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BIODEGRADATION OF BRASSICA HAULMS BY WHITE ROT FUNGUS *PLEUROTUS ERYNGII*

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Abstract

The white rot fungus *P. eryngii* was grown on chemically and hot water treated agrowaste *Brassica* haulms. The fungus degraded lignin, cellulose, hemicellulose and carbon content of both chemically as well as hot water treated waste and produced in turn the edible and nutritious fruiting body. The progressive breakdown of lignin, cellulose and hemicellulose was correlated with apparent increase in the activities of lignolytic, cellulolytic and hemicellulolytic enzymes. Lignin degraded at faster rate during the vegetative phase and at slower rate during reproductive phase whereas, cellulose and hemicellulose depleted slowly during vegetative phase and rapidly during reproductive phase. The carbon content of the agrowaste decreased while, the nitrogen content increased and the C/N ratio came closer during degradation of the waste. Hot water treated substrate supported better production of enzymatic activity and degraded more efficiently than chemically sterilized substrate. The total yield and biological efficiency of the mushroom was the maximum on the hot water treated substrates.

Key words: Biodegradation, *Brassica* haulms, *Pleurotus eryngii*.

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Abbreviations: **BE**: biological efficiency; **CMCase**: carboxy methyl cellulase; **CTAB**: cetyl trimethyl ammonium bromide; **GF/C**: glass microfibre filter; **IARI**: Indian Agricultural Research Institute; **MEA**: malt extract agar; **PPO**: polyphenol oxidase.

INTRODUCTION

The recent thrust in utilization of agricultural wastes into value added products has led to extensive studies on biodegradation and bioconversion of these wastes by fungi. *Brassica* haulms is an agricultural by-product generated in large quantity in India. In India about 6.71 million tons mustard crops are produced annually. This leads to generation of huge quantity of *Brassica* haulms. These are lignocellulosic in nature and consist of three main polymeric constituents – cellulose, hemicellulose and lignin. Major portion of these haulms remain unutilized and either left to natural degradation or burnt in the field leading to severe environmental aggression and wastage of resource. Biodegradation of *Brassica* haulms by *Pleurotus eryngii* is significant as it not only leads to formation of simpler compounds but also results in protein rich food.

Pleurotus eryngii is an edible mushroom, which confers advantages over other mushrooms for its capability to grow on non-fermented lignocellulosic wastes and produce in turn fruit bodies with higher nitrogen content. The aim of present investigation was to study the

biodegradation of agricultural wastes and production of nutritional food.

MATERIALS AND METHODS

The culture and its maintenance

The pure culture of *Pleurotus eryngii* used in the present investigation was 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

Brassica haulms (*Brassica compestris*) was used as a substrate for the cultivation of *Pleurotus eryngii*. This substrate was treated and sterilized by hot water treatment and chemical sterilization method.

Substrate Pretreatments

Hot water treatment

In this treatment, hot water was used for the sterilization of substrate. The substrate was completely dipped in water (50 lit for every 10 kg dry substrate) in a drum. The substrate was allowed to stay in water for 20 hours. After that excessive water was drained out. This was followed by complete dipping of the substrate in hot water (temperature 70-80^{0}C) for one hour. Then water was drained out and substrate was evenly spread on platform till the cooling of substrate. These hot water treated substrate was ready for spawning.

Chemical sterilization

In the chemical sterilization, the substrate was soaked in water (50 lit for every 10 kg dry substrate) containing 200 ppm each of nuvan and bavistin in a drum. The substrate was allowed to stay in water for twenty hr. After that excessive water was drained out, the substrate was evenly spread on slanted clean platform for about one hour to further remove free water. This chemically sterilized substrate was 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 the bag was tied with rubber band and 12 holes of about 1cm diameter was 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, treatments was filled and kept in mushroom house on the iron racks on the bricks.

