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BIODEGRADATION OF SUGARCANE BAGASSE BY *Pleurotus citrinopileatus*

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Abstract

The chemically as well as hot water treated agrowaste sugarcane bagasse was subjected to degradation by *Pleurotus citrinopileatus*. 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. Biodegradation of the waste in terms of loss of lignin, cellulose and hemicellulose showed positive correlation with cellulases, xylanase, laccase and polyphenol oxidase (PPO) activity of the fungus. During mycelial growth of the fungus, lignin degradation was faster and during fructification, lignin degradation was slower than cellulose and hemicellulose. The carbon content of the sugarcane bagasse decreased while, nitrogen content increased 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 maximum on the hot water treated substrates. Degradation of the hot water treated sugarcane bagasse was better and faster than chemically treated substrates.

Key words: Biodegradation, *Pleurotus citrinopileatus,* Sugarcane bagasse.

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INTRODUCTION

Sugarcane bagasse is an agricultural byproduct generated in large quantity in India. About 244.8 million tons of sugarcane is produced annually in India. This leads to generation of huge quantity of bagasse. These are lignocellulosic in nature and are formed by three main polymeric constituents - cellulose, hemicellulose and lignin. Large quantity of these bagasse remained unutilized and either left to natural degradation or burnt in the field leading to severe environmental aggression and wastage of resource. Biodegradation of bagasse by *Pleurotus citrinopileatus* is significant as it not only leads to formation of simpler compounds but also results in protein rich food.

The white rot fungus *Pleurotus citrinopileatus* 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 effective biodegradation of agricultural waste and production of nutritional food.

MATERIALS AND METHODS

The culture and their maintenance

The pure culture of *Pleurotus citrinopileatus* used in the present investigation was procured from Directorate of Mushroom Research (DMR), Chambaghat, Solan (H.P.). Throughout the study, the culture was maintained on malt extract agar (MEA) medium at 23-25^{0}C and was subcultured 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. Wheat grain spawn was prepared following the method of Singh *et al* (2011). The preparation of spawn involved soaking of wheat grain in water followed by mixing of buffers, sterilization and inoculation with pure culture of *Pleurotus citrinopileatus* 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 mycelium growth.

Preparation of Substrate

The selected agrowaste sugarcane bagasse was used for the cultivation of *Pleurotus citrinopileatus*. 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 liters for every 10 kg dry chopped substrate) in a drum. The substrate was allowed to stay in water for 20 hours. After that excess water was drained out. After draining, the substrate was again completely dipped 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 liter for every 10 kg dry substrates) containing 200 ppm each of nuvan and bavistin in a drum. The substrate was allowed to stay in water for 20 h. 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 polythene 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.

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 5 days interval three bags for each treatment were removed for enzyme assay. The content of a set of three bags were mixed uniformly. 10 gram sample was homogenized in 100 ml of 50 mM sodium acetate buffer (pH 5.0) for cellulose and hemicellulose assay, while 10 gram sample was homogenized in 50 mM 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 study.

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 a residue was dried at 105^0C 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_2SO_4 and the mixture was placed in water bath at 30^0C for 1 h and made up 30 ml with distilled water and then hydrolyzed in autoclave for 1 h. The hot solution was filtered though GF/C and lignin residues were washed with hot water. The GF/C was then dried at 105^0C and finally lignin was deducted from 200 mg. The remaining was cellulose.

Extraction of Extracellular enzyme

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 assays.

Enzyme assays

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, carboxymethyl cellulose (CMC) 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 waterbath at 45^0C for 6 h and then 1 ml of alkaline $CuSO_4$ was added in each test tube and again kept in boiling waterbath at 100^0C for 20 minutes. Then solutions were taken out of waterbath and 1ml of arsenomolybdate solution was mixed in each test tube. Final volume was made up 10 ml of each test tube with the help of distilled water. After cooling for 30 minutes at room temperature absorbance was read at 540 nm, using 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 p-nitrophenyl-β-d-glucopyranoside (PNPG) in 0.1 M acetate buffer pH 5.0 was added. The reaction mixture was incubated at 45^0C for 1 h. After incubation period 1.5ml 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 1 ml of enzyme filtrate and 3 ml 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 $h^{-1}ml^{-1}$ and for laccase and PPO change in absorbance by 0.001 $min^{-1}ml^{-1}$.

