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EXTRACELLULAR XYLANASE PRODUCTION BY *PLEUROTUS* SPECIES ON LIGNOCELLULOSIC WASTES UNDER *IN VIVO* CONDITION USING NOVEL PRETREATMENT

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Abstract

The production of extracellular xylanase by three species of *Pleurotus* species i.e. *P. florida*, *P. flabellatus* and *P. sajor caju* was studied under *in vivo* condition during their cultivation on pretreated lignocellulosic wastes. Neem (*Azadirachta indica*) oil and ashoka (*Saraca indica*) leaves extract were used for pretreatment of paddy straw and wheat straw. Between these two wastes, paddy straw pretreated with neem oil, supported better xylanase production than wheat straw. Initially, xylanase production was low but it increased in subsequent days and reached at peak on 25th day of cultivation of *Pleurotus* species. Thereafter, there was decrease in the activity of the enzyme. On 25th day of incubation *P. florida* produced maximum xylanase on neem oil pretreated paddy straw i.e. 10.59 $Uh^{-1}ml^{-1}$. Among the three species, *P. florida* showed maximum enzyme activity followed by *P. flabellatus* and *P. sajor caju*.

Key words: Lignocelluosic wastes, novel pretreatment, xylanase, *Pleurotus* Spp.

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INTRODUCTION

Lignocellulose is the most abundant renewable biomass available in nature which comprises of cellulose, hemicellulose and lignin (8). Major hemicellulosic constituent of lignocellulosic material is xylan and it is the most abundant renewable polysaccharide after cellulose. It is a potential resource for producing many valuable products (7). Xylan is a heterogeneous polysaccharide composed of β -1,4 linked xylose chains with branches containing arabinose and 4-O-methyl glucuronic acid. Complete hydrolysis of xylan involves the synergistic action of an array of main and side chain cleaving enzymes, among which xylanases (1,4 β-D-xylan xylanohydrolase (EC 3.2.1.8)) play a key role (3). Xylanase (EC 3.2.1.8) is a class of enzymes which break the hemicellulose by degrading the linear polysaccharide the beta-1,4-xylan into xylose (2).

In view of the harmful effects of chemicals, research efforts have been initiated to exploit the fungicidal potential of botanicals to replace chemicals for disease management in mushroom culture. The studies on management of *Trichoderma* green mold through botanical extracts were conducted to organic farming (10). Water extracts of plant parts of *Azadirachta indica*, *Chrysanthemum* spp. and *Tagetes erecta* inhibited the growth of *Trichoderma viride* and *T. harzianum* in the range of 20 - 25% and 7- 28%, respectively and stimulated the growth on *Agaricus bisporus* mycelium markedly under *in vitro* studies. Recently, neem has received substantial attention particularly in terms of its potential for insect pest control. Neem, *Azadirachta indica*, belongs to family Meliaceae, is native to arid regions of the Indian subcontinent. Antifungal, antiviral and nematicidal property of this plant has been exploited in rural India since time immemorial. It has been age old practice in rural India to mix dried neem leaves with stored grains or to place them among warm clothes to repel insects.

Xylanases are present in fungi for the degradation of plant matter into usable nutrients. Commercial applications of xylanase include the chlorine-free bleaching of wood pulp prior to the paper making process, and the increased digestibility of silage (in this aspect, it is also used for fermentative composting)(1). Apart from its use in the pulp and paper industry, xylanases are also used as food additives to poultry, in wheat flour for improving dough handling and quality of baked products, for the extraction of coffee, plant oils, and starch, in the improvement of nutritional properties of agricultural silage and grain feed, and in combination with pectinase and cellulase for clarification of fruit juices and degumming of plant fiber sources such as flax, hemp, jute, and ramie.

Xylanases are produced either by solid-state fermentation (SSF) or submerged fermentation. The use of SSF as a method of production for xylanase could offer some apparent economic and engineering advantages over the classical submerged fermentation. These include high concentration of the product and simple fermentation equipment as well as low requirement for aeration and agitation during enzyme production. The solid substrates used not only supply the nutrients to the microbial cultures, but also serve as an anchorage to the cell. Generally, carbon source has been very cost effective in the production of xylanase and cost can be reduced using the agrowastes that are abundant and considered as the best substrates for SSF processes (12).

