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Full Length Research Paper

Varieties of ligno-cellulolytic enzymes and proteins of wood ear mushroom

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The enzyme potential of different mushrooms varied differently and differed greatly based on the nature of the substrates used for cultivation. But in common, all mushrooms produce both cellulases and laccase, more particularly, for easy degradation and utilization of the substrate. High level of cellulolytic enzyme activities namely: exo β-1, 4 glucanase and endo β-1,4 glucanase was observed in beds prepared with paddy straw + wheat bran (3:1), whereas high level of lignolytic enzyme activity namely, laccase was observed in paddy straw + wheat bran (1:1). Protein profile studies of *Auricularia polytricha* were compared with those of *Pleurotus platypus, Pleurotus florida,* and *Pleurotus eous*. SDS - PAGE analysis of mycelium of different mushrooms revealed the presence of 12-14 protein bands in *A. polytricha*. Common protein bands were expressed at 14 kDa, 25 kDa and 49 kDa in all the mushrooms.

Key words: *Auricularia polytricha*, bioefficiency, cellulases, laccase, protein.

INTRODUCTION

Auricularia auricula was the first artificially cultivated mushroom in China, and also in the world. The Chinese classical agricultural book 'Tang Ben Cao', written in about A.D. 600, described its cultivation method. *Auricularia polytricha* is widely distributed throughout the tropical and subtropical regions of the world (Zoberi, 1972). Nowadays, *Auricularia* mushrooms are among the top four most important cultivated mushrooms in the world, grown mainly in China and Southeast Asia, with a world annual production of 420,000 tons (Yan et al., 1999). Wood ear mushrooms (*Auricularia* spp.) are commonly cultivated in Asia. Plastic bag cultivation is gaining popularity due to the scarcity of suitable logs and the ease with which different species of *Auricularia* can be cultivated on sawdust. The technology can be expected to spread in the near future. There are many *Auricularia* species of which *A. polytricha, A. fuscosuccinea* and *A. auriculu-judea* are the most commonly grown.

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A. polytricha is the most suitable species to cultivate in tropical regions where temperatures are high (Well *et al*., 1984). *A. polytricha* mushrooms are rich in protein, but both qualitative and quantitative analysis of proteins evealed great intra and interspecies differences. This variation can also be seen from their different nutritional values. The heterogeneity of the structural and physiochemical properties of the mushroom proteins may be the main cause of differences. SDS - PAGE analysis of different strains gave an idea on protein pattern between the strains. The analysis of fruit bodies contains 20, 18 and 16 protein bands in button, stipe and pileus. The farm waste without degradation in soil causes the loss in texture, water holding capacity and nutrient status. The waste has adverse effect on its alkalinity and acidity due to which the pH has been increased. The organic carbon, phosphorus and potassium are not made available due to their complex nature. With this background, the present study has been formulated to undertake the degradation of agricultural waste which breaks down the high molecular weight fraction of lignin and cellulose by *A. polytricha*. However, *A. polytricha* is one of the most suitable fungal organisms for producing

Table 1. Mycelial and morphological characters of *A. polytricha*.

S/N	Portion	Character
1	Colony character	Creamy white in colour, later turn into light brown in colour
2	Spore colour and size	White spore, 16-18×6-8 µm in size
3	Fruiting body and Shape	Ear-shaped and flattened types
4	Stipe	Short stalk (about 7 mm long), lateral, rudimentary.
5	Cap	Rubbery (when fresh), thin, brown, ear-like, 2-15 cm broad.
6	Flesh	Thin
7	Veil	Absent

Table 2. Physiological requirements for cultivation of *A. polytricha*.

S/N	Period of growth	Temperature (°C)	Relative Humidity (%)
1	Spawn run	24±2	80-85
2	Primordial initiation	20±2	80-85
3	Fruiting body development	22±2	80-85

protein rich food from various agro wastes without composting.

The literature on the cultivation of *A. polytricha* is very few in India and only a limited work has been attempted. Hence, the present work was initiated with the objectives to study the presence of different ligno-cellulolytic enzymes and proteins of wood ear mushroom.

