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Improved Yield of Ligno-Cellulolytic Enzymes on Oyster Shell Powder Added Typha Weed Substrate by *Pleurotus florida*

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Abstract

Solid state fermentation (SSF) of different combinations of Typha weed (*Typha latifolia*) substrate (TPS) and oyster shell powder (OSP) including TPS+OSP (100+0), TPS+OSP (99+1), TPS+OSP (97+3), TPS+OSP (95+5), TPS+OSP (92+8) and TPS+OSP (90+10) was conducted employing white-rot fungus *Pleurotus florida*. The influence of variable combinations was analyzed on different parameters viz., production of fungal ergosterol, enzymes (CMCase, xylanase, glucosidase, xylosidase, Mn-peroxidase and laccase) and specific protein. The highest ergosterol (371µg/g) was recorded in the TPS+OSP (95+5) combination. The highest CMCase activity (307 IU/g) and peroxidase activity (79 IU/g) were recorded in TPS+OSP (95+5) combination, whereas the highest xylanase (258 IU/g), β-glucosidase activity (116IU/g) and β-xylosidase (42 IU/g) activities were achieved in the sets of TPS+OSP (97+3). Moreover, highest laccase (801 IU/g) was recorded in TPS+OSP (92+8) set. The protein content analyzed showed highest protein (147 IU/g) in TPS+OSP (95+5) set. The lower concentration of OSP remarkably supported the production of all enzymes including ergosterol and concentration of protein, while lower levels were not supportive. In consequence, the use of OSP at their lower concentrations is recommended for the improvement of solid state fermentation and enzyme production.

Keywords: Oyster shell powder; *Typha latifolia; Pleurotus* spp; Laccase; Mn-peroxidase

Introduction

Enhanced demand of unique lignocellulolytic enzymes before multipurpose applications realized their potential and enforced for their improved commercial production using cheaper resources. White-rot basidiomycetes are strongly capable to synthesize the relevant hydrolytic (cellulases and hemicellulases) and the unique oxidative network of ligninolytic extracellular enzymes [1]. *Pleurotus* is a versatile genus belonging to white-rot basidiomycete fungi and well known for their complexity of the enzymatic system and prominent lignocellulolytic property, member of this genus can colonize a wide range of natural lignocellulosic wastes [2]. *Pleurotus* spp. bears unique ability to produce extra cellular lignocellulolytic enzymes including laccase and Mn-peroxidase [3], xylanase [4], CMCase, β-glucosidase and β-xylosidase. The cluster of these enzymes have a vibrant potential of applications in various industries including chemicals, fuel, food pulp and paper, textile and laundry, animal feed, and agriculture [5]. Moreover, these have shown enormous biotechnological potential as they can be used in a wide variety of lignocellulose degradation [6] and detoxification of agro-industrial residuals with high phenolic contents [7].

Production of enzymes is a process totally dependent on the culture nutrients and the nature of basal substrate used. Lignocellulosic substrates are generally used as low-grade domestic fuel and burnt negligently or thrown away, which create post disposal problems in our surroundings [2,8]. Disposal of plant based lignocellulosic materials through burning causes environmental pollution as they release high level of CO_2 as well as it is the cause of unnecessary wasting of large amount of organic materials [9]. Therefore, the development of a biotechnological process to convert agro industrial wastage up to a non-harmful level will have environmental importance [10,11]. *Typha latifolia* an excellent hyper-accumulator macrophyte grows worldwide in highly contaminated aquatic sites [12] and provides huge size of lignocellulosic biomass which remains unused. This large biomass of lignocelluloses may be utilized as the best source of fermentation processes for the biotechnological conversion into enzymes and other relevant products.