Biological efficiency

At the stage of pinhead (primordia) appearance, perforation was made to facilitate the formation of full-fledged fruit body. The pinheads were allowed to grow their full size and the mature fruit bodies were picked up before the edge of the cap started curling. The fruit bodies were harvested by twisting them so that broken pieces of mushroom did not remain in the substrates and adjacent smaller fruit bodies was not disturbed. After first harvest the polythene was cut open and the substrates were sprayed with water according to the atmospheric conditions. The yield was expressed as of fresh fruit bodies produced per bag. Biological efficiency (B.E.) was calculated as the percentage conversion of dry substrates to fresh fruit bodies following Chang *et al* (2) i.e.

$$\text{Bilogical efficiency (BE)} = \frac{\text{Fresh weight of mushroom}}{\text{Dry weight of substrate}} \times 100$$

Sample collection

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 cellulase and hemicellulase assay, while another 10 g of sample was homogenized in 50mM phosphate buffer (pH 6.0) for laccase and PPO assay. Homogenized samples were filtered through Whatman No.1 filter paper and filtrate was used for enzymatic studies (8).

Cellulose, Hemicellulose and Lignin estimation

The method of Jayme and Lang (10) was followed for cellulose, hemicellulose and lignin estimation. It included two major steps: (a) Digestion of sample and (b) estimation of protein by Bradford method (1). For digestion, acid detergent solution (0.5M H_2S0_4, 2% CTAB and 72% H_2SO_4) was used.

Hemicellulose

Dried sample (0.5 g of 20 mesh powder of the substrate under estimation) was digested with acid detergent solution. The digested sample was filtered with Glass micro fiber filter (GF/C). Filtrate was analyzed by Bradford method to calculate protein. Then residue was dried at 105^{0}C and its weight was deducted from 0.5 g (initial weight of lignocellulose).

Lignin and cellulose determination

Two hundred mg of sample (left after filtration) was mixed with 2 ml of 72% H_2S0_4 and the mixture was placed in water bath at 30^{0}C for 1 hr, and made up 30 ml with distilled water and then hydrolyzed in autoclave for 1 hr. The hot solution was filtered through GF/C and lignin residues were washed with hot water. The GF/C was then dried at 105^{0}C and finally lignin was deducted from 200 mg, the remaining was cellulose.

Extraction of Extracellular enzymes

Samples of substrate were collected at regular interval of 5 days and extracted in acetate buffer (pH 5.0) for cellulolytic and hemicellulolytic enzymes and in phosphate

buffer (pH 6.0) for lignolytic enzymes. Filtrate of extraction was used for enzyme assay.

Enzyme assay

Cellulases and xylanase were assayed by the method of Sandhu and Kalra (17). 0.5 ml enzyme extract was mixed in 0.5 ml of substrate. Substrates used for exoglucanase (FPase, EC 3.2.1.91), endoglucanase (CMCase, EC 3.2.1.4) and xylanase (EC 3.2.1.8) were Whatman filter paper no.1, carboxymethylcellulose and xylan respectively, prepared in 0.1 M acetate buffer separately. For exo-1,4 β-glucanase, 8 disc of 0.6 cm diameter Whatman filter paper No.1 was used as substrate and 0.5 ml of 0.1 M acetate buffer was mixed. All solutions were taken in triplicate. Solutions were kept in the water-bath at 45^0C for 6 hrs and then 1 ml of alkaline $CuSO_4$ was added in each test tube and again kept in boiling water-bath at 100^0C 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, UV-visible spectrophotometer (Elico SL 164). For β-glucosidase (E.C. 3.2.1.21) 0.5 ml of appropriate dilution of culture filtrate and 0.5 ml of α-p-Nitrophenylglycerol (PNPG) in 0.1 M acetate buffer pH 5.0 was added. The reaction mixture was incubated at 45^0C for 1 hr. After incubation period 1.5 ml of 10 % sodium carbonate solution was added to each test tube, and absorbance was read at 425 nm. The amount of reducing sugars released was estimated using glucose standard. Laccase (EC 1.10.3.2) was assayed following by Dhaliwal *et al* (5) using a reaction mixture consisting of 1ml of enzyme filtrate and 3ml of guaicol substrate prepared in 0.1M sodium phosphate buffer (pH 6.0), while PPO (EC 1.10.3.1) was assayed using the methodology of Rai and Saxena (13) consisting of 1ml of enzyme extract and 3 ml of catechol prepared in 0.1 M sodium phosphate buffer (pH 6.0). Change in absorbance was observed at 495 nm. The units used for cellulases and xylanase is µ mole glucose release $ml^{-1}hr^{-1}$ and for laccase and PPO change in absorbance by 0.001 $ml^{-1}min^{-1}$.

