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Assessment of Parameters Influencing Rice Straw Associated Mycelial Growth of *Pleurotus ostreatus* MTCC 142 and a Wild Isolate of *Pleurotus ostreatus*

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Abstract

Pleurotus ostreatus, commonly known as the oyster mushroom, is a medicinally and biotechnologically important edible mushroom. The present study focuses on assessment of various parameters which may influence the optimum mycelial growth of Pleurotus ostreatus MTCC 142 and a wild isolate of Pleurotus ostreatus. Solid-state cultivation of the mushroom was carried out using rice straw as the substrate. Indirect methods of measurement such as cellulolytic activity and total protein content were used to determine the level of rice straw associated mycelial growth. In case of Pleurotus ostreatus MTCC 142 the highest protein yield was recorded in rice straw supplemented with 1% (w/v) potato starch and 0.5% (w/v) peptone, with an initial moisture ratio of 1:10, whereas, the highest cellulase production was associated with rice straw supplemented with 1% (w/v) lactose and 0.5% (w/v) tryptone, with an initial moisture ratio of 1:5, when inoculated with one mycelium plug. For the wild isolate of Pleurotus ostreatus the highest protein content and cellulase activity were recorded in the substrate supplemented with 1% (w/v) lactose and 0.5% (w/v) beef extract, with an initial moisture ratio of 1:10, when inoculated with two mycelial plugs.

Keywords: *Pleurotus ostreatus*, mycelia, rice straw, substrate.

Introduction

Solid-state fermentation (SSF) may be defined as any fermentation process carried out on moist solid substrate which provides physical support and nutritional requirement in the absence of free flowing liquid[1]. SSF can be employed in the production of various biomolecules like enzymes, organic acids, aromatic compounds, antibiotics and enriched foodstuff[2,3].

The white-rot basidiomycete fungus *Pleurotus ostreatus*, popularly known as the oyster mushroom, is a commercially important edible mushroom, which possesses high nutritive value and medicinal properties. It has been reported to have several applications like production of lignocellulolytic enzymes and animal feed supplements from agronomic wastes[4-6]. The oyster mushrooms can be easily cultivated under semi-controlled conditions in a small space by utilizing various agro-industrial residues as substrate[7].

During the course of a fermentation process, determination of microbial growth is an essential parameter to understand the metabolism and overall yield of a desired metabolite[8]. In case of SSF, direct measurement of the mycelial growth is difficult due to ramification of fungal hyphae into solid substrate particles. Therefore, different indirect methods of measurement were developed. Some indirect methods for determination of fungal biomass in SSF include measurement of cell components like chitin, ergosterol, glucosamine, nucleic acids and protein, scanning electron microscopic observation, metabolic activity like oxygen uptake and release of carbon dioxide and synthesis of extracellular enzymes[9].

The type of fungal strain and cultural conditions can significantly affect the content of different cell components[10], which in turn may influence the fungal growth and metabolite production in a fermentation process. Therefore, this study was undertaken with the objective to assess various parameters that may influence optimum mycelial growth of *P. ostreatus* during solid-state cultivation on rice straw.

Material and Methods

Chemicals and reagents: Microbiological media used in this study were obtained from Himedia Laboratories Pvt. Limited (Mumbai, India). The analytical grade chemicals and reagents were purchased from Loba Chemie (Mumbai, India), Qualigens Fine Chemicals (Mumbai, India) and s. d. Fine-Chem Ltd. (Mumbai, India).

Source of fungal strains: A standard strain of *P. ostreatus* MTCC 142 was obtained from Microbial Type Culture Collection, Chandigarh, India. A wild isolate of basidiomycete fungus, morphologically identified as *P. ostreatus*, was obtained from a decomposing tree trunk on Kuruva Island (11°49'18"N,

76°5'32"E) in Wayanad District of Kerala, India. The fungal strains were maintained on glucose yeast extract (glucose, 10 g; yeast extract, 5 g; per liter of distilled water; pH 5.8) agar plates at 4°C until use.

Source of rice straw: Rice (paddy) straw was procured from local market in Bangalore city. The straw was washed several times under running tap water, dried in hot air oven at 50°C and cut into 1 cm pieces. This was utilized as the substrate for fungal growth.

