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Purification and biochemical characterization of endoglucanase from *Penicillium pinophilum* MS 20

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Cellulases find increasing prominence in sustainable production of fuel and feedstock from lignocellulosic biomass. The purification and biochemical characterization of individual components of cellulase complex is important to understand the mechanism of their action for the solubilization of crystalline cellulose. In this study, an extra-cellular endoglucanase isolated from culture filtrate of *Penicillium pinophilum* MS 20 was purified to homogeneity by ammonium sulphate precipitation, ion-exchange chromatography and gel filtration. The purified endoglucanase (specific activity 69 U/mg) was a monomeric protein with molecular mass of 42 kDa, as determined by SDS-PAGE. The endoglucanase was active over a broad range of pH (4-7) with maximum activity at pH 5 and showed optimum temperature of 50°C. It retained 100% activity at 50°C for 6 h and half- lives of 4 h and 3 h at 60°C and 70°C, respectively. The kinetic constants for the endoglucanase determined with carboxymethyl cellulose as substrate were V_{max} of 72.5 U/mg and apparent K_m of 4.8 mg/ml. The enzyme also showed moderate activity towards H_3PO_4 swollen cellulose and *p*-nitrophenyl β-D-glucoside, but no activity towards filter paper, Avicel and oat spelt xylan. The activity was positively modulated by 47, 32 and 25% in the presence of Co^{2+}, Zn^{2+} and Mg^{2+}, respectively to the reaction mixture. The wide pH stability (4-7) and temperature stability up to 50°C of endoglucanase makes the enzyme suitable for use in cellulose saccharification at moderate temperature and pH.

Keywords: *Penicillium pinophilum* MS 20, Endoglucanase, Carboxymethyl cellulose, Purification, Biochemical properties.

Lignocellulosic biomass is considered as major feedstock for the production of ethanol. Cellulose, the main constituent of lignocellulosic biomass is the most abundant and renewable source of energy. The potential importance of cellulose hydrolysis in the context of conversion of plant biomass to fuels and chemicals is widely recognized. Cellulose is an unbranched linear polymer of glucose molecules linked by β-1, 4-glycosidic bonds which in turn forms higher order febrile structure. Because of the crystalline nature and complex composition cellulose cannot be utilized directly by most of the microorganisms. In nature, however, there are diverse group of microorganisms, including cellulolytic bacteria and filamentous fungi which produce extra-cellular cellulases to hydrolyze and metabolize insoluble cellulose. These cellulases are either free or cell-associated.

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*Abbreviations***:** CMC, carboxymethyl cellulose; DNS, dinitrosalicylic acid; PDA, potato dextrose agar; PNPG, *p*-nitrophenyl β-D-glucoside.

Cellulose hydrolyzing enzymes are classified in three types based on mode of catalytic action: (i) endoglucanases or 1, 4-D-glucan-4-glucanohydrolases (EC 3.2.1.4), (ii) exoglucanases or 1,4-D-glucan glucanohydrolases (EC 3.2.1.74), and (iii) β-glucosidases or β-glucoside glucohydrolases (EC 3.2.1.21). Endoglucanases act randomly at internal amorphous sites in the cellulose polysaccharide chain initiating the cellulose degradation by generating oligosaccharides of various lengths and consequently new chain ends. Exoglucanases attack the chain ends, liberating either glucose or cellobiose as major products, while β-glucosidases complete the hydrolysis by converting cellobiose and cello-oligosaccharides into glucose[1]. The co-operative action of the constituent enzymes hydrolyzes crystalline cellulose to smaller oligosaccharides and finally to glucose.

The cellulase system from *Trichoderma* sp. has been the most extensively studied among the cellulolytic fungi[2]. However, the amount of cellulase secreted by this fungus is insufficient for conversion of cellulose to glucose, hence there is an increasing demand for the production of cellulase in conversion

of cellulose to glucose for subsequent production of fuel ethanol[3].

Endoglucanases have many useful applications in biopolishing cotton fabric, improving the processing of paper pulp, de-inking paper, enhancing the efficiency of laundry detergents and increasing the utilization efficiency of plant materials in animal feed manufacture[4]. They also play key role in increasing the yield of the fruit juices, beer filtration, oil extraction and in improving the nutritive quality of bakery products and animal feed[5]. These industrial applications necessitate endoglucanases to be sufficiently robust and stable under conditions of intended industrial applications. Hence obtaining endoglucanases with new physiochemical properties is an important endeavor[6].

