



Cultivation of Oyster Mushroom and Recovery of Value Added by-products from Biodegradable Lignocellulosic Waste Materials by Solid State Fermentation

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Abstract

In the present study, the edible fungus *Pleurotus sajor-caju* was investigated for its ability to grow on different lignocellulosic substrates namely, water hyacinth, wood chips and paddy and to produce various lignocellulolytic enzymes such as cellulase, xylanase and pectinase. The production pattern of the extracellular enzymes, total proteins and reducing sugar level was studied during the growth of this fungus under Solid State Fermentation (SSF) for a period of 56 days. Wheat grains were rapidly utilized and consumed by the fungus as a simple carbon source and were used for spawn formation. Different lignocellulosic complex carbon sources served as substrates and exhibited better induction process for more enzyme biosynthesis and production. The maximum activity of enzymes was obtained on 28th and 56th day of culture growth. The total yield of mushroom grown on different lignocellulosic substrates and levels of cellulose, pectinase and xylanase was also detected.

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Introduction

Large amount of lignocellulosic wastes are generated through forestry and agricultural practices, paper-pulp industries, timber industries and many agro industries. Currently, much of these lignocellulosic wastes are disposed by biomass burning or shredded and composted which results in environmental pollution. Fungal bioconversion of wood is an important agent responsible for the environmental carbon circulation (Kirk and Farrell 1987, Eriksson *et al.*, 1990) and moreover by making use of appropriate bioconversion technology, these wastes can be converted into variety of value added products. Various lignocellulosic substrates and white-rot fungi have been used successfully in submerged and

solid-state fermentation for lignocellulolytic enzyme production. The type and composition of lignocellulosic substrate appear to determine the type and amount of enzyme produced by basidiomycetes (Elisashvili *et al.*, 2006). Compared with submerged fermentation, solid-state fermentation provides certain advantages of fungal enzyme production with the aspect of application in bioprocesses such as biobleaching, biopulping, bioremediation etc (Stajic *et al.*, 2006).

It has also been shown that, during solid-state fermentation of lignocellulosic materials, some fungi produce a different set of enzymes compared with synthetic liquid culture. Many of the mushroom species have the potential to produce and secrete a wide

spectrum of enzymes, like cellulases, hemicellulases, xylanases, lignin peroxidase, manganese peroxidase and laccases which enable them to thrive over a range of lignocellulosic wastes and produce protein rich biomass of fruiting bodies. During cultivation on lignocellulosic substrates enzyme activities change when shift between substrate colonization and fructification stages of mushroom growth (Omoanghe and Mikiashvili, 2009).

Mushrooms are very nutritious products which are rich in crude fiber and protein. Mushrooms also contain low fat, low calories and good vitamins. In addition, many mushrooms possess multi-functional medicinal properties. (Heleno *et al.*, 2010; Mattila *et al.*, 2001). In the present study, three biodegradable lignocellulosic waste materials namely, Water hyacinth (*Eichhornia crassipes*) a fast growing perennial aquatic weed widely distributed throughout the world. Wood chips and paddy were used as substrate for oyster mushroom cultivation using *Pleurotus sajor-caju* and recovery of value added byproducts such as various enzymes, mushrooms and reducing sugar was studied and compared.

Materials and Methods

Culture

Culture of *Pleurotus sajor-caju* was obtained from the Department of Biotechnology, Pillai's college New Panvel.

Substrates

Fresh and healthy Water Hyacinth whole plants were collected from a freshwater pond of Palm Beach road, Nerul, wood chips were obtained from carpenter's shop and paddy straw was obtained from APMC market, Vashi, Navi Mumbai.

Mushroom production

Processing of substrate

The water hyacinth was thoroughly washed several times with tap water to remove adhering dirt. Samples of stem, petiole and leaf of the fresh water hyacinth selected as the substrate material were chopped into small pieces (~1-2 cm), blended to small particles (~3-5 mm), and finally dried in a hot air oven at 80°C for 3-4 hours. Similarly, wood chips and paddy straw were also chopped into small pieces and washed extensively with

tap water to remove soil particles and dried in hot air oven at 80°C for 3-4 hours.

