

Sequestration and purification of essential enzyme from *Pleurotus florida* compost waste and its application studies

S Karthika Devi¹, N Prabhu^{1*}, S Monika¹, N M Swapna¹, S Vaishali¹, T Gajendran²

¹Department of Biotechnology, Vivekanandha College of Engineering for Women, Tiruchengode, Tamil Nadu, India, ²Department of Biotechnology, Bharathidasan Institute of Technology, Anna University, Tiruchirappalli, Tamil Nadu, India

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ABSTRACT

Spent oyster mushroom (*Pleurotus florida*) compost as tremendous source for isolation of industrial significant enzymes such as amylase, protease, and cellulase. This study was conducted to achieve efficient extraction of lignocellulolytic enzymes amylase (EC 3.2.1.1), cellulase (EC 3.2.1.4), and protease (EC 3.4.21.14) from spent oyster mushroom (*P. florida*) compost waste. Optimal enzyme recovery was achieved when spent oyster mushroom compost wastes and concentrated by acetone precipitation. The purification was performed by column chromatography. The enzymes such as cellulase, amylase, and protease released from oyster mushroom (*P. florida*) compost waste were showed activities of 15.78 U/ml, 3.42 U/ml, and 0.042 U/ml, respectively. These were utilized in various industrial and environmental applications such as starch processing in potato waste from food industry using amylase and biotreatment of cotton waste using cellulase.

1. INTRODUCTION

Macrofungi of oyster mushroom (*Pleurotus florida*) have the capability to produce a high variety of intracellular and extracellular enzymes which can degrade agro-waste materials contain complex organic compounds into simple low molecular weight compounds. These are water soluble and remarkable nutritive value [1]. The enzymes secreted from mushrooms are nonspecific, and it can decompose a wide variety of structurally different compounds [2]. Basically mushroom like basidiomycetes of fungal cell does not have chlorophyll like plants, so it cannot produce their own food using carbon dioxide, mineral ions, and water for photosynthesis as do green plants. Mushrooms are heterotrophs and obtain their nutrients by absorbing soluble inorganic and organic materials from agro substrates such as wood logs, manure composts, or other organic synthetic composts.

Approximately two million tons of spent mushroom compost waste are produced yearly by mushroom farms, with almost 1.2 million tons generated from oyster mushrooms. This high amount of spent mushroom compost waste is incompatible for reuse in mushroom production; therefore, it is used either as garden fertilizer or deposited

in landfills, which pollutes the environment. Spent oyster mushroom (*P. florida*) compost wastes contain fungal mycelia, extracellular enzymes, and unused lignocellulosic compounds [3]. The waste from oyster mushroom farms has been used in novel processes such as production of value-added products such as biogas production, bulk enzyme extraction, and production of organic fertilizer; animal feed supplements and used in degradation of pentachlorophenol [4-6]. The utilization of oyster mushroom compost waste is benefit for human health and the environment because the agricultural waste from mushroom farms used as raw materials for various processes.

The current investigation was performed to isolate and partial purification of the various industrial enzyme from oyster mushroom (*P. florida*) compost waste, which is utilized in various environmental applications such as starch hydrolysis of potato waste and biotreatment of cotton waste.

2. MATERIALS AND METHODS

2.1. Mushroom Bed Preparation

Oyster mushroom (*P. florida*) bed was prepared using rice straw waste from agro-industry in Tiruchengode, Namakkal (Dt) in Tamil Nadu. The autoclavable polypropylene bags containing layers of rice straw were inoculated with *P. florida* mushroom spawn, which was incubated at $25 \pm 28^\circ\text{C}$ for 25 days in darkroom. After 25 days of incubation, the mycelia were fully covered in whole bed and produce mushroom flushes. After third or fourth yield, old mushroom bed was used as a source for crude industrial enzyme extraction.