Penicillium pinophilum produces complete cellulase which hydrolyses highly ordered cellulose producing glucose as a major end product[7]. Study of individual components of cellulase complex is important to understand the mechanism and synergistic action of the enzymes for efficient cellulose degradation. The present investigation reports the purification and biochemical characterization of an endoglucanase from *P. pinophilum* MS 20.

Material and Methods

Microorganism and culture conditions

A new strain of *Penicilllium*, *P. pinophilum* MS 20 was isolated from wood and the ITS sequence was deposited in NCBI Gene Bank under accession number JN222359 (manuscript communicated). The strain was maintained on potato dextrose agar (PDA) slants with 0.2% dextrose at 28°C.

Production of endoglucanase

Enzyme production was carried out in modified Mandel and Weber medium[8] containing (g/l): KH_2PO_4, 2.0; $CaCl_2$, 0.3; $MgSO_4$, 0.3; urea, 1.5; $(NH_4)_2SO_4$, 7.0; yeast extract, 05; peptone, 1.25; 10% Tween 80, 10 ml, trace metal solutions ($FeSO_4$, 5 mg; $ZnSO_4$, 1.4 mg; $MnSO_4$, 1.56 mg; $COCl_2$, 2.0 mg) and cellulose powder, 25.0. The pH of the medium was adjusted to 5.5. The culture was grown at 28°C for 96 h on a rotary shaker maintained at 200 rpm. Samples were withdrawn after an interval of 24 h, centrifuged at 10000 rpm for 15 min at 4°C and the supernatant was checked for endoglucanase activity.

Enzyme assay

Endoglucanase activity was measured by incubating 1 ml of assay mixture containing 0.5 ml of 1% (w/v) of carboxymethyl cellulose (CMC) and 0.5 ml of suitably diluted enzyme in 0.05 M acetate buffer (pH 4.8) for 30 min at 50°C. Enzyme and reagent blanks were also simultaneously incubated with test samples. The reducing sugar formed was estimated by the dinitrosalicylic acid (DNS) method. One unit of enzyme activity was defined as the amount of enzyme producing 1 µmol of reducing sugar per min under assay conditions[9]. The filter paper activity (FPAase) of the culture filtrate was measured according to standard procedure recommended by Commission on Biotechnology, IUPAC[10]. The *p*-nitrophenyl β-D-glucosidase (PNPGase) activity was determined by method described by Ghose and Bisaria[11].The protein concentration was determined according to the method of Lowry *et al*[12] using bovine serum albumin as a standard.

Purification of endoglucanase

The culture filtrate obtained after 96 h of incubation was subjected to fractional ammonium sulphate precipitation (40-60% saturation). The resultant precipitated proteins were collected by centrifugation at 10000 rpm for 15 min. The precipitate was dissolved in 0.05 M sodium phosphate buffer, pH 7, dialyzed against same buffer with several changes for 8 h and applied to a DEAE-Sephadex column (3 × 20 cm), previously equilibrated with 0.05 M sodium phosphate buffer, pH 7. The column was extensively washed with the same buffer and elution was carried out by a linear gradient of 0-0.5 M sodium chloride in sodium phosphate buffer, pH 7 at a flow rate of 15 ml/h. The fractions having maximum specific activity towards CMC were pooled and concentrated by ultrafiltration through Amicon UM-10 membrane. The concentrated sample was applied on to a Bio-Gel P-100 column (2 × 100 cm), previously equilibrated with 0.05 M sodium phosphate buffer, pH 7. Elution was carried out using the same eluent at a flow rate of 12 ml/h. The fractions (1.5 ml each) having maximum specific activity were pooled and concentrated. The enzyme was loaded on to HPLC gel filtration TSK G 2000 SW pre-packed column (7.5 mm × 600 mm) and eluted with 0.05 M sodium phosphate buffer, pH 7 at a flow rate of 0.7 ml/min.

Determination of molecular mass of endoglucanase

The molecular mass of the purified endoglucanase was estimated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli[13], followed by silver staining. The molecular weight markers used were: β-amylase (Mr 205,000), Alcohol dehydrogenase (Mr 97,400), bovine serum albumin (Mr 66,000), ovalbumin (Mr 43,000) and carbonic anhydrase (Mr 29,000). The gel was stained by silver staining method. A plot of log molecular weight of the standard protein markers against relative mobility gives the molecular weight of the protein.

