

Characterization of an Extracellular Cellulose-Hydrolyzing Enzyme Complex from a Thermotolerant Strain of *Aspergillus* sp.

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Abstract *Aspergillus* sp. CX-1 strain grown on microcrystalline cellulose resulted in the accumulation of high levels of cellulase and xylanase activities that were higher by two to four folds than those from the conventional commercial producer, *Trichoderma reesei* QM9414. *Aspergillus* sp. CX-1 demonstrated greater thermostability and better catalytic characteristics of total cellulase activity (FPase) as compared to *T. reesei* and *Aspergillus niger* F-2039.

Key words: Cellulase, xylanase, fungi, *Aspergillus*

Filamentous fungi that secrete large amounts of hydrolytic enzymes have considerable importance in the carbon turnover in nature and have attracted a great deal of attention due to their industrial applications [2, 5]. Cellulases and xylanases have great potential in textile, food, alcohol, pulp and paper industries, animal feed, and fuel production [12, 20].

The most costly step for enzymatic hydrolysis of insoluble natural wastes is in the actual production of microbial enzymes. To make biomass hydrolysis processes economically applicable and less expensive, highly efficient fungal strains, that can produce high extracellular concentrations of enzymes with good physicochemical and catalytic characteristics in short periods of time, are needed [9, 12]. Thus, the biotechnology industry has developed an increasing appetite for novel microorganisms which are useful for a variety of purposes including as sources of enzymes [6].

The physical heterogeneity of a lignocellulosic substrate, and the complexity of a multi-component cellulose and hemicellulose hydrolyzing enzyme system present a

formidable problem to investigators. Crystalline cellulose can be degraded by three main types of enzymes: 1) exo-1,4- β -D-glucanase (FPase, EC 3.2.1.91); 2) endo-1,4- β -D-glucanase (CMCase, EC 3.2.1.4); and 3) β -glucosidase (cellobiase, EC 3.2.1.21) [2, 13, 21]. In addition, four enzymes are required for the hydrolysis of hemicellulose; among these enzymes, 1,4- β -xylanase (EC 3.2.1.8) appears to be the dominant enzyme [5]. Thus, all the components are coordinately expressed and obviously have a common regulatory mechanism [8]. However, comparison of data from different laboratories remains difficult due to a wide range of methods used for measuring cellulase activity and to various culture conditions used [3, 17].

Taking these factors into account, the characteristics of different cellulases and xylanases from the new strain of *Aspergillus* sp. CX-1 were compared with those of the conventional commercial producers of cellulolytic enzymes, *Trichoderma reesei* and *Aspergillus niger*, in a parallel experiment using the same carbon and nitrogen sources and the same enzyme assay methods recommended by the Commission on Biotechnology (IUPAC) [7, 10].

This paper summarizes the characterization of extracellular hydrolases; cellulase and xylanase produced by the new filamentous fungus isolate, *Aspergillus* sp. CX-1.

A filamentous fungus strain exhibiting cellulolytic and xylanolytic activities was isolated from soil near a hot spring in the Ashkhabad city (Turkmenistan) area. The fungus was partly identified as *Aspergillus* sp. (strain CX1). Cellulolytic fungi, *T. reesei* QM9414 and *A. niger* F-2039, were obtained from the All-Russia Culture Collection, Moscow, Russia. The strains were cultured on the agar Mandels medium, supplemented with 0.1% peptone, 0.2% Tween 80, and strips of filter paper [13]. The conidia inoculums of all the fungal strains were pregrown on a liquid Mandels' medium with 0.5% glucose. The fungi were cultured in Erlenmeyer flasks

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filled with 10% of their volume under shaking condition (200 rpm) for 20 h, at 41°C for *Aspergillus* sp. and 28°C for *T. reesei* and *A. niger*. For cellulase and xylanase production, the mycelia were used further to inoculate the above media containing 2% (w/v) microcrystalline cellulose (MCC, Avicel, American Viscose Co., U.S.A.) as a sole carbon source. Incubation was carried out in batch conditions for 3–5 days.

