# PRODUCTION OF CHITOSAN FROM OYSTER MUSHROOM FOR α-AMYLASE IMMOBILIZATION

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### Abstract

Experiments were carried out to produce chitosan from a locally available mushroom and to use the produced chitosan as a matrix to immobilize α-amylase. On the basis of morphological characteristics and the sequence of the internal transcribed spacer region of the nuclear rDNA, the fungal sample was identified as *Pleurotus ostreatus*. The average crude chitin content in the dried fruit body of *P. ostreatus* was 24.11%. The average yield of chitosan from *P. ostreatus* was 163.3 mg/g dry weight and the degree of deacetylation of the produced chitosan was 73.42%. This chitosan was used as a matrix to immobilize α-amylase. The diameter of the α-amylase immobilized beads ranged from 1839 - 2273 μm. The amount of reducing sugar produced from starch by using free α-amylase and chitosan-immobilized α-amylase was 1.710 and 1.508 mg/ml, respectively. Immobilized enzyme produced only 11.81% less reducing sugar than that of the soluble enzyme in the first cycle. However, immobilized α-amylase was easily recovered from the product and reused for two more cycles which was not possible with the same soluble free enzyme. Considering the total production of reducing sugar in three cycles, chitosan-immobilized α-amylase was found to be more productive and cost-effective than conventional soluble enzymatic reaction.

### Introduction

Chitin, a polymer of N-acetyl-D-glucosamine, is found in shrimp, crabs, lobster and fungi. The main derivative of chitin is chitosan. Chemically, chitosan is a linear polymer of β (1 - 4) linked 2-amino-2-deoxy-β-D glucopyranose units together with some proportion of N-acetylglucosamine units and it is easily derived by alkaline N-deacetylation of chitin (Tharanathan and Kittur 2003). The main difference between chitin and chitosan is the percentage of the acetyl group present in their chemical structure. If the percentage of N-acetylglucosamine is more than 50%, it is chitin and if this percentage of N-acetylglucosamine is less than 50%, the material is called chitosan (Viarsagh *et al.* 2010).

There are two advantages of using chitosan over chitin for biotechnological purpose. Firstly, to dissolve chitin, highly toxic solvents as lithium chloride and dimethylacetamide are used whereas chitosan is readily dissolved in diluted weak acids. Secondly, chitosan possesses positive ionic charges, which give it the ability to chemically bind with negatively charged macromolecules (Li *et al.* 1992). In this respect, chitosan has received increasing commercial interest as a suitable material for enzyme immobilization.

The production of chitosan from crustacean chitin is poorly eco-friendly. This is because crustacean chitin possesses high amount of $CaCO_3$ which releases environment polluting $CO_2$ during the extraction processes. Another setback is the availability of raw crustacean shells is basically seasonal and depends on the geographical location. These problems also cause variability in the chitosan production. Therefore, it is necessary to explore other more eco-friendly approach

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for the production of chitinous polysaccharides. This work was, therefore focused on the production of chitosan from easily available oyster mushroom and its application in enzyme immobilization.

In order to make enzyme utilization more favorable in biotechnological processes, different methods for cost reduction have been put into practice and immobilization is one of them. Immobilized enzymes are more stable than free ones and can be reused for several times. Immobilization is defined as imprisonment of the enzyme in the support or matrix (Kawaguti *et al.* 2006). A suitable matrix should possess reactive functional group, high affinity to proteins, non-toxicity and mechanical stability. The macromolecule chitosan possesses many of these properties and thus it may be used to immobilize enzyme.

The enzyme α-amylase hydrolyses the α-1, 4-glycosidic bond that holds the glucose units together in a starch. This enzyme is widely used in food, textile, paper and detergent industries. The local industries are dependent on imported α-amylase. Furthermore, the free form of this enzyme is not reusable and hence the local industries import huge amount of this enzyme. In the present study, the environment friendly fungal chitosan was used to immobilize this widely used enzyme. In Bangladesh, some studies were done on the extraction and characterization of chitin and chitosan from shrimp (Nessa *et al.* 2010, Hossain and Iqbal 2014) but study on fungal chitosan is almost absent. Therefore, the present study was done to produce chitosan from a locally available oyster mushroom and to use it as a matrix for immobilization of α-amylase.

