Detection and Characterization of New Thermostable Endoglucanase from *Aspergillus awamori* Strain F 18

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Abstract

Aspergillus awamori although an efficient producer of many hydrolytic enzymes, has not been exploited commercially for enzyme production. An extracellular endoglucanase (EG) from culture filtrates of *Aspergillus awamori* strain F 18 was 12 fold purified by ammonium sulphate precipitation followed by DEAE-anion exchange chromatography. The purified enzyme had an optimum pH of 4.0, temperature tolerance of 60C and exhibited a K_M value of 17.24 mg/ml and V_{max} of 28.8 µg glucose/min/IU. This novel enzyme was thermostable retaining 65% of activity after incubation at 60C for 2 h. SDS-polyacrylamide gel electrophoresis revealed the monomeric enzyme to have a molecular weight of about 43 kDa. The purified monocomponent enzyme characterized in this study had low k_M value and showed activity over a broad range of pH (4-9) and good thermostability. This newly detected endoglucanase has indicated its potential for commercial use in industries.

Key words: *Aspergillus awamori,* Endoglucanase , paddy straw, solid state fermentation

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The utilization of cellulosic biomass is a subject of worldwide interest in view of the rapid depletion of oil reserves and growing food shortage (Gong et al 1999). The conversion of cellulosic mass to fermentable sugars through biocatalysts such as cellulases, derived from cellulolytic microorganisms has been suggested as a feasible process and this offers potential to reduce the use of fossil fuels. (Dale 1999; Lynd et al 1999). Lignocellulose is the most abundant renewable natural product in the biosphere. Annual production of cellulose is estimated to be 10^{10} tonnes/year (Singh and Hayashi 1995). Successful utilization of cellulosic materials as renewable carbon sources is dependent on the development of economically feasible processing technologies for enzymatic hydrolysis of cellulosic materials to low molecular weight products such as hexoses and pentoses. This involved development of technologies for cellulase production.

A cellulasic enzyme system consists of three major components: endo-ß-glucanase (EC 3.2.1.4), exo-ß-glucanase (EC 3.2.1.91) and ß-glucosidase (EC 3.2.1.21) (Bhat 2000). Endo-ß-glucanase or 1,4-ß-D-glucan glucanohydrolase, commonly known as carboxymethylcellulase (CMCase) and denoted as Cx is involved in the random scission of cellulose chains yielding glucose and cello-oligosaccharides, exo- ß - glucanase or 1,4- ß - D-glucan cellobiohydrolase, commonly known as filterpaperase (FPase) and denoted as C1 is involved in the exo-attack from the non-reducing end of cellulose with cellobiose as the primary end product. The hydrolysis of cellobiose to glucose is carried out by ß –glucosidase. Among the three major components of the cellulasic enzyme system, endoglucanase is considered to be the most important component, since its action leads to the formation of non-reducing ends necessary for the action of FPase and cellobiase (Eriksson and Wood 1985). In view of the potential use of this endoglucanase in cellulose waste recycling and many other related industries, considerable attention has been focused on bulk production of endoglucanase..

Endoglucanses are produced by a wide array of microorganisms viz., bacteria, fungi, yeasts, actinomycetes, algae and myxobacteria (Tengerdy and Szakacs 2003; Krishna 2005). The filamentous fungi are of particular importance, because of the relative ease and cost-effectiveness of production (Wood and McCrae 1982; Oliveira 2006). Although, *Aspergillus awamori* has been shown to be an efficient producer of hydrolytic enzymes (Botella et al 2005) not much work has been made to characterize these enzymes which in turn can help into devise the proper technologies for

industrial exploitation of endoglucanase. Therefore, the purpose of this study was to purify the endoglucanase from *A. awamori* F 18 characterize the physicochemical characteristics, including molecular weight, optimum pH and temperature, K_M and V*max* and analyze the stability of the enzyme over a range of temperatures and pH.

Materials and Methods

Inoculum preparation. *Aspergillus awamori* F 18, a soil isolate from Microbiology divisional collection was grown on potato dextrose agar medium for 5 d at 30 C. A spore suspension of 2 x 10^6 spores/ml was prepared using sterilized distilled water containing Tween 80 (1%) used as inoculum.