Determination of optimum pH, temperature and stability of endoglucanase

The optimum pH of endoglucanase was determined by estimating the enzyme activity in a reaction mixture with different pH buffers (3-9) under standard assay conditions. The buffer systems used were: 0.05 M citrate buffer (pH 3), 0.05 M acetate buffer (pH 4-5), 0.05 M phosphate buffer (pH 6-7), 0.05 M Tris-HCl buffer (pH 8) and 0.05 M carbonate-bicarbonate buffer (pH 9). Estimations of endoglucanase activity at different temperature (40-70°C) were carried under standard assay conditions.

The pH stability of the enzyme was measured by incubating 2.5 U of enzyme for 1 h at 50°C in a buffer of desired pH. The temperature stability was determined by incubating 2.5 U of enzyme at different temperatures for different time intervals and then estimating the residual activity under standard assay conditions.

Determination of kinetic parameters

The Michaelis-Menten constant (K_m) and maximal reaction velocity (V_{max}) of purified endoglucanase were determined by measuring the enzyme activity with CMC in concentrations ranging from 1 to 10 mg/ml. From the double-reciprocal plots, kinetic constants were calculated according to Lineweaver-Burk plot.

Substrate specificity

The endoglucanase activity against various polysaccharides was determined by incubating 1 ml of the reaction mixture containing 0.5 ml suitably diluted enzyme in acetate buffer (0.05 M, pH 4.8) in the presence of different substrates for 30 min at 50°C. The substrates used were: CMC, filter paper, *p*-nitrophenyl β-D-glucoside (PNPG), H_3PO_4 swollen cellulose, cellulose, Avicel and oat spelt xylan.

Effect of metal ions on endoglucanase activity

The effect of metal ions on the endoglucanase activity was determined by incubating the reaction mixture with various metal ions at two different concentrations (1 and 10 mM) for 30 min at 50°C and relative activities were determined using CMC as substrate in 0.05 M acetate buffer, pH 4.8. The experiment was carried out in duplicates and the mean value was considered as the final percent relative activity. Endoglucanase activity in absence of metal ions was taken as 100%.

Results and Discussion

Purification of endoglucanase

The extra-cellular culture filtrate of *Penicillium pinophilum* MS 20 showed CMCase, 9.6l, FPAase, 2.5; and PNPGase, 7.31 U/ml. The applications of *P. pinophilum* MS 20 enzymes in hydrolysis of various cellulosic substrates have been reported[7]. Several other cultures are reported to produce various cellulolytic activities under submerged fermentation. The following cellulase activities have been reported from *Aspergillus niger*: CMCase, 0.54; FPAase, 1.63; and β-glucosidase, 1.02 U/ml[14]. The reported CMCase, FPAase and PNPGase activities from *P. janthinellum* are: 21.5, 0.55 and 2.3 IU/ml, respectively. The reported cellulolytic activities in *Trichoderma viridae* are: CMCase, 33.8; FPAse, 0.88; and BGL, 0.33 U/ml[15].

The culture filtrate of *P. pinophilum* MS 20 was subjected to fractional ammonium sulphate precipitation (40-60% saturation), followed by DEAE-Sephadex anion-exchange column chromatography and gel filtration on Bio-Gel P-100. Endoglucanase was purified to homogeneity with specific activity of 69 U/mg and the percent recovery of 3.6 (Table 1).

Table 1—Purification of endoglucanase from *Penicillium pinophilum* MS 20

Steps	Total volume (ml)	Total protein (mg)	Endoglucanase activity (U/ml)	Total units	Specific activity (U/mg)	Recovery (%)	Fold purification
Culture filtrate	100	80.74	9.6	960	11.9	100	1
Ammonium sulphate precipitation	12	30.1	53.1	638.1	21.2	66.5	1.78
DEAE-Sephadex	4	2.2	25.3	100.8	45.7	10.5	3.82
Bio-Gel P-100	2	0.5	17	34.6	69	3.6	5.8

One unit of enzyme activity was defined as the amount of enzyme producing 1 µmol of reducing sugar per min under assay conditions.

The enzyme was found to be homogeneous and gave a single band of 42 kDa, as revealed by SDS-PAGE (Fig. 1). The endoglucanase showed a single peak on HPLC which confirmed the purity of enzyme (data not shown). The endoglucanase (EG2) from *P. janthinellum* has shown a molecular mass of 43.9 kDa[6]. The reported molecular masses of endoglucanases from various microorganisms vary widely and are found to be in the range of 11-100 kDa, however, the most typical are 30-55 kDa[6].

pH, Temperature optima and stability of endoglucanase

The endoglucanase showed optimum activity at pH 5 and retained 85% of the activity at pH 4. It showed maximum stability at pH 5 and exhibited 60% and 83% of residual activity at pH 3 and 4, respectively. At pH 6 and 7, the enzyme retained 90% and 69% of activity, respectively. The pH profile of the enzyme showed maximum stability in the pH range of 4-7 (Fig. 2). For most of the endoglucanases from *Penicillium* sp., the optimum pH lies in the range of 4-5, however, the reported optimum pH range is 4-6.5 for the endoglucanases from other microorganisms. Acidic cellulases have application in the non-ionic surfactant-assisted acidic deinking of old news print and old magazines. The use of acidic cellulases during deinking is advantageous, as it improves pulp freeness and repulping efficiency[16].