Carbon and Nitrogen estimation

Carbon was determined by Walkley and Black (24) and nitrogen of lignocellulosic wastes was determined by Microjeldal 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 flask 2 to 3 times. Then it was allowed to stand for 30 minutes. 200 ml distilled water was then added followed by addition of 10 ml concentrated Phosphoric acid and 1 ml indicator and titrated against $FeSO_{4.}$

Nitrogen

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

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 minute 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 colour of solution changed from greenish blue to green.

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 were used and their average was taken as quantitative measures for determining percentages of cellulose, hemicellulose, carbon, nitrogen and biological efficiency as well as activities of extracellular enzymes.

RESULTS

Table 1, illustrates the biodegradation of cellulose, hemicellulose and lignin content of sugarcane bagasse at mycelial growth, during fructification and after harvesting (spent compost). The cellulose, hemicellulose and lignin content of untreated sugarcane bagasse were estimated at 42.53, 31.16 and 20.0 percent, respectively. The rate of degradation of cellulose and hemicellulose of hot water as well as chemically treated sugarcane bagasse during vegetative growth of *P. citrinopileatus* was slower than lignin. The degradation of cellulose, hemicellulose and lignin during vegetative phase was observed as 7.14, 9.72, 41.0 percent in hot water treated substrate and 3.78, 3.72, 32.35 percent in chemically treated substrates 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. citrinopileatus* in comparison to chemically treated substrate. However, there was no appreciable difference in the rate of degradation of lignin in the differently treated substrate.

Table 2, depicts the percentage of carbon, nitrogen and their ratio in the substrate at different stage of growth of the fungus. Carbon content and C/N ratio of sugarcane bagasse decreased while nitrogen content increased. The decrease in carbon content and increase in nitrogen content was slightly more in hot water treated substrate than chemically sterilized substrate.

Table 3, shows the activities of cellulolytic, hemicellulolytic and lignolytic enzymes produced by *P. citrinopileatus* on sugarcane bagasse during its cultivation. The result showed that the activity of cellulase and xylanase was lower during vegetative phase and higher 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. citrinopileatus*. 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 mean yield of *P. citrinopileatus* from three flushes (fresh weight) on different lignocellulosic wastes and their biological efficiency is given in Table 4. The mean yield of *P. citrinopileatus* during first flush per 120 g of dry weight of sugarcane bagasse was observed to be 95 g and 60 g, second flush was recorded as 45 g and 40 g and third flush was found to be 30 g and 20 g on hot water and chemically treated brassica haulms, respectively. The biological efficiency of *P. citrinopileatus* was recorded as 140.16% and 100.00% on hot water treated chemically treated brassica haulms, respectively.

DISCUSSION

The fast degradation of lignin and slow depletion of cellulose and hemicellulose during mycelial growth and slow degradation of lignin and fast depletion of cellulose and hemicellulose during fruit body formation in the present investigation revealed the differential requirement of the fungus *P. citrinopileatus* during different phase of its growth. Same pattern of biodegradation of lignocellulosic wastes by various species of *Pleurotus* have been reported (4, 7, 12, 18, 19, 20, 24 and 25). These observations suggested that the cellulose and hemicellulose serve as energy source for the formation of fruit bodies. In the present investigation hot water treated sugarcane bagasse degraded more efficiently by *P. citrinopileatus* than chemically treated sugarcane bagasse. The probable reason is that under high temperature hydrogen bond of cellulose got disrupted leading to formation of amorphous cellulose which are more susceptible to fungal attack and thereby degradation. Similarly, the 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 and 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

Table 1. Biodegradation of cellulose, hemicellulose and lignin of sugarcane bagasse agrowastes by Pleurotus citrinopileatus.

	CELLULOSE %				HEMICELLULOSE %				LIGNIN %			
	A	B	C	D	A	B	C	D	A	B	C	D
Hot water	42.53	39.49 (7.14)	34.51 (18.85)	23.41 (44.95)	31.16	28.13 (9.72)	24.52 (21.30)	21.65 (30.51)	20.0	11.72 (41.0)	9.52 (53.4)	8.51 (57.5)
Chemical	42.53	40.92 (3.78)	36.23 (14.81)	27.43 (35.38)	31.16	27.0 (3.72)	25.17 (19.25)	22.41 (28.08)	20.0	13.52 (32.35)	9.52 (52.5)	9.0 (55.0)

A=Untreated substrate B= At the completion of spawn run C= After fructification D= Spent compost Figure in bracket shows Percent loss

Table 2. Percent carbon, nitrogen and their Ratio in sugarcane bagasse agrowastes during growth of Pleurotus citrinopileatus.