Preparation of substrates for inoculation

Water hyacinth, Wood chips and Paddy straw were soaked for 3 hours in hot tap water containing 5% CaCO₃ for pH adjustment to 6.5-7.9 which is an optimal pH for *Pleurotus* (Iqbal and Shah, 1989). After 3 hours, the excess water was again drained off. All the three substrates were then sterilized by autoclaving at 121°C for 1 hour.

Spawn preparation

Whole wheat grains were washed with tap water to remove dirt and floating seeds. These wheat grains were then soaked in hot tap water overnight for the grains to become soft. Excess water was then removed from the grains. The grains were mixed with 0.5 % CaCO₃ on dry weight basis (to adjust pH). These treated grains were then filled in bottles and autoclaved for two consecutive days at 121°C for 1 hour. These grains were then inoculated with actively growing mycelium of the *P. sajor-caju* from potato dextrose agar slants. These bottles were then incubated at (27±2) °C in dark for mycelial growth for 12 to 15 days.

Substrate inoculation

The spawn of *P. sajor-caju* was used to inoculate the sterile substrate. 500 gm of each substrate was inoculated with 200 gm of spawn. The plastic bags were sterilized by wiping with alcohol. Spawning was done in layers by alternately layering the substrate with spawn. The procedure was followed until the plastic bag was full. These bags were then sealed and placed in well ventilated dark room at 25-27°C as growth of mycelium does not require light. After three days of incubation, fine holes were made into each spawned bag for proper aeration.

Fruiting

When the bags were fully invaded with mycelia they were taken out of the dark room and kept exposed to light. The plastic bags were cut open on the sides without disturbing the bed and sprayed with water twice a day using a hand sprayer. The mass of substrate was kept undisturbed for the appearance of fruiting bodies. The spray of water was discontinued a day before the harvest

of the fruiting bodies. The mushrooms were collected in three flushes.

Preparation of enzyme extract

For enzyme extraction, 10 gm of each autoclaved substrate was inoculated separately into zip-lock pouch with 4 gm spawn. Seven such pouches of each substrate were prepared. The pouches were also incubated in dark room at 25-27°C with maintenance of proper humidity. These pouches were sacrificed after every 14 days interval for extraction of different enzymes.

The colonized substrate from the pouches was transferred into 250 ml Erlenmeyer flasks and 100 ml acetate buffer (pH 5.4) was added to these. The flasks were then kept on shaker for 2 hours. After 2 hours the extracts were filtered through muslin cloth. The filtrates were centrifuged at 5000 rpm for 10 minutes. The enzyme extracts were then refrigerated for further analysis of enzymes.

Analyses

Cellulase assay

Determination of cellulase activity was based on the colorimetric quantification (at 540 nm) of free glucose released by the hydrolysis of cellulose using substrate.

One IU is the amount of enzyme that liberates one μmol glucose per minute under test conditions (i.e., pH 5.4, temperature 37°C and substrate concentration).

FPase, CMCCase, B-glucosidase assay

Cellulase activity was measured as filter paper activity units. Filter paper activity is a combined assay for endo and exo β -1, 4 glucanase. 1 ml of enzyme extract was incubated with Whatman no. 1 filter paper strip (1.0 cm x 6.0 cm) and 2 ml of 0.1 M acetate buffer (pH 5.4) for 1 hour at 50°C. 1 ml of enzyme extract was incubated for 30 min at 50°C with 1 ml of 1% CMC (for CMCCase) and 1 % Salicin (for β -glucosidase) prepared in 0.1 M acetate buffer. Final volume was made up to 3 ml with buffer. The reaction was terminated by adding 3 ml of DNSA reagent and the mixture was boiled for 5 minutes and cooled. The absorbance was read at 540 nm using colorimeter. Control was run without filter paper strip to apply corrections for the reducing sugars present in sample. This absorbance was translated by plotting

against standard curve to get μg of glucose to calculate units of enzyme activity.