MATERIALS AND METHODS

Collection and maintenance of wood ear mushroom

Surveys were conducted in the hills of Nilgiris, Shervoys and Lower Pulneys during rainy season. A wood ear mushroom (*Auricularia* sp.) was collected from coffee plantations of Horticultural Research Station, Yercaud. The fungus was isolated and maintained in the PDA medium for future study.

Identification of the wood ear mushroom

The naturally collected mushroom was identified based on the morphological characters namely, cap character, cap diameter, colour, stem length and spore characters. In addition, mycelial characters in the agar medium were also used for the identification (Table 1).

Physiological requirements for the growth of *A. polytricha*

Beds were prepared using paddy straw and other substrates following the method described by Sharma et al. (2006) with modifications. Paddy straw was wetted thoroughly with water overnight (16-18 h). Two 50 g of paddy straw with 60% of moisture was filled in poly propylene bags with wheat bran at the ratio of 3:1 and sterilized in autoclave at 20 lbs pressure for 2 h. After sterilization, the bags are inoculated with 100 g spawn and incubated at different temperatures. The relative humidity of 80-85% was maintained constantly. The temperatures of 20±2°C, 24±2°C and 28±2°C for spawn run, temperatures of 16±2°C, 20±2°C and 24±2°C for primodial initiation and temperatures of 20±2°C, 22±2°C and 24±2°C for the fruiting body development were maintained. Three replications were maintained. The observations of the days for spawn run, days for pin head formation, number of fruiting bodies, weight of the fruiting bodies and total yield were recorded (Table 2).

Selection of suitable substrate for spawn production

The spawns were prepared using different substrates namely, sorghum grain alone and in combination with saw dust, rice bran and wheat bran. Sorghum grain was mixed with rice bran, wheat bran and saw dust at the ratio of 3:1 and 1:1 for comparison. The spawn substrate was filled in polypropylene bags of size 11" × 5" (150 gauge thickness), plugged with cotton and autoclaved at 20 lbs pressure for 2 h. Then the spawn bags were inoculated with a 9 mm disc of fungal culture and incubated at room temperature (28±2°C). Three replications were maintained, the days for which complete spawn run in different substrates was recorded. The best combination for early spawn run was used for future spawn preparation:

$$\text{Bio efficiency \%} = \frac{\text{Total weight of harvested mushroom} \times 100}{\text{Dry weight of the substrate used}}$$

Extraction of enzymes

One gram of fresh mushroom was ground in 1 ml 0.1 M

sodium phosphate buffer (pH 6.5). The homogenate was centrifuged at 10,000 g for 15 min at 4°C within 5 min. The supernatant collected was used for enzyme assays (Sadasivam and Manickam, 1992).

Assay of enzymes

Exo-ß-1, 4 glucanase

Exo-ß-l, 4 glucanase was assayed based on its activity on filter paper discs. Filter paper (Whatman No. 1) was cut into four mm diameter discs by a paper sponge to ensure uniform surface area of the substrate in each tube. The enzyme source (0.5 ml) in 0.1 M sodium citrate buffer at pH 5.8 was added to 32 mg of the filter paper. The mixture was incubated at 50°C for 1 h and the amount of reducing sugar was determined. The enzyme activity was measured following the procedure of Miller (1972) and expressed as µ mol of glucose released /ml of the extract per min.

Endo-ß-1, 4 glucanase

Dinitrosalicylic acid method suggested by Miller (1972) was followed for the estimation of endo-β-1, 4 glucanase activity. Production of reducing sugars in µg per ml of extract was determined. Carboxy methyl cellulose solution (1%) at 0.45 ml was mixed with 0.05 ml of enzyme source.

The mixture was incubated at 5°C for 15 min, followed by placing the enzyme substrate mixture in warm water bath (55°C) for 5 min. While the tubes were still immersed in water bath, 1 ml of 40% potassium sodium tartrate solution was added and cooled to room temperature (28-30°C).The volume was made up to 5 ml with distilled water (pH 7.0). The absorbance of the sample was measured at 540 nm in a spectrophotometer (GS5703AT).

