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INDUSTRIALLY IMPORTANT ENZYMES FROM SPENT OYSTER MUSHROOM BED WASTES

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ABSTRACT

Discarded oyster mushroom beds were examined for their bioactive compounds and industrially important enzymes. Spent oyster mushroom beds were used as substrate by micro organisms such as *Bacillus megaterium, Pseudomonas aeruginosa, Proteus vulgaris, Bacillus amyloliquefaciens and Micrococcus* sp were used production of industrially important enzymes and bioactive compounds. Bioactive compounds such as total sugars and reducing sugars were produced by *B. amyloliquefaciens. B. megaterium, B. amyloliquefaciens* showed positive result in lipid hydrolysis. *Microorganisms* such as *B. megaterium, P. aeruginosa, P. vulgaris, B. amyloliquefaciens, and Micrococcus* sp used spent oyster mushroom bed as good source for production of industrially important enzymes such as laccase, lignin peroxidase , manganese peroxidase, cellulase, protease, amylase, pectinase, xylanase and catalase.

KEY WORDS: Spent oyster mushroom beds, *B. amyloliquefaciens*, laccase, cellulase, amylase, xylanase.

INTRODUCTION

Today mushrooms are becoming more and more popular amongst people as a continental or Chinese delicacy. As the number of diabetic patients is increasing steadily in India, mushrooms can supplement a good diet for these patients with low calories and high protein value. Due to their fast growth and simple cultivation without need of any chemical fertilizers or pesticides, mushroom farming is becoming a very popular cottage industry (Nidadavolu et al., 2010). The government is encouraging it as a small-scale industry and giving subsidiaries for mushroom cultivation. Using agricultural waste materials, rural women are cultivating mushrooms in their spare time and even in small extra spaces in their homes. Hence, this has developed as a village industry and is earning respectable amounts of money to support rural families. It is becoming one of the important activities of the Development of Women and Children in Rural Areas groups formed in the villages to help themselves. The program has been revolutionary in all districts of the state of Andhra Pradesh and is becoming very popular in other states of India. The mushroom production technology is considered a very attractive and useful program because it not only recycles agricultural wastes such as paddy, jowar, maize straw, cotton waste, groundnut waste, and oil cake but also produces highly nutritious mushroom for human consumption. It also gained significance because it is low-tech and manageable in normal conditions. With a simple 2-day training process, illiterate woman can easily practice the preparation of mushroom beds with agricultural wastes, their sterilization methods, spawning, casing, cropping, and so on. Previously, villagers were afraid of the mushrooms and never ventured to touch them (Nidadavolu et al., 2010).

Their reasons were, first, that they did not know how to distinguish between edible and poisonous mushrooms, and, second, that the appearance of poisonous mushrooms (colored or spotted) in the house or agricultural fields was considered a bad omen. Now in the changed conditions, people are fascinated with the production and are cultivating mushrooms, for which they are receiving attractive payment.

The use of mushrooms for their bioactive compounds has been in practice for a long time, since these mushrooms can provide compounds that have nutritional, medicinal, and biological importance. However, the isolation of bioactive compounds from discarded mushroom beds is not in common practice. When our scientific group visited a village, we found huge piles of discarded mushroom beds on the outskirts of the village and saw that their disposal was becoming a problem. To solve this disposal problem and to get "wealth out of waste" (WOW), the present investigation was undertaken. Collection of these discarded beds from many villages and extraction of industrially important enzymes and bioactive compounds is the topic of this paper.

METHODOLOGY

Microorganism Obtained

The bacterial strains such as Bacillus megaterium (MTCC-428), Pseudomonas aeruginosa (MTCC-424), Proteus vulgaris (MTCC-1771), *Bacillus amyloliquefaciens (MTCC-1270) and Micrococcus* sp (MTCC-7527) were obtained from the Microbial Type Culture of Collection, Chandigarh gene bank, India. The culture was maintained in Luria broth.