The deficiency of several substrates may be strengthened and improved by the deliberative supplementation using external compounds [13]. The process can be stimulated by adding a variety of inducing substrates [14] and metals. The oyster shells are the biological wastes rich in different nutritional constituents; thus may be used as additives with basal substrates. Application of oyster-shell powder increases organic matter, available phosphorus, and exchangeable cations concentrations. This is an elevated quality conditioner consists of calcium carbonate and many micronutrients [15]. It provides a long-lasting, steady release of nutrients to help regulate pH levels improves nutrients uptake and enhance substrate tilt [16].

The present study was conducted concerning bioconversion of Typha (*Typha latifolia*) plant substrate into valuable enzymes with reference to the supplementation of oyster shell powder. Under this study influence of the powdered oyster shells was evaluated on the enzyme profile, soluble protein and production of ergosterol of *Pleurotus florida* during solid substrate fermentation.

Materials and Methods

Microorganism and its maintenance

The white-rot fungus *Pleurotus florida* was employed in the present

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study. The culture was obtained from the, Mushroom Training and Research Centre (MTRC), Veer Bahadur Singh Purvanchal University, Jaunpur, UP, India. It was maintained on Potato Dextrose Agar (peeled, sliced and boiled potato, 200 g; dextrose, 20 g; agar, 20 g l^{-1}) and Yeast Extract Agar medium (maltose, 10 g; peptone, 8 g; yeast extract, 2.5 g; agar, 20 g l^{-1}) [17]. The organism was maintained by sub-culturing fortnightly on the above said media at 22 ± 1 ºC in culture tubes.

Inoculum preparation

Inoculum was prepared by culturing fungus on solid culture medium in Petri plates. Fungal disc were picked from the edge of young and growing fungal colony using sterile cork borer measuring 7 mm diameter. Three fungal discs were transferred in each 250 ml Erlenmeyer conical flask containing different combination of substrate. These conical flasks were incubated at 22±1 ºC and regularly shaken to avoid clump formation.

Preparation of substrate

Fully developed *Typha latifolia* plants were collected from the nearby waterlogged areas of the Veer Bahadur Singh Purvanchal University campus. These were cut into small pieces (<5 mm) and sundried followed by oven drying (60ºC; 45 min) to complete removal of moisture. Completely dried small pieces of Typha plant were employed as basal substrate for the solid substrate fermentation in 250 mL Erlenmeyer conical flasks.

Preparation of oyster shell powder

Oyster shells were collected from the Gomati river bank (Barabanki, Uttar Pradesh, India) during summer season in the months of May-June. Every piece of oyster shells were washed, broken into small pieces and ground (size>1 mm) to fine powder by the treatment of liquid nitrogen. Furthermore, the oyster shell powder (OSP) was sterilized chemically using 2% (v/v) formalin before supplementation.

Supplementation regime

To evaluate the influence of different ratio of TPS and OSP both constituents were accurately weighed and mixed to compose different combinations including TPS+OSP (99+1), TPS+OSP (98+2), TPS+ OSP (97+3), TPS+ OSP (95+5), TPS+ OSP (92+8) and TPS+ OSP (90+10). These combinations of different ratio were filled into 250 mL sized Erlenmeyer conical flasks organizing different experimental set and TPS+OSP (100+0) as control (un-supplemented) to precede investigations. The basal substrate (TPS) and supplement (OSP) were chemically sterilized in separate by using 2% (v/v) of formalin and 0.5% (w/v) bavistin.

Solid state fermentation

The Erlenmeyer flask of different substrate combinations were prepared and plugged with cotton plugs. The cotton-plugged sets were then autoclaved at 20 psi for 60 min. The Erlenmeyer flask were shaken to break clumping and cooled. Furthermore, sterilized and cooled sets were inoculated with fungal discs and incubated at 22±1°C in BOD incubator to conduct solid substrate fermentation under controlled conditions. During incubation, the flasks were shaken regularly, to uniformly mix fungal mycelia and fully cover the substrate.