Substrate for solid state fermentation. Paddy straw obtained from Indian Agricultural Research Institute farm was washed, dried at 105 C for 24 h and used as substrate. The physiochemical properties of the straw were evaluated before use.

Enzyme production. Solid state fermentation was carried out in 250 ml Erlenmeyer flasks containing 5.0 g of paddy straw and 15 ml Reese's mineral medium (Reese and Mandel 1963). The flasks were sterilized at 15 psi for 30 min for two consecutive days and inoculated with 2 ml of fungal spores. Following incubation for 5 d, 100 ml of citrate buffer (0.05 M, pH 4.8) was added to the flasks and kept under mild stirring (120 rpm) for 1 h. The slurry was filtered through muslin cloth, followed by Whatman filter paper No. 1. The crude extract was used for estimation of endoglucanase enzyme activity.

Enzyme and other assays. Endoglucanase activity of cell free culture filtrates was estimated by the method described by Ghose et al (1983) using carboxymethyl cellulose 2% (w/v) dissolved in citrate buffer (0.05 M) of pH 4.8 as a substrate. One IU of activity on the substrate was defined as µ mole of glucose released per min at 50 C after 30 min using a glucose standard curve. Total soluble protein and reducing sugars were estimated by the method described by Lowry et al (1951) and Miller (1959), respectively.

Enzyme purification. All operations were done at 0–4 C. After solid state fermentation, the crude filtrate was centrifuged at 12000 rpm for 15 min to remove any suspended material and supernatant was subjected to ammonium sulphate precipitation in two steps - 0-30% saturation and 30-90% saturation. The proteins precipitated at 30-90% saturation which exhibited a max activity was suspended in citrate buffer pH 5.5 and dialyzed against the same buffer at 4 C for 24 h. The enzyme solution from the first step was applied to a DEAE cellulose column (1.5 x 12 cm) that had been equilibrated with 20 mM NaCl citrate buffer (pH 5.5). After the column was washed thoroughly with the buffer, a linear gradient elution (with a flow rate of 30 ml/h) was made with the buffer containing NaCl added @ 0 to 1.5 M. Fractions of 3 ml each were collected and those active fractions with endoglucanase were pooled, concentrated by dialysis and then dissolved with citrate buffer (0.05 M) of pH 4.8.

Activity staining by native PAGE. Endoglucanase activity at various purification steps was detected by developing the zymogram. Caboxymethyl cellulose (Low viscosity) 0.2 % was incorporated in the resolving gel (native PAGE) solution to detect the endoglucanase in gel. Upon completion of electrophoresis, the gel was stained with 0.2% (w/v) Congo red dye and checked for clear bands against a dark background.

Enzyme characterization. *Determination of optimum pH and pH stability of enzyme.* The optimum pH of the cellulase was determined by measuring its activity after incubation at different pH (3-10) of the reaction mixtures for 30 min. The various buffers used included 50 mM citrate buffer (pH 3-6), sodium phosphate buffer (pH 7-8) and glycine-NaOH buffer (pH 8.5 to 10). For the determination of pH stability of the enzyme, pretreatment in buffers of 4 and 5 pH was done at 50 C, 60 C for 2 h respectively and was followed by measuring the residual endoglucanase activity under standard assay conditions.

Determination of optimum temp and thermal stability of enzyme. The optimum temp of the enzyme was determined by incubating the reaction mixture at 30-80 C for 30 min. The temperature stability of the enzyme was detected by incubating enzyme at 50-70 C at 60 and 120 min. The samples were removed periodically and assayed for residual endoglucanase activity under standard assay conditions.

Determination of molecular weight of purified enzyme. The molecular weight of the enzyme was determined by SDS-PAGE following the method of Laemmli (1970), using a separating gel of 10% (w/v) acrylamide. Phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa) and carbonic anhydrase (31 kDa) were used as standard markers.