Substrate Collection

Collection of waste mushroom beds

The oyster mushroom bed waste was collected from Mushroom farming house, Srimad Andavan Arts and Science College, Trichy. The substrate was dried in sun shade & made into powder by means of mechanical blenders.

Preparation of Extract

A total of 42 g of dried oyster mushroom bed was soaked in 200 ml of distilled water for one day. After filtration and centrifugation the obtained supernatant was used for further studies. This extract was used as broth for the production of enzymes by micro organisms such as *B. megaterium, P. aeruginosa, P. vulgaris, B. amyloliquefaciens, and Micrococcus* Sp. **(**Nidadavolu et al., 2010**)**

Total Soluble Sugars Estimation

All samples were standardized to a standard volume with 80% ethanol the sample was centrifuged at 2000 rpm for 5 minutes. To 0.5 ml of supernatant, 0.5 ml of ethanol was added. One ml of phenol solution was added and 5ml of concentrated H_2SO_4 was added through the side wall of tubes and mixed well. Optical density was read at 490nm (Buysse and Merckx, 1993).

Reducing Sugars

Standard glucose was prepared with concentration of 20µg-100µg and a standard graph was prepared. Various test tubes containing (0.2 ml-1ml) working standard glucose was taken and made up to 3 ml with distilled water. Two ml of DNS reagent was added and heated in boiling water bath at 80°c for 15 minutes and cooled. OD value was observed at 580nm. Pipetted out 1ml of culture and 1ml of extract in test tubes and equalized the volume to 3ml the test material. To the test tube, 3ml of DNS reagent was added and heated in boiling water bath at 80°c for 5mins. When the contents of the tubes are still warm, then added 1ml of 40% Rochelle salt solution and cooled. OD value was observed at 580nm. **(**Miller et al., 1959)

Lipid Estimation

The Tributrin agar medium was prepared. Hundred micro litre of test sample was pipetted out and inoculated into the surface of sterile agar plate. The L-rod was dipped in alcohol and sterilized. This sterile L -rod was placed on the surface of agar. The sample was spread on the surface of agar medium and incubated at 37°c for 24 -48hrs. Lipid hydrolysis was evidenced by appearance of oil droplets and halo (clearance) around colony.

Protein Estimation

Various concentrations (10µl-100µl) of the bovine serum albumin stock solution (2mg/ml) were prepared out and made up to the volume 0.5 ml with distilled water. Then 4.5 ml of the coomassive brilliant blue reagent was added and mix gently. Optical density was observed after 5 mins.

Enzyme assays

Laccase, Lignin peroxidase and Manganese peroxidase

Laccase activity was measured by taking the optical density of the reaction mixture prepared by mixing 0.5 ml of distilled water, 1 ml of sodium acetate buffer (pH 4.5), and 0.5 ml of substrate solution (46 mM guaiacol) to 0.5 mL of crude enzyme extract at 440 nm for up to 90 s with time intervals of 30 s (Coll et al., 1993)

Lignin peroxidise activity was evaluated by following the same procedure as for laccase, but 0.5 ml of hydrogen peroxide was also added to the mixture.

Manganese peroxidise activity was assayed using 0.5 ml of sodium tartrate buffer (pH 5), 0.5 ml of 100 µM guaiacol, 1 ml of distilled water, 0.1 ml of culture filtrate, and 0.5 ml of hydrogen peroxide (30% w/v) containing a reaction mixture and by reading its optical density at 465 nm. For these 3 enzymes, 1 activity unit was defined as the amount of enzyme necessary to oxidize 1 µ mol substrate/min.

Cellulase assay

Cellulase activity was assayed by determination of the reducing sugars released from carboxymethyl cellulase (CMC). A volume of 0.5 ml of the culture supernatant fluid was incubated with 1 ml of 2% CMC in 0.05 M sodium acetate buffer (pH 4.8) at 50 °C for 10 min. The reduced sugar product was assayed using the dinitrosalicylic acid method using glucose as the sugar standard. The activity was expressed in µg/ml of extract (Mandels et al., 1976).