D- Glucose at 20 to 100 µg/0.5 ml of distilled water (pH 7.0) was prepared and used to plot the standard graph. Comparing the standard graph, actual amount of glucose released in the enzyme substrate mixture was calculated and the enzyme activity was expressed as µ mol of glucose released/ml of the extract per min.

Laccase

Laccase activity was determined by the method suggested by Frochner and Ericksson (1974). Assay mixture containing 5 ml of 1.0 M guaiacol in 0.1 M sodium phosphate buffer was pipetted into test tube and incubated at 25°C. Then, 0.1 ml enzyme source was added. After 5 min, absorbance was determined at 470 nm.

The enzyme activity was expressed as 0.001 OD change/ min/ ml of the extract in per min (Sadasivam and Manickam, 1992).

Polyphenol oxidase

Polyphenol oxidase activity was estimated by the method described by Sadasivam and Manickam (1992). Assay mixture consisting of 2.5 ml of 0.1 M phosphate buffer (pH 6.0) and 0.3 ml of catechol solution (0.01 M) was taken in a cuvette and placed in a spectrophotometer set at 495 nm and the absorbance was adjusted to zero. The cuvette was removed and 0.2 ml enzyme source was added. After shaking, the cuvette was placed immediately in spectrophotometer (GS5703AT). The changes in absorbance for every 30 s up to 3 min were recorded. The enzyme activity was expressed as 0.001 OD change /min/ml of extract.

Protein profile studies

Protein profiling of A. polytricha and other edible mushrooms

To study the protein profiling nature of different mushrooms, Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS – PAGE) was conducted following the method. Mycelial discs of 9 mm diameter of *A. polytricha* and other mushrooms: *P. florida, P. platypus* and *P. eous* were inoculated in Potato Dextrose Broth (PDB) separately. Ten days old mycelial mats of different mushrooms were harvested individually and the moisture content was reduced by pressing in between sterile filter paper. One gram of dried mycelial mat was macerated thoroughly with one ml of 0.1 M sodium phosphate buffer (pH 7.0) in a chilled pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was taken and stored at -20°C.

For SDS- PAGE studies, samples were heated in boiling water bath at 100°C for 5 min and incubated at 4°C for 30 min. The samples at 30 µg were loaded into the wells of polyacrylamide gels (Sigma – Aldrich Techware system, Sigma, USA). The gel was loaded with standard medium range marker protein (14 to 97 k Da) in the sample buffer (Fermentas Life Sciences Pvt. Ltd., India was used). The electrophoresis was started with 85-100 V and increased to 150 V after the dye had moved into the separating gel. The gels were immersed in 0.2% Coomassie brilliant blue (R 250) staining solution overnight with uniform shaking. The staining solution was absorbed by the proteins. The gels were transferred to destaining solution following the procedure of Sadasivam and Manickam (1992). The protein profiles of different mushrooms were compared with the marker protein and conclusion was made.

RESULTS AND DISCUSSION

The results indicated that the optimum temperature for spawn run of *A. polytricha* was 24±2°C with a relative humidity of 80-85%. The temperature of 20±2°C was the

Table 3. Selection of best substrate for spawn production of *A. polytricha*.

S/N	Substrate	Days for complete spawn run
1	Sorghum grain	50.6[d]
2	Sorghum grain+rice bran (3:1)	47.0[f]
3	Sorghum grain+wheat bran (3:1)	36.0[e]
4	Sorghum grain+ saw dust (3:1)	51.0[b]
5	Sorghum grain+rice bran (1:1)	55.3[c]
6	Sorghum grain+wheat bran (1:1)	53.0[c]
7	Sorghum grain+saw dust (1:1)	56.6[a]
	CD (P=0.05)	2.96

Mean of three replications. Means followed by common letters are significantly different at the 5% level by LSD.

