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Some Chemical and Biochemical Changes in Straw Constituents During Growth of *Pleurotus flabellatus* (Berk & Br) Sacc.

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Summary. The quantitative changes in constituents of rice straw during different stages of growth of the fungus *Pleurotus flabellatus* were investigated. Cellulose, hemicellulose(s), lignin, total carbon and total nitrogen showed a continuous decrease from inoculation until the end of fruit body harvesting, whereas free sugars, total ash and C/N ratio increased. As calculated on constant ash basis, 14 and 13.9% of cellulose, 6.6 and 7% of hemicellulose(s) and 4 and 1.5% of lignin were decomposed during the mycelial growth and fructification respectively. Total N decreased by 0.16 and 0.23% during the mycelial growth and fructification respectively. The progressive breakdown of cellulose and hemicellulose(s) was correlated to an apparent increase in the activities of celullase and hemicellulase(s). The trend in development of cellulases and β-glucosidase activities in the substrate during different stages of its growth was demonstrated.

Introduction

Pleurotus flabellatus (Berk & Br) Sacc. (Basidiomycetes) was isolated from the wood of *Ficus bengalensis* in Mysore and its successful cultivation has been reported by Zakia Bano and Srivastava (1962). This fungus is cultivated for its white attractive spathulate edible fruit bodies of mild flavour. Besides *Pleurotus ostreatus* and *Pleurotus sajor-caju* cultivated in India, *P. flabellatus* is also catching the commercial interest of mushroom growers due to the simplicity of its cultivation and white attractive fruit bodies. It has also been found that rice straw is the best and most economic substrate for the cultivation of *P. flabellatus* (Zakia Bano et al., 1978).

There are few reports related to the nutrition and chemical changes brought about by *Agaricus bisporus* (Waksman and Nissen, 1932; Gerrits, 1968), *Pleurotus florida* (Zadražil, 1974) and *P. sajor-caju* (Kandaswamy and Ramaswamy, 1976) in the substrate constituents during their growth. Toyama and Ogawa (1974) have studied the production of cellulolytic and oxidising enzyme activities of *P. ostreatus* and *P. cornucopiae* along with many other wood rotters under various cultural conditions.

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As far as the authors know, no such data are available for changes during the growth of *P. flabellatus* on rice straw. Hence, it was thought worthwhile to report a preliminary examination of some of the chemical and biochemical changes brought about by this fungus in rice straw constituents during different stages of its growth from inoculation until the end of fruit body harvesting in the present study.

Materials and Methods

Culture Maintenance and Growth Conditions. Pure cultures of *P. flabellatus* isolated from the fruit bodies cultivated during the last three years were maintained on malt extract agar at 25°C. Using these cultures, inoculum was prepared on wet chopped (2–3 cm long) rice straw aseptically.

Preparation of Substrate. Rice straw (*Oryza sativa*) chopped to a length of 2–3 cm was soaked in water (40 litres of water for every 2.5 kg dry chopped straw) at room temperature (22°–28°C) for 18 h. After draining away the excess water, every 3 kg of the wet chopped straw containing 78% water was mixed with 100 g of a 3-month-old inoculum and 25 g horse gram (*Dolichos biflorus*) powder (mill size ca. 0.5–1 mm) and filled into 150 gauge polyethylene bags (30 x 40 cm) with perforations (of 1 cm diam.) at intervals of 7 cm. The mouth of the bag was tied with a piece of thread. Eight such replicates of the inoculated substrate were prepared starting with a dry weight straw of 5 kg at a time. The inoculated substrate was incubated at an ambient temperature of 22°–28°C and relative humidity of 55%–75%. The substrate showed an inside temperature of 24°–31°C during the incubation period (mycelial growth). As primordia started emerging from the polyethylene perforations the bags were cut open exposing the straw substrates. The exposed blocks were watered heavily and yield noted on the third day after primordia formation. The yield was expressed as g of fresh fruit bodies (containing 90% water) produced kg^{-1} dry straw. The experiment was repeated thrice.

Samples from the substrate collected at different stages of growth, before inoculation (0 day), after inoculation (0 day), during mycelial growth (6th day), after mycelial growth, i.e., when primordia were just formed (12th day), and after harvesting the fruit bodies (15th day) were used in further investigations.

Isolation of Carbohydrates. Freeze dried samples (25 g each) of the substrates at different stages of growth mentioned above were taken in triplicates. The isolation of carbohydrates, i.e., 70% alcohol soluble sugars, total hemicelluloases (A + B) and alkali insolubles, was carried out as described by Wankhede and Tharanathan (1976). The typical scheme of isolation of carbohydrates from one of the samples is given in Fig. 1.