Protease assay

Various concentrations (20-100µl) of standard solution were taken in five tubes. Then 0.3 ml of 0.5% azocasein and 100µl of 10mM $CaCl_2$ was added.0.4 ml of buffer. Then incubated at 37 °C for 1 hr and allowed to cool. Then 1ml of TCA was added. 1.8 N of NaOH was added. The optical density was read at 425 nm. 100µl of test sample was taken and 100 µl of 10mM CaCl2 and 0.4 ml of buffer were added. Incubated at 37°C for 1 hr. One ml of TCA and 1ml of NaOH were added. Optical density was read at 425 nm.

Alpha Amylase assay

Various concentrations (100mg/ml) of maltose were taken as standard. Then it will make up to 10 ml of distilled water. In these test tubes 0.5 ml of standard solution was taken. 5 ml of starch solution was added and incubated for water bath for 90°c at 10 mins. Then 5 ml of HCl solution and 1 ml of iodine solution were added to each tube. Optical density was read at 640nm. To 5ml of extract solution and 0.5 ml of sample was added and incubated the reaction mixture for 90° C at 10mins. Then 5ml of 0.1 N HCl and 1 ml of iodine solution was added. Optical density was read at 640 nm.

Beta Amylase Assay

Test sample of 500 µl was taken and 200µl of soluble starch was added. Then 200 µl of enzyme extract was added and then volume is made up to 300 µl using phosphate buffer. Then it is incubated for 15 mins to 30 mins. Then 300 µl of DNS solution was added and mixed well. Then, incubated in boiling water bath for 15 mins. Optical density was read at 575nm (Miller et al., 1959)

Pectinase assay

Two hundred micro litre of 1% pectin solution was taken and 2 ml of sodium citrate buffer was added. Then 1 ml of reaction mixture was added and incubated at 35°C ±1°C for 25mins.After incubation 0.5 ml of sodium carbonate solution was added. Then 3ml of DNS reagent was added and incubated in boiling water bath for 10-15 mins. After incubation 20ml of distilled water was added and Optical density was read at 570nm.

Xylanse assay

Five hundred microlitre of sample and 5ml of extract solution was added & mixed gently and incubated in water bath at 50°c for 5 mins. After incubation 3ml of DNS was added to each tube and incubated in water bath at 80°c for 10 mins. OD was read at 550 nm. Various concentration (0.2 to 1ml) of working standard sample was taken and incubated in water bath at 50°c for 5 mins. Then 3 ml of DNS solution was added to each tube. Again test tubes were incubated for 10 mins at 80°C. Optical density was added at 550 nm.

Tabe 1 Enzymatic activities of mushroom bed extract

Parameters	*Bacillus megaterium*	*Pseudomonasa eruginosa*	*Proteus vulgaris*	*Bacillus amyloliquefaciens*	*Micrococcus sp*
Laccase (U/ml)	0.7±0.035	0.24±0.012	0.49±0.0245	0.45±0.0225	0.89±0.0445
Lignin Peroxidase (U/ml)	0.116±0.0058	1.75±0.0875	0.068±0.0034	0.553±0.027	0.3±0.015
Manganese Peroxidase (U/ml)	1.29±0.0645	0.95±0.0475	0.395±0.0197	0.874±0.043	0.988±0.049
Cellulase (U/ml)	0.46±0.023	0.45±0.0225	0.4±0.02	0.42±0.021	0.45±0.0225
Protease (U/ml)	0.19±0.0095	0.2±0.01	0.19±0.0095	0.22±0.011	0.16±0.008
Alpha amylase (U/ml)	0.29±0.0145	0.29±0.0145	0.25±0.0125	0.59±0.295	0.27±0.0135
Beta amylase (U/ml)	0.86±0.043	0.962±0.048	0.876±0.0438	1.014±0.050	0.955±0.047
Pectinase (U/ml)	0.309±0.0154	0.327±0.0163	0.098±0.0049	0.249±0.012	0.239±0.011
Xylanase (U/ml)	0.11±0.0055	0.12±0.006	0.15±0.0085	0.17±0.0075	0.16±0.008
Catalase (U/ml)	0.17±0.0085	0.46±0.023	0.5±0.025	0.76±0.038	0.23±0.0115