### Materials and Methods

For extraction of chitin and then production of chitosan, mushroom sample was collected from Savar, Dhaka, Bangladesh (Fig. 1). The enzyme α-amylase (1,4 α-D-glucan glucano-hydrolase, EC 3.2.1.1) was purchased from Sigma-Aldrich. The study was conducted in the laboratory of Microbiology and Industrial Irradiation Division, Institute of Food and Radiation Biology, Atomic Energy Research Establishment, Savar, Dhaka and in the Department of Botany, Jahangirnagar University (JU), Department of Biotechnology and Genetic Engineering, JU, Bangladesh.
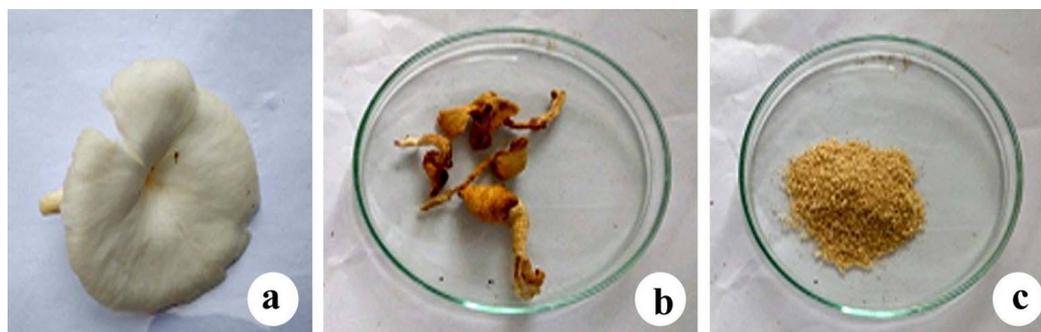


Fig. 1. The mushroom sample: (a) Front view, (b) dried fruit body and (c) powdered fruit body.

The preliminary identification of the sample was done based on the shape, diameter, margin, texture and color of the pileus, gill attachment to stipe, gill spacing, stipe length, stipe diameter, stipe color and stipe texture. To confirm the identity of the fungal sample, molecular identification based on DNA sequence of the internal transcribed spacer (ITS) region of the nuclear rDNA was done. Nuclear DNA was extracted from the fruit body of the mushroom by using Maxwell DNA Kit (Promega, USA) according to the manufacturer's instruction. The concentration of the purified

DNA was measured by using NanoDrop Spectrophotometer (Model: ND2000, Thermo Scientific, USA).

The internal transcribed spacer (ITS) region of the nuclear rDNA was amplified by polymerase chain reaction (PCR). The primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') was used as described by White *et al.* (1990). After PCR reaction, agarose gel electrophoresis was done to resolve the PCR product and determine its size. Electrophoresis was carried out at 100 V for 30 min. The gel was then viewed under a gel documentation system and photographed. The PCR product was purified and the DNA sequencing was done at 1st base Laboratory, Malaysia. After sequencing the resulted nucleotide sequences were edited and compared using Basic Local Alignment Search tool (BLAST) of National Center for Biotechnology Information (NCBI). The obtained DNA sequence of this study (GenBank accession No. MT672600) was compared with the ITS sequence of related species by ClustalW using MEGA 7.0 (Kumar *et al.* 2016) and a phylogenetic tree was constructed based on Tamura and Nei (1993).