Production of fungal mycelia: Solid-state cultivation of *P. ostreatus* was carried out using the rice straw. One gram of dry straw was filled in a glass culture tube measuring 2.5 cm in diameter and 15 cm long, moistened with 5 ml mineral salt solution containing (g/l) KH_2PO_4 0.2; $MgSO_4.7H_2O$ 0.1; NH_4Cl 0.3; $CaCO_3$ 1.0 and distilled water, at initial pH 5.8 and autoclaved[11]. The sterile tubes were cooled and each tube was aseptically inoculated with one mycelial plug (6 mm) from seven days old culture plate of *P. ostreatus*. The inoculated tubes were subjected to static incubation in dark at 25±1ºC for 10 days[12,13].

Determination of mycelial growth: Following incubation the rice straw colonized with fungal mycelia was homogenized with 0.2 M phosphate buffer (pH 6) and filtered through coarse filter paper[14]. The filtrate was centrifuged at 5000 rpm for 20 min at 4ºC. For determination of straw associated mycelial growth indirect methods of measurement such as cellulolytic activity and total protein content were used. Cellulase assay of the supernatant (crude enzyme) was performed by dinitrosalicylic acid (DNS) method using 1% (w/v) buffered carboxy methyl cellulose (CMC) as substrate and glucose as standard[15,16]. One unit of cellulase activity was defined as the amount of enzyme required to release 1 µmol of reducing sugar per minute per gram of dry substrate under standard assay conditions and expressed as U/gds. The total protein yield in the supernatant was quantified by the method specified by Lowry *et al.*[17], using bovine serum albumin as standard and expressed as mg/gds.

Effect of nutritional parameters: The effect of carbon supplements on fungal growth was investigated by incorporating glucose, fructose, sucrose, maltose, lactose, mannitol, starch and malt extract into mineral salt solution at 1% (w/v) concentration. The effect of different organic (yeast extract, beef extract, peptone, tryptone, soyabean meal, casein, urea) and inorganic (ammonium sulphate, ammonium nitrate, sodium nitrite) nitrogen sources were determined at 0.5% (w/v) concentration. Inoculated tube without any carbon or nitrogen supplementation was used as control.

Effect of cultural parameters: The effect of initial moisture ratio was determined by adding mineral salt solution to 1 g of rice straw at different ratio: 1:1, 1:2, 1:3, 1:5, 1:7, 1:10 and 1:15 (w:v). The effect of inoculum size was evaluated by inoculating the tubes with different numbers of mycelial plugs (1, 2, 3, 4 and 5). A sterile tube without inoculum was used as control.

Statistical analysis: Effect of each parameter was studied in triplicate and the data have been graphically presented as mean ± standard deviation of triplicates (n = 3). All the graphs have been prepared using Microsoft Excel 2007.

Results and Discussion

P. ostreatus is an economically important edible mushroom that is widely cultivated throughout Asia and Europe[18]. They are consumed mainly because of their delicious taste and high nutritive value as they are rich in protein, carbohydrate, minerals, vitamins and low fat contents[19]. *P. ostreatus* also possesses medicinal value as it exhibits hematological, antiviral, antitumor, antibiotic, antibacterial and hypo-cholesterolic properties[4,20]. *P. ostreatus* has many industrial applications as potential degrader of various xenobiotics and industrial pollutants[6].

Substrate for fungal cultivation: *P. ostreatus* can be successfully cultivated on cheap, lignocellulosic wastes such as soyabean, rice and wheat straw, sugarcane bagasse, corn cobs, sawdust, waste paper, cotton stalks and whole grains[21]. In India, rice straw is one of the major crop residues used as cattle feed and mushroom cultivation owing to its easy availability and lower cost. Therefore, in the present study, a standard strain and a wild isolate of *P. ostreatus* were cultivated on rice straw in order to study the cultural parameters affecting the mycelial growth. Chopped rice straw supplemented with mineral salt solution supported good growth of *P. ostreatus* within 10 days of incubation as observed from the mycelial run colonizing the straw. This might be due to the presence of high moisture content within the straw fibres along with a decent level of utilizable lignin and cellulose. *P. ostreatus* synthesizes lignocellulolytic enzymes like laccases and cellulases. This result is in accordance with that of an earlier study which reported good biomass production capacity of chopped paddy straw inoculated with five different species of *Pleurotus*[22].