Xylanase assay

1 ml of enzyme extract was incubated with 1 ml of 0.1 M acetate buffer (pH 5.4) and 1 ml of 0.5% (wt/vol) Birchwood xylan prepared in 0.1 M acetate buffer (pH 5.4). Reaction mixture was incubated at 40°C for 30 min. The amount of reducing sugar released was determined by Miller's method using D-xylose as standard. One unit of xylanase is defined as the amount of enzyme which releases one μmol of D-xylose per minute under assay conditions.

Pectinase assay

1 ml of enzyme extract was incubated with 1 ml of 0.1 M acetate buffer (pH 5.4) and 1 ml of 0.5% pectin prepared in 0.1 M acetate buffer (pH 5.4). Reaction mixture was incubated at 40°C for 30 min. The amount of reducing sugar released was determined by Miller's method using D-galacturonic acid as standard. One unit of pectinase is defined as the amount of enzyme which releases one μmol of reducing sugars per minute under assay conditions.

Determination of free reducing sugars

The amount of free reducing sugars in a known volume (1ml) of enzyme extract was determined by DNS method of Miller (1959).

Protein determination

Protein content in both the enzyme extract and mushroom was determined colorimetrically using Bradford's method (1976) by measuring optical density at 600 nm. The amount of protein ($\mu\text{g}/\text{ml}$) was calculated using standard curve of Bovine Serum Albumin (BSA).

Determination of lignin content

Gravimetric determination of lignin in cultivated substrates was estimated according to (Sun *et al.*, 1996 and Adsul *et al.*, (2005). 10 gm of air-dried substrate was fragmented into small pieces and suspended in 200 ml 1 % (wt/vol) aqueous solution of NaOH. The mixture was autoclaved at 121°C for 1 hr in 500 ml Erlenmeyer flask. The residues were collected and extremely washed by tap water until neutral pH and then dried at 80 °C for 48

hr and weighed. The loss of weight is corresponded to lignin content.

Moisture content

A known amount of mushroom sample was taken in a dry, pre weighed china dish. The china dish with sample was weighed before and after drying at 80°C in an oven. Moisture content of substrate sample was calculated using formula:

$$\text{Moisture content} = \frac{A-B}{A} \times 100$$

Where A: wet weight of the sample and B: oven dried weight of the sample.

Determination of organic carbon

The organic carbon in the three substrates was determined by Walkey and Black method.

Determination of phosphorus content

The phosphorus content in the substrates was determined by Fiske-Subbarow Acid molybdate method.

Nitrogen estimation

Nitrogen values can be converted to crude protein using the formula: Crude protein = (N x 6.25) (Jones *et al.*, 1942).

Hence, N= crude protein/ 6.25.

Results and Discussion

Cultivation of mushroom presents an economically important biotechnological industry that has developed all over the world. It is estimated that more than 10 million metric ton of edible and medicinal mushrooms were produced in 2004 in various countries (Royse, 2005). Mushroom can convert the huge lignocellulosic wastes into a wide variety of products (edible or medicinal food, feed and fertilizers), protecting and regenerating the environment.

Under present investigation *P. sajor caju* was cultivated on different substrates and incubated at 25-30° C along 56 days. Pouches of each substrate were sacrificed after every 14 days of incubation. Enzymes, reducing sugar and protein were estimated in enzyme extract obtained from these sacrificed pouches.

The activity of FPase (exoglucanase) was observed on 0th day of incubation in water hyacinth and paddy whereas it was not detected in case of wood chips. This could be due to the hydrolyzed wheat grains used for spawn preparation of *P. sajor-caju*. Maximum FPase activity was found on 56th day of incubation in wood chips (4 IU/ml) followed by paddy (2 IU/ml). In case of water hyacinth, maximum activity (1 IU/ml) was observed on 28th day which further decreased with further incubation. Sherief *et al.*, (2010) observed maximum exoglucanase activity on rice straw and sawdust after 20 days which also showed induction quite late. Thus, maximum FP ase (exoglucanase) activity was reported in wood chips on 56 days of incubation.