At first chitin was extracted from the mushroom and then converted to chitosan. For production of chitosan the methods described by Johney *et al.* (2017) and Yang *et al.* (2017) were followed with some modifications. The solubility of chitosan was tested at different concentrations of (0.2, 0.5 and 1%) acetic acid at 50°C on a magnetic stirrer. Acid-base titrametric method described by Domard and Rinaudo (1983) with some modifications was used to determine the degree of deacetylation (DD) of chitosan.

For testing the potentiality of using the produced chitosan as a matrix to immobilize enzyme, a widely used enzyme α-amylase was used considering its industrial application, cost and availability. The beads were formed by dipping the α-amylase, chitosan and sodium alginate solution to 0.2 M $CaCl_2$ solution using a syringe. The beads were kept into $CaCl_2$ solution for 30 minutes. Then the beads were separated by using sieve and washed twice with distilled water and kept at 4°C for further use. Twenty (20) enzyme-immobilized beads were randomly selected for determination of their size. The diameter of the beads (μm) was measured under a microscope (Micros, Austria), integrated with the Olympus CellSens Entry software.

The enzymatic activity of the free and immobilized α-amylase was measured by using starch as a substrate. In brief, 5 ml of 1% starch solution was added to the α-amylase immobilized chitosan beads. In another tube 5 ml of 1% starch solution was added to 1 ml distilled water instead of the enzyme and it served as a negative control. For positive control, 1 ml of 30 U α-amylase was added to a tube containing 5 ml of 1% starch solution. The tubes were incubated at 37°C for 30 minutes. Two ml solution from each sample was taken into fresh test tube. Freshly prepared 2 ml dinitrosalisylic acid (DNS) reagent and 1 ml Rochelle salt solution were added to the tubes. The optical density of the solution was measured at 575 nm by using a UV-VIS spectrophotometer.

The reusability of the immobilized α-amylase was determined by repeated batch experiments. At the end of each cycle the immobilized enzyme was removed, washed with distilled water and the reaction medium was changed with fresh substrate solution. The assay was carried out for 3 cycles under standard assay conditions.

**Results and Discussion**

The PCR conditions and the primers (ITS4 and ITS5) used in this study successfully amplified the target region of the DNA. The PCR produced a single band of expected size (Fig. 2). The PCR product was purified and DNA sequencing was performed. A pair-wise comparison of the obtained DNA sequence of *Pleurotus ostreatus* with the available DNA sequences of the

GenBank was performed using NCBI-BLAST (Altschul *et al.* 1990).The BLAST analysis result revealed that the ITS rDNA sequence of the mushroom sample used in this study is 99% identical to that of *P. ostreatus* strains (ICMP 11678, ICMP 11679, NAMA 2017-414) previously deposited to GenBank of NCBI by other researchers. The phylogenetic tree constructed using other representative species of the genus *Pleurotus* revealed that the mushroom sample used in this study is closely related to other strains of *P. ostreatus* (Fig. 3).
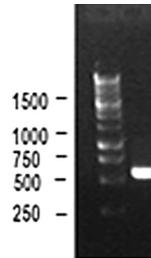


Fig. 2. Agarose gel electrophoresis of PCR product. The PCR product was electrophoresed in 0.8% agarose gel visualized under UV after staining with ethidium bromide. The bands in the left lane represent DNA size marker.
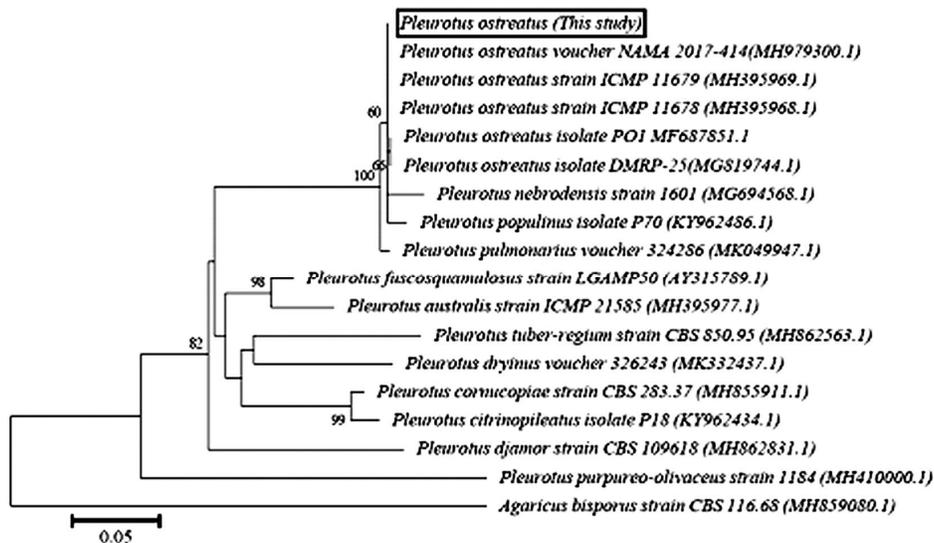