Preparation of Enyzme Extract. Fresh substrate (25 g, ~75 to 80% moisture) was taken in triplicates and homogenised in 250 ml of 50 mM sodium acetate buffer, pH 5.4, and filtered through muslin cloth. The fibrous residue (8 g) was collected. The filtrate was clarified by centrifugation at 4000 *g* for 15 min and the volume made to 250 ml (C) with the same buffer. The fine residue left over after centrifugation (0.25 g) was extracted with fresh amounts of the above buffer and the volume made to 50 ml (R) after centrifuging for 15 min as above. The fibrous residue was also extracted, centrifuged and the volume made to 150 ml (F) with the same buffer. The typical scheme of extraction of enzyme is shown in Fig. 2. All operations were carried out at 5° ± 1°C.

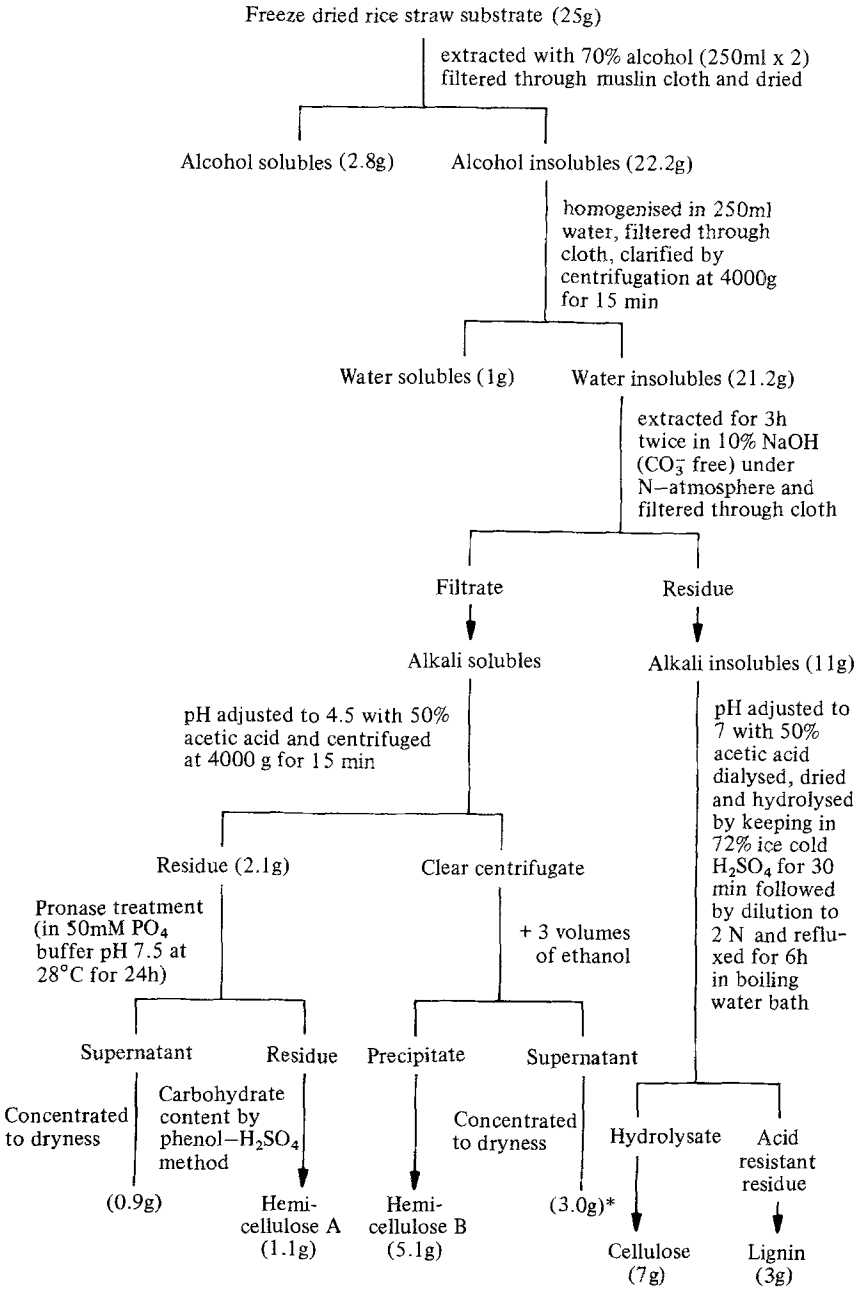

Fig. 1. The isolation of carbohydrates from rice straw (before inoculation)
*Did not respond to phenol-H_2SO_4 test

