2 same pattern of enzyme activity was found on hot water and chemically treated substrates and maximum FPase activity was found on

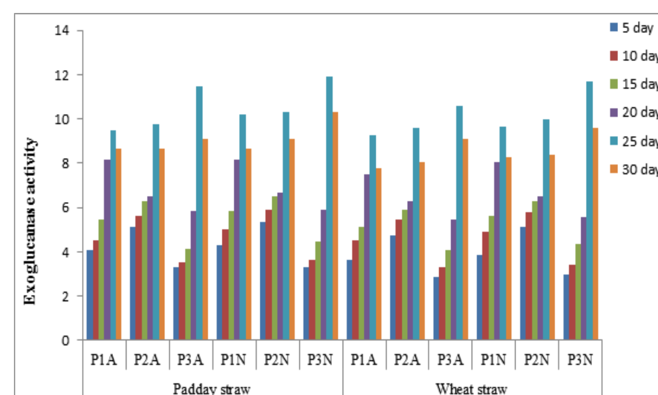

Figure 1. *In vivo* Exoglucanase activity (unit/ml/hr) of *Pleurotus* species on plants extract treated Paddy and Wheat straw.

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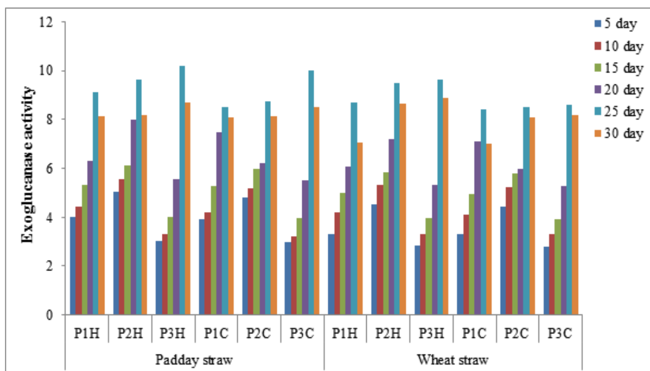

Figure 2. *In vivo* Exoglucanase activity (unit/ml/hr) of *Pleurotus* species on Hot water and chemically treated Paddy and Wheat straw.

the 25th day on paddy straw and wheat straw. On hot water treated paddy straw and wheat straw the enzyme activity was observed as 10.20 and 9.62 unit/ml/hr and on chemically treated paddy straw and wheat straw 10 and 8.60 unit/ml/hr, respectively.

It is evident from the observations that *P. flabellatus* showed maximum activity of exoglucanase followed by *P. florida* and *P. sajor-caju*. Among all treatments, neem oil treated substrates supported maximum enzymatic production followed by aqueous extract of ashoka leaves, hot water and chemical treatment. Between the two substrates paddy straw supported better enzyme production than wheat straw.

Endo - 1, 4- β- glucanaseor CMCase(EC 3.2.1.4)

The endoglucanase activity of *Pleurotus* species on plant extracts, hot water and chemically treated substrates is presented in figure 3 and 4. This enzyme (CMCase) also exhibited low activity in the initial stage like exoglucanase (FPase).Similar to FPase a gradual increase in CMCase activity was observed till the 25th day of cultivation on paddy and wheat straw.Maximum CMCase activity was observed as 68.15 and 65.19 unit/ml/hr on ashoka leaves treated paddy straw and wheat straw and 70.96 and 70.37 unit/ml/hron neem oil treated paddy and wheat straw, respectively (figure 3).Similarly, on hot water treated paddy and wheat straw maximum endoglucanase activity was recorded as 68 and 65.1 unit/ml/hr and on chemically treated paddy and wheat straw it was 65 and 59 unit/ml/hr, respectively on the 25th day of cultivation.