Effect of nutritional parameters: In a fermentation process, the measurement of microbial biomass is often necessary since metabolic activity is strongly related to the growth rate and the actual biomass present[3]. Owing to the difficulty in estimating the mycelial growth in SSF involving fungi, indirect method of measurement was employed. Since *P. ostreatus* is a potent producer of cellulase, the cellulolytic activity was determined along with total protein yield. Sugars in general facilitate the growth and proliferation of saprophytic fungi and thus result in an increase in biomass. Among the various carbon supplements added to the substrate, lactose and potato starch supported maximum cellulase (0.39 U/gds) and protein (1.4 mg/gds) synthesis, respectively, in case of *P. ostreatus* MTCC 142. This finding is in perfect correlation with a report suggesting the usage of lactose as an inducer in commercial production of cellulase[23]. Glucose, on the other hand, demonstrated the lowest

cellulase (0.01 U/gds) and protein (0.95 mg/gds) synthesis. This could be due to the phenomenon of catabolite repression initiated by glucose, resulting in decreased synthesis of enzymes[24]. Other carbon supplements such as fructose, sucrose, maltose and malt extract showed good mycelial development which could be correlated with the cellulolytic activity and protein content. In case of the wild isolate of *P. ostreatus*, lactose resulted in highest cellulase (0.44 U/gds) and protein (1.68 mg/gds) yield, whereas potato starch showed the lowest cellulase and protein yield, presented in figure 1 and figure 2. A previous study also suggested that supplementation of the substrate with carbon sources can enhance the production of lignolytic enzymes and contribute satisfactory fungal growth and biomass accumulation[25]. Nitrogen supplements are incorporated into fermentation medium in order to facilitate better biomass synthesis and subsequently higher metabolite secretion. Nitrogenous compounds are utilized by the microbial cells for the synthesis of nucleotides, amino acids, proteins, enzymes and other metabolites[26]. Tryptone and peptone supported optimal synthesis of cellulase (0.27 U/gds) and protein (1.5 mg/gds), respectively, in case of *P. ostreatus* MTCC 142. Interestingly, beef extract resulted in maximum cellulase (2.99 U/gds) and protein yield (1.5 mg/gds) for the wild isolate of *P. ostreatus,* depicted in figure 3 and figure 4.

It may be interpreted from these results that organic nitrogen supplements facilitated better mycelial growth in both the *Pleurotus* strains. This could be attributed to the naturally occurring amino acids, peptides, vitamins and accessory growth factors in these organic supplements, which would have been effectively utilized for mycelial proliferation. Comparatively, the inorganic nitrogen supplements resulted in moderate level of fungal growth, wherein, the least was recorded with sodium nitrite. The carbon to nitrogen (C/N) ratio has a marked influence on the yield of microbial biomass[27]. In the present study a C/N ratio of 2.0 stimulated good mycelial growth, probably due to better assimilation of these supplements by the developing mycelia. It was also noticed that in most instances the mycelial productivity in the supplemented straw was comparatively higher than in the unsupplemented control straw.

Effect of cultural parameters: Moisture in the culture medium greatly affects the mycelial growth rate and metabolic activity. In case of SSF with filamentous fungi, the available moisture in the substrate provides much needed turgor pressure which enables better penetration of hyphal tip into solid substrate[28]. For this reason adequate level of moisture is required for efficient uptake of nutrients. In case of *P. ostreatus* MTCC 142, it was noted that initial moisture ratio of 1:5 and 1:10 showed the highest cellulase activity (0.29 U/gds) and protein yield (1.95 mg/gds), respectively, as illustrated in figure 5 and figure 6.

