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Purification and characterization of theromohalophilic chitinase producing by halophilic *Aspergillus flavus* isolated from Suez Gulf

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ABSTRACT

This study aimed at production of chitinase enzyme from marine waste. Out of 27 fungal isolates, *Aspergillus flavus* (AUMC 13576) obtained from El-Sokhna has proved to be the most potent strain for chitinase production with activity 620.54 U/l using colloidal chitin as carbon source. The enzyme was purified consecutively by ammonium sulphate precipitation, Sephadex G-100 gel filtration column and DEAE - Cellulose A52 ion exchanger chromatographic column. The molecular weight of purified chitinase was estimated to be 30 KDa by SDS-PAGE. The purified enzyme was exposed for different properties investigations. The purified chitinase gave the highest activity (1368.8 U/l) within temperature rang (60 °C) at optimum pH 7.5 and 0.9 g/l of the substrate concentration. The kinetic measurements as Km and Vmax values of the enzyme were determined to be 0.18 g chitin/ml and 274.31 U/l, respectively. The enzyme showed thermal stability at 50 °C for 15 min. in salinity concentrations (NaCl) up to 0.8 M. Among different tested heavy metals, $MnCl_2$ and $FeSO_4$ boosted the activity positively. These results indicate the potential of mesophilic *A. flavus* (AUMC 13576) in the production of chitinases employing shrimp as an ideal substrate.

Introduction

Chitin is considering the most important natural polymer in the world. The monomer unit composed of β 1–4 N-acetyl glucose amine (Anuradha and Revathi, 2017). Chitin has many sources in nature, marine waste is one of them especially the crustacean shell. Such waste could be accumulated on the coastal line as a pollutant (Olsen et al., 2014).

Chitin can be hydrolyzed into oligomeric and monomeric components using biological processes. Chitinase enzymes are type of enzymes, which hydrolyze linkage of chitin polymers with converting long chain of high molecular weight compounds to other short chain of low molecular weight ones. Bacterial and fungal microorganisms play a significant role for production of chitinase enzyme (Ekundayo et al., 2016). *Paenibacillus* sp. and *Streptococcus thermophilus* have been used for production of chitinase enzymes by living on the chitin from marine shrimp and crab waste as the carbon source (Kao et al., 2009; Mao et al., 2013). Also, *Mortierella* sp. and *Rhizopus nigricans* have been applied for production of chitinases using chitin as substrate (Jeraj et al., 2006; Kim et al., 2008).

Aspergillus species are a dominant species specialized in degrading chitin as feeding from many reports mentioned so far. Notably, degradation of chitin is occurred during the development of fungal cell wall. *A. terreus* and *A. niger* LOCK 62 were found to be the efficient source for chitinase production having versatile applications (Ghanem et al., 2010; Maria and Urszula, 2012). In this study, a chitinolytic fungus *A. flavus* (AUMC 13576) isolated from Suez Gulf sediment was investigated for chitinase production, purification and characterization.

Materials and methods

Isolation and identification of chitinolytic fungal isolates

Sediment samples were isolated from Gulf of Suez, Egypt (Krishnaveni and Ragunathan, 2014). Twenty seven (27) fungal

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isolates were isolated by serial dilutions of soil samples and plated on potato dextrose agar (PDA) and sabouraud dextrose agar (SDA) media under condition 50% sea water (Tallapragada and Venkatesh, 2017). All isolates were purified and tested for chitinase production using the following medium ingredients; colloidal chitin 3.5 g/L; K_2HPO_4, 3.0 g/L; yeast extract, 3.5 g/L; peptone, 2.0 g/L; KCL, 3.0 g/L; NaNo3, 3.0 g/L, $MgSo_4$, 0.5 g/L; $FeSo_4$ 0.01 g at pH 6.2 and 30 °C (Narayanan et al., 2013). Fungal isolate was identified for species level by Assuit University Mycology center. The isolate was deposited in NCBI and released by 23 June 2018 under accession number MH373680 version 1.

Estimation of protein content

The exochitinase measurements were implemented according to Lowry et al. (1951).

