Partial purification and characterization of a highly thermostable and pH stable endoglucanase from a newly isolated *Bacillus* strain M-9

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Cellulases to be employed for lignocellulose bioconversion for biofuel (ethanol) industry or for production of other specialty chemicals must be extremely robust to withstand harsh industrial conditions like extremes of temperature, pH, presence of inhibitory agents etc. In the present study the *Bacillus* strain M-9 isolated from decomposing rice bran produced significant amount of endoglucanase after 72 h of fermentation. Endoglucanase was partially purified by ammonium sulphate precipitation (20-50% saturation) and characterized for few properties. The endoglucanase showed activity over broad range of temperatures (30-100°C) with maximum activity at 60°C. The enzyme retained 50 and 39% of the maximum activity at 90 and 100°C, respectively. The enzyme was thoroughly stable at 50-70°C for 30 min-1 h. The endoglucanase showed activity over a wide range of pH (3-10) with maximum activity at pH 5. Detergents (SDS, Tween 80), metal ions (Zn$^{2+}$, Pb$^{2+}$) and EDTA caused little to moderate reduction in enzyme activity. The endoglucanase was purified to the extent of 9.06 folds by salt precipitation and DEAE-cellulose chromatography. Endoglucanase showed molecular weight of approximately 54 kDa as examined by SDS-PAGE. High thermostability and acid/alkalinstability of the endoglucanase reflect potential commercial significance of the enzyme.

**Keywords**: Endoglucanase, *Bacillus* strain M-9, Thermostable, Acid/alkalinstable

Cellulose represents the most abundant renewable natural product in the biosphere with an estimated annual production of $4.0 \times 10^7$ tons$^1$. The proportion of cellulose in plant tissues ranges from 20 to 45% of dry weight. Much of the cellulose in nature exists as waste paper$^1$ or as waste material from agriculture industry in the form of stalks, stems and husk$^2$. Cellulose is an unbranched glucose polymer, composed of anhydro-D-glucose units, which can be hydrolyzed by cellulolytic enzymes produced by bacteria and fungi. By means of chemical or bioconversion methods, it is possible to transform this insoluble polymer into glucose, an excellent substrate for industrial fermentation. Cellulose is frequently found in close association with other compounds, such as hemicellulose, lignin and other polysaccharides, which make its bioconversion more difficult$^3$. Potential of cellulose as an alternative energy source has stimulated research into bioconversion processes which hydrolyse cellulose to soluble sugars for feedstock in alcoholic fermentations and other industries for the production of specialty chemicals. A number of biomass conversion methods have been proposed *viz.*, acid hydrolysis, pyrolysis and enzymatic hydrolysis, but the latter being environmentally safe and can be performed at normal temperature and pH is the most preferred method$^4$. Three component cellulases are involved for complete degradation of cellulose: 1) endoglucanase (EC 3.2.1.4) cleaves the β-1,4- linkage in amorphous region of cellulose to yield long chain oligosaccharides, 2) cellobiohydrolase (EC 3.2.1.19) cuts in exo-manner on oligosaccharides to produce cellobiose, a dimer of glucose, and finally 3) β-glucosidase (EC 3.2.1.21) hydrolyses cellobiose to yield glucose. Cellulases have got very high biotechnological potential for applications in different industries, such as food, brewery, wine, pulp and paper, textile, detergent, feed and agriculture$^4$. Cellulases are used in cotton preparations, wool and dyeing treatment and in effluent treatment. Due to their vast applications and ever increasing demand, novel cellulases with better process suitability, high specific activity, and better specificity and stability are being discovered from new lineages of cellulolytic organisms. Different microorganisms including bacteria, yeast and fungi are capable of producing cellulases$^4$$^8$. Majority of studies on cellulase production have been focused on fungi with relatively lesser emphasis on bacterial sources for cellulase
production\textsuperscript{4,6-8}. Bacteria, due to their extremely high natural diversity have the capability to produce highly thermostable, alkalistable enzyme complement, and may serve as highly potent sources of industrially important enzymes\textsuperscript{4,6}. One of the prerequisite for the enzymes to be employed for industrial applications is that they must be robust enough and highly stable under hostile conditions of industrial processes like extremes of temperature and pH. For instance, for the successful application of cellulases in detergent industry, enzymes must have alkaline pH optima, similarly for lignocellulose transformation, in pulp and paper industry or in feed industry, highly thermostable cellulases with acid/alkalinstability are desirable. Many researchers have documented production of thermostable and alkalistable cellulases from different microorganisms\textsuperscript{4,6,8,10}. In this communication, the purification and characterization of highly thermostable and acid/alkalinstable endoglucanase from a newly isolated bacterium \textit{Bacillus} strain M-9 is reported.