While in case of the wild isolate, initial moisture ratio of 1:10 demonstrated maximum cellulase (0.34 U/gds) and protein (2.09 mg/gds) synthesis. It can be deciphered from these findings that moderate to high initial moisture ratio is beneficial for good colonization and optimum mycelial growth. The yields of cellulase and protein progressively increased with an increase in initial moisture ratio, probably because availability of adequate moisture content initiated rapid uptake of water leading to hyphal cell elongation and elaborate mycelial run within the saturated straw fibres. The good water holding capacity of rice straw also facilitated better mycelial development. This observation may be further explained by a previous study on microbial biomass and its activity in birch litter which reported a strong correlation between moisture content and respiration. An initial increase in the respiration rate was recorded in the continuously wet samples which became relatively constant thereafter. Their study also established a strong relationship between microbial respiration potential and biomass formation[29]. A high initial moisture ratio of 1:15 demonstrated decreased enzymatic activity which could be attributed to the development of anoxic condition affecting the growth rate of *P. ostreatus*.

The initial load of microbial inoculum also influences the final yield of the metabolites. One mycelial plug of *P. ostreatus* MTCC 142 resulted in maximum cellulase (0.2 U/gds) and protein yield (1.5 mg/gds), whereas, two mycelial plugs of the wild isolate of *P. ostreatus* elucidated maximum cellulase (0.22 U/gds) and protein (1.5 mg/gds) synthesis. When 3 to 5 mycelial plugs were used cellulase and protein synthesis showed an inconsistent decrease, indicated in figure 7 and figure 8. This decrease beyond a certain level could be due to depletion of nutrients and/or reduction in oxygen potential resulting from a high inoculum size.

The solid-state cultivation of *P. ostreatus* on agro-industrial byproducts is not only economical but also facilitates good mycelial growth. Apart from producing a nutritious food, the practice of mushroom cultivation has been found to improve the straw quality and the mycelia associated straw may act as a good source of mycoprotein for cattle feed[30]. Due to large content of nitrogen, phosphorus and potassium the spent straw may be used as manure[31].

Conclusion

It can be concluded that through supplementation of rice straw with suitable carbon and organic nitrogen sources, along with regulation of initial moisture content and inoculum size, mycelial growth of *P. ostreatus* may be improved.

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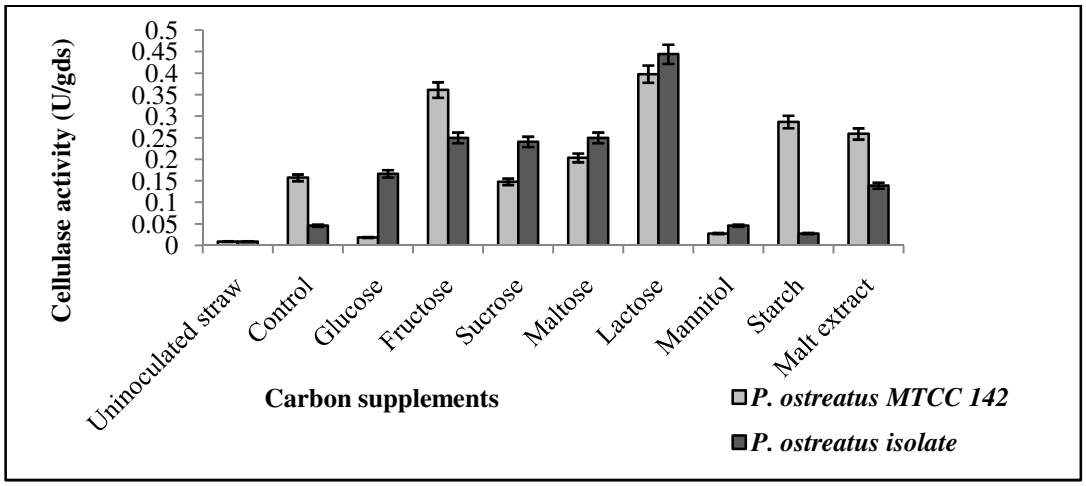