P. sajor-caju culture grown on water hyacinth and wood chips showed endoglucanase or CM Case activity from 0th day of incubation whereas it was not detected in case of paddy as substrate at this stage. The CM Case activity gradually increased till 28th day in both water hyacinth and wood chips. In case of paddy as substrate, the CM Case was induced at a later stage. The peak values of CM Case activity were found on 56th day and were recorded as 12.66 IU/ml in case of both substrates wood chips and paddy. Maximum activity on water hyacinth (12.33 IU/ml) was observed on 28th day which is induced much earlier.

β -glucosidase activity was observed in water hyacinth and wood chips after 7 days of incubation however the activity was observed in paddy on 0th day. Maximum β -glucosidase activity was observed on 56th day in both wood chips and paddy and was found to be 4 IU/ml and 7 IU/ml respectively. In case of water hyacinth maximum β -glucosidase activity was observed on 28th day and was recorded as 9.33 IU/ml.

It has been observed that the maximum cellulase activity was induced at an earlier stage in water hyacinth when compared to wood chips and paddy. This could be attributed to the lesser lignin content in water hyacinth as compared to other two substrates (Table 1) (Fig. 1-3).

Xylanase activity in case of water hyacinth was found to increase gradually from 0 to 56 days and maximum xylanase production was observed on 56th day which was recorded as 21.5 IU/ml. Early xylanase production was observed with wood chips as substrate compared with other two lignocellulosic materials used in this study. This result is in agreement with the result obtained by Sherief *et al.*, (2010). This could be due to inductive effect of hemicellulose on xylanase production.

Hemicellulose in wood chips is more than paddy straw and water hyacinth (Table 1). In case of both wood chips and paddy, there was a drop in xylanase production as observed on 14th day after which the activity increases significantly. Maximum xylanase activity was observed on 56th day in both substrates. The activity was found to be 11.5 IU/ml and 12.5 IU/ml on wood chips and paddy respectively (Fig. 4). This is in agreement with the results obtained by Techapun *et al.*, (2003) who reported that paddy straw was not a good inducer of xylanase. Studies carried out by Sherief *et al.*, (2010) showed that optimum xylanase production was recorded after 20 days in case of sawdust and after 45 days in case of rice straw. Earlier studies indicated that optimum xylanase production was detected after 16 days by *P. ostreatus* grown on wheat straw (Garzillo *et al.*, 1994. Qinnghe *et al.*, (2004) indicated that corncob which contains high level of xylan (33%) has been used as an induction substrate for different xylanases production by *P. ostreatus*.

High level of pectinase activity was observed initially on 0th day of incubation in all the three substrates. This could be attributed to spawn on wheat grains. The pectinase activity gradually fell from 0 to 14 days of incubation. After 14th day, the activity was again found to increase gradually till 56th days (Fig. 5). The rise and fall in the activity could be related to the shift in carbon source used by *P. sajor-caju*. Initially *P. sajor-caju* utilised wheat grains as carbon source. When wheat grains were used up, the organism switched over to the lignocellulosic material (substrate) as carbon source. Maximum pectinase activity was observed on 56th day in all three substrates. The activities were 51.33 IU/ml, 29.03 IU/ml and 31 IU/ml on water hyacinth, wood chips and paddy respectively. Much work on pectinase production by *Pleurotus* species has not been carried out previously. Our aim of studying this enzyme is due to its importance and wide use in food industry during extraction and clarification of fruit juice, tissue maceration and also in paper and pulp industry, waste management, animal feed and textile industry. A.A. Sherief *et al.*, (2010) showed that *P. ostreatus* pectinase was optimally produced after 35 days in case of sawdust and after 20 days in case of rice straw.