**Experimental Procedure**

**Chemicals, media and media components**

All the chemicals, media and media components used were of analytical grade obtained from Sigma Chemicals Ltd, USA; HiMedia Laboratories Ltd.; Ranbaxy Fine Chemicals Ltd. India; Qualigens Fine Chemicals Ltd. India; and Merck and Co. Inc., USA.

**Isolation and screening for cellulytic bacteria**

A total of ten bacterial strains were isolated from decomposing rice bran samples collected from Rice Mills located in the R.S. Pura region of Jammu (J & K, India). For isolation of bacteria, 5-10 g sample was inoculated into nutrient broth (pH 8-11) and incubated at 45°C for 24-48 h. The enriched samples were plated on nutrient agar and isolated colonies were purified further on nutrient agar. All the cultures were maintained in the culture collection centre of the Fermentation Biotechnology Laboratory of Department of Biotechnology, University of Jammu, Jammu. For determining cellulase producing ability of the isolates, the bacterial strains were streaked on CYPE-agar (carboxymethyl cellulose or CMC 1.0, peptone 0.5, yeast extract 0.5, K\textsubscript{2}HPO\textsubscript{4} 0.1, MgSO\textsubscript{4} 0.02 andNaCl 0.5%; pH 9-10) and plates were incubated at 37°C for 24 h. Then the bacterial colonies were subjected to congo red staining for determining their cellulase producing ability as described earlier\textsuperscript{11}.

**Endoglucanase production, assay and total protein estimation**

Overnight grown culture of bacteria was inoculated into CYPE broth which had same composition as that of CYPE-agar excluding agar. Submerged fermentation for cultivation of the organism and hence enzyme production was carried out in 250 mL of CYPE broth contained in Erlenmeyer flask of 500 mL capacity, under shaking conditions (200 rpm) at 45°C (Innova, New Brunswick, USA). The enzyme activity was assayed after different time intervals. Crude enzyme was obtained by centrifugation (Sigma, 3K 30) of a suitable volume of fermentation broth at 10,000 × g for 5 min at 4°C and the supernatant was considered equivalent to crude enzyme and was used for assaying endoglucanase activity after appropriate dilution.

Endoglucanase activity was assayed by using 0.5% (w/v) CMC as the substrate (in tris buffer 50 mM, pH 8) and measuring the release of reducing sugars at 50°C by dinitrosalicyclic acid method\textsuperscript{12} using a calibration curve for D-glucose. One unit of endoglucanase activity was defined as the amount of enzyme which produces one µmol of reducing sugar (glucose equivalent) per min under assay conditions. Protein content in the supernatant was estimated by the method of Lowry \textit{et al.}\textsuperscript{13} using bovine serum albumin (BSA) fraction V as standard.

**Partial purification of endoglucanase**

Crude enzyme preparation obtained after cultivation of the organism under submerged fermentation in shake flask was subjected to ammonium sulphate precipitation to obtain different saturation levels (20-100%). Then each fraction was tested for protein content and endoglucanase activity. The fractions which showed significantly high activity were pooled and dialysed. For dialysis the enzyme preparation was filled in dialysis bag and suspended in the same buffer in which the ammonium sulphate precipitated enzyme was dissolved. Dialysis was done for 12 h and the buffer out side the dialysis bag was replaced with the fresh buffer after every 3 h. Then the dialyzed preparation was used for characterization of endoglucanase for some properties.

**Influence of temperature and pH on endoglucanase activity**

Enzyme assay was carried out at different temperatures (30-100°C) to determine the effect of temperature on activity of endoglucanase. For determining the effect of pH on enzyme activity the pH of enzyme assay mixture was varied from 3 to 10
using citrate, acetate, phosphate, tris and carbonate-bicarbonate buffers (50 mM).

**Thermostability and pH stability of the endoglucanase**

Thermostability of endoglucanase was determined by pre-incubating the enzyme preparation at 30-90°C for different time periods, and then assaying the residual activity. For determining pH stability, enzyme was pre-incubated with buffers of varying pH (4-9) for different time periods and residual activity was analyzed.