*Corresponding Author

N Prabhu,

Department of Biotechnology,
Vivekanandha College of Engineering for Women,
Tiruchengode - 637 205, Tamil Nadu, India.
Email: prabhu.aut.26@gmail.com

2.2. Plate Assay for Various Enzymes

Oyster mushroom (*P. florida*) was screened for various industrial enzymes using agar plate method with the following compositions.

2.2.1. Amylase test

Oyster mushroom (*P. florida*) mycelia stage was tested for amylase production by starch hydrolysis. Starch agar medium contains peptone, 5 g; beef extract, 3 g; starch, 20 g; agar, 15 g; and distilled water, 1000 ml, which was inoculated with oyster mushroom (*P. florida*) mycelia stage and incubated at 28°C for 8 days. After 8 days of incubation, the mycelial growth containing agar plate was flooded with iodine solution. After 1 day of incubation, the clear zone around mycelia indicated the production of amylase [7].

2.2.2. Carboxymethyl cellulose (CMC) agar medium for cellulase test

Cellulase enzyme production from mycelia stage of *P. florida* was tested using CMC agar medium. In this media contain malt extract, 20 g/l; agar, 15 g/l; carboxymethyl cellulose sodium salt, and 10 g/l mycelia stage of *P. florida* were inoculated in CMC agar plate and incubated at 28°C for 8 days. After 8 days of incubation, the plates were flooded with 0.2% of aqueous Congo Red Solution, then which was destained with 1 M NaCl for 15 min. The appearance of yellow areas around the mycelia indicates the presence of cellulase activity [8].

2.2.3. Gelatin agar medium for protease test

For protease test, the *P. florida* mycelia were inoculated on casein agar medium (g/l) (gelatin, 20; casein, 10; sodium chloride, 10; sodium bicarbonate, 1; and agar 15) and incubated at 28°C for 8 days. The presence of protease activity was determined by protease agar plate flooded with mercuric chloride solution (20% HCl was mixed in 15% mercuric chloride solution). The presence of clear zone surrounding the mushroom mycelia indicates protease activity [9].

2.3. Collection of Oyster Mushroom (*P. florida*) Compost Waste

The oyster mushroom (*P. florida*) compost waste was collected from Mushroom farm, Vivekanandha College of Engineering for Women, Tiruchengode, Namakkal (Dt) in Tamil Nadu.

2.4. Preparation of Crude Enzyme Extract

The oyster mushroom (*P. florida*) compost waste (25 g, ~75–85% moisture) was taken and homogenized using mortar and pestle in 250 ml of 50 mM sodium acetate buffer, pH 5.4, and filter through muslin cloth. The filtrate was clarified by centrifugation at 4000 rpm for 15 min. The obtained supernatant was used for further studies [10].

2.5. Protein Estimation

The total protein content of crude enzyme extract from oyster mushroom (*P. florida*) compost waste was determined by Lowry's method. To 1000 µl of the sample, 4.5 ml of alkaline copper sulfate reagent was added, mixed well and allowed to stand at room temperature for 10 min, then 0.5 ml of diluted Folin–Ciocalteu's phenol reagent was added, and after 30 min of incubation at room temperature, absorbance was measured at 660 nm. The protein content in sample was calculated from a standard curve prepared with bovine serum albumin as a standard [11].

2.6. Estimation of Enzyme Activities

2.6.1. Amylase assay

Amylase activity was measured by estimation of reducing sugar released from starch. In this assay, a volume of 1 ml of the crude

enzyme extract was incubated with 1 ml of 1% starch solution (pH 6.9) at 30°C for 30 min. Then, 1 ml DNS reagent was added into the reaction mixture, which was kept in a boiling water bath for 10 min. After 10 min incubation, the solution was cooled at room temperature, and the optical density of reaction mixture was read at 540 nm [12].