Determination of ergosterol by HPLC

Mycelial ergosterol was determined as per standard protocol described by Martin et al., [18] using high pressure liquid chromatography (HPLC). For this 20 g of fermented substrate was taken and masticated using mortar and pestle. It was transferred into the centrifuge tube kept in ice and added with 1 ml of absolute alcohol. The preparation was stirred for 1min and centrifuged (Remi, India) for 5 min at 14,000 × g. The supernatant thus obtained was collected and filtered using nitrocellulose filter (Millipore). The filtrate obtained was analyzed using HPLC (Shimadzu LC-20AT) for ergosterol. The HPLC was equipped with Biorad Aminex HPX-87H ion exchanger column with a guard column (Micro-Guard Refill Cartridge, Bio Rad, Richmond, CA).

Preparation of crude enzyme extract

Crude enzyme extract was prepared by crushing 10 g of fermented substrate with 10 ml of 0.1M phosphate buffer (pH 6.8). Mycelia and solid substrate were removed by the filtration followed by centrifugation at 10,000 × g in a cooling centrifuge (Remi, India) for 5min. In consequence, the supernatant obtained was used as crude enzyme extract for enzyme assays.

Enzyme assay

Investigations regarding enzyme analyses were performed using standard protocols. For carboxymethyl cellulase activity [19], assay mixture was prepared by adding 0.5 ml culture filtrate and 0.5 ml of 1% (w/v) carboxymethyl cellulose (Merck) solution to 1 ml of 100 mM phosphate buffer and followed by 3.0 ml 3, 5-dinitrosalycylic acid reagent. The mixture was incubated at 22°C for 30 minutes and 1 ml of 40% (w/v) Rochelle salt (sodium-potassium tartrate) solution was added to terminate reaction and kept in boiling water bath for 15 min to develop colour. The intensity of red colour was measured spectrophotometrically at 575 nm. The enzyme activity was expressed as unit/liter 1µmol glucose released per min per liter. For xylanase activity [19], assay mixture contained 0.5 ml enzyme extract to 0.5 ml of 0.25 % (w/v) xylan (Oat spelt, sigma) solution to 1 ml of 100 mM of phosphate buffer and 3.0 ml DNS reagent and incubated at 22°C. Reaction was stopped by Rochelle salt and intensity of colour was measured at 575 nm. The enzyme activity was expressed as unit per liter as 1µ mol D-xylose released per min per liter. The β-glucosidase and β-xylosidase activities were assayed [20] by measuring the amount of p-nitrophenol liberated from p-nitrophenyl β-glucopyranoside (PNPG) (Sigma) and p-nitrophenyl β-xylopyranoside (PNPX) (Sigma) substrates respectively. The assay mixture containing 0.9 ml of 0.1% (w/v) PNPG and 0.1 ml enzyme extract was incubated at 22°C for 10 min. The reaction was terminated by adding 1 ml of 2% sodium carbonate solution, and the liberated p-nitrophenol was measured spectrophotometrically at 400 nm. The enzyme activity was expressed as IU/g (1µ mol p-nitrophenol released per min l^{-1}) of enzyme extract.

The manganese peroxidase (MnP) activity was determined spectrophotometrically using 3 mM phenol sulfonpthalein (Merck) as substrate. The reaction mixture contained: 0.2 ml buffer enzyme preparation, 2 mM H_2O_2 and phenol sulfonpthalein, it was incubated for 5 minutes at 22±1^{0}C. The reaction was terminated by using 2M NaOH. The absorbance was monitored at 610 nm [21]. The enzyme activity was expressed as IU/g. One unit of enzyme was defined as the amount required for oxidizing 1µ mol of phenol sulfonpthalein per min. The assay of laccase activity was performed at 30°C using 20 mM ABTS (2, 2 -azino-bis (3-ethylbenzathiazoline-6- sulfonate), Merck) as the substrate [22]. The assay mixture (1 ml) contained 780 µl of distilled water, 20 µl of ABTS from the stock of 20 mM, 150 µl of sodium acetate buffer (pH-5.6) and 50 µl of culture supernatant as enzyme. The absorbance of assay mixture was monitored at 420 nm. The enzyme activities were expressed as international units (IU), one

unit was defined as the amount of enzyme required to produce 1µmol product per min at 30°C and presented as IU/g.