Initial high reducing sugar content in the culture filtrate (0 day) may be due to the hydrolysis of wheat grains during autoclaving and hydrolysis of grains by *P. sajor-caju* during spawn development. Maximum production of reducing sugar released then gradually decreased thereafter and an increase in the β -glucosidase activity

was seen (Fig. 6). There was a decrease in the concentration of reducing sugar after 28 days when the fruiting bodies start to appear. This could be due to degradative enzymes from mushroom growing on the three substrates including cellulase and xylanase act on the hemicellulosic components converting them into mixture of solubilized sugars, mainly oligomer. The sugars are then available as essential nutrients for growth of mushrooms (Anakalo Kihumbu, 2008). Mushrooms utilize reducing sugar for growth and thus prevent feedback inhibition of degradative enzymes like β -glucosidase (Ryu and Mandels, 1980). Thus an increase in cellulase activity was seen. In case of water hyacinth and wood chips maximum values of reducing sugar were obtained on 14th day which were 1200 μ g/ml and 990 μ g/ml respectively. Highest value of reducing sugar in paddy was obtained on 28th day which was recorded as 1260 μ g/ml.

Protein pattern showed gradual and significant increase in total unspecified protein production by *P. sajor-caju* and correlated positively with fermentation periods. The value of protein reached maximum on 28th day of incubation for water hyacinth (174 μ g/ml) and paddy (188 μ g/ml) and on 56th day of incubation for wood chips (124 μ g/ml) (Fig. 7). Sherief *et al.*, (2010) observed that the value of protein reached maximum after 40 days of incubation for rice straw and after 45 days incubation for sawdust. The observed increase in protein content during mushroom growth and fruiting period indicates a positive substrate bioconversion (Alemawor *et al.*, 2009) (Table 2). Most of the extracellular fungal enzymes produced during bioconversion process by the growing fungus are proteinaceous in nature, thus the spent enzymes could contribute some amount of protein to the substratum (Kadari, 1999).

Protein, the most important constituent of food material is found in oyster mushroom. The total protein content in dry powder of mushroom cultivated on different substrates was estimated by Bradford's method (1976). The highest content of protein was found in mushroom grown on water hyacinth which was followed by wood chips and then paddy (Table 3). Chang and Miles (1988) reported that the range of protein in oyster mushroom was 19-35 %. Findings of present experiment differed from finding of Chang and Miles (1988) but are in accordance with those obtained by Sarker *et al.*, (2007). Badshah *et al.*, (1994) and Zaman (2004) found that the protein content of mushroom is affected by nutrient status of the substrate.

Table.1 Composition of different lignocellulosic wastes

Lignocellulosic waste	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Wood chips	40-50	20-40	14-19
Paddy	29.45	24.7	16.78
Water hyacinth	23.0	35.0	7.5

Table.2 Comparison of bioconversion efficiency of lignocellulosics into value added products

Value Added Products	Water hyacinth	Wood chips	Paddy
1. Mushroom			
• Yield	10.9	11	14
• Protein	17.2	11.5	9.9
2. Enzymes(IU/ml)			
• Cellulase			
FPase	1	4	2
CMCase	12.33	12.66	12.66
β-Glucosidase	9.33	4.66	7
• Pectinase	51.33	29.03	31
• Xylanase	21.5	11.5	12.5
3. Reducing Sugar (µg/ml)	1200	990	1260
4. Protein (µg/ml)	174	124	188

Table.3 Effect of different substrates on protein content of oyster mushroom

Substrates	Protein Content (Gm %)	Yield (%)
Water hyacinth	17.2	10.9
Wood chips	11.5	11
Paddy	9.9	14

Table.4 Comparison of carbon, nitrogen and phosphorus content of different substrates

Substrates	Carbon (%)	Nitrogen (%)	C:N ratio	Phosphorus
Water hyacinth	15	1.32	7.30	78.5
Wood chips	22.98	0.703	32.69	6
Paddy	46.8	0.69	67.82	24