The optimum temperature of purified endoglucanase was found to be 50°C (Fig. 3). At temperatures 40°C and 50°C, the enzyme retained complete activity for almost 4 h and 2 h, respectively. The half-life of the enzyme at 60°C and 70°C was 4 h and 3 h, respectively (Fig. 4). The wide pH stability (4-7) and temperature stability up to 50°C of

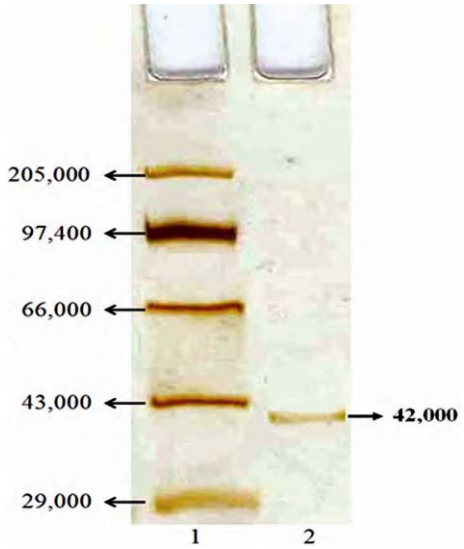

Fig. 1—Molecular mass determination of endoglucanase [10% SDS-PAGE visualized by silver staining: lane 1, standard molecular marker proteins; and lane 2, purified endoglucanase]

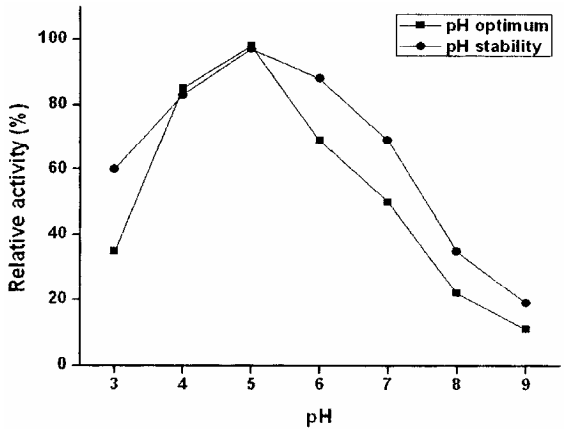

Fig. 2—pH optima and pH stability of endoglucanase [The pH stability was measured by incubating 2.5 U of the enzyme for 1 h at 50°C in a buffer of desired pH]

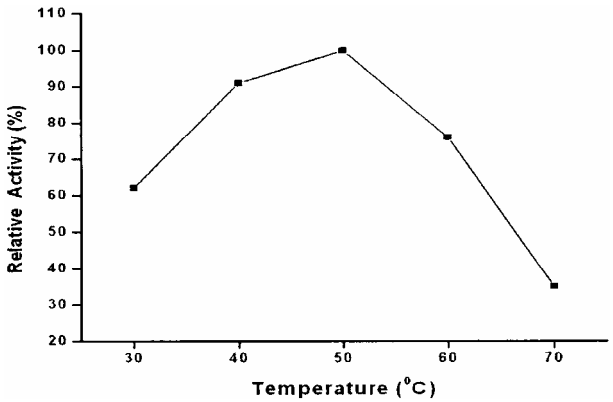

Fig. 3—The optimum temperature of endoglucanase in 0.05 M acetate buffer, pH 5 [The enzyme activity was determined by incubating 2.5 U of enzyme in the range of 40-70°C]