The results of endoglucanase clearly shows that neem oil treated substrates supported maximum activity of endoglucanase followed by ashoka leaves extracts, hot water and chemical treatment. Between the two substrates, better endoglucanase activity was seen on

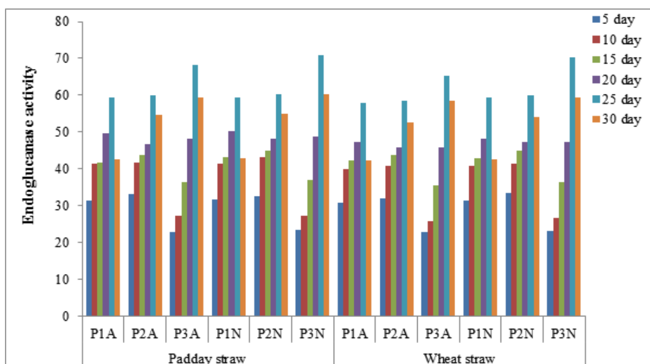

Figure 3. *In vivo*Endoglucanase activity (unit/ml/hr) of *Pleurotus* species on plants extract treated Paddy and Wheat straw.

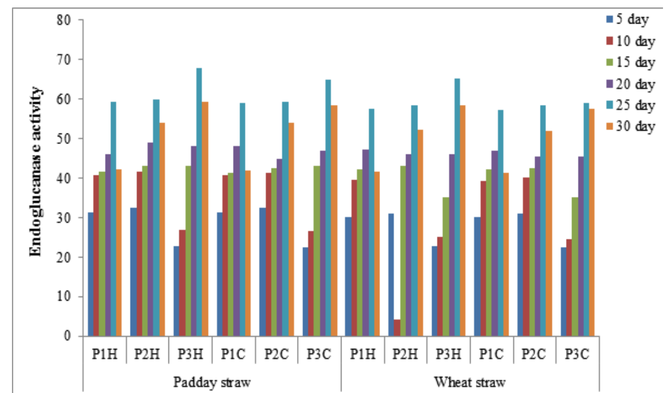

Figure 4. *In vivo* Endoglucanase activity (unit/ml/hr) of *Pleurotus* species on Hot water and chemically treated Paddy and Wheat straw. P1= *P. sajor- caju,* P2= *P. florida,* P3= *P. flabellatus,* H= hot water, C= chemical, A= aqueous extract of ashoka leaves, N= neem oil.

paddy straw than wheat straw.

β-Glucosidase (EC 3.2.1.21)

Figure 5 and 6 show the *in vivo* β-glucosidase activity of *Pleurotus* on differently treated substrates. The result shows that like edoglucanase and exoglucanase, the activity of β-glucosidase was lower in the initial stage of cultivation of *Pleurotus*. The activity of the enzyme increased after 5th day and reached at peak on 25th day. Maximum β-glucosidase activity by *P. flabellatus* on 25th day of cultivation was observed as 7 and 6.4 unit/ml/hr on ashoka leaves treated paddy straw and wheat straw and 7.15 and 6.5 unit/ml/hr on neem oil treated paddy straw and wheat straw, respectively (figure 5). Similar pattern of production of β-glucosidase was observed on hot water and chemically treated substrates. On hot water treated paddy straw and wheat straw the enzyme activity was observed as 6.5 and 6.3 unit/ml/hr

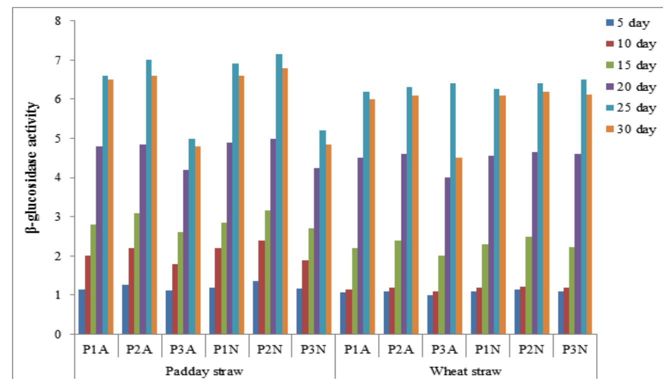

Figure 5. *In vivo* β-glucosidase activity (unit/ml/hr) of *Pleurotus* species on plants extract treated Paddy and Wheat straw.