Fig.1 Cellulase activity with water hyacinth

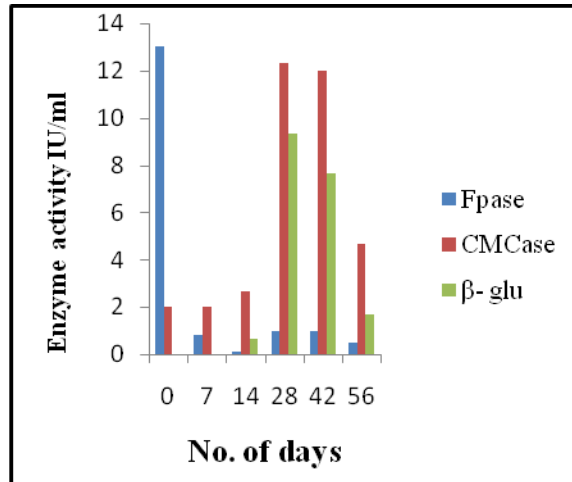


Fig.2 Cellulase activity with wood chips

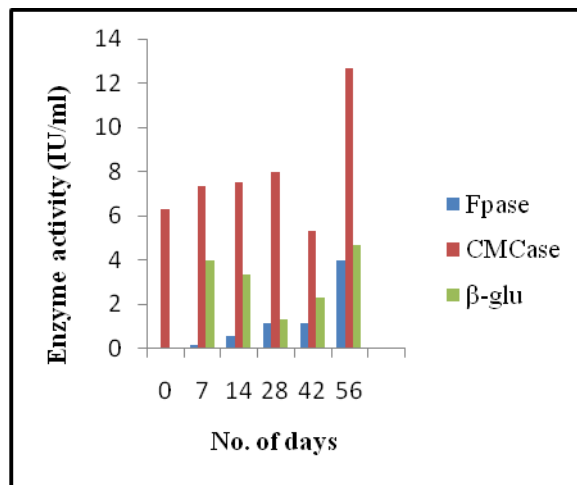


Fig.3 Cellulase activity with paddy

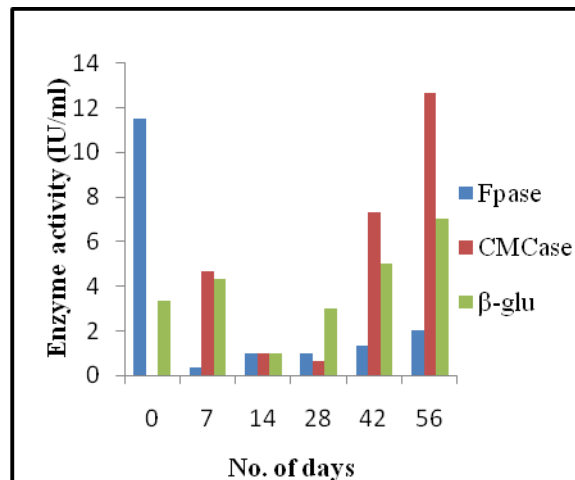


Fig.4 Xylanase activity on different substrates

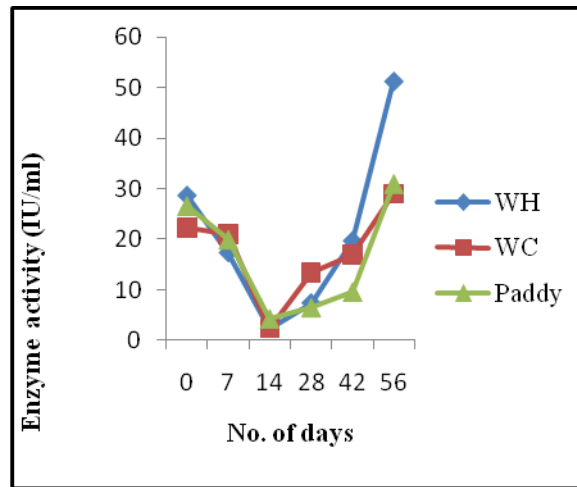


Fig.5 Comparison of pectinase activity on different substrates

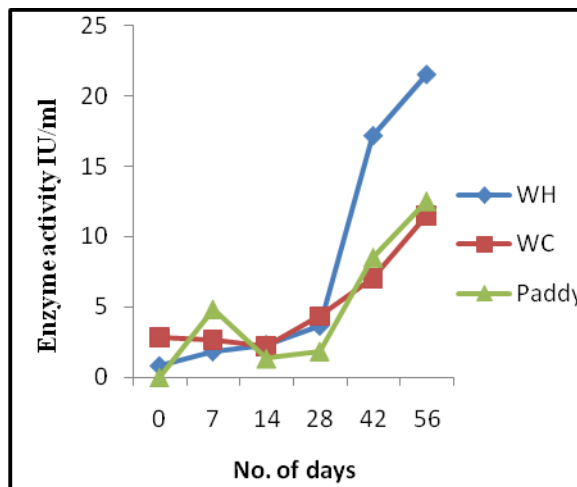


Fig.6 Comparison of reducing sugar released during fermentation

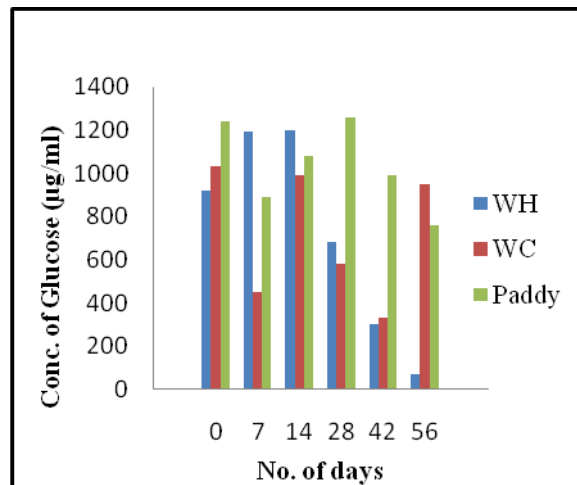
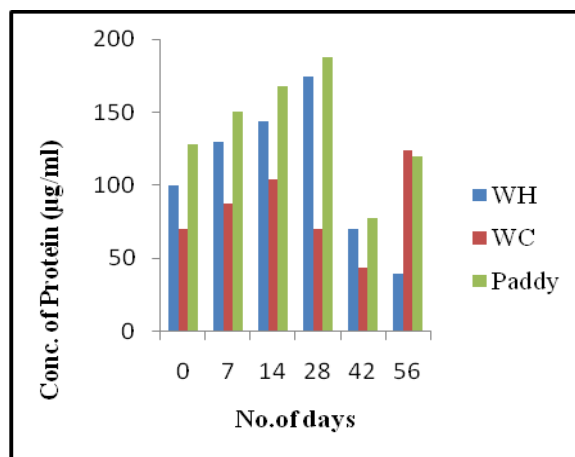


Fig.7 Comparison of protein produced on different substrate



The nitrogen and phosphorus content was found to be high in water hyacinth whereas high carbon content was found in paddy (Table 4). The C: N ratio was found to be higher in paddy than other two substrates used. Hence, more yield of mushroom was obtained using paddy as substrate.

An attempt was made to utilize the water hyacinth, *Eichhornia crassipes*, as a substrate for oyster mushroom cultivation and a good yield was achieved. This was related to the ideal C:N ratio and low lignin content. This technology could be very cheap and it is also a method of helping to eradicate a troublesome aquatic weed.