Fig. 3. Phylogenetic tree showing the relationship between *P. ostreatus* used in this study (GenBank accession No. MT672600) with other fungi. The tree was constructed by maximum likelihood method based on Tamura and Nei (1993) integrated in the MEGA7.0 software (Kumar *et al.* 2016). The phylogenetic tree was tested with 1000 bootstrap replicates.

The chitin content in the dried *P. ostreatus* ranged from 23.40 to 24.90% (Table 1). Similar level of chitin was also extracted from *Aspergillus niger* (Wu *et al.* 2005). However, Yen and Mau (2006) reported higher level (25.08 - 36.72%) chitin recovery from *Lentinula edodes*. In a previous study it was reported that yield of chitosan varies depending on the source material. For example, the yield of chitosan from *Laccaria amethysta, Cantharell escibarius, Laccarial accata*

and *Hericium erinaceus* was 18.80, 11.77, 7.18 and 61.11%, respectively (Elem and Uraku 2017). The yield of chitosan obtained in this study ranged from 15.20 to 17.45%. This yield was within the range (14.14 - 23.69%) reported in *Lentinula edodes* (Yen and Mau 2006).

**Table 1. Chitin content of the *P. ostreatus* and productivity of chitosan.**

| Experiment no. | Initial amount (g) | Crude chitin | | Chitosan produced | | DD chitosan (%) |
|---|---|---|---|---|---|---|
| | | (g) | (%) | (g) | (%) | |
| 1 | 20 | 4.81 | 24.05 | 3.27 | 16.35 | 72.73 |
| 2 | 20 | 4.98 | 24.90 | 3.49 | 17.45 | 74.73 |
| 3 | 20 | 4.68 | 23.40 | 3.04 | 15.20 | 72.81 |
| Av. | 20 | 4.82 | 24.11 | 3.26 | 16.33 | 73.42 |

The degree of deacetylation (DD) is one of the most important parameters that affect the physico-chemical properties of chitosan. The DD of the chitosan produced in the present study ranged from 72.73 to 74.73% (Table 1). Elem and Uraku (2017) suggested that the large positive charge density due to high DD makes chitosan useful for biomedical and industrial applications, particularly as a carrier, support for enzyme immobilization and drug delivery. The observed DD value of the chitosan indicates that it may also be used for the said purposes.

Use of soluble enzymes has several disadvantages. For example, soluble enzymes cannot be reused, unstable and more sensitive to process conditions (Shi *et al*. 2011). For these reasons, application of solid phase catalysts has received more importance in the last decades. Advantages of immobilized enzymes are that the generally expensive can be used repeatedly in successive batches or the process can eventually be carried out in a continuously operating reactor (Biro *et al*. 2008).
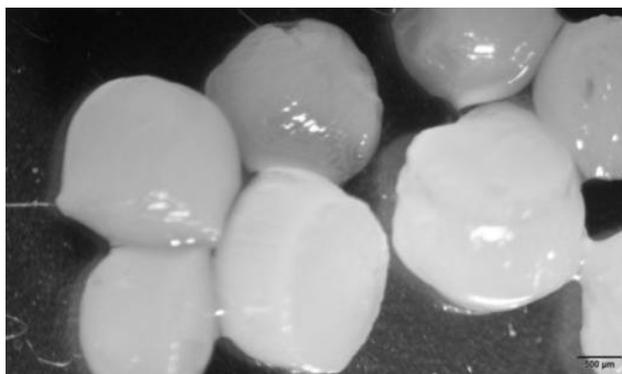