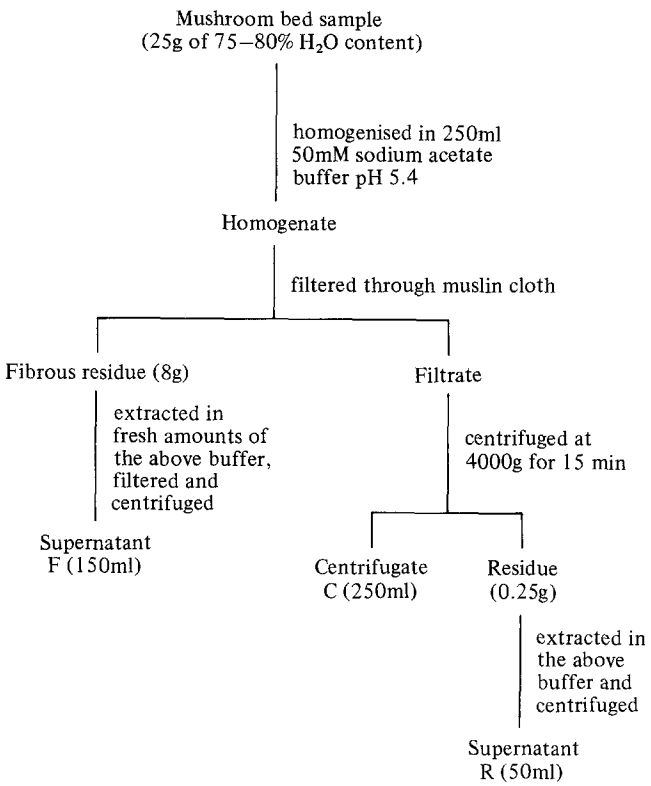

Fig. 2. Extraction and fractionation of enzymes from the fungal substrate

Enzyme Assays. Hemicellulase activity was assayed according to the method of Dekker and Richards (1975) as described by Wankhede et al. (1977), using 0.5% of hemicellulose B of rice straw as the substrate.

Carboxymethyl cellulase (CMC-ase) activity, cotton activity (C_1) and filter paper degrading (FPD) activity were estimated as described by Mandels and Weber (1969) using carboxymethyl cellulose, absorbent cotton and Whatman No. 1 filter paper strips (1 x 6 cm) respectively.

β-glucosidase activity was determined by the method of Wood (1968) using cellobiose as the substrate and the glucose released after incubation was quantitated by the method of Dahlquist (1961).

One unit of activity of the hitherto described enzyme assays was defined as mg free sugar released g^{-1} dry weight of the substrates at different stages of growth.

Aryl β-glucosidase activity was assayed as described by Petterson et al. (1963) using p-nitrophenyl β-D-glucoside as the substrate and the unit of activity was defined as mg p-nitrophenol liberated g^{-1} dry weight of the substrate.

Analytical Methods. Total carbohydrates in 70% alcohol solubles and the hydrolysate of the alkali insolubles in Fig. 1 were estimated by the method of Dubois et al. (1956). Pentosan content was estimated according to the method of Cerning and Guilbot (1973).

Direct estimation of cellulose was carried out according to Updegraff (1969). Ash, crude protein (Total N x 6.25) and also the direct estimation of lignin were determined by A.O.A.C. methods (1975). Protein, total reducing sugars and total carbohydrates of the enzyme samples were estimated by the method of Lowry et al. (1951), Nelson (1944) and Dubois et al. (1956) respectively.

Loss of Organic Matter. Direct dry weights of the substrate at different stages of fungal growth were determined through knowledge of the net fresh weight and moisture content. Loss of organic matter was calculated as the percentage difference between the dry weight of substrate during inoculation and that of other stages of the fungal growth.

Results and Discussion

The fungus *Pleurotus flabellatus* takes about 12 days to produce the primordia which grow into mature fruit bodies in the next 3 days (fructification) at the experimental conditions given in the text. Since there was a variation of yield from 300 g to 500 g fresh fruit bodies (containing 90% water) kg^{-1} dry straw, substrates that yielded 400 g fresh fruit bodies kg^{-1} dry straw were taken for all the analyses 'after harvest of fruit bodies,' to ensure comparison among the analytical values obtained.