Determination of soluble protein

Total soluble protein in solid state cultures were determined by the Bradford [23] method using bovine serum albumin as the protein standard. All experimental analyses were performed in triplicate sets (n=3).

Results and Discussion

Influence of OSP on fungal ergosterol

Mycelial ergosterol was considerably influenced because of the OSP supplementation at different levels with TPS as basic substrate. As observed highest ergosterol (371 µg/g) content was recorded in the combination of TPS+OSP (95+5) which was more than 5 folds higher to the content of ergosterol (74 µg/g) recorded in the control set. The next second highest ergosterol content (366 µg/g) was obtained in TPS+OSP (97+3) combination and the lowest amount (82 µg/g) in the set of TPS+OSP (90+10). As compared the supplementation of OSP at their higher concentrations adversely affected the ergosterol content, while lower levels remarkably supported and stimulated the ergosterol (Figure 1).

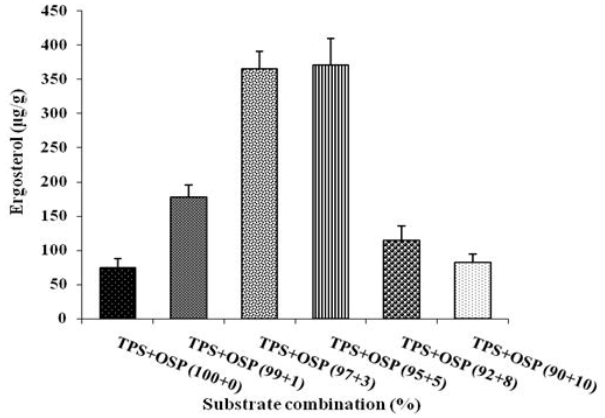

Figure 1: Influence of different OSP concentrations [0(TPS+OSP; 100+0), 1(TPS+OSP;99+1), 3(TPS+OSP; 97+3), 5(TPS+OSP; 95+5), 8(TPS+OSP; 92+8) and 10 (TPS+OSP; 90+10) %] on mycelial ergosterol of *Pleurotus florida* during solid state fermentation of Typha plant powder.

Influence of OSP on the profile of lignocellulolytic enzymes

Enzyme activities of six different lingo-cellulolytic enzymes studied were variably influenced due to the supplementation of OSP at variable concentrations. The highest CMCase activity (307 IU/g) was recorded in the set of TPS+OSP (95+5) combination and the lowest (42 IU/g) in the TPS+OSP (90+10) set. However, un-supplemented set (TPS (100) showed 158 IU/g of CMCase. As it was observed; lower concentrations of OSP led to enhance CMCase activity; whereas, higher concentrations found to influence negatively (Figure 2a).

The production of xylanase was too considerably influenced by the OSP supplementation. The highest xylanase activity recorded was 258 IU/g in the TPS+OSP (79+3) combination which was followed to 189 IU/g in TPS+OSP (99+1) set however, lowest xylanase (101 IU/g) activity in TPS+OSP (90+10) set (Figure 2b). The response of OSP at the lower levels less than 3% (w/w) found supportive and improved xylanase production in comparison to un-supplemented set.

The activity of β-glucosidase was also analyzed in response to the supplementation of OSP at different levels. The β-glucosidase activity was remarkably influenced by the additions of OSP. The highest activity (116 IU/g) was noted in TPS+OSP (97+3) set and lowest 32