Figure-1
Effect of carbon supplements on cellulase activity of *P. ostreatus*

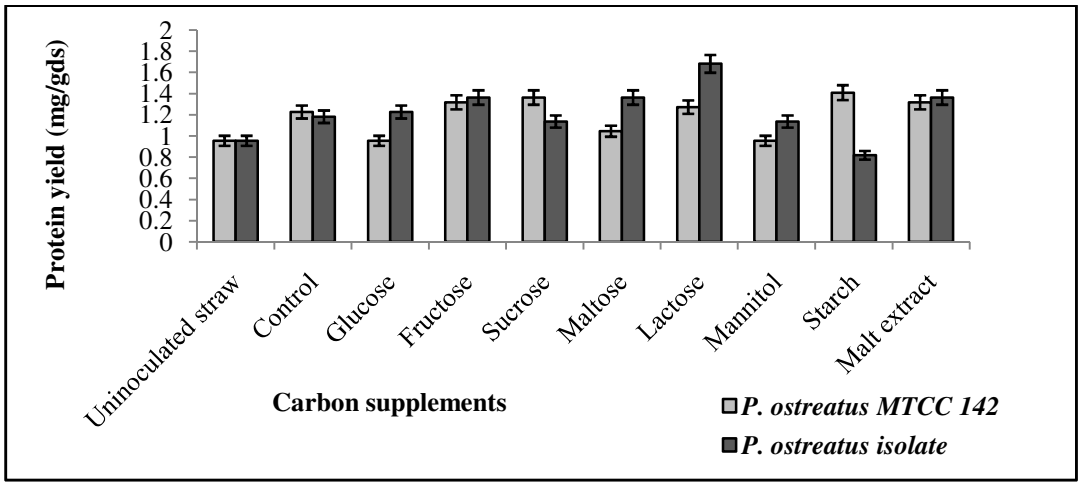

Figure-2
Effect of carbon supplements on protein yield of *P. ostreatus*

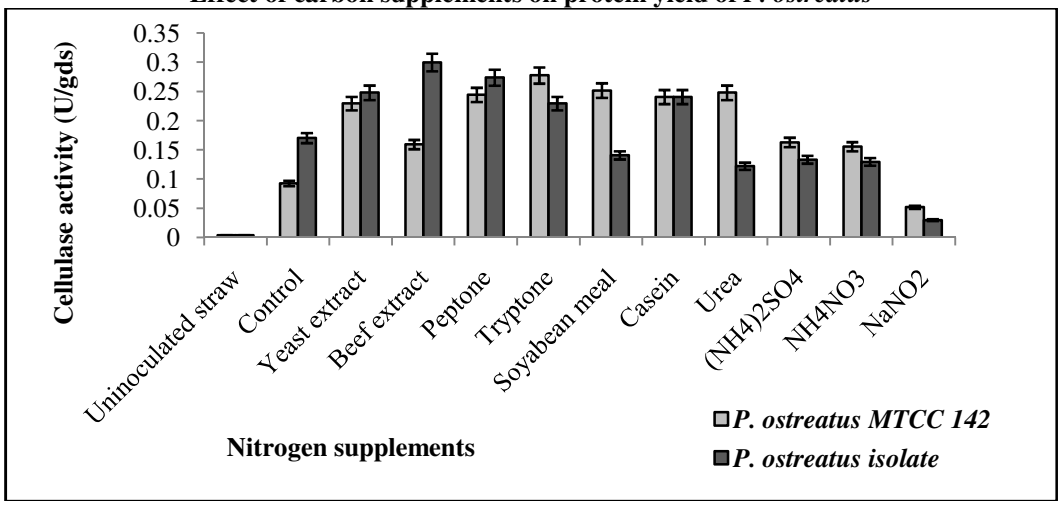

Figure-3