The direct analytical values of the substrate at different stages of growth for free sugars, cellulose, hemicellulose(s), lignin, water solubles, total C, total N, C/N ratio and total ash are given in Table 1a. These values give an idea of the percentage of these components present at that particular moment, but do not give any information concerning the increase or decrease of the components during the course of the fungal growth. Further, the total ash was found to show a relative increase from inoculation until the end of fruit body harvesting, because there was a constant utilization of organic matter. However, the total amount of ash could be assumed to remain constant all the time without forgetting that a fraction of it also entered into the developing fruit bodies. Hence all the analytical values were converted on a constant ash basis and all percentages expressed as percentages of the dry matter during inoculation (Table 1b).

Changes in Cellulose, Hemicellulose(s),and Lignin. It is evident from Table 1b, that 14.0% of cellulose and 6.6% of hemicellulose(s) were decomposed during the 12 day mycelial growth. During the next 3 days of fructification, 13.9% of cellulose and 7.0% of hemicellulose(s) were decomposed. These relative percentages of cellulose and hemicellulose(s) decomposition during the two phases of growth imply that *P. flabellatus* utilises cellulose preferentially over hemicellulose(s) and also indicate the rate of metabolic activity is high during the last 3 days of fruit body build up (fructification). The above findings are in good agreement with the results reported by Gerrits (1968) for *A. bisporus*, but contradict those of Kandaswamy and Ramaswamy (1976) where during the growth of *P. sajor-caju* on rice straw, cellulose is reported to be actively utilised during mycelial growth and during fructification its utilisation was negligible.

The apparent lignin content increase after the fruit body harvest shown in Table 1a is due to the constant use of other organic constituents of rice straw and an increase in the ash content. Calculating on equal ash basis as Gerrits (1968) has done, it was found that lignin content decreased by 4% during the first 12 days of mycelial growth and by 1.5% during the next 3 days of fructification (Table 1b). This demonstrates the

Table 1a. Changes in rice straw constituents[a] (%) during growth of *P. flabellatus*

Rice straw constituents	Stages of fungal growth				
	Before inoculation (0 day)	After inoculation (0 day)	During mycelial growth (6th day)	After mycelial growth (12th day)	After harvest of fruit bodies (15th day)
Total carbohydrates	63.4	63.0	60.4	58.2	52.1
70% alcohol soluble sugars[b]	0.78	0.86	1.10	1.72	3.15
Pentosans	23.9	23.2	21.6	18.8	15.6
Total hemicellulose(s) (A + B)[b]	24.8	24.2	22.2	19.8	16.9
Cellulose[b]	28.0	27.5	23.8	19.0	10.0
Cellulose	36.2	35.5	32.1	24.2	12.1
Lignin[b]	12.0	12.0	14.0	12.6	16.5
Lignin	23.0	23.0	24.0	21.4	28.0
Water solubles[b]	15.2	16.0	18.2	21.2	27.1
Total carbon	25.4	25.2	24.2	23.3	20.8
Total nitrogen	0.63	0.62	0.58	0.52	0.37
C/N ratio	40.3	40.7	41.7	44.8	56.3
Total ash	11.0	11.0	11.8	12.4	17.6

[a]The above values are the mean of three replications on dry weight basis
[b]The values were obtained by the fractionation procedure of Fig. 1

Table 1b. Changes in rice straw constituents [a] (%) during the growth of *P. flabellatus* converted on equal ash basis

Rice straw constituents	Stages of fungal growth				
	Before inoculation (0 day)	After inoculation (0 day)	During mycelial growth (6th day)	After mycelial growth (12th day)	After harvest of fruit bodies (15th day)
Total carbohydrates		63.0	56.3	51.6	32.6
70% alcohol soluble sugars[b]		0.86	1.00	1.53	1.97
Total hemicellulose(s) (A + B)[b]		24.2	20.7	17.6	10.6
Cellulose		35.5	29.9	21.5	7.6
Lignin		23.0	22.4	19.0	17.5
Water solubles		16.0	16.9	18.8	16.9
Total carbon		25.2	22.6	20.7	13.0
Total nitrogen		0.62	0.54	0.46	0.23
C/N ratio		40.7	41.9	45.0	56.5
Total ash		11.0	11.0	11.0	11.0
Loss of organic matter		0.0	7.0	10.0	36.0

[a]The above values are the mean of three replications on dry weight basis
[b]The values were obtained by the fractionation procedure of Fig. 1

lignoclastic property of *P. flabellatus*, a property also observed in *P. florida* (Zadražil, 1974), *P. ostreatus* (Ulezlo et al., 1975) and *P. sapidus* (Daugulis and Bone, 1977). The greater decomposition of lignin during mycelial growth increases the accessibility of cellulose for easy degradation during fructification and it is well known that lignin is a barrier for the microbiological attack of cellulosic materials (Millett and Baker, 1975).

