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Unveiling the potential of metal-tolerant fungi for efficient enzyme production

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ABSTRACT

The present study first time reports the utilization of metal tolerant fungi as a potential source for efficient enzyme production. The purification and characterization of alkaline protease from an indigenous zinc-metal tolerant fungal isolate *Aspergillus flavus* NJP08 has been demonstrated. The specific activity of enzyme was determined as 89.1 U mg^{-1} which is found to be the highest among the reported *Aspergillus flavus* isolates so far. The protease was purified 55.87 fold up to homogeneity and identified as "alkaline protease" with a molecular mass of 33 kDa. The N-terminal sequence was GLTTQKSAPWGLG which showed high similarity with other reported proteases of genus *Aspergillus*. Physico-chemical characterization of enzyme revealed an estimated half-life of >20 h with aliphatic and GRAVY index values of 79.65 and −0.161 respectively, depicting high thermo-stability and secretory nature of protease. The protease was active within the temperature range of 25–50 °C with an optimum temperature of 50 °C and was stable in the pH range of 6.0–11.0. The enzyme was activated by Ca^{2+} and Fe^{2+} ions, partially inhibited by Cu^{2+} ions and strongly inhibited by PMSF. High enzyme stability in presence of various detergents further strengthens enzyme applicability in industrial applications.

1. Introduction

Proteases (E.C.3.4.21) are one of the three largest groups of commercial enzymes accounting approximately up to two-third share of the worldwide industrial enzyme market [1]. Among proteases, alkaline proteases are of immense economic importance as they can withstand high temperature, pH, non-aqueous and denaturing conditions. They are widely used in laundry and dish washing detergent, cosmetics and food processing with expanding research applications in diagnostic reagents, peptide synthesis and synthetic organic chemistry [2]. The protease activity affected severely by various factors such as pH, temperature, surfactants, bleach systems, and mechanical handling that eventually determine its stability.

Alkaline proteases can be obtained from a wide range of sources such as animals, plants and microorganisms (bacteria, moulds, yeasts) as well as mammalian tissues. However, from a commercial perspective, high enzyme yield and economical downstream processing are highly desirable. To compensate the industrial demand for an alkaline protease possessing properties to overcome these challenges, there is a constant need to search for new enzyme sources. In this regard, microorganisms remain the preferred choice due to high yield of purified proteases and their wide range of biochemical and catalytic properties [3]. At present, a major proportion of commercially available alkaline proteases are derived from *Bacillus* strains, although the potential of using fungi as a promising alternative is now being progressively comprehended [3,4]. Fungi are an attractive source of proteases due to their inexpensive cultivation, ease of handling and suitability for genetic manipulation. Moreover, fungi are capable to grow on economical media and often secrete bulk quantities of enzymes which offer convenient downstream processing [5–7]. Filamentous fungi have received particular attention owing to their highly diverse biochemical and enzyme systems that empowers them to survive in extreme and diverse habitats. Among filamentous fungi, genus *Aspergillus* has been recognized as one of the dominant and economically important group of fungi. Previously, we reported an abundance of the genus *Aspergillus* in zinc metal rich regions of

Abbreviations: BCA, bicinchoninic acid; CBB, coomassie brilliant blue; DEAE cellulose, diethylaminoethyl cellulose; EDTA, ethylene diamine tetraacetic acid; GRAVY, grand average of hydropathy; MALDI–TOF-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; NCBI, National Centre for Biological Information; PAGE, polyacrylamide gel electrophoresis; PMSF, phenyl methyl sulfonyl fluoride; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulphate; TCA, trichloro acetic acid.

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Zawar mines, Udaipur, India [8]. The study was well supported by other reports demonstrating remarkable ability of filamentous fungi to bind metallic elements [9–11]. The binding of metal ions can be achieved by various processes ranging from physicochemical interactions, such as absorption or adsorption of metals and their chelation by extra- or intracellular proteins; regulating metal uptake and/or efflux by intracellular sequestration and compartmentalization [12,13]. In particular, secreted proteins play a vital role in extracellular co-precipitation of metals; a common phenomenon to tackle the high metal concentration among filamentous fungi. In the present investigation, screening of fungi from naturally occurring metal rich regions was performed with a viewpoint to recognize new isolates capable of producing active and stable secreted proteins (enzymes) in extreme conditions. The present study demonstrates the ability of a metal-tolerant fungus *Aspergillus flavus* NJP08 isolated from zinc mines of India to secrete enormous amount of proteins, especially proteases.

2. Materials and methods

2.1. Fungal isolates and culture conditions

In the present study, all the fungal isolates were isolated from rhizosphere soil samples of zinc metal rich regions of the Zawar mines, Udaipur, India (24°21′ N, 73°44′ E). Briefly, rhizospheric soil samples were collected from naturally grown plants (*Calotropis procera* and *Tephrosia purpurea*) and isolation of fungi was carried out on Martin Rose Bengal Agar medium (HiMedia, Mumbai, India, pH 7.2) after serial dilutions of pooled soil samples. The complete details of isolation and identification methods can be obtained from our previous report [8]. The selected fungus *A. flavus* NJP08 has been deposited with the MTCC, Institute of Microbial Technology, Chandigarh, India (NCBI Accession No. 10829).