The three species of *Pleurotus* selected in the present investigation are edible mushrooms with high protein content, medicinal and nutritional value. They are capable of producing extracellular enzymes which degrade lignocellulosic wastes into useful resources. Plant extracts were used in the pretreatment of substrate to inhibit the growth of unwanted microorganisms in *Pleurotus* cultivation. The main objective of pretreatment is practical exclusion of unwanted microorganisms (i.e. sterilization) to avoid the use of chemicals and loosens the structure of lignocellu-

losic material so that a large surface area can be available for the action of lignocellulolytic enzymes. The present work was taken up with the aim to test the ability of *Pleurotus* species to produce xylanase on plant extracts pretreated locally available plenty of paddy straw and wheat straw.

MATERIALS AND METHODS

The cultures and their maintenance

The pure culture of *Pleurotus florida, P. flabellatus and P. sajor caju* used in the present investigation were procured from IARI, New Delhi. Throughout the study the culture was 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

Preparation of substrate

Paddy straw and wheat straw were used as substrates for the cultivation of *Pleurotus* spp. These substrates were treated and sterilized by plant extracts.

Plant extract treatment

During present investigation, the lignocellulosic wastes were treated with two plant extracts *i.e.* aqueous extract of ashoka (*Saraca indica*) leaves and neem (*Azadirachta indica*) 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.

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 xylanase assay. Homogenized samples were filtered through Whatman No.1 filter paper and filtrate was used for enzymatic studies.

Enzyme assay

Xylanase was assayed by the method of Sandhu and Kalra (11). 0.5 ml enzyme extract was mixed in 0.5 ml of substrate. Substrate used for xylanase (EC 3.2.1.8) was xylan, prepared in 0.1 M acetate buffer. All solutions were taken in triplicate. Solutions were kept in the water-bath at 45^{0}C for 6 h and then 1 ml of alkaline $CuSO_4$ 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).

RESULTS

Two types of plant products i.e. neem oil and ashoka leaves extracts were used for pretreatment of substrates for cultivation of *Pleurotus* spp alongwith xylanase production. Different lignocelulosic substrates showed different rate of xyalanse activity but followed a fixed pattern. In this study we used paddy straw and wheat straw, both are major crops in Eastern Uttar Pradesh of India. In vivo xylanase activities of *Pleurotus* species on plant extract treated paddy straw are presented in figure 1. Low activity of the enzyme by all the *Pleurotus* species on neem oil as well as aqueous extract of ashoka leaves treated paddy straw was observed on the 5th day of incubation which was in the range of 5.26- 6.44 $Uh^{-1}mL^{-1}$. The enzyme showed slight increase in its activity on the 10th day and exhibited its peak value on 25th day. Among the three species *P. florida* (10.59 $Uh^{-1}mL^{-1}$) showed the maximum xylanase activity on neem oil treated paddy straw followed by *P. flabellatus* (8.88 $Uh^{-1}mL^{-1}$) and *P. sajo-caju* (8.52 $Uh^{-1}mL^{-1}$). Similar pattern of xylanase activity was recorded on aqueous extract of ashoka leaves treated paddy straw. However enzyme activity was a bit lower than on neem oil treated substrate. On ashoka leaves treated paddy straw also *P. florida* (10.30 $Uh^{-1}mL^{-1}$) showed maximum xylanase activity followed by *P. flabellatus* (8.74 $Uh^{-1}mL^{-1}$) and *P. sajor-caju* (8.37 $Uh^{-1}mL^{-1}$). After 25th day there was continuous decrease in the activity of the enzyme.

Figure 2 shows the *in vivo* xylanase activities of *Pleurotus* spp. on plant extract treated wheat straw. The pattern of the enzyme production remains almost same as on paddy straw except the activity of the enzyme was lower on wheat straw than on paddy straw.