Michaelis constant (K_M) and maximal velocity (V_{max}) determination. The purified enzyme was incubated with substrate (CMC) conc and the reducing sugar produced was measured colorimetrically at 575 nm with DNSA reagent. The V_{max} and K_m were calculated from double-reciprocal plots according to the method of Lineweaver and Burk (1934).

Table 1. Chracteristics of the endoglucanase enzyme from *Aspergillus awamori* strain F 18 at different steps of purification

Step	Volume (ml)	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Purification Fold	Yield (%)
Crude Extract	300	150	180.3	0.83	1	100
$(NH4)_2SO_4$ precipitation	15	105	63	1.67	2	70
DEAE-anion exchange chromatography	2	25	1.2	20.83	12.5	23.8

Results and Discussion

The biological breakdown of cellulose is catalyzed by a complex enzymatic system, involving the concerted action of three enzymes, among which endoglucanase constitutes a key enzyme. In the current investigation, an attempt was made to purify and characterize the endoglucanase enzyme produced by *Aspergillus awamori* strain F-18, as a prelude to its potential utilization in industry.

The substrate used i.e. rice straw, for the crude enzyme production had an organic carbon 48%, total nitrogen 0.5%, cellulose 41.3%, hemicellulose 21.4% and 12.1% lignin. Rice straw, being a recalcitrant plant material, is thereby considered a waste; however it has been shown to be a promising substrate for production of different hydrolytic enzyme via solid state fermentation as the cellulose component is three-folds higher than lignin (Sinegani et al 2005; Jatinder et al 2006; Mishra et al 2007). *A. awamori* has been widely used in the production of such enzymes *via* SSF on other solid supports (Koutinas et al 2001; Botella et al 2005).

Purification of endoglucanase. The purification scheme for endoglucanase included three steps (Table 1). The data indicated that the purified endoglucanase showed a 12.5fold higher specific activity with 23.8% overall yields, than crude enzyme. Jatinder et al (2006) have detected two isoforms of endoglucanase in the zymogram of *Melanocarpus* sp. produced by solid state fermentation of rice straw and wheat straw.

Ammonium sulphate precipitation has been widely used for the concentration of the protein from dilute samples. Peshin and Mathur (1999) recovered 40% β-glucosidase activity at 60-90% saturation from crude extract using ammonium sulphate precipitation method. Similarly Usama and hala (2008) recovered 79% of endoglucanase activity using ammonium sulphate precipitation (70% saturation) from crude filtrate *Aspergillus niger* grown on water hyacinth under soild state fermentation. In our study, we recovered 70 % endoglucanase yield with 2fold purification.

Concentrated enzyme fractionated on DEAE-Cellulose (Fig. 1), followed by elution of the fractions (32-40) with 1.5 M NaCl citrate buffer (pH 5.5) from third peak revealed significant endoglucanase activity (20.83 IU/mg) with a low yield of 23.8%. This may be because of the acidic nature of the endoglucanase which resulted in strong electrostatic interactions between the enzyme and matrix. Similar results were obtained by Bakare et al (2005) who used DEAE- Sephadex anion exchange chromatography to purify the cellulase from *Pseudomonas fluorescens*. A single band on the zymogram (Fig. 2a) and on SDS PAGE (Fig. 2b) confirmed the presence of only a single endoglucanase that was free of contaminating β-glucosidase, and or xylanase activity in the purified product.

Optimum pH and pH stability of enzyme. The purified endoglucanase was active in a broad pH range of 3 to 9 with pH optima 4.0 at 50 C (Fig. 3 a) which is in concurrence with the observations on other microbial cellulases reported by different workers (Heikinheimo and Buchert 2001; Fowler et al 2001). The enzyme retained 63% of its activity at pH 8.0 indicating its potential for commercial use. Kang and Rhee (1995) also reported alkaline active carboxymethyl cellulases from a *Cephalosporium* strain. In our study, the endoglucanase was quite stable and retained around 70% activity even after incubation for 2h at optimum pH 4.0 (Fig. 3 b).