Catalase assay

One milli litre of 3% H_2O_2 was taken in test tube. One drop of culture was added and bubble formation was observed (Aebi, 1984).

Table 2 Bioactive compounds of mushroom bed extract.

Parameters	*Bacillus megaterium*	*Pseudomonas aeruginosa*	*Proteus vulgaris*	*Bacillus amyloliquefacien*	*Micrococcussp*
Total sugars (U/ml)	10.5±0.525	10.8±0.6	10.2±0.51	11±0.9	10.2±0.7
Reducing sugar(U/ml)	3.48±0.175	3.44±0.15	3.32±0.14	3.52±0.18	3.48±0.17
Lipid hydrolysis (U/ml)	Appeared	Not appeared	Appeared	Appeared	Not appeared
Protein (U/ml)	0.09±0.0045	0.1±0.005	0.1±0.005	0.08±0.004	0.1±0.005

RESULTS

Oyster mushroom bed was taken as a substrate sources and micro organisms such as *Bacillus megaterium, Pseudomonas aeruginosa, Proteus vulgaris, Bacillus amyloliquefaciens and Micrococcus* sp were used in production of industrially important organisms and bioactive compounds. Industrially important enzymes such as laccase, lignin peroxidase, manganese peroxidase, cellulase, protease, alpha amylase, beta amylase, pectinase, xylanase and catalase were produced by these micro organisms. Micrococcus sp showed higher laccase activity. Using mushroom bed waste as substrate, Pseudomonas aeruginosa showed lignin peroxidase activity of 1.75 U/ml. Manganese peroxidase was produced by *Bacillus.megabacterium* of 1.29 U/ml. Cellulase and protease was similarily produced in same range of 0.4(U/ml) and 0.1U/ml respectively. Bacillus amyloliquefaciens showed higher activity in both alpha and beta amylase. Pectinase was produced by Pseudomonas aeruginosa in higher amount. Using mushroom bed as good source Bacillus amyloliquefaciens showed higher activity in xylanase and catalase production. Bioactive compounds such total sugar and reducing sugar was present in Bacillus amyloliquefaciens. From this it is concluded that, the mushroom bed is a good source of production various industrially important enzymes and bioactive compounds.