2.6.2. Cellulase assay

Cellulase activity was assayed using cellulose as the substrate. In this assay, cellulose was dissolved in 10% NaOH at a cold temperature (4°C). 1 ml of crude enzyme extract was incubated in 1 ml of dissolved cellulose (pH 4.8) at 50°C for 10 min. The activity of reaction mixture was determined using DNS assay method, various concentration of 1% glucose used as a standard for this assay [12].

2.6.3. Protease assay

Protease activity was determined in the crude enzyme extract from *P. florida* compost waste using 1.5% casein in 0.2 M Tris-HCl buffer, pH 7.2. In this assay, 1 ml of 1.5% casein was added in 1 ml of crude enzyme extract, which was incubated at 37°C for 10 min. 3 ml of 5% TCA was added in the reaction mixture and was incubated at 37°C for 10 min. After 10 min of incubation, the reaction mixture was centrifuged at 10,000 rpm for 10 min, and 0.5 N of NaOH, 0.6 ml of Folin reagents were added and incubated for 30 min, Optical density of reaction mixture was read at 660 nm [13].

2.7. Acetone Precipitation

The enzymes in crude extracts were further precipitated by the gradual addition of chilled 60% (v/v) acetone with continuous stirring and kept in a cold temperature at 4°C for 24 h. The precipitated protein was collected by centrifugation at 12,000 rpm at 4°C for 30 min and dissolved in Tris-HCl buffer (pH 8.0). The sample was dialyzed against the same buffer using acetylated dialysis bag [14].

2.8. Purification of Various Enzymes

The purification of various industrial enzymes was done using a silica gel chromatography column. This was equilibrated with 50 mM potassium phosphate buffer (Buffer A-pH 7). The presence of dialyzed enzyme solution (30 µl) in the column was washed with 4 ml of Buffer A. Elution was done by distilled water. The active fractions of different enzymes such as cellulase, amylase, and protease were detected at 540 nm, 420 nm, and 660 nm, respectively.

2.9. Storage Stability of Crude Enzymes

The storage stability of crude industrial enzymes was analyzed using 1 ml of crude extract and was mixed with 1 ml of 50 mM sodium acetate buffer (pH 5.8) and stored at 4°C. The crude enzymes activity were analyzed every day up to 5 days.

2.10. Application Studies

2.10.1. Starch processing of potato waste

Potato (59 g) was homogenized using distilled water, which was treated with 100 ml of crude enzyme extract from *P. florida* compost waste. One flask containing potato homogenate with the addition of 100 ml distilled water was act as control. The starch degrading capability of crude enzyme extract was analyzed using dinitrosalicylic acid method [12].

2.10.2. Biotreatment of cotton waste

Cotton waste (1.5 g) was treated with 100 ml of crude enzyme extract from oyster mushroom (*P. florida*) compost waste in non-agitated and

agitated systems at pH 5.8 (50 mM Sodium acetate buffer). The flask was incubated at 37°C for 24 h. After enzymatic treatment, the cotton waste was washed twice in hot distilled water to deactivate the enzyme and then air-dried.

Weight loss of cotton waste from crude enzymatic treatment was determined by weighing the sample before and after the treatment following 24 h of treatment at 37°C. Reducing sugar from the sample was determined by dinitrosalicylic acid method [12]. Enzymatic treated and untreated cotton morphology differences were investigated using light microscope.

3. RESULTS AND DISCUSSION

3.1. Mushroom Bed Preparation

Oyster mushroom (*P. florida*) bed was prepared using rice straw as substrate. After 25 days of incubation at dark condition, the first stage of mushroom flush was formed. After 3rd and 4th yields, loss of nutrients results in the formation of compost which was used as a substrate for various industrial enzyme extractions.

3.2. Enzymatic Screening of Oyster Mushroom (*P. florida*) Using Agar Plates

After incubation of various agar plates for 8 days at 28°C for the industrial enzymatic tests, the following results were obtained [Table 1]:

It was seen that the oyster mushroom (*P. florida*) showed that the best industrial enzymatic activities. Among the three enzymes, the protease enzyme from *P. florida* was showed less activity.