Carbon and Nitrogen estimation

Carbon was determined by Walkley and Black (24) and nitrogen of lignocellulosic wastes was determined by Micro kjeldahl method from oven dried powdered samples of zero days at completion of spawn run, after first flush and after cropping (spent compost).

Carbon

0.5 g crushed and dried sample was taken in 500 ml flask. Two blanks were included to standardize $FeSO_4$ solution. 15 ml $K_2Cr_2O_7$ solution was added. This was followed by rapid addition of 20 ml of concentrated H_2SO_4 and swirling of flask 2 to 3 times. Then it was allowed to stand for 30 min. 200 ml distilled water was then added followed by addition of 10 ml concentrated Phosphoric acid and 1ml indicator and titrated against FeSO4.

Nitrogen

Digestion of sample

2.0 g dried and crushed sample was taken in 500 ml Kjeldahl flask. 10 ml of digestion mixture and 20 ml of concentrated sulphuric acid was added. The flask was heated for 4 to 6 hr in a digestion fume hood until clean solution is obtained. The solution was made up to 100 ml with distilled water.

Distillation

Dry distillation assembly was used. 10 ml of digested aliquots was taken in a modified Markman apparatus along with an equal volume of 45% NaOH. Hot steam was allowed to pass through mixture for 5-10 min and distillate was collected in 150 ml conical flask containing 20 ml of 4% Boric acid with 1 drop of mixed indicator (0.5% Bromocresol green and 0.1% methyl red in 95% ethyl alcohol). The color of solution was changed greenish blue to green.

Titration

The distillate was titrated against 0.1 N HCl. One blank was run without sample. One standard solution of ammonium chloride was also titrated against 0.1 N HCl.

Throughout the experiments three replicates of each analysis was used and their average was taken as quantitative measure for determining percentage of cellulose, hemicellulose, carbon, nitrogen and biological efficiency as well as activities of extracellular enzymes.

RESULTS

The biodegradation of cellulose, hemicellulose and lignin content of *Brassica* haulms at mycelial growth, during fructification and after harvesting (spent compost) is given in Table 1. The cellulose, hemicellulose and lignin content of untreated *Brassica* haulms were estimated at 31.08%, 30.0% and 26.65 %, respectively. The rate of degradation of cellulose and hemicellulose of hot water as well as chemically treated *Brassica* haulms during vegetative growth of *P. eryngii* was slower than lignin. The degradation of cellulose, hemicellulose and lignin during vegetative phase was observed as 21.2%, 18.76% and 36.43% in hot water treated substrate and 19.14%, 16.26% and 32.45% in chemically treated substrate in the given order. The rate of degradation of cellulose and hemicellulose increased sharply during fruit body development. Contrary to this, lignin degradation was faster during vegetative phase and slower during fructification. Cellulose and hemicellulose content of hot water treated substrate degraded more efficiently and effectively by the *P. eryngii* in comparison to chemically treated substrate. However there was no appreciable difference in the rate of degradation of lignin in the differently treated substrate.

The activities of cellulolytic, hemicellulolytic and lignolytic enzymes produced by *P. eryngii* on brassica haulms during

cultivation are given in Table 3. The result showed that the activity of cellulase and xylanase was low during vegetative phase and high during fruit body formation. CMCase (EC 3.2.1.4) activity was more than FPase (EC 3.2.1.91) at all the stages of growth of *P. eryngii*. β-glucosidase (E.C. 3.2.1.21) acmayed later than CMCase and FPase. However laccase (EC 1.10.3.2) and PPO (EC 1.10.3.1) appeared and peaked earlier than the cellulases and xylanase. Their activities were higher during vegetative phase and lower during fructification stage. The activity of laccase was more than the PPO at all the stages of growth of the fungus. Hot water treated substrate supported production of more enzymes than chemically sterilized substrate.

The percentage of carbon, nitrogen and their ratio in the substrate at different stage of growth of the fungus is presented in Table 2. Carbon content and C/N ratio of *Brassica* haulms decreased while nitrogen content increased. The decreases in carbon content and increase in nitrogen content was slightly more in hot water treated substrate than chemically sterilized substrate.