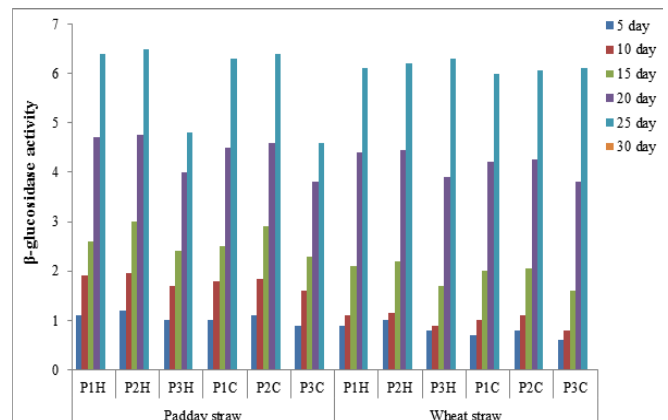

Figure 6. *In vivo* β-glucosidase activity (unit/ml/hr) of *Pleurotus* species on Hot water and chemically treated Paddy and Wheat straw.

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and on chemically treated paddy straw and wheat straw 6.4 and 6.1 unit/ml/hr, respectively.

Discussion

It is clear from the observations that maximum activity of exoglucanase,endoglucanase and β-glucosidase were observed on neem oil treated substrates followed by ashoka leaves extracts, hot water and chemically treated substrates. Between the two substrates, paddy straw supported better enzyme production followed by wheat straw. It was further observed that *P. flabellatus* showed maximum activity of endoglucanase followed by *P. florida* and *P. sajor-caju*.

The observations of cellulolytic enzymes productionindicate that in the initial stage of fungal growth FPase, CMCase and β-glucosidase exhibit low activity. However, activities of these enzymes increased in the latter stages of growth. The detection of appreciable activities of cellulolytic enzymes in the latter stages of mycelial growth demonstrates the capability of the fungus of causing cellulolysis only after the hydrolysis of lignin by laccase and PPO. Hence, it is evident from these observations that *Pleurotus* mycelium utilized lignin by the action of the lignolytic enzymes in the beginning followed by the utilization of cellulose by action of cellulases. Lignin probably inhibits the production and action of cellulolytic enzymes. These observations are in agreement with Rai and Saxena(8) and Singh (9) who recorded increased activity of cellulases by *Pleurotus* species in liquid culture during latter growth phase. A similar trend was reported in *L. edodes*(10) and *A. bisporus* (11).

Gerrits (12), Hedger and Basuki (13), Kirk and Fenn (14) also observed that the removal of lignin precedes the cellulolysis in the degradation of lignocelluloses by the basidiomycetes. Dhaliwal *et al*. (15) suggested that degradation of cellulose is induced only after lignin degradation during growth of *P. ostreatus* under *in vitro* study and recorded maximum level of laccase after 12 days and cellulase after 14 days. Burla *et al.* (16) reported maximum cellulase level on the seventh day of mycelial growth of *P. ostreatus*; strain *florida* in the *in vitro* studies.

Singh *et al.* (17, 18) and Pandey *et al.* (19) reported that cellulolytic enzyme exhibited maximum activity in the latter stage of growth of *Pleurotus* species on lignocellulosic and vegetable wastes under *in vitro* condition. In *Volvaeriella volvacea* the course of cellulase production on paddy straw, cotton waste and carboxymethyl cellulose was studied by Kapoor *et al*. (20) who observed maximum enzyme activity on 5th to 7th days. Among exoglucanase and endoglucanase enzymes, endoglucanase always exhibited more activity than exoglucanase at all the stages of growth of *Pleurotus* species.

Paddy straw supported maximum enzyme production followed by wheat straw. This may be because of soft and swollen nature of paddy straw due to presence of more of amorphous and less of crystalline form of lignocelluloses. Hence, paddy straw become more susceptible to fungal attack and facilitates the entry of various elements to growing mycelium, which further induce extracellular enzyme production.

Other articles in this theme issue include references (21-36).

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