From these results, the presence of protease activity was determined using mercuric chloride solution. The clear zone around the mushroom mycelia indicates the protease activity of *P. florida* [Figure 1a]. The activity of cellulase enzyme from *P. florida* was analyzed by Congo red aqueous solution. The appearance of yellow areas around the mycelia indicates the presence of cellulase activity [Figure 1b]. The iodine solution was used to detect the amylase enzyme activity from *P. florida*. Clear zone around the mushroom mycelia indicates the presence of amylase activity [Figure 1c].

Many reports suggested that the secretions of industrial enzymes in mushrooms are used for its fruiting body formation [15]. Reports from Slawinska and Kalbarczyk 2011 show that the cellulase enzyme activity in some *Pleurotus* species is more than the amylase activity; hence, the same result was recorded in the current study [16]. Protease enzyme from various fungal sources is important in pharmaceutical industries for various medical applications [17].

3.3. Quantification of Enzymes

Oyster mushroom (*P. florida*) compost waste contains lot of industrially important enzymes such as amylase, cellulase, and protease [Figure 2]. Among the three enzymes, activity of cellulase was found to be higher than other enzymes and found to be 15.78 U/ml, respectively. Amylase enzyme activity from crude extract of *P. florida* compost waste was 3.42 U/ml. The activity of protease enzyme from crude extract of compost waste was 0.042 U/ml. From this result, the oyster mushroom (*P. florida*) compost waste as a good source for extraction of lot industrially important enzymes.

Similar to this observation, the waste produced from oyster mushroom forms for extraction of industrially important enzymes that have

Table 1: Screening of various industrial enzymatic activity of oyster mushroom (*Pleurotus florida*).

Enzymes	Substrates	Test reagent	Plate appearance	Zone size (mm)
Protease	Gelatin	Mercuric chloride solution	Clear zone around the mycelium	2
Cellulase	Cellulose	Congo red solution	Appearance of the yellow area around the mycelium	-
Amylase	Starch	Iodine solution	Clear zone around the mycelium	6



Figure 1: Plate assay for different industrial enzymes (a) plate test for cellulase, (b) plate test for protease, and (c) plate test for amylase

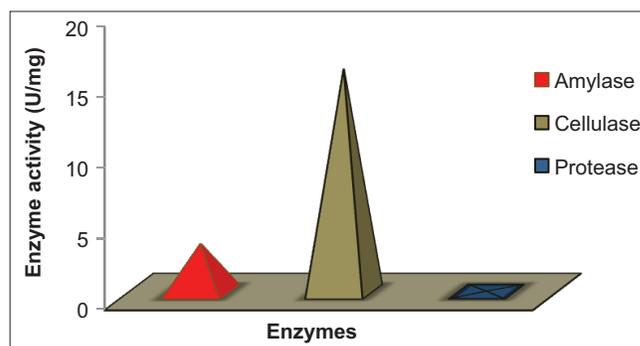


Figure 2: Total activity of enzymes

previously been studied [18]. The reutilization of oyster mushroom compost waste was reduce a lot of environmental problems such as disposals of bulk density of waste into the soil, stability, surface crust, temperature changes, pH changes in soil, aeration, and water retention capacity of soil [19]. Many investigations reported that the spent oyster mushroom compost has a plenty of extracellular enzymes. The highest specific activity of cellulase (19.2 U/mg) was obtained using 50 mM sodium acetate (pH 5.8) extraction buffer. Low specific activity (4.17 U/mg) of amylase enzyme was obtained from *P. florida* compost waste. Very low specific activity (0.05 U/mg) of protease enzyme was obtained from crude extract. By this study, it was concluded that oyster mushroom (*P. florida*) compost waste as a good source for extraction of industrially important enzymes [Figure 3].

3.4. Storage Stability Test for Industrial Enzymes

From this study, the storage stability of crude amylase enzyme from oyster mushroom (*P. florida*) compost waste was decreased after 5 days of incubation at 4°C in pH 5.6 [Figure 4].