**Ion-exchange chromatography, SDS-PAGE and molecular weight of endoglucanase**

Dialysed enzyme preparation obtained after ammonium sulphate precipitation was subjected to ion exchange chromatography using DEAE–Cellulose column (20 × 1 cm). The dialyzed enzyme preparation was loaded on DEAE-Cellulose column which was pre-equilibrated with acetate buffer (50 mM, pH 5.5). The column was washed first with equilibration buffer, and then bound proteins were eluted using linear gradient of 0.1-1.0 M NaCl (in acetate buffer) at a flow rate of 1 mL per min. The fractions (2.5 mL each) were collected and assayed for endoglucanase activity and those showing high activity were pooled, and used for SDS-PAGE analysis. Resolving gel consisted of 12.5% polyacrylamide in Tris-HCl (1.5 M, pH 8.8), while stacking gel consisted of 4.5% polyacrylamide in Tris-HCl (1.0 M, pH 6.8).

**Results and Discussion**

**Time course of endoglucanase production**

Of the ten bacterial strains tested for cellulase producing ability, five were found to be positive for cellulase production on CYPE-agar plates. Among the five cellulase producing isolates, bacterial isolate M-9 showed maximum activity and this isolate was identified by morphological, biochemical and physiological examination and was ascribed to genus *Bacillus* and designated as *Bacillus* strain M-9. Highest activity (3720 IU/L) was observed after 72 h of submerged fermentation; significantly high enzyme activity was present even after 24-48 h of fermentation (2750-3100 IU/L), however, after 72 h activity got slightly decreased (2930 IU/L) as shown in Fig. 1.

There are many reports of cellulase production by bacterial species isolated from various natural sources. Optimum time for maximum enzyme production by different bacterial sp. has been reported to be quite variable. Different *Streptomyces* spp. have been reported to produce maximum cellulase after 72-120 h of fermentation. However, *Bacillus subtilis* produced maximum cellulase after 10 h of fermentation. Maximum enzyme production stage of the organism largely depends upon the type of microbial strains and their genetic make up, and on cultural and environmental conditions employed during growth of the organism.

**Partial purification of endoglucanase**

Ammonium sulphate precipitation of crude enzyme resulted in maximum activity at 20-50% ammonium sulphate saturation. Precipitated protein was dissolved in small quantity of acetate buffer (50 mM, pH 5.5) and dialyzed, and the dialyzed preparation was used for studying some properties of enzyme. Ammonium sulphate at saturation levels of 40-60% has been used for cellulases or xylanases purification from different microbial sources. However, *Pseudomonas fluorescens* cellulase was purified at 90% ammonium sulphate saturation.

**Effect of temperature on endoglucanase activity**

Partially purified enzyme preparation showed activity over a broad range of temperature (30-100°C) with the maximum activity at 60°C (2970 IU/L). Presence of significant activity even at elevated temperatures of 100°C (approx. 39% of the maximum) and 90°C (about 50% of the maximum) indicates that enzyme may be of great commercial value (Fig. 2). Industrial processes are generally carried out at elevated temperatures, and enzymes being fragile molecules are mostly unstable under such conditions, therefore, enzymes with high temperature optima are desired for industrial applications. Thermostability profile of the enzyme showed that enzyme was thoroughly stable at 60°C;
on either side of this point lowered stability was displayed by the enzyme, but nonetheless sufficient activity was present even when the enzyme was pre-incubated at 80-90°C for 30 min - 1 h (Fig. 3). This further indicates that enzyme might comply with the industrial process requirements as it possessed prolonged stability under high temperature which is the desired feature for industrial applications.

Generally, microbial cellulases from different sources have been found to have temperature optima of about 35-50°C\(^\circ\)\(^1,2,16,22\). However, *Bacillus licheniformis* cellulase was found to be moderately thermostable with optimum activity at 65°C, and retained 90% of the original activity\(^6\) for one hour at 60°C. *Streptomyces* sp. CMCase showed varying degree of stability\(^1,10,16\) at 50-70°C, at pH 10 over 1-3 h. Microbial cellulases with varying thermostability (50-70°C) for different time periods have been documented\(^2,20,23\). For industrial applications, highly thermotolerant enzymes are required, for which either the natural microflora may be screened or the enzyme may be tailored by protein engineering so that it can withstand and work at elevated temperatures during process conditions\(^10\).