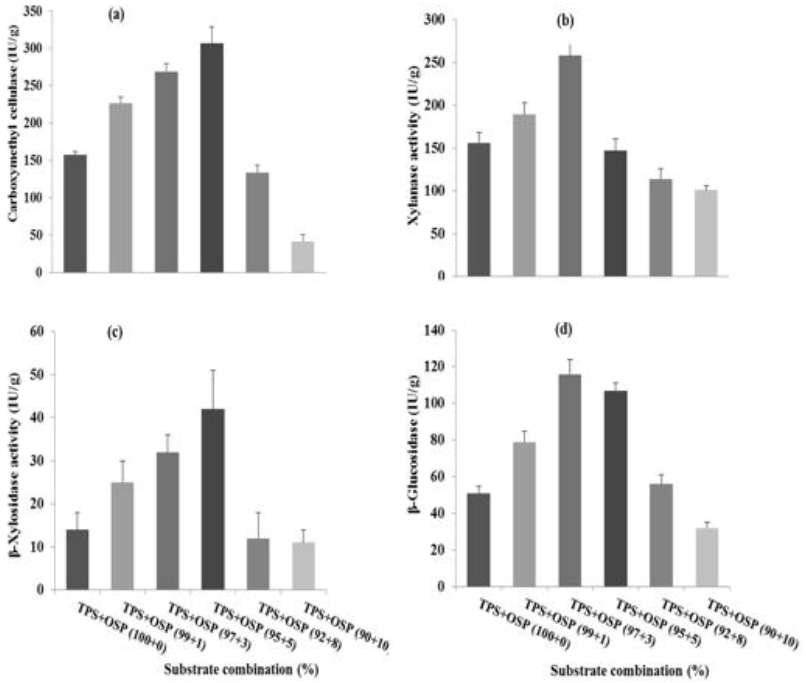

Figure 2: Influence of different OSP concentrations [0(TPS+OSP; 100+0), 1(TPS+OSP;99+1), 3(TPS+OSP; 97+3), 5(TPS+OSP; 95+5), 8(TPS+OSP; 92+8) and 10 (TPS+OSP; 90+10) %] on (a) Carboxy methyl cellulase, (b) Xylanase (c) β-xylosidase (d) β-glucosidase of *Pleurotus florida* during solid state fermentation of Typha plant powder.

IU/g in the set of TPS+OSP (90+10) which was comparatively lower than un-supplemented TPS+OSP (100+0) set of experiment (Figures 2c and 2d).

Analyses on β-xylosidase activities showed highest 42 IU/g in the TPS+OSP (97+3) combination which was three folds greater than control set as it was 14 IU/g; however, lowest activity (11 IU/g) was recorded in TPS+OSP (90+10) (Figure 2c).

Enzyme profile of peroxidase was as well remarkably influenced after the OSP supplementation. The lower concentrations lesser than 5% (w/w) supplementation positively influenced and increased the peroxidase activity, however it was concurrently reduced in the OSP levels higher to 5% (w/w). As observed the highest peroxidase activity (79 IU/g) was recorded in TPS+OSP (95+5) followed by 71 IU/g in TPS+OSP (93+3) and lowest 26 IU/g in the TPS+OSP (90+10) set. The control set of the study resulted 46 IU/g of Mn-peroxidase (Figure 3).

Increasing the concentration of OSP up to 8% (w/w) the TPS+OSP (92+8) showed peak (801 IU/g) value of laccase activity. The second highest laccase activity (714 IU/g) was represented in the set of TPS+OSP (95+5) combination; whereas, it was negatively influenced at higher levels. The un-supplemented set represented 370 IU/g laccase activity (Figure 4).

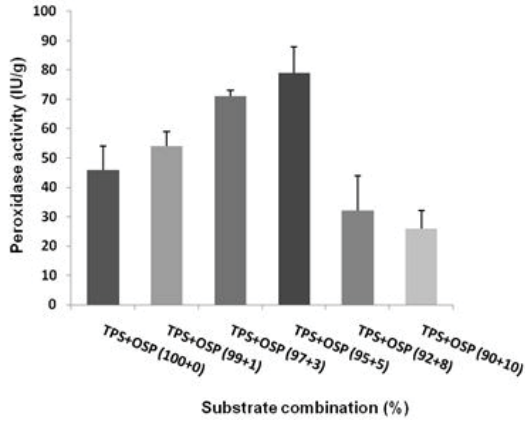

Figure 3: Influence of different OSP concentrations [0(TPS+OSP; 100+0), 1(TPS+OSP;99+1), 3(TPS+OSP; 97+3), 5(TPS+OSP; 95+5), 8(TPS+OSP; 92+8) and 10 (TPS+OSP; 90+10) %] on laccase profile of *Pleurotus florida* during solid state fermentation of Typha plant powder.