DISCUSSION

Under *in vivo* condition xylanase activity by all the three species of *Pleurotus* in the present work showed a gradual increase during mycelial growth and a sharp increase during fructification on both substrates. Neem oil treatment shows much better response on all substrate than aqueous extracts of Ashoka leaves. Maximum xylanase activity

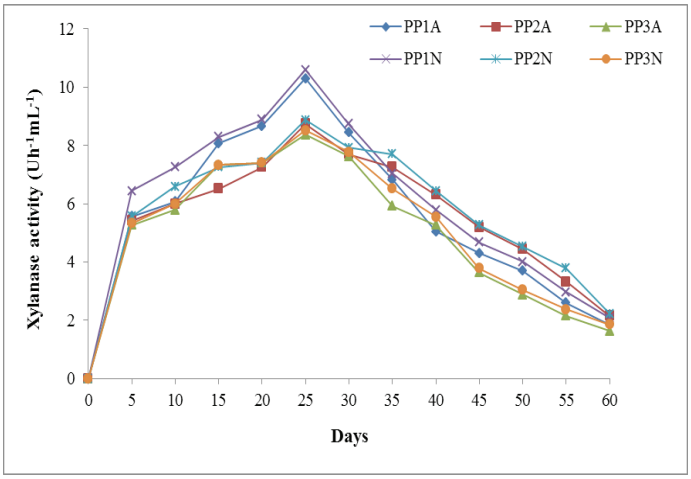

Figure 1. *In vivo* xylanase activity ($Uh^{-1}ml^{-1}$) of *Pleurotus* species on plant extract treated paddy straw.

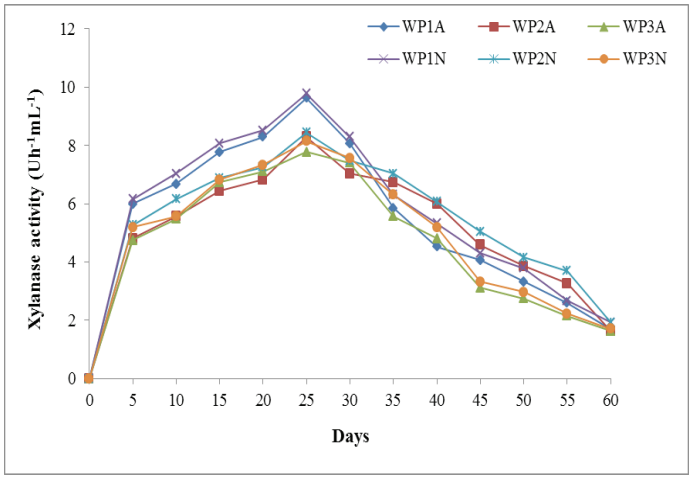

Figure 2. I*n vivo* xylanase activity ($Uh^{-1}ml^{-1}$) of *Pleurotus* species on plant extract treated wheat straw.

was shown by *P. florida*, followed by *P. flabellatus* and *P. sajor caju* on neem oil treated substrates. Between the two substrates paddy straw supported maximum xylanase production followed by wheat straw. Maximum xylanase activity was observed on paddy straw as well as wheat straw on 25th day in *in vivo* condition.

The low activity of xylanase during the beginning of incubation could probably be due to the fact that the xylanase was repressed by the lignolytic enzyme, which was active in the beginning as reported by Singh et al. (14,15,16). This also suggests that hemicellulose is not utilized in the beginning by the growing mycelium. In the experiment xylanase exhibited low activity on the 5th day of incubation on each substrates and maximum activity on 25th day on paddy straw as well as wheat straw.

The increase in xylanase activity by the 10th day and 15th day of incubation is suggestive that the enzyme was active in hydrolyzing hemicellulose in the latter stage. However, Ghosh and Nandi reported higher xylanase activity during initial phase of growth of *Pleurotus* species on water hyacinth (6). Singh and Singh *et al.* reported low xylanase activity in the initial stage of growth of *Pleurotus* and high activity in the latter stage on vegetable and other lignocellulosic wastes (16,17). Maximum xylanase activity was shown by *P. florida* on neem oil treated substrates. Maximum xylanase production was observed on wheat straw.

However, contradictory result were found by Ghosh and Nandi, Chen *et al.* who reported that xylanolytic activity was crucial for the vegetative growth of fungus (4,5,6). Ahlawat *et al.* also observed that during the cultivation of *Volvariella volvacea* xylanase activity was crucial during vegetative growth of fungus and found more on 10th day grown on paddy straw (1).

The probable reason for the better production of xylanase on plant extracts (neem oil and ashoka leaves extract) treated substrate could be that the plant extract contained some elements or substances which when taken up by the growing mycelium induced better enzyme activities. Thus, lignocellulosic materials which are available in plenty can be utilized as resource for production of xylanase.

Other articles in this theme issue include references (18-45).

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