Supernatants from batch cultures of all the cellulolytic fungal strains used in this study were separated using filtration and centrifugation, and then divided into four fractions for exo-1,4- β -D-glucanase, endo-1,4- β -D-glucanase, β -glucosidase, and 1,4- β -D-xylan xylanohydrolase assays. All the enzymes were assayed by measuring the rate at which they produced reducing sugars from their respective substrates. The activities of exo-1,4- β -D-glucanase towards Whatman No. 1 filter paper (50 mg), endo-1,4- β -glucanase towards carboxymethyl cellulose (FMC Corporation, 10 mg), and β -glucosidase towards salicin (Sigma, 10 mg) were measured as described by Wood and Bhat [21]. Xylanase activity was determined as described elsewhere [11]. Enzyme and substrate were included in all assays. To measure the exo-1,4- β -D-glucanase activity (also known as FPase, total cellulase, or true cellulase activity), we used the method recommended by IUPAC [10]. One ml of 0.05 M citrate buffer, pH 4.8, was added to 0.5 ml of culture filtrate containing enzyme suitably diluted in the same buffer and a strip of Whatman No. 1 paper (1 \times 6 cm). The reaction mixtures were incubated at various temperatures (25–70°C) for 60 min, and then 3 ml of DNS reagent was added to stop the reaction, which were thereafter transferred to a boiling water bath for 5 min, and then to cold water. The color formed was read in a spectrophotometer at 540 nm. Enzyme (culture filtrates) blanks and glucose standards were treated exactly the same way.

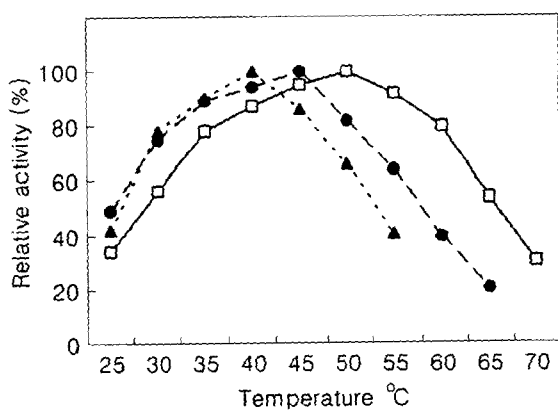


Fig. 1. Effect of temperature on exo-1,4- β -D-glucanase activity. Temperature profile of exo-1,4- β -D-glucanase (total cellulase, FPase) secreted by different fungi. The reaction mixtures containing culture filtrates were incubated at various temperatures (25–70°C) for 60 min. \square - \square *Aspergillus* sp. CX1; \triangle - \triangle *T. reesei* QM9414; \bullet - \bullet *A. niger* F-2039.

The unit (U) of each of cellulase complex enzyme and xylanase activity was defined as the amount of the enzyme needed to liberate 1 mole of reducing sugars per minute per milliliter of supernatant under appropriate conditions.

Initially, the temperature and pH optima for assaying the exo-1,4- β -D-glucanase (FPase) activities were examined in the culture liquids of the three different cellulolytic fungi grown in batch conditions (Figs. 1 and 2). The enzyme secreted by *Aspergillus* sp. CX1 had a temperature optimum of 50°C; however, *T. reesei* and *A. niger* have temperature optima at 40 and 45°C, respectively (Fig. 1). The temperature profiles for the cellulase activities of the three fungi coincided with the temperature optima for growth. The pH optimum of the enzyme activity of *Aspergillus* sp. CX1 was less acidic than those of *T. reesei* and *A. niger* (Fig. 2). The hydrolytic potentials of the three fungal strains were then compared to establish the maximal activities of exo-1,4- β -D-glucanase, endo-1,4- β -glucanase, β -glucosidase, and xylanase in culture filtrates after 3–4 days of batch cultivation with MCC as a carbon substrate. As shown in Table 1, the highest cellulolytic and xylanolytic activities among the fungal strains used were produced by *Aspergillus* sp. CX1. This fungus appeared to exhibit complete cellulase and xylanase activities, including high β -glucosidase and xylanase activities.

The amounts of different cellulases and xylanase produced by *Aspergillus* sp. CX1 (Table 1) were above the average, compared to the yields of other molds described in literature [1, 4, 15].