Optimum temp and thermal stability of enzyme. Our studies on the thermal stability revealed the novelty of the enzyme, as it was found to be more heat stable compared to other reported *Aspergillus* endoglucanases (Peshin and Mathur 1999; Hurst et al 1977) as the heat stability curves of endoglucanase (Fig. 3 c) revealed that the enzyme retained 63% and 61% of its activity even after 2h of incubation at 50 C and 60 C, respectively (Fig. 3 d).

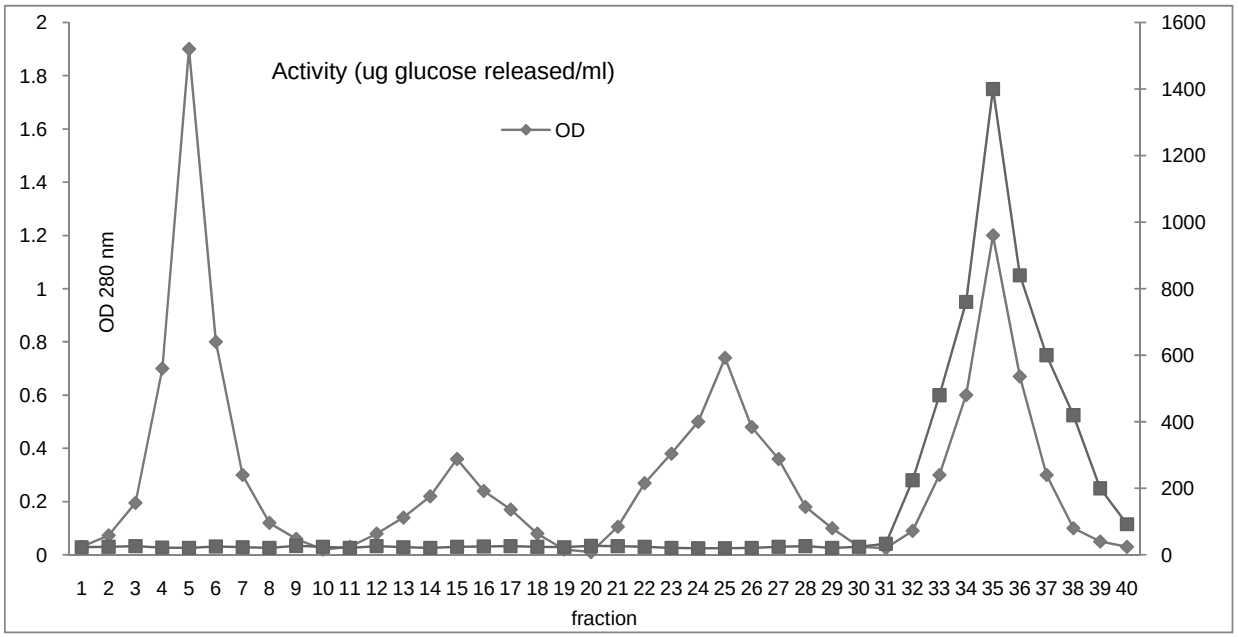


Fig. 1 Purification of endoglucanase by DEAE-Anion exchange chromatography

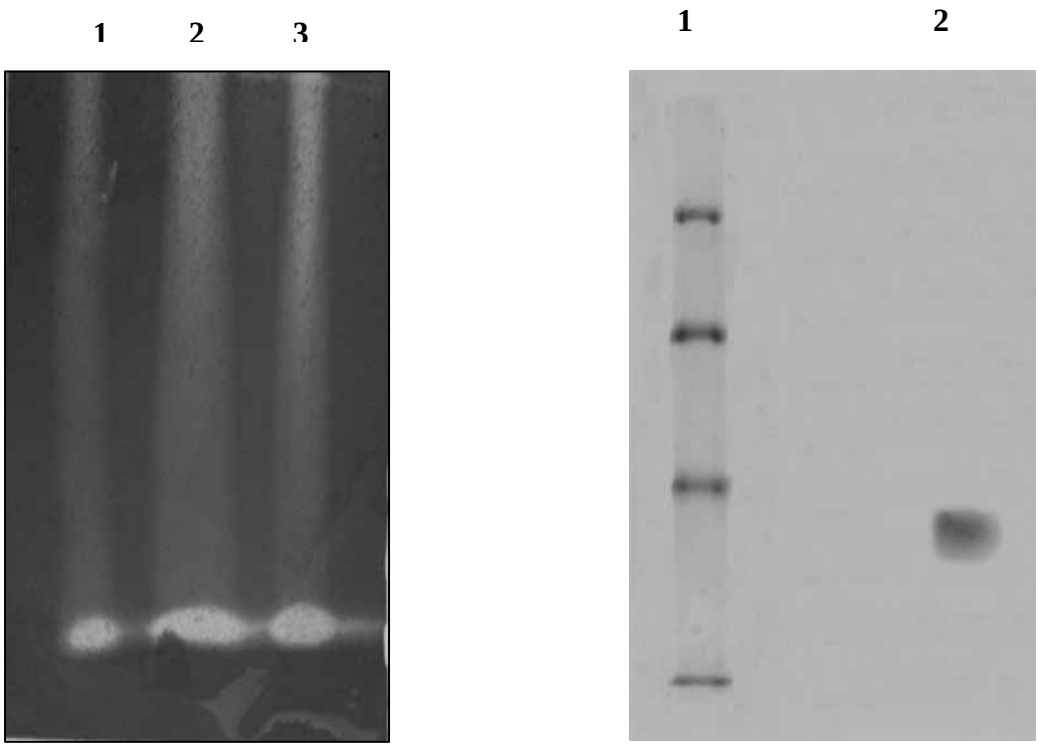
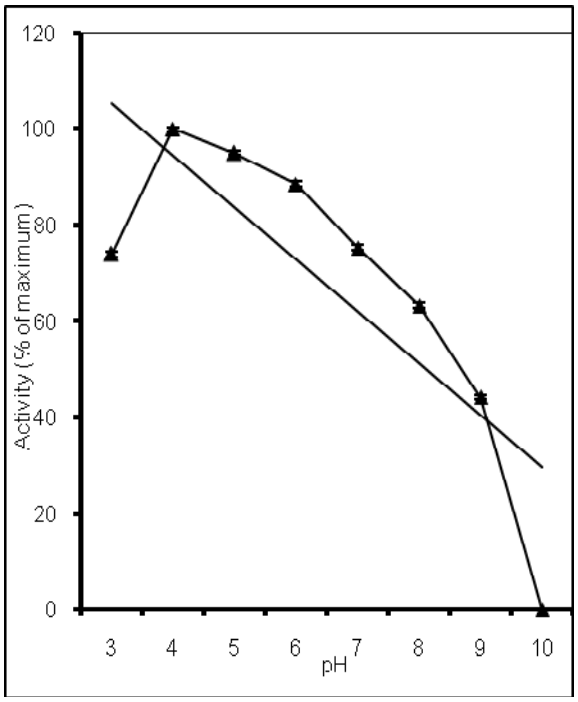
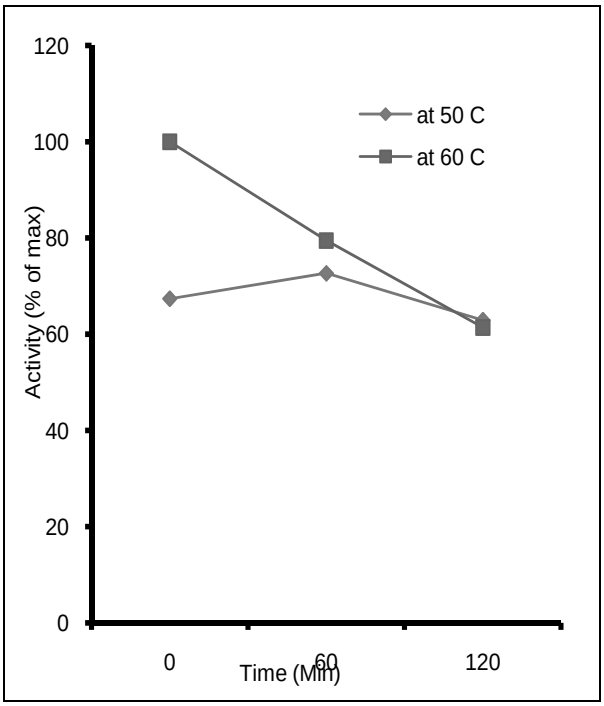
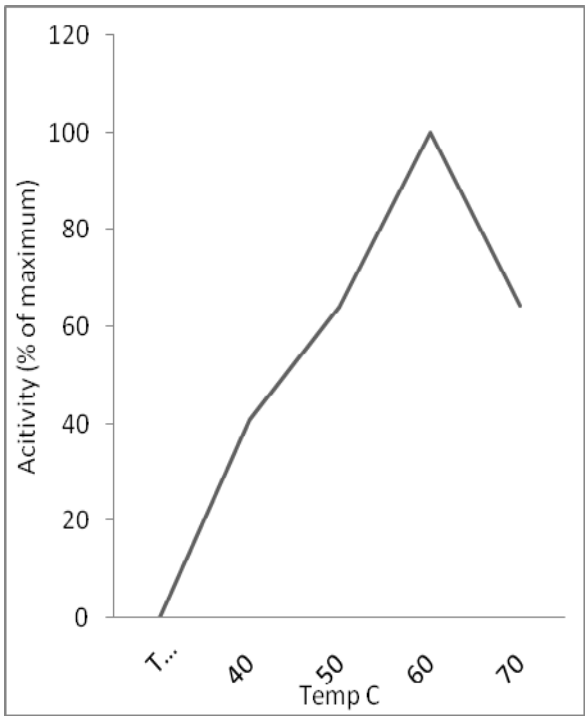