3.5. Purification of Different Enzymes

Figure 5 shows the result of each step of different enzymes purification. Activities of purified enzymes using gel filtration chromatography were cellulase (7.65 U/ml), amylase (0.45 U/ml), and protease (0.001 U/ml), respectively [Figure 5].

3.6. Biotreatment of Cotton Waste

From this study, the crude enzymatic treatment of cotton waste was evaluated by weight losses of waste cotton before and after treatment. The weight loss value caused by crude enzyme extract from *P. florida* compost waste is shown in Table 2. The presence cellulase enzyme in the crude extract caused the weight loss (0.1 g) in the cotton waste, after 24 h of incubation.

Table 2: Biotreatment of cotton waste.

Sample	Incubation time (h) and reducing sugars (mg/ml) (h)		
	1	12	24
Control (50 mM sodium acetate buffer)	-	-	-
Crude extract	109.40	144.90	199.64

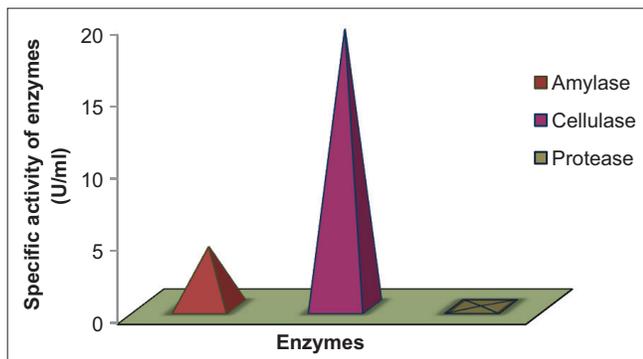


Figure 3: Specific activity estimation of enzymes

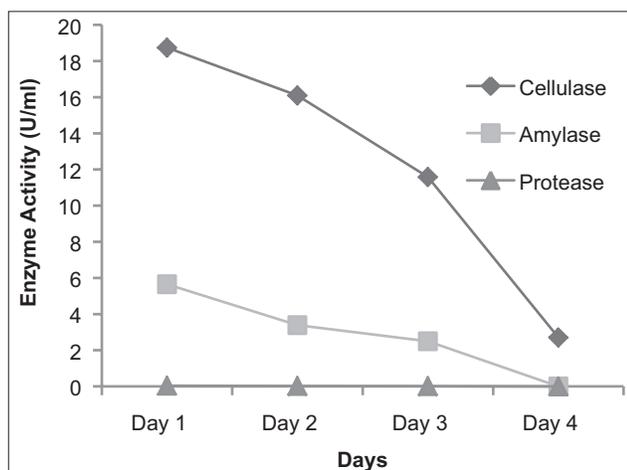


Figure 4: Storage stability of industrial enzymes

The sample from cotton waste treated with crude cellulase was yield measurable reducing end groups from cotton carbohydrates. Table 2 shows the time dependence of reducing sugar liberation during the crude enzymatic treatment. Csiszar *et al.*, 2001, reported that the amount of released reducing sugar by cellulase and hemicellulase enzymes increases with the time of enzymatic treatment [20]. The present investigation also shows the same result; the reducing sugar production from sample of cotton waste treated with crude enzyme extract from *P. florida* compost waste was increased based on their increased reaction time.

The morphology changes in the crude enzyme from *P. florida* compost waste treated cotton sample have been studied with a light microscope. Figure 6 shows the surface morphology of untreated cotton waste from crude enzymatic treated cotton waste. The surface of the crude enzymatic treated cotton sample was varied from untreated cotton sample. During enzymatic treatment the presence of cellulase enzyme in the crude extract causes wall damages in the cotton fiber, which occur due to the presence of cellulose molecules in the primary wall of the cotton. Due to this reaction weight loss of cotton and production of reducing end groups were formed.