2.2. Preparation of fungal cell free filtrate

The stock culture was inoculated in 100 mL of MGYP medium (0.3% malt extract, 1% glucose, 0.3% yeast extract, 0.5% peptone; pH 7.0) in 250 mL Erlenmeyer flasks and incubated at 28 °C on an orbital shaker (150 rpm) under dark conditions. After 72 h, fungal mycelia were separated by centrifugation (8000 rpm, 10 min and 4 °C) and washed thrice with sterile water to remove all traces of media. Typically, 10 g fungal biomass (fresh weight) was suspended in Erlenmeyer flasks containing 100 mL of sterile de-ionized water and further incubated for 72 h under darkness. After incubation, the biomass was separated by filtration using Whatman filter paper no. 1 and the fungal cell free filtrate containing extracellular secretion was collected to evaluate extracellular protease activity and stored at 4 °C until further use.

2.3. Protease assay

Protease assay was carried out using azocasein as a substrate to quantitate the alkaline protease in fungal cell free filtrate, and also throughout the course of enzyme purification [14]. Briefly, the reaction mixture was prepared by mixing 400 μl of azocasein (5 mg mL^{-1} in 0.1 M Tris–HCl buffer, pH 8.0) and 100 μl of enzyme solution, followed by incubation at 37 °C for 20 min. The reaction was terminated by adding 500 μl of 20% (w/v) TCA, mixed thoroughly, and incubated at 37 °C for 20 min. The tubes were centrifuged at 10,000 rpm for 5 min at ambient temperature to separate unutilized azocasein. Supernatant (800 μl) was collected and diluted with 1 N sodium hydroxide solution in a 1:1 ratio and absorbance was measured at 440 nm using a JASCO V-630 UV–Visible spectrophotometer. Control experiments were executed in an identical manner except that the enzyme was added after the addition of TCA reagent. One unit of specific activity was expressed as micrograms of azocasein hydrolysed per hour per milligram of protein.

2.4. Protein precipitation and estimation

The complete precipitation of protein was achieved by gradual addition of solid ammonium sulphate to a final concentration of 80% (w/v) to crude fungal cell-free filtrate containing extracellular proteins. The mixture was gently stirred overnight at 4 °C and the resulting precipitate was collected after centrifugation at 12,000 rpm for 10 min at 4 °C. The protein pellet obtained thereafter was suspended in copious amount of sterile Milli-Q water and placed in a dialysis bag (12 kDa cut off) made up of cellulose acetate membrane. The bag was immersed in 50 mM phosphate buffer (pH 7.2) and dialyzed gently for 24 h at 4 °C by repeatedly changing the buffer. The dialysate was concentrated with Amicon centrifugal filter units and stored at −70 °C in aliquots.

The concentration of protein was determined using BCA method as per the manufacturer's protocol using bovine serum albumin as standard [15]. One hundred μL of sample was added to 2 mL of BCA reagent and incubated at 37 °C for 10 min. Absorbance was measured at 562 nm on a Jasco V-630 UV–visible spectrophotometer and protein concentration was determined by comparison to a standard curve.

2.5. Protein purification

Protease purification was carried out by following steps. The concentrated protein solution obtained after dialysis step was subjected to gel filtration chromatography on a Sephadex G-75 column (1 cm × 50 cm) equilibrated with 50 mM Tris-HCl (pH 8.0) buffer. Fractions of 10 mL were collected at a flow rate of 30 mL h^{-1} with the same buffer and analyzed for protein content (280 nm Abs) and protease activity. Fractions showing protease activity were subjected to anion exchange chromatography on a DEAE cellulose column (1.5 cm × 20 cm) equilibrated with 50 mM Tris–HCl buffer (pH 8.0). After washing the column with equilibration buffer, elution was carried out with a linear gradient of potassium chloride in the range of 0–0.5 M in the same buffer. Fractions (2 mL) were collected at a continuous flow rate of 20 mL h^{-1} and analyzed for protein concentration (280 nm Abs) and protease activity. Fractions showing protease activity were collected and concentrated by lyophilization after desalting. All the purification steps were conducted at temperatures not exceeding 4 °C.

2.6. SDS-polyacrylamide gel electrophoresis

SDS-PAGE was carried out to determine the molecular weight of enzyme as described by Laemmli [16]. A combination of 12% separation gel and 5% stacking gel with Tris-glycine running buffer (pH 8.3) was used for polyacrylamide gel electrophoresis. After electrophoresis, gels were stained with 0.25% Coomassie brilliant blue (CBB) R-250 in 45% methanol–10% acetic acid for 3 h and de-stained overnight with 45% methanol–10% acetic acid solution.

2.7. Native PAGE and zymogram

Native PAGE was carried out in conjunction with SDS-PAGE, except that the sample was not heated and SDS (reducing agent) was left out. Protease zymogram was also developed with adequate modifications as per the protocol of Leber and Balkwill [17]. Briefly, after electrophoresis, the gel was submerged in 100 mM glycine–NaOH buffer (pH 10.0) containing 2.5% (v/v) Triton X-100 for 30 min at 4 °C with agitation. Subsequently, the gel was washed