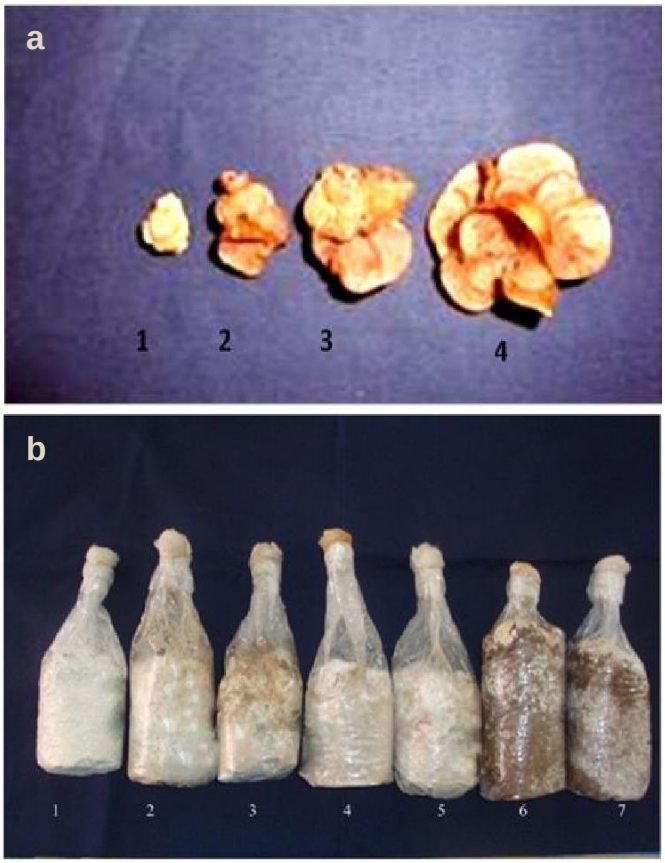

Figure 1. (a) Different growth stages of *Auricularia polyticha*. 1. Development; 2. Primordial initiation; 3. Fruiting body development; 4. Maturation. (b) Selection of suitable substrate for spawn production. 1. Sorghum grain; 2. Sorghum grain + wheat bran (3:1); 3. Sorghum grain + wheat bran (1:1); 4. Sorghum grain + rice bran (3:1); 5. Sorghum grain + rice bran (1:1); 6. Sorghum grain + saw dust (3:1); 7. Sorghum grain + saw dust (1:1).

optimum for primordial initiation, while fruiting body development was the best at 22±2°C (Table 3).

Spawn vigour is one of the main factors in deciding good cropping and yield of mushrooms (Figure 1). The success of mushroom cultivation and its yield depend to a large extent on the type and purity of spawn used. The effect of different substrates individually and in combination was studied on the growth of *A. polytricha*

Figure 2. Cultivation of *A. polytricha* on different substrates.

Table 4. Cultivation of *A. polytricha* on different bed substrates.

Treatment	DFSR	DFPF	Fruiting bodies (avg. no/ bag)	Fruiting body (avg. wt g)	Yield (g/bed)	Biological efficiency (%)
Paddy straw	27.0^{b}	39.0^{C}	25.4^{cd}	5.40^{b}	137.6^{a}	55.04
Paddy straw + wheat bran (3:1)	21.0^{b}	33.3^{d}	25.4^{cd}	6.10^{a}	155.0^{b}	62.00
Paddy straw + rice bran (3:1)	28.0^{b}	39.6^{c}	29.4^{a}	5.00^{c}	147.0^{c}	58.80
Paddy straw + saw dust (3:1)	33.0^{a}	43.0^{ab}	25.8^{bcd}	5.10^{bc}	131.6^{bc}	52.60
Paddy straw + wheat bran (1:1)	32.6^{a}	41.3^{bcc}	27.7^{abc}	4.50^{d}	125.0^{d}	49.70
Paddy straw + rice bran (1:1)	35.3^{a}	45.0^{a}	28.5^{ab}	4.00^{e}	114.3^{e}	45.72
Paddy straw + saw dust (1:1)	34.5^{a}	46.0^{a}	23.7^{d}	4.30^{de}	102.3^{de}	40.90
CD (P=0.05)	4.55	3.10	3.05	0.31	4.50	