The mean yield of *Pleurotus eryngii* from three flushes (fresh weight) on different lignocellulosic waste and their biological efficiency is given in Table 4. The mean yield of *P. eryngii* during first flush per 105 g of dry weight of *Brassica* haulms was observed as 125 g and 75 g, second flush was recorded as 70 g and 25 g and third flush was found as 40 g and 25 g on hot water and chemically treated *Brassica* haulms, respectively.

The biological efficiency of *P. eryngii* was 223.80% and 119.04% on hot water treated and chemically treated *Brassica* haulms, respectively.

DISCUSSION

The rapid degradation of lignin and slow degradation of cellulose and hemicellulose during vegetative phase and slow degradation of lignin and fast depletion of cellulose and hemicellulose during fructification in the present investigation revealed the differential requirement of the fungus *P. eryngii* during different phases of its growth. Similar pattern of biodegradation of lignocellulosic wastes by various species of *Pleurotus* have been reported (4, 7, 12, 18, 19). These observations suggest that the cellulose and hemicellulose serve as an energy source for the formation of fruit bodies. In the present investigation hot water treated *Brassica* haulms degraded more efficiently and effectively by *P. eryngii* than chemically treated substrate. The probable reason for this could be that under high temperature hydrogen bond of some of cellulose of *Brassica* haulms got disrupted leading to formation of amorphous cellulose from the crystalline cellulose which was more susceptible to fungal attack and thereby degradation. Similarly disruption in some of the bonds of lignin and hemicelluloses under aforesaid condition could have made the substrate vulnerable to fungal attack.

The decrease in carbon content of the substrate in the present study at the completion of the spawn run, after first flush in the spent compost could probably be because of bioconversion and biodegradation of organic compounds. The increase in nitrogen content during growth of the mushroom may be either because of its ability to fix atmospheric nitrogen or due to the presence of some nitrogen fixing bacteria in the compost. Other workers (3, 8, 9, 15, 16) also noted an increase in nitrogen content of the residues in *Pleurotus* bed and suggested that *Pleurotus* species have the ability to fix nitrogen from air. However Kurtzman (11) visualized that increase in nitrogen content of the compost is because of the presence of nitrogen fixing bacteria in the bed. Contrary to this Rajarathnam *et al* (14) observed that nitrogen content of rice straw compost decreased during cultivation of *P. flabellatus*.

The gradual increase in the activities of cellulolytic enzymes (FPase, CMCase and β-glucosidase) along with xylanase during vegetative phase and sharp increase during fructification in the present investigation can be correlated with slow depletion cellulose and hemicelluloses in the vegetative phase and fast depletion in reproductive phase. This further strengthens the view that cellulose serves as an energy source for the formation of fruit bodies in *Pleurotus* species. Similar results have also been reported in many other species of *Pleurotus* on various lignocellulosic substrates (2, 22, 23). Maximum activity of laccase and polyphenol oxidase during vegetative phase of growth of *Pleurotus eryngii* can be directly correlated with degradation of lignin in this stage. Elisashvili *et al* (6), Chang *et al* (2) and Singh *et al* (21, 22) also reported high activity of these enzymes

Table 1. Biodegradation of cellulose, hemicellulose and lignin of *Brassica* haulms by *Pleurotus eryngii* instead of Biodegradation of cellulose, hemicellulose and lignin of brassica haulms by *Pleurotus eryngii*.

Treatment	CELLULOSE %				HEMICELLULOSE %				LIGNIN%			
	A	B	C	D	A	B	C	D	A	B	C	D
Hot water	31.08	24.49 (21.20)	19.86 (36.10)	13.10 (57.85)	30.0	24.37 (18.76)	20.83 (30.56)	19.00 (36.66)	26.65	16.94 (36.43)	14.43 (45.85)	13.65 (48.78)
Chemical	31.08	25.13 (19.14)	21.21 (31.75)	19.23 (38.12)	30.0	25.12 (16.26)	21.33 (28.9)	20.27 (32.43)	26.65	18.00 (32.45)	15.83 (40.60)	14.83 (44.35)

A=Untreated substrate, B= Substrate after the completion of spawn run, C= Substrate after fructification, D= Spent compost, Figure in bracket shows Percent loss.