Enzymatic assay

The exochitinase activity of samples was estimated according to the assay described by Miller (1959). Upon DNS method, 100 ul of enzyme sample solution was added to 100 ul of the colloidal chitin substrate, and then incubated in a water bath for 20 min at 40 °C. After incubation, 100 ul was discarded and the other 100 ul reaction mixture was stopped by adding 400 ul of DNS reagent followed by boiling for 5–10 min. The developed color was measured at 575 nm. The enzyme unit was defined as the amount of enzyme that released 1 mg of reducing sugar per min. under the given condition with respect to N-Acetyl glucosamine, the calibration curve was from 0 to 400 ug/ml (Cańtizares-Macías et al., 2001; Suwanto et al., 2010; Miller, 1959)

Crude enzyme preparation

The fungal cells were kept aside from the culture medium by filtration and centrifugation at 8500 rpm for 15 min. in cooling centrifuge. The clear supernatant was considered as crude enzyme solution. After 7 days of incubation at 28 °C using colloidal chitin as substrate prepared from shrimp chitin (sigma aldrich), the fungal cells were kept aside from the culture medium by filtration and centrifugation at 8500 rpm for 15 min. in cooling centrifuge. The clear supernatant was considered as crude enzyme solution.

Partial purification of chitinase enzyme(s) produced by A. flavus (AUMC 13576)

Ammonium sulphate, acetone and ethanol fractionation using different concentrations (30%, 50%, 70% and 90%) of each precipitant in a sequential manner were applied for partially purifying the target enzyme protein. The precipitants were applied for the supernatant of 7-dayes-old of *A. flavus* (AUMC 13576), grown on sucrose, yeast extract and peptone medium at pH 6.2 and 30 °C. protein content and enzyme activity were measured for each fraction.

Column chromatography

Partial purified sample was loaded to DEAE-cellulose A52 (2.6 x 20 cm) column. Different salinity of tris 0.05 M HCl and pH (7.5) were applied and the elution was preceded in a stepwise gradient of NaCl (0–1.0%) at a flow rate (0.5 ml/min). the chosen fractions were applied for Sephadex G-100 column (1.6 × 30 cm). the purification column was eluted with tris 0.05 M HCl and pH (7.5) at rate 0.4 ml/min. likely, the fractions of 3 ml were collected and the protein content with chitinase activity of each fraction were determined (Jenifer et al., 2014). The purified beak was chosen for determining the properties of chitinase enzyme.

Molecular weight

SDS-PAGE with 10% acrylamide gel was applied to assure the integrity of purified eluted protein and to specify approximately the molecular weight (Kim et al., 2008). Prestained Standard protein marker, broad range ranging from 7 to 175 KDa (New England BioLabs), was applied.

Different properties of purified chitinase

Effect of pH, temperature, and salinity

Effect of different pH ranges on enzyme activity as pH rang (2.0–6.0) with citrate phosphate buffer and pH rang (6.0–9.0) with sodium phosphate buffer were estimated. Also, different temperatures ranging from 30 °C to 70 °C, as well as different salinity ranging from 0.2 to 2 M were applied to get the maximum activity. (Sarkar et al., 2017; Wang and Yeh, 2008; Patil et al., 2013). In addition, different metal ions; Co^{+2} (CoNO3), Fe^{+2} (FeCl3), Cu^{+2} (CuSO4), Mn^{+2} (MnCl2), Zn+ (ZnNO3), Mg^{+2} (MgSO42), Fe^{+2} (FeSO4) and EDTA at 0.01 M concentration were applied for purified enzyme dissolved in 0.05 M phosphate buffer at pH 7.5 (Gao et al., 2015).

Kinetic parameters and thermal stability

K*m* and Vmax were calculated based on incubation of purified enzyme with different substrate concentrations (0.1–1.2%) in terms of colloidal chitin (Patil et al., 2013). Thermal stability was measured for purified enzyme by incubation of the enzyme at the desired temperatures for varied intervals (15, 30, 45, and 60 min.) in 25 mM sodium phosphate buffer (Dai et al., 2011).

Results

The total of 27 isolated fungi were purified and tested for chitinase production according to the clearance zone resulted from colloidal chitin degradation. Four isolates were selected for further screening using sucrose, yeast extract and peptone broth medium for the chitinase activity. The isolates were found to grow sea water medium otherwise no growth were found so that 50% sea water was used as a growth key in the constituent medium. Likely, chitin was found to be a key factor for growing the four isolates and it's a must to be a part of medium ingredient. The most potent isolate was identified based on morphological characterization to be *A. flavus* (AUMC 13576) with GenBank: MH373680.1.

Fractional precipitation

As indicated in Fig. 1, ammonium sulfate precipitation partially purified the enzyme through different fractions. The most convenient one was fraction 90% where the highest activity (2337.1 U/l) was obtained as well as the protein content. Other fractions gave a lower protein recovery accompanied with very weak enzyme activity. On the other hand, other precipitating agents representing in ethanol and acetone solvents gave a denatured proteins with different fractions resulted in no activity. Upon that, 90% ammonium sulfate fraction was selected to partially purify the chitinase enzyme from the strain.

Deae-cellulose column

The loaded partially purified enzyme on DEAE cellulose A-52 column was eluted in a sum of 34 fractions as indicated in

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