Effect of nitrogen supplements on cellulase activity of *P. ostreatus*

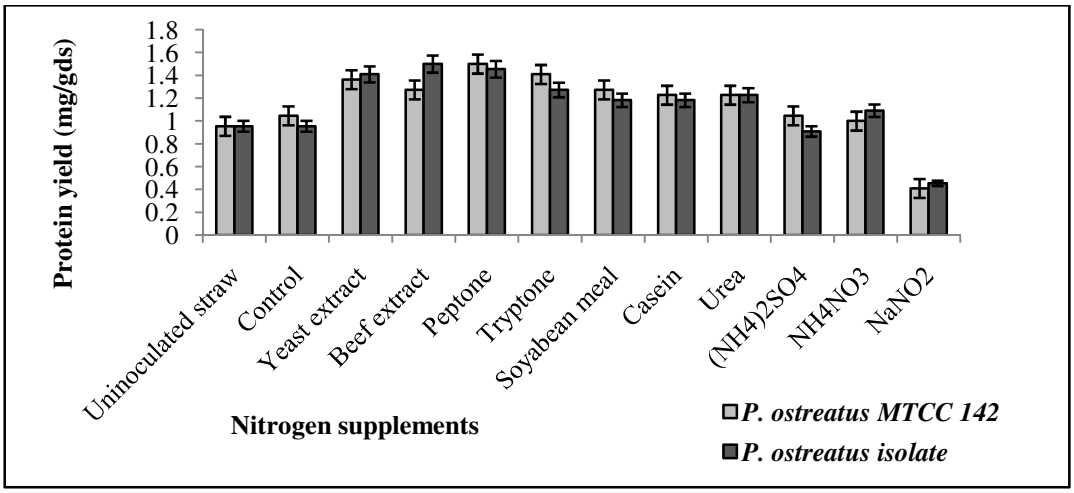

Figure-4
Effect of nitrogen supplements on protein yield of *P. ostreatus*

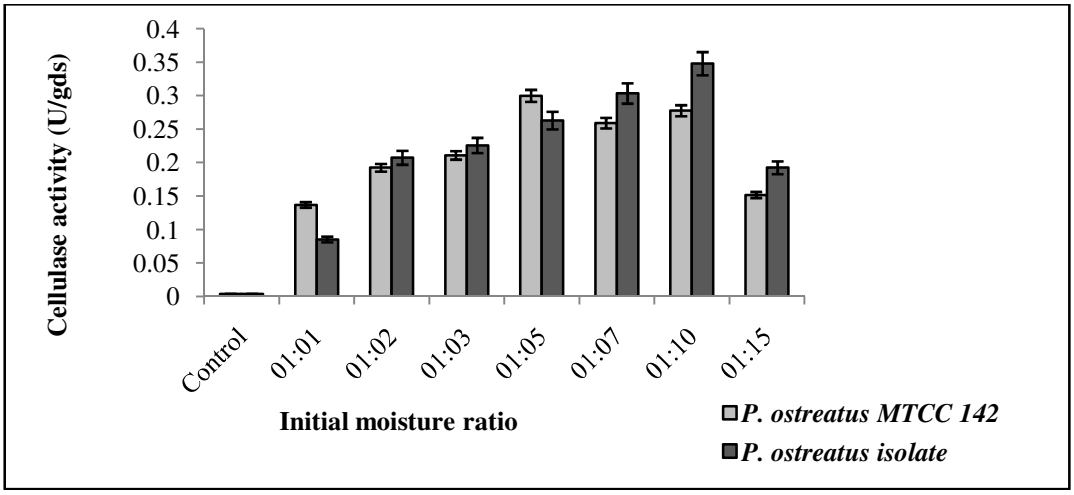

Figure-5
Effect of initial moisture ratio on cellulase activity of *P. ostreatus*