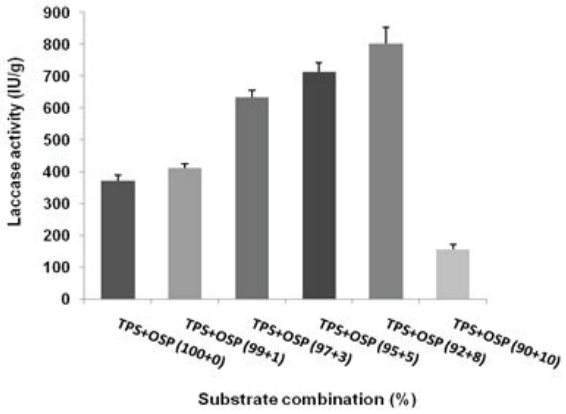

Figure 4: Influence of different OSP concentrations [0(TPS+OSP; 100+0), 1(TPS+OSP;99+1), 3(TPS+OSP; 97+3), 5(TPS+OSP; 95+5), 8(TPS+OSP; 92+8) and 10 (TPS+OSP; 90+10) %] on Mn-peroxidase profile of *Pleurotus florida* during solid state fermentation of Typha plant powder.

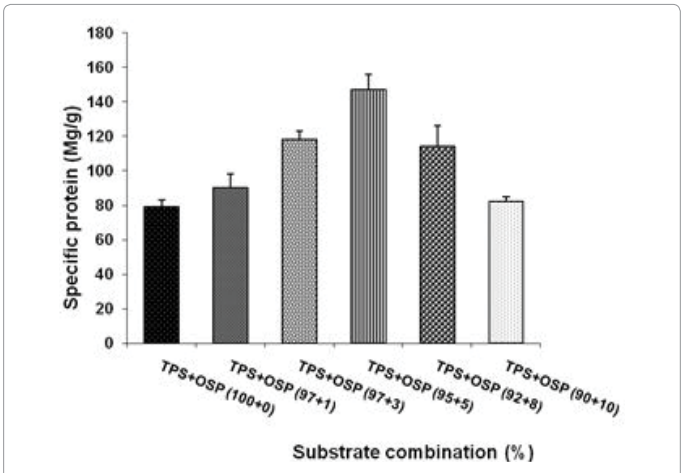

Figure 5: Influence of different OSP concentrations [0(TPS+OSP; 100+0), 1(TPS+OSP;99+1), 3(TPS+OSP; 97+3), 5(TPS+OSP; 95+5), 8(TPS+OSP; 92+8) and 10 (TPS+OSP; 90+10) %] on specific protein of *Pleurotus florida* during solid state fermentation of Typha plant powder.

Influence of OSP on soluble protein

The concentration of protein in fermented culture was remarkably influenced because of the supplementation of OSP at different combination of the added substrate. The utmost amount of protein content (147mg/g) was recorded in TPS+OSP (95+5) combination which was followed to (118 mg/g) in TPS+OSP (97+3). The lowest protein content (82 mg/g) was achieved in the TPS+OSP (90+10) combination. On the basis of the comparative observations it was apparent that lower concentrations of OSP up to a level of 5% were supportive; however higher concentrations found suppressive of protein synthesis (Figure 5).