3.7. Starch Processing of Potato Waste

In this work investigated that the amylase enzyme from *P. florida* compost waste extract has potential to degrade starch present in the potato waste. The activity of crude amylase from potato waste containing sample was 0.015 U/ml. This was analyzed by DNS assay method [12]. Many research investigated that crude amylase from various microbes was improves the hydrolyzing of various starches from agro-waste [21-23].

From this study investigates, the crude amylase from *P. florida* compost waste showed a low level of activity [Table 3]. Hence, the activity of amylase in crude extract was decreased after 3 days of incubation with potato waste.

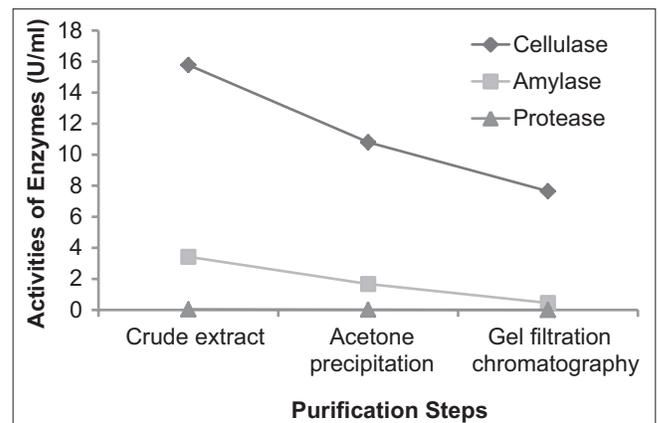


Figure 5: Purification of industrial enzymes

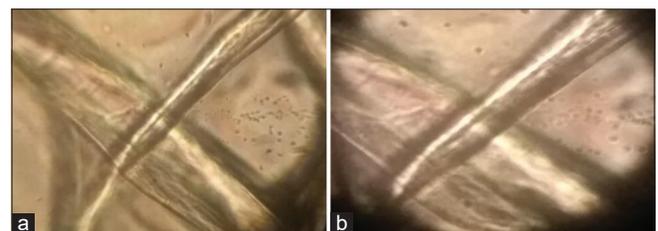


Figure 6: Morphology analysis (a) cotton without treatment of crude extract, (b) cotton treated by crude extract

Table 3: Starch processing in potato waste.

Sample	Incubation time (h)	Temperature (°C)	pH	Crude amylase activity (U/ml)
Control	1	37	7.4	-
Crude extract	1	37	7.4	0.015

4. CONCLUSION

The investigation of present work concluded that the oyster mushroom (*P. florida*) compost waste as a good source for extraction of extracellular industrially important ligninolytic enzymes such as amylase, cellulase, and protease. The enzymatic activity of oyster mushroom *P. florida* mycelia was screened using different agar media. Higher activities of amylase and cellulase were observed, whereas very less protease activity was observed in mycelial growth on protease agar media. The total protein content of crude extract from compost waste and acetone purified sample was analyzed by Lowry's method. The protein content of crude sample was 0.82 mg/ml and acetone precipitated sample was 1.25 mg/ml. The activity of crude industrial enzymes from *P. florida* compost waste extract was analyzed by various quantitative enzyme assay. The crude cellulase enzyme was showed highest activity of 15.78 U/ml, amylase enzyme from crude sample was showed lower activity of 3.42 U/ml. Very low protease activity was shown in crude sample about 0.042 U/ml. The presence cellulase and amylase enzyme in crude extract from *P. florida* compost waste were utilized in biotreatment of cotton waste and starch hydrolysis in potato waste containing water sample. The desizing of cotton waste was analyzed by weight loss determination, reducing sugar quantification, and microscopic study on morphology analysis of cotton surface. In summary in this work, the various industrial enzymes were extracted from oyster mushroom (*P. florida*) compost waste and which was utilized diverse environmental applications such as biotreatment of cotton waste and starch hydrolysis in potato waste.

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