Subs. & Spp.	**First Day**			**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	42.87	0.68	63.04	38.17	0.79	48.31	34.42	1.21	28.44	20.52	2.11	9.72
Chemical	43.97	0.71	61.92	41.46	0.76	54.55	35.13	0.95	36.97	21.63	1.90	11.38

C = Carbon percent N = Nitrogen percent C/N = Carbon and nitrogen ratio

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*.

In the present investigation activity of cellulolytic enzyme (FPase, CMCase and β-glucosidase) along with xylanase showed gradual increase in vegetative phase and sharp increase during fructification. This can be correlated with slow depletion of cellulose and hemicellulose component in the vegetative phase and their fast depletion in reproductive phase. This observation further supports 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 *Pleu-*

Table 3. Activity of cellulases (U h-1ml-1), xylanase (U h-1ml-1), laccase (U min-1ml-1) and PPO (U min-1ml-1) by Pleurotus citrinopileatus on sugarcane bagasse

Enzyme	Treatment	DAYS											
		5	10	15	20	25	30	35	40	45	50	55	60
FPase	H	7.92	8.51	12.82	9.62	8.96	3.03	2.22	1.82	1.33	0.73	0.62	0.60
	C	2.29	9.33	10.29	12.51	12.11	11.33	9.85	5.85	3.92	2.96	2.75	1.69
CMCase	H	21.85	34.74	32.96	23.11	19.33	18.59	8.4	8.14	5.37	3.23	2.96	2.32
	C	4.96	5.70	14.29	21.48	19.59	18.0	16.88	8.92	2.29	1.88	1.02	0.95
β-glucosidase	H	0.52	1.20	1.90	4.94	9.33	4.94	3.58	2.31	1.02	0.50	0.49	0.28
	C	0.55	0.89	2.91	3.32	8.69	3.67	2.88	1.45	0.44	0.33	0.22	0.18
Xylanase	H	6.40	6.66	11.40	12.88	12.27	11.62	5.22	4.66	3.21	2.45	2.32	2.07
	C	1.40	3.48	9.48	11.86	9.70	7.33	5.55	4.81	3.62	2.37	2.13	1.81
Laccase	H	8.96	8.81	13.68	9.68	8.79	7.59	3.42	2.89	1.49	0.39	0.23	0.12
	C	6.0	6.11	8.29	5.98	3.67	2.31	0.37	0.29	0.21	0.09	0.03	0.0
PPO	H	0.72	1.72	5.36	2.26	2.18	1.16	1.06	1.04	0.64	0.34	0.24	0.21
	C	0.78	1.98	4.25	2.56	1.46	0.33	0.29	0.39	0.35	.018	0.13	0.07

H = Hot water treatment; C = Chemical treatment

Table 4. Yield performance and biological efficiency (B.E) of Pleurotus citrinopileatus on sugarcane bagasse agrowastes.

Treatment	Dry weight	Wet Weight	I flush	II flush	III flush	Total weight	B.E %
Hot water	120	500	95	45	30	170	140.16
Chemical	120	500	60	40	20	120	100

rotus on various lignocellulosic substrates (2, 22, 23, 24, and 25). Maximum activities of laccase and polyphenol oxidase during vegetative phase of growth of *Pleurotus citrinopileatus* can be directly correlated with degradation of lignin in this stage. Elisashvili *et al* (6), Chang *et al* (2), Singh *et al* (21, 22 and 24) also reported high activity of these enzymes during the colonization stage and declined activity during primordia formation. The observations of present work revealed that hot water treated sugarcane bagasse supported the fast mycelial growth during cultivation of *P. citrinopileatus*. 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 of the *P. citrinopileatus* was seen on hot water treated substrate than on chemically treated substrate. Singh (18) reported that cumulative yield remained higher on *Pleurotus* species autoclaved substrates than chemically sterilized substrates.

Other articles in this theme issue include references (27-54).

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