Changes in Total C, N and C/N Ratio. From the direct analytical values of Table 1a it is observed that total C and total N showed a gradual decrease during the mycelial growth but disappeared more rapidly during the formation of fruit bodies. Hence, the C/N ratio also showed a gradual increase till the formation of primordia, and a sudden increase during fruit body formation. These results show some dissimilarities from those of Gerrits (1968) on *A. bisporus*, Zadražil (1974) on *P. florida* and Rangaswamy et al. (1975) on *P. sajor-caju* who reported an increase in the N content of the substrate during their growth (even after fruit body harvesting) and progressive decrease of C/N ratio from inoculation until the end of fruit body harvesting.

The decrease in the N content by 0.08% during the first six days of mycelial growth (Table 1b) may be due to such processes as deamination during the fungal metabolism (while utilising proteins/amino acids from the substrate) and a similar decrease of 0.08% N observed on the 12th day of incubation was possibly due to its incorporation into the primordia which were being formed.

Detection and Variation in the Activities of Carbohydrases. While assaying the hemicellulase(s) and CMC-ase activities from all the 3 fractions of the enzyme preparation viz. C, F, and R, it was found that most of the activity was concentrated in the C fraction and hence this fraction was selected as a source of crude enzyme in all the subsequent assays. Unless otherwise mentioned, the efficiency of the enzyme extractions in acetate buffer (50 mM) in the pH range of 4 to 6 indicated that the extractions were very efficient at pH 5.4 and all the enzymes studied were stable at this pH.

As evident from Fig. 3, CMC-ase and hemicellulase activities showed a gradual increase during mycelial growth and a steep increase during fructification and this can be correlated to the simililar decrease in the amounts of the corresponding polysaccharides. Also it was found that the activity of CMC-ase was always greater than hemicellulase at any stage of the fungal growth and this is in consonance with higher amounts of cellulose getting depleted from rice straw as compared to hemicellulose(s).

According to Reese et al. (1950), for the complete saccharification of crystalline cellulose all the three essential enzymes of the cellulolytic complex, C_1, C_x and β-D-glucosidase, are required. Hence, we studied here the trend in development of these enzymes during the different growth stages of the fungus. The results are shown in Fig. 4. It was observed that C_1, C_x (Fig. 3) and FPD activities showed a progressive increase throughout the growth period while β-D-glucosidase activity indicated an increase on 0 day followed by a decline but increased abruptly during later stages of the fungal growth. β-glucosidase activity could also be assayed as aryl glucosidase.

Carbohydrases in the Inoculum. The 3-month-old fungal inoculum raised on chopped straw under aseptic conditions did not show CMC-ase or hemicellulase activity, but contained fairly high amounts of β-glucosidase activity. Also it had rich amounts of soluble carbohydrates and reducing sugars (Table 2). The absence of polysaccharase system

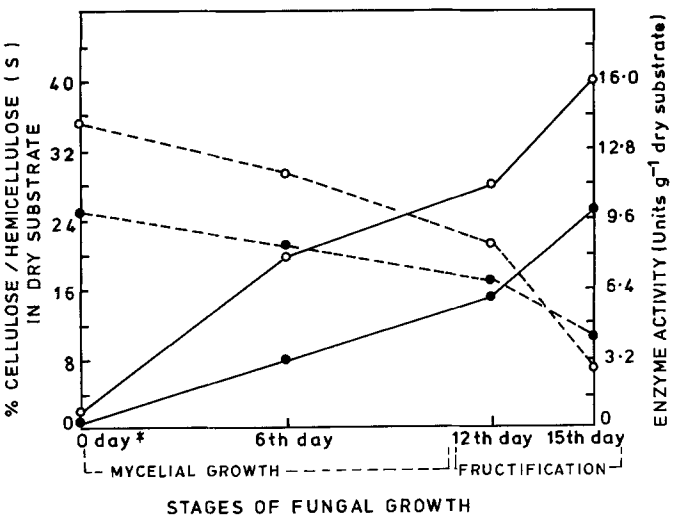

Fig. 3. Decomposition of cellulose (○——○) and hemicellulose(s) (●——●) in the fungal substrate correlated to apparent increase in the activities of carboxymethyl cellulase (○——○) and hemicellulase(s) (●——●), during cultivation of *P. flabellatus*. *Enzyme assays:* 1 ml enzyme + 1 ml 0.5% CMC + 2 ml 50 mM Na acetate buffer pH 4.8, incubated at 50°C for 30 min (CMC-ase). 1 ml enzyme + 1 ml 0.5% hemicellulose + 2 ml 50 mM Na acetate buffer pH 5.4 incubated at 37°C for 60 min [hemicellulase(s)]
*4 h after inoculation