Discussion

White-rot fungi and especially *Pleurotus* spp. are highly adaptable to grow on a wide variety of agro-industrial lignocellulosic wastes because of their capability to synthesize relevant hydrolytic (cellulases and hemicellulases) and unique oxidative (ligninolytic) extracellular enzymes [24]. Various agricultural substrates may contain significant concentrations of soluble inducers, have been successfully used in submerged and solid-state fermentation for improving ligninolytic enzyme production [24-26]. Typha weed is a versatile hyper accumulator plant most abundantly grows in water logged areas. This grows more successfully where water bodies are most dominantly contaminated by the industrial wastes and harmful pollutants like heavy metals. This plant is considered to be almost useless in the society and most often destroys and recycled on the same place. In the present study we have used Typha weed substrate as basal substrate for the purpose of its efficient utilization in biochemical processes.

In order to observe the influence of substrate combination on the ergosterol production; the fungus was studied. According to the observations, lower concentrations of the oyster shell powder resulted positive responses but to a limit; however higher concentration of OSP could not support the ergosterol synthesis. The OSP dominantly contains $CaCO_3$ as major constituent, however some other minerals *viz*., Al_2O_3, MgO, Na_2O_3, P_2O_5 and SiO_2 presents as traces [15,16]. The stimulating nature of the OSP at low levels might be due to the presence of these micronutrients, while, addition at high levels is repressive that might because of the minerals accumulation at high amounts. In other hands it is speculated that enhanced supplementation of additives

may increase the temperature of substrate that can suppress or kill the mycelia [15,27] and reduces growth rate [16]. Due to suppressed growth of death of mycelia may cause reduction in ergosterol synthesis because ergosterol is one of the major constituents of fungal mycelia. A similar trend to those of the growth rates was also recorded with ergosterol production. Generally, ergosterol production is linked to the growth of mycelia [28,29] with a direct correlation of data between ergosterol and growth rate. The higher growth rate behind low level of OSP supplementation in the set represented a higher ergosterol yield. Similar observations were also reported earlier by Han et al. [30].

In a different experimental observation influence of OSP and its variable combinations with basal substrate were elucidated on the production of ligno-cellulolytic enzymes (CMCase, xylanase, β-glucosidase, β-xylosidase, laccase and Mn peroxidase). The enzyme activities were remarkably produced and variably influenced after the OSP supplementation. Similar observations were also reflected in a previous study [16] in which lower levels led to stimulation; while higher levels suppressed the enzyme activities. The addition of OSP generally rich in calcium ions provides the porosity to the substrate [31,32] and rapid growth of mycelium with limited lower levels. In addition it is known that oyster shell protein too possess several amino acids mainly valine, alanine, glutamine, serine, proline, lucien, tyrosine and lysine etc. [33]. Thus supplementation of OSP provides these amino acids which might work as nitrogen source in culture medium and stimulated the yield of enzymes at lower levels. However, higher levels of OSP may increase excess of substrate pH and consequently unfavorable temperature due to high N level in substrate that probably results to suppress the growth and to end with low yield of enzymes.

In an ultimate observation of the study it was observed that the production of protein content in culture was improved due to the supplementation of OSP but it was in a limit of up to 5% (w/w) of OSP concentration, while higher concentration reduced the yield of protein content in the culture. Similar observations were also recorded in an earlier study [16]. This observation is quite interlinked with the findings of the previous parameters including production of ergosterol or growth and the yield of few enzymes. Since the growth and ergosterol was remarkably influenced which has been too influenced the protein content in culture. Similarly, it can be postulated with the production of several enzymes and the yield of protein contents.

Conclusion

The Typha plant substrate and oyster shells are the easily available waste substrate cum supplements which can be exploited for their biotechnological conversion in quality products like ligno-cellulolytic enzymes of multipurpose uses. Based on the findings of the present study it can be concluded that Typha plant substrate is a very good substrate choice. In addition lower levels of OSP are supportive to improve the yield of ligno-cellulolytic enzymes. Therefore, the use of OSP and TPS in solid substrate fermentations for the production of useful enzymes is recommended.

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