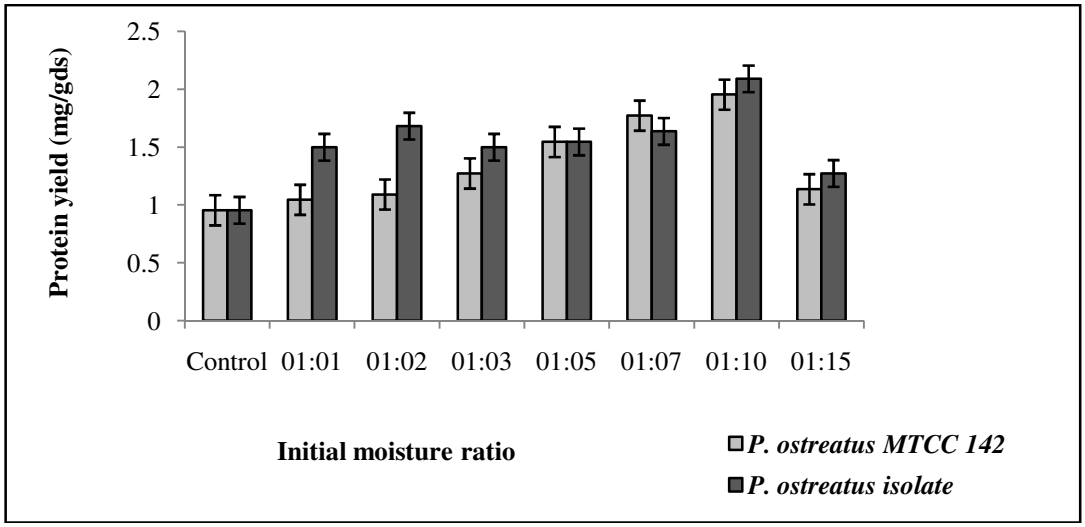

Figure-6
Effect of initial moisture ratio on protein yield of *P. ostreatus*

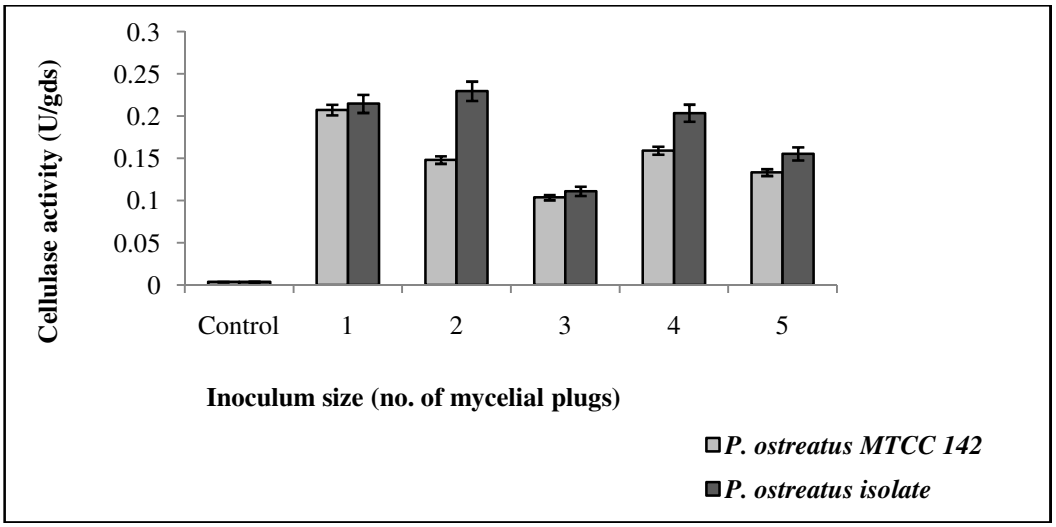

Figure-7
Effect of inoculum size on cellulase activity of *P. ostreatus*

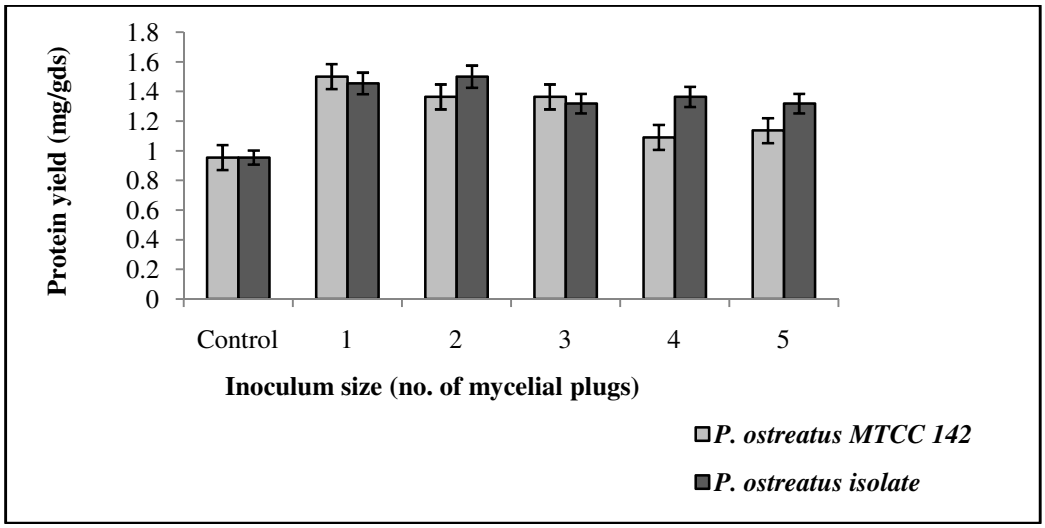

Figure-8
Effect of inoculum size on protein yield of *P. ostreatus*

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