DISCUSSION

Similar to our present observations, the wastes produced by mushroom forms for extraction of bioactive compound have previously been studied (Nidadavolu et al 2010). Furthermore, mushroom bed extract have been analyzed for possible utilization as valuable bioactive compounds. The separation of sugars, lipids, and diversified enzymes from mushroom bed extracts is a novel concept for the creation of WOW. Reutilization of spent mushroom substrate (SMS) and the separation of bioactive compounds with soil properties like bulk density, stability, surface crust, temperature changes, infiltration rate, aeration, and water retention capacity have been utilized. It had noticed that in our study that temperature and water content played an important role in the extraction of compounds and enzymes and the addition of straw to the soil caused an increase in the number of total bacteria, actinomycetes, and fungi of the rhizosphere (Shukry et al., 1999). The yield of green gram increased in plots previously supplied with mushroom spent rice straw. Earlier research reported that mushroom compost waste can be used as an alternative fertilizer to farm yard manure in strawberry growing (Ozguven, 1998) .Various reports have shown that mushroom compost waste can be a good culture substrate when it is mixed with soil as a farm yard fertilizer or used alone in order to replenish the physical condition of the soil .The results obtained in the present study clearly indicate that improvement in the physical and chemical status of the soil is closely associated with the compounds released by spent mushrooms and the subsequent discharge of a variety of enzymes during the decomposition process and the SMS is an added advantage for the improvement of soil fertility. Recycling of waste from the cultivation of Pleurotus sajor-caju gave a significant yield of Pleurotus sajor-caju on starch-, peptone-, and wheat bran-supplemented SMS. Recycling of waste mushroom substrate for mushroom cultivation has been carried out using the sawdust from waste shiitake bed log for the cultivation of Pleurotus cornucopiae (Nakaya, 1997). Pretreatment of waste mushroom beds and methods for converting the same to yield sugars and ethanol has been patented. In accordance with prior work , the determination of laccase enzymes from mushroom bed extracts is an innovative technology for the separation of sugars from lignocellulosic materials (which are not suitable for food or fodder) for the large scale production of bioethanol to improve petrol. A change in the physicochemical properties of recycled spent mushroom compost through vermicomposting by epigeic earthworms Eisenia foetida and E. andrei was reported. Thermal treatment technologies were compared to determine an appropriate method of recovering energy from 2 wastes, spent mushroom compost and coal tailings (Finney et al., 2009). As the ingredients for growth media for microorganisms are enormously increased by SMS, different bioactive compounds can effectively substitute for costly commercial media. Our present efforts were successful in the cultivation of 4 white rot fungi and establishment of production efficiencies of lignolytic enzymes during 7 and 14 days of incubation time. It has been concluded that certain bioactive compounds and industrial enzymes can be extracted from these waste beds. These waste beds can be used for the production of bioethanol, bioenergy, and biogas. Since these waste beds are ecofriendly, very cheap, easily extractable, and proven to be good media, these extracts can be used directly as media for the growth of microorganisms in comparison to cost-effective synthetic media. By our study, it is concluded that micro organisms such as Bacillus megaterium, Pseudomonas aeruginosa, Proteus vulgaris, Bacillus amyloliquefaciens, and Micrococcus. sp used spent oyster mushroom bed as good source for production of industrially important enzymes such as laccase, lignin peroxidase, manganese peroxidase, cellulase, protease, amylase, pectinase, xylanase and catalase. Bioactive compounds such as total sugars, lipids and protein were also produced in higher amount.

SUMMARY

The substrate from different mushrooms varies in its physical, chemical and biological properties and each one has its own specific utility. The spent substrate from oyster mushroom bed has been found to be nutritionally rich in N: P: K contents and having high cation exchange capacity, it has the ability to replace Farm Yard Manure for the purpose of raising horticultural and cereal crops The strains such as B. megaterium, P. aeruginosa, P. vulgaris, B. amyloliquefaciens, and Micrococcus sp were bought from MTCC and cultured. Mushroom beds were used as substrate for production of various industrially important enzymes and bioactive compounds. Total sugar and reducing sugars were higher in B. amyloliquefaciens. Lipid hydrolysis content was appeared in B. megaterium, P. vulgaris, and B. amyloliquefaciens.The high amount of protein content was present in P. aeruginosa followed by P. vulgaris and Micrococcus sp. Using mushroom bed as substrate, micro organisms such as B. megaterium, Pseudomonas aeruginosa, Proteus vulgaris, Bacillus amyloliquefaciens, and Micrococcus sp has ability to produce industrially important enzymes. Micrococcus sp showed higher laccase activity, where lignin peroxidase was higher in Pseudomonas sp. B. megaterium produced manganese peroxidase with activity of 1.29(U/ml). Cellulase and protease were closely similar in all five organisms .Amylase, catalase and xylanase activities were higher in Bacillus amyloliquefaciens. The results indicate that these waste mushroom bed extracts can be used as cost-effective media for the growth of microorganisms and for the production of bioactive compounds, industrial enzyme and ethanol.

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