Mean of three replications. Means followed by common letters are significantly different at the 5% level by: LSDDFSR - Days for spawn run; DFPF - Days for pinhead formation.

and the results are presented in Figure 2. Among the different substrates tested, sorghum grain + wheat bran (3:1) was found to be the best as it recorded 36 days for complete spawn run, followed by sorghum grain + rice bran (3:1) (47 days) and sorghum grain (50.6 days). Sorghum grain + saw dust (1:1) supported the least growth as it recorded 56.6 days for spawn run. Thiribhuvanamala et al. (2005) successfully produced the spawn of *A. polytricha* on sorghum grain, rice bran, wheat bran and saw dust with different combinations. Sharma and Puttoo (2004) reported that the grain substrates were better than the straw substrates for the spawn production in *P. sajor-caju*. Geetha et al. (2002) tested thirteen substrates for the spawn production in *P. florida, P. djamor, P. citrinopileatus* and *P. fossulatu* and found that sorghum was found to be the best for sporophore development, total yield and number of mushrooms.

The substrate, paddy straw + wheat bran (3:1) was found to be the best one as it recorded 21.0 days for spawn run and 33.3 days for pin head formation. The same combination also recorded the highest yield of 155 g with 62% bioefficiency. The results from Table 4 revealed that in general, mixing of paddy straw with wheat bran, rice bran and saw dust was found to induce more growth of *A. polytricha*. Among the substrates, paddy straw + wheat bran (3:1) was found to be the best one as it recorded 21.0 days for spawn run and 33.3 days for pin head formation (Figure 3). The same combination also recorded the highest yield of 155 g with 62% bio-efficiency. The mean fruiting body weight was also

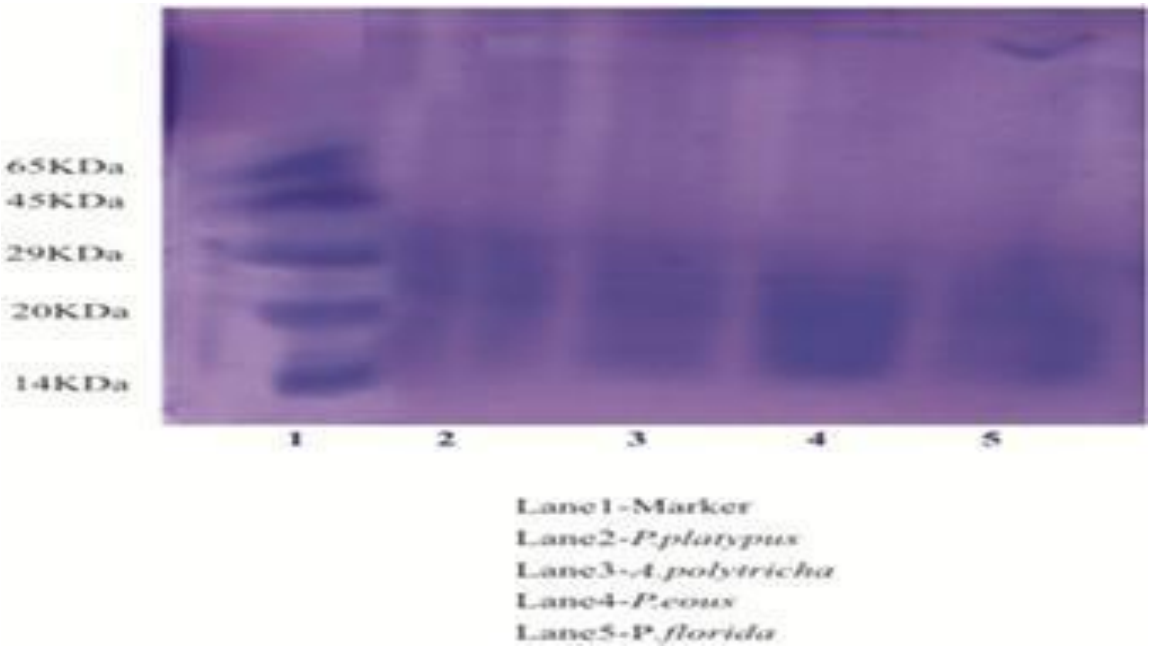

Figure 3. Sodium Dodecy sulphate- Poly Acrylamide Gel Electrophoresis for the protein profile of *Auricularia polyticha*.