Table 2. Percent carbon, nitrogen and their ratio in *Brassica* haulms during growth of *Pleurotus eryngii* instead of Percent carbon, nitrogen and their ratio in brassica haulms during growth of *Pleurotus eryngii*

Treatment	0 Days			At Completion of spawn run			After First flush			Spent compost		
	C	N	C: N	C	N	C: N	C	N	C: N	C	N	C: N
Hot water	39.38	0.79	49.84	30.64	1.23	24.91	26.47	1.83	14.46	16.38	2.34	7.00
Chemical	39.50	0.89	44.38	31.17	1.13	27.58	26.52	1.79	14.81	13.53	2.27	5.96

C= carbon, N= nitrogen

Table 3. Activity of cellulases (U ml^{-1} h^{-1}), xylanase (U ml^{-1} h^{-1}), laccase (U ml^{-1} min^{-1}) and PPO (U ml^{-1} min^{-1}) by *Pleurotus eryngii* on *Brassica* haulms instead of Activity of cellulases (U $h^{-1}ml^{-1}$), xylanase (U $h^{-1}ml^{-1}$), laccase (U $min^{-1}ml^{-1}$) and PPO (U $min^{-1}ml^{-1}$) by *Pleurotus eryngii* on Brassica haulms.

Enzyme	Treatment	DAYS											
		5	10	15	20	25	30	35	40	45	50	55	60
FPase	H	4.66	8.04	20.25	14.44	7.18	6.14	4.14	3.77	3.18	3.08	2.69	2.12
	C	2.51	6.14	11.62	12.25	18.62	8.88	7.77	6.22	6.00	3.00	2.91	2.19
CMCase	H	6.66	16.59	26.18	39.37	29.37	18.29	9.11	6.88	5.85	5.65	4.96	4.01
	C	3.92	6.59	9.77	22.85	19.00	10.22	8.51	8.83	7.40	5.75	4.26	3.29
β-glucosidase	H	0.74	2.52	2.58	8.49	15.08	15.23	8.52	1.78	1.59	1.53	1.17	0.21
	C	1.51	1.92	2.94	3.80	5.13	9.15	8.44	5.00	2.90	1.64	0.99	0.67
Xylanase	H	6.44	12.11	24.88	20.48	13.29	13.18	8.07	7.33	4.22	4.12	3.76	3.03
	C	5.76	13.07	15.25	15.88	15.37	14.25	13.59	12.66	10.07	9.39	8.13	5.79
Laccase	H	0.41	1.59	3.87	6.40	4.76	1.93	0.72	0.62	0.57	0.42	0.33	0.23
	C	0.59	2.56	3.78	3.48	2.11	1.85	1.46	0.57	0.29	0.20	0.20	0.12
PPO	H	0.22	1.36	3.30	0.90	0.81	0.73	0.54	0.34	0.33	0.32	0.27	0.22
	C	0.23	1.32	3.21	2.43	1.30	0.88	0.63	0.46	0.34	0.34	0.31	0.25

Table 4. Yield performance and biological efficiency (B.E) of *Pleurotus eryngii* on *Brassica* haulms instead of Yield performance and biological efficiency (B.E) of *Pleurotus eryngii* on brassica haulms.

Substrate and treatment	Dry weight	Wet Weight	I flush	II flush	III flush	Total weight	B.E %
BH	105	500	125	70	40	235	223.80
BC	105	500	75	25	25	125	119.04

H = Hot water treated, C = Chemically treated, B = Brassica haulms

during the colonization stage and declined activity during first primordia formation.

It is revealed from the results that hot water treated *Brassica* haulms supported the fast mycelial growth during cultivation of *Pleurotus eryngii* selected in this investigation. This was because of the presence of more amorphous lignocellulosic material which is easy to be attacked by the fungus mycelia. Hence better spawn run, yield and biological efficiency was seen on hot water treated substrate than on chemically treated substrate. Singh (18) reported that cumulative yield and biological efficiency of *Pleurotus* species remained higher on autoclaved substrates than chemically sterilized substrates.

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Other articles in this theme issue include references (25-40).

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