Fig. 4. Photograph of the α-amylase immobilized beads. The beads were observed under a microscope integrated with Olympus CellSens Entry software. Scale bar = 500 µm.

Enzymes can be immobilized on various supports. In this study, chitosan obtained from *P. ostreatus* was successfully used to produce α-amylase immobilized beads (Fig. 4).The diameter of the enzyme immobilized beads produced was found to range from 1839 - 2273 µm (Fig. 5) which is smaller than that reported by Biro *et al*. (2008). Portaccio *et al*. (1998) and Spagna *et al*. (1998) successfully used chitosan for immobilization of α-L-arabinofuranosidase and β-galactosidase, respectively. The α-amylase immobilized in chitosan beads retained their activity indicating that

the use of chitosan in enzyme immobilization might not have destroyed the catalytically active tertiary structure of α-amylase. Norouzian (2003) emphasized that the preservation of the catalytically active tertiary structure of the enzyme is important in immobilizing the enzyme.

Reusability is one of the advantages of enzyme immobilization. Reusability of the α-amylase immobilized beads was determined for two more cycles. The amount of reducing sugar produced using free enzyme (α-amylase) was 1.710 mg/ml and using the immobilized α-amylase was 1.508 mg/ml (Table 2). Immobilization has reduced only 11.81% reducing sugar production in the first cycle. However, most importantly the immobilization caused the enzyme to be used for at least three cycles which was not possible with free enzyme. Considering the total production of reducing sugar in three cycles, immobilization of α-amylase was found more productive and cost-effective.
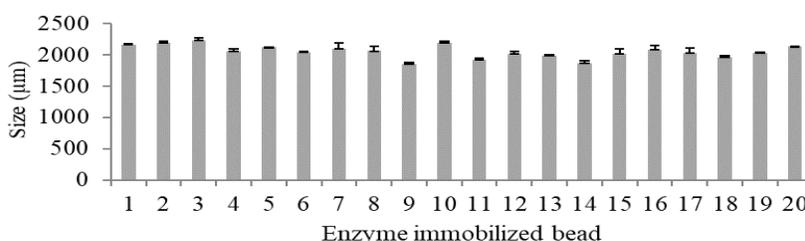


Fig. 5. Size of the α-amylase immobilized beads. Twenty beads were randomly selected and their size was measured from three directions.

**Table 2. Amount of reducing sugar produced by immobilized and soluble free α-amylase.**

| Sample | Amount of reducing sugar (mg/ml) produced | | | |
|---|---|---|---|---|
| | 1st cycle | 2nd cycle | 3rd cycle | Total |
| Starch and α-amylase immobilized beads | 1.508 | 1.315 | 1.250 | 4.073 |
| Starch and free α-amylase | 1.710 | -* | -* | 1.710 |

*Soluble free α-amylase was not reusable after 1st cycle.

Based on the above discussion, it can be concluded that the locally available *Pleurotus ostreatus* is a good source of extracting chitin and then producing chitosan. Furthermore, the chitosan produced from the chitin of *P. ostreatus* can be used in immobilization of α-amylase. The immobilized enzyme was usable for 3 cycles instead of one time use of soluble free enzyme. The cumulative total production of reducing sugar from starch by using the immobilized α-amylase was 238% more than the reducing sugar produced by free α-amylase. Thus, immobilization of α-amylase using chitosan may be useful in industrial sector. However, further research to optimize the conditions will be required before such endeavor.

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