Table 5. Studies on the activities of different lignocellulolytic enzymes of *A. polytricha*.

Substrate	Exo ß-1,4 glucanase (µ mol of glucose released / ml of the extract) per min	Endo ß-1,4 glucanase (µ mol of glucose released / ml of the extract) per min	Laccase (Change in absorbance/ min / ml of the extract) per min	Polyphenol oxidase (Change in absorbance/ min / ml of the extract) per min
Paddy straw	34.30^{dc}	38.60^{bc}	43.00^{d}	0.20^{f}
Paddy straw + wheat bran (3:1)	48.00^{a}	47.00^{a}	47.00^{c}	1.61^{b}
Paddy straw + rice bran (3:1)	43.00^{b}	41.30^{c}	51.00^{b}	2.71^{a}
Paddy straw + saw dust (3:1)	33.00^{d}	38.30^{c}	41.00^{d}	0.66^{d}
Paddy straw + wheat bran (1:1)	41.30^{b}	43.00^{b}	56.60^{a}	0.94^{c}
Paddy straw + rice bran (1:1)	36.60^{c}	33.00^{d}	35.03^{e}	0.84^{c}
Paddy straw + saw dust (1:1)	25.60^{e}	28.00^{e}	21.30^{f}	0.36^{e}
CD (P=0.05)	3.17	3.12	3.35	0.16

Mean of three replications CD - critical difference at 0.05%.

maximum (6.10 g) in the same treatment with 25 fruiting bodies.

The results of the present study revealed that the mushroom samples collected from the beds prepared with paddy straw + wheat bran (3:1 ratio) recorded the highest level of Exo ß-1,4 glucanase (48 µ mol of glucose/ml/min) and Endo ß-1,4 glucanase (47 µ mol of glucose/ml/min) followed by beds prepared with paddy straw + wheat bran (3:1 ratio) (43.00 and 41.30 respectively). Contrastingly, the activity of laccase was the highest (56.60 change in absorbance/min/ml) in the mushroom samples for paddy straw + rice bran 1:1 ratio. Polyphenol oxidase activity was the maximum for paddy straw + rice bran 3:1 ratio (2.71 change in absorbance/min/ml). On the whole, the cellulolytic enzyme activity was high in paddy straw + wheat bran 3:1 ratio beds and lignolytic enzyme activity was high in beds prepared into paddy straw + rice bran 1:1 ratio (Table 5). According to Ratcliffe et al. (1994), various species of mushroom manifest different enzymatic activity.

The major components of agro wastes like cellulose, hemicelluloses and lignin were degraded by a variety of

cellulases are involved in the degradation of cellulose component of the substrate and they play a major role in determining the rate of colonization and thereby the yield (Chang et al., 1993). The present study with four mushrooms also revealed that there is only minor difference noticed between them in protein profiles and *A. polytricha* is found to have 10-12 bands (Figure 3). SDS – PAGE analysis of different mushrooms, revealed the presence of 12-14 protein bands in *A. polytricha*. Common protein bands were expressed at 14 kDa, 25 kDa and 49 kDa in all the mushrooms. Proteins and amino acids are the most important constituents of fruiting bodies than other nutrients such as carbohydrates, vitamins and minerals. Gel electrophoretic analysis of fruiting bodies of *C. indica* at developmental stages revealed that 20, 18 and 16 protein bands were present in button, stipe with well differentiated pileus and stipe with tiny pileus respectively (Purkayastha and Nayak, 1981).

Conclusion

The study on the lignocellulytic enzymes of *A. polytricha* provides useful information for degradation of agricultural waste by balancing the C:N ratio. As such, the degradation of agricultural waste breaks down the high molecular weight fraction of lignin and cellulose by *A. polytricha*. The protein studies of *A. polytricha* are very useful for identifying the antioxidant and medicinal property.

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