Fig. 2a (left) = Zymogram of endoglucanase of *Aspergillus awamori* strain F 18: lane 1 = conc crude extract; lane 2: ammonium sulphate precipitated 30-90 % fraction; lane 3: purified endoglucanase
b right = Molecular weight determination of endoglucanase by SDS- PAGE: lane 1 - standard proteins (top to bottom) phosphorylase (97.4 kd); albumin (66 kd); ovalbumin (45 kd); and carbonic anhydrase (30kd); lane 2 = purified endoglucanase







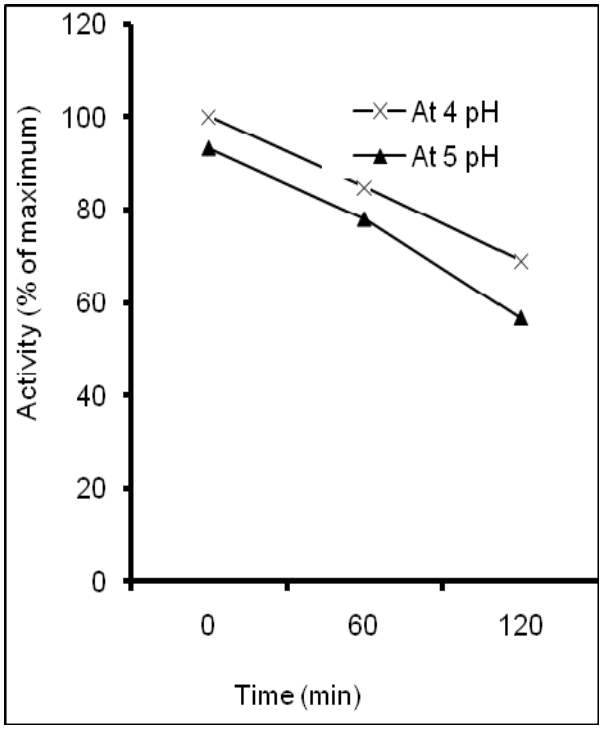


Fig. 3a-d. Determination of different properties of endoglucanase

The present investigation illustrates the promise of the newly detected endoglucanase produced by *A. awamori* which is active over broad range of pH (4-9), thermostable. Such enzymes have potential for commercial use in detergent and textile industry where mono-component enzyme formulations of fungal endoglucanases are the preferred choice because they are required in low quantities and do not show action over crystalline regions of cotton fibres (Schülein M 1997).

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