Effect of pH on endoglucanase activity

Activity assay of endoglucanase was done in reaction mixture at varying pH by using appropriate buffers. It was found that enzyme has got activity over a broad range of pH (Fig. 4). Maximum activity was expressed at pH 5 (2600 IU/L), however, significantly high activity was shown by the enzyme at pH 4 (2110 IU/L), 6 (2040 IU/L) and 7 (1880 IU/L), and even under high alkaline pH of 8-10 (1240-1680 IU/L). Although maximum stability of the endoglucanase was found at pH 5 but the enzyme was found to be thoroughly stable at pH 4-6 where it retained more than 70-83% of the maximum activity for 30 min- h incubation, however, stability decreased more drastically at pH 7-9 but nonetheless, considerable activity was still present i.e. 35-58% of the maximum (Fig. 5). Optimum pH of 4.5-8.0 has been reported for different microbial cellulases\(^1,2,24\). Similar to present observations, *Bacillus licheniformis* cellulase was found to be more stable under acidic conditions.
conditions. Alkalophilic Bacillus spp. cellulases have been reported to have optimal activities between pH 8 and 9, and show stability over a broad pH of 6-12. However, Streptomyces sp. cellulases were found to be optimally active at pH of 5.5-7. Similar to present results, a novel Streptomyces sp. CMCase was reported to have high activity over a broad range of pH (5-10), and remained stable for 1 h in the presence of commercial detergent (Tide, pH 11) at 40°C. Many industrial processes are operated at extremes of pH (either acidic or alkaline), therefore, the enzyme complement must suit the process requirements and must be capable of withstanding such harsh conditions for prolonged periods or at least during the process time.

Effect of additives on enzyme activity

Enzyme assay was performed in presence of detergents (SDS, Tween 80), metal ions (Zn\(^{2+}\), Pb\(^{2+}\)) or EDTA at final concentration of 10 mM. It was found that all of these additives lead to reduction in enzyme activity (by 31-63%) as compared to the control (Fig. 6). Nonetheless, the enzyme showed considerable activity even in presence of these additives indicating robust nature of the enzyme which is one of the most desirable features for industrial enzymes. Detergents might be involved in altering the structural and conformational characteristics of enzymes. EDTA has been reported to cause reduction in enzyme activity of Pseudomonas fluorescens cellulase. Zn\(^{2+}\) ions caused activity reduction of Caldibacillus cellulovorans cellulase.

DEAE-Cellulose chromatography

Partially purified enzyme preparation (using ammonium sulphate precipitation) was subjected to DEAE-Cellulose chromatography for further purification. DEAE-Cellulose chromatography resulted in purification fold of 3.47 to 9.06 (Fig. 7). Sinorhizobium fredii cellulase was purified by 9.08 folds using ion exchange chromatography. Similarly, Pseudomonas fluorescens cellulase was purified by 24-25 folds by using ammonium sulphate precipitation and ion exchange chromatography.

SDS-PAGE and molecular weight determination

Fractions from DEAE-Cellulose column which showed the highest activity were pooled and subjected to SDS-PAGE for determination of molecular weight of the protein. Purified enzyme preparation showed only one band corresponding to molecular weight of 54 kDa.

Fig. 6—Effect of various additives on activity of endoglucanase

Fig. 7—Elution profile of endoglucanase from DEAE-Cellulose column

Fig. 8—SDS-PAGE analysis of Bacillus strain M-9 endoglucanase. M-indicates molecular weight markers, and P-shows purified endoglucanase band of molecular weight approx. 54 kDa.
approximately 54 kDa (Fig. 8). Different researchers have reported varying molecular weights of cellulases from different organisms. *Pseudomonas fluorescens* cellulase displayed molecular weight of 36 kDa\(^1\). Similarly, cellulases of molecular weight of 36 kDa and 23 kDa have been reported from *Aspergillus niger*\(^2\). Cellulase from *Sinorhizobium fredii* showed molecular weight of 94 kDa\(^20\), while that from *Caldibacillus cellulovorans* displayed the molecular weight of the 85 kDa\(^26\). Thus molecular weight of cellulases is quite variable among different microorganisms.

**Conclusions**

It may be concluded from this work that the organism *Bacillus* strain M-9 has the potential to produce highly thermostable and acid/alkalistable endoglucanase which could have potential applications for wide range of industries. However, further work on optimization of cost effective substrates (mainly carbon and nitrogen sources among other components) for bulk production of enzymes must be initiated, and the molecular basis of high thermostability and acid/alkalistability of the cellulases must be investigated in depth.

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**References**