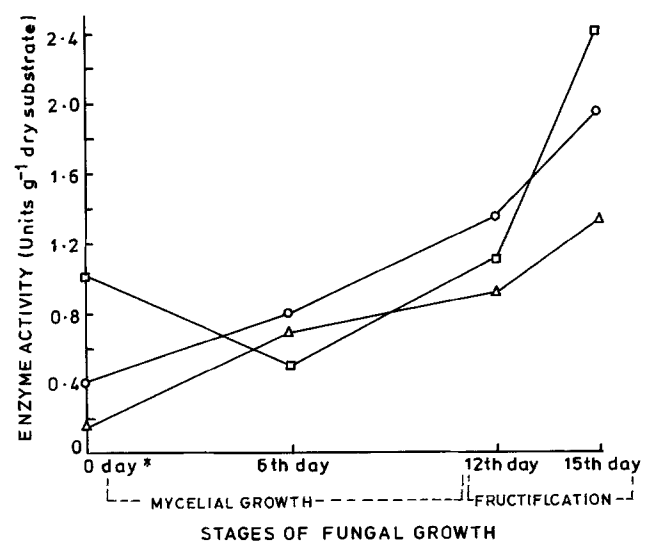

Fig. 4. Development of cotton activity (○——○), filter paper activity (△——△) and β-glucosidase activity (□——□) in the *P. flabellatus* substrate. *Assay conditions:* 1 ml enzyme + 2 ml 50 mM sodium acetate buffer pH 4.8. + 50 mg absorbent cotton incubated at 50°C for 24 h (○——○). + 50 mg Whatman No. 1 filter paper strips, incubated at 50°C for 60 min (△——△). + 1 ml 0.04% cellobiose in the above buffer incubated at 40°C for 20 min (□——□)
*4 h after inoculation

Table 2. Carbohydrase activities in the inoculum and inoculated rice straw at 0 h, after 2 h and 4 h of inoculation

Carbohydrates	Activity (units g^{-1} dry substrate)			
	Inoculum	Inoculated rice straw		
		at 0 h	after 2 h	after 4 h
CMC-ase	Nil	Nil	Nil	0.40
Hemicellulase(s)	Nil	Nil	Nil	0.30
β-glucosidase	0.60	1.00	1.28	1.50
Aryl glucosidase	0.48	0.80	0.96	1.20
Total reducing sugars[a]	1.60	0.10	0.116	0.124
Total carbohydrates[a]	14.40	6.40	6.720	7.200

[a]In the enzyme samples, as mg g^{-1} dry weight of the substrate

can possibly be due to the presence of good amounts of soluble carbohydrates and reducing sugars. This also provided indirect evidence that, at least for some period in the 3-month-old duration of inoculum preparation, the polysaccharase should have been active enough to accumulate the estimated rich amounts of broken-down carbohydrates. However, after about 4 h of inoculation, the inoculated rice straw started exhibiting CMC-ase and hemicellulase(s) activities (Table 2). This shows the inductive nature of the polysaccharases.

Changes in Dry Weight of the Starting Substrate and Biomass Produced. Keeping the DWS (dry weight of starting substrate) as 100%, after the harvest of fruit bodies the initial mass was reduced to 64%. That about 4% of the dry weight straw was converted into fruit bodies (400 g fresh fruit bodies, containing 90% water, kg^{-1} dry straw) and the rest of the matter disappeared (i.e., 32%) should account for the products like H_2O, CO_2, etc., thrown out during the fungal metabolism.

To conclude, *P. flabellatus* utilised more nitrogen during fruit body formation and a proper supply of the right nitrogen source to the substrate at this stage (i.e., at primordia formation) should help to increase the yield. The spent straw was rich in soluble carbohydrates and lignin. It had also a fairly high polysaccharase activity which can be used further for the saccharification of cellulosic wastes (e.g., paper or cotton wastes) and the production of single cell proteins therefrom.

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