In the present study, the potential of lignocellulosic waste materials like water hyacinth, wood chips and paddy for the cultivation of Oyster mushroom (*P. sajor caju*) was tested. During the present research, it was observed that *P. sajor caju* is able to grow on different lignocellulosic waste materials causing their degradation by producing a bulk of inducible lignocellulases. The nutritional composition of the mushroom *Pleurotus sajor-caju* grown on the hyacinth lignocelluloses compares favorably with that got when the mushroom is grown on common substrates. Cultivation of *Pleurotus sajor-caju* combined with waste utilization can be an economical and harmless method of waste disposal. It has nutritional benefits especially where nutritive foods are scarce and costly but lignocellulosic wastes are abundant. Enzyme production using *Pleurotus* spp. through SSF of lignocellulosic wastes holds a great promise in future. The protein content of mushroom grown on water hyacinth is higher as compared to other two substrates. Enzymes like cellulase, xylanase act on hemicellulosic components of wastes and convert them to simple form of sugars like oligomers which are then utilized for formation of fruiting bodies (mushroom). The production of oyster mushrooms on water hyacinth by SSF is an effective method for production of nutritional and protein rich food which also help in waste management. The findings suggest that using

hyacinth substrates does not have detrimental effect in terms of the proximate composition of mushroom.

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