Aspergillus sp. CX-1 exhibited both high cellulase and xylanase activities on cellulose. It appeared that the same transcriptional activator regulates both xylanolytic and

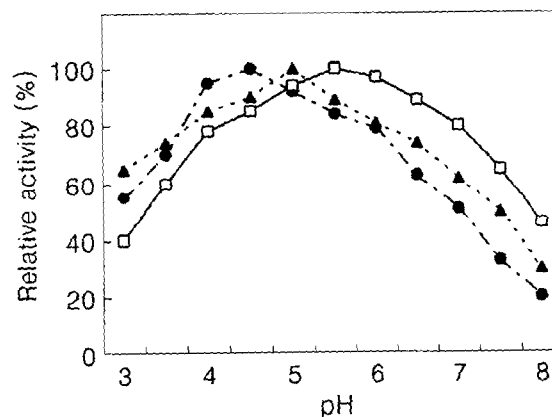


Fig. 2. Effect of pH on the exo-1,4- β -D-glucanase (total cellulase, FPase) activity of different fungi.

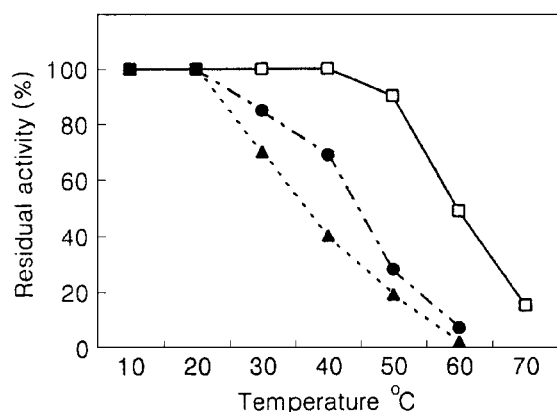
The reaction mixtures containing culture filtrates were incubated in acetate, citrate, phosphate, and borate buffers (0.05 M) in the pH range of 3.0–8.0 at the optimal temperature for each particular fungal cellulase. \square - \square *Aspergillus* sp. CX1; \triangle - \triangle *T. reesei* QM9414; \bullet - \bullet *A. niger* F-2039.

Table 1. A comparison of cellulase and xylanase activities in crude culture filtrates of several cellulolytic fungi¹.

Organism	Enzyme activities (U/ml) ²			
	Endo- β -glucanase	Exo- β -glucanase	β -Glucosidase	Xylanase
<i>Aspergillus</i> sp. CX1	26.4	0.48	3.96	19.2
<i>T. reesei</i> QM9414	9.9	0.12	0.97	4.3
<i>A. niger</i> F-2039	5.6	0.16	1.8	8.1

¹Cultures were grown for various times until maximum activities were detected in the culture filtrates. The activities of the four enzymes were measured in the same culture filtrate of the particular fungal strain.

²Activities were measured and calculated according to the recommendations of IUPAC [10].

**Fig. 3.** Temperature stability of exo-1,4- β -D-glucanase (total cellulase, FPase) secreted by different fungi.

The assaying of cellulase activities was performed at the optimal temperature for each particular fungal cellulase \square - \square *Aspergillus* sp. CX1; \blacktriangle - \blacktriangle *T. reesei* QM9414; \bullet - \bullet *A. niger* F-2039.

endoglucanase gene expression as recently shown in some other fungi [19].

To examine the thermal stability of the three fungal strains, the culture filtrates were preincubated at various temperatures, and samples were taken after 3 h to compare the residual activity. As shown in Fig. 3, the enzyme produced by *Aspergillus* sp. retained full activity up to 45°C; thereafter, the activity decreased sharply. In contrast, *T. reesei* and *A. niger* enzymes exhibited much less thermal stability.

Recent techniques developed in constructing new recombinant strains using genetic engineering methods have been frequently applied in biotechnology [4, 14, 16, 22]. Although these techniques are more expensive, there is a limitation for mass production and the viability is also often diminished. Hence, emphasis is still placed on isolating new fungal strains from nature with highly effective stable hydrolytic enzymes, and better physicochemical and catalytic properties than those currently available. It was shown elsewhere that the isolated strains of *Aspergillus* possess a very active multicomplex cellulase-xylanase system [18].

The data presented in this paper demonstrate that the new fungus strain, *Aspergillus* sp. CX1, secretes a complete

set of cellulase complexes and also xylanases with high activities, and its thermal stability is better than those from the more widely studied sources of extracellular cellulolytic enzymes, *T. reesei* and *A. niger* [1, 4, 15, 16, 19]. Accordingly, further studies are needed on this strain due to an economic point of view.

Strain development, optimization of culture conditions, and mode of cultivation can considerably increase cellulase yield as well as the stability and resistance of cellulolytic systems against chemical inhibitors [23].

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