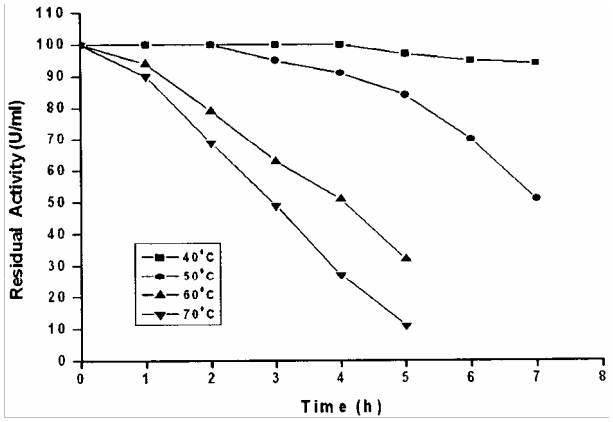

Fig. 4—Thermal stability of endoglucanase [2.5 U of enzyme was incubated in 0.05 M acetate buffer (pH 5) at different temperatures for different time intervals and residual activity was determined]

Table 2—Biochemical characteristics of endoglucanases from some microbial sources

Microorganism	Molecular mass (kDa)	PI	Optimum temperature (°C)	pH	K_m (mg/ml)	V_{max} (U/mg)
Penicillium pinophilum[5] IMI87160ii	25	7.4	50-60	4-5	-	-
	39	4.8	50-60	-		
	62.5	4.1	50-60	4- 5		
	54	3.7	50-60	4- 5		
	44.5	4	65-75	-		
Streptomyces lividans[17]	46	3.3	50	5.5	4.2	24.9
Fusarium oxysporum[18]	34	-	50	6	23.2	22.5
Thermotoga neapolitana Cel A[19]	29	4.6	95	6	0.97 [a]	69.2
T. neapolitana Cel B[19]	30	4.1	106	6-6.5	0.3 [a]	18.4
Gloeophyllum sepiarium (EGS)[20]	45.1	3.8	59	4.1	7.6 [b]	-
G. trabeum (EGI)[20]	40.5	3.1	62	4.2	6.3 [b]	-
Rhizopus oryzae REC 1[21]	41	-	55	5-6	-	-
R. oryzae REC 2[21]	61	-	55	5-6	-	-
Melanocarpus sp. MTCC 3922 EG I[22]	40	4.0	50	6	20	9.54×10^2 [c]
Melanocarpus sp. MTCC 3922 EGII[22]	50	3.6	70	5	13.3	2.2×10^2 [c]
Aspergillus terreus[23]	25		60	2		
A. oryzae[24]	25	-	55	4.4	20	854
Penicillium pinophilum KMJ601[25]	37	-	28	5	1.08	225.6
P. pinophilum MS 20 (Present work)	42	-	50	5	4.8	72.5

a, mM, b, g/ l, c, μmol/min/mg protein

endoglucanase from *P. pinophilum* MS 20 makes the enzyme suitable for use in cellulose saccharification at moderate temperature and pH.

Kinetic parameters and substrate specificity

The apparent K_m and V_{max} of the purified endoglucanase were 4.8 mg/ml and 72.5 U/ml, respectively. The enzyme was active towards CMC (69 U/mg) and also showed moderate activity towards H_3PO_4 swollen cellulose and PNPG, but no activity towards filter paper, Avicel and oat spelt xylan. Some endoglucanases have also been reported from various microorganism having higher catalytic activity towards CMC and negligible activity towards other linear polymers. Table 2 lists the biochemical properties of endoglucanases from various microbial sources.

Based on ITS sequence homology the present strain was identified as *P. pinophilum* MS 20 (manuscript communicated). As compared to earlier report of endoglucanse from *P. pinophilum*[5], the endoglucanase in present study did not show iso-enyme pattern. The molecular mass and optimum temperature of endoglucanse in the present study was 42 kDa and 50°C, respectively. The iso-enzymes of endoglucanases from *P. pinophilum* IMI87160ii have shown different molecular masses with optimum temperature in the range 50-75°C [5].

Table 3—Effect of metal ions on endoglucanase activity

Metals	Relative activity (%)	
	1 mM	10 mM
Control	100	100
Co^{2+}	138.14	147.52
Zn^{2+}	124.45	132.12
Ca^{2+}	83.23	94.60
Mg^{2}	121.16	125.19
K^{+}	96.21	104.44
Na^{+}	66.33	72.58
Cu^{2+}	34.40	28.26
Hg^{2+}	34.22	9.34
Fe^{2+}	62.39	51.84
Pb^{2+}	68.74	46.22
Ni^{2+}	64.55	52.57
Mn^{2+}	75.26	67.22
Cd^{2+}	56.30	43.14

Effect of metal ions on endoglucanase activity

The relative endoglucanase activity was increased by 47%, 32% and 25% in the presence of $CoCl_2$, $ZnSO_4$ and $MgSO_4$ at the concentration of 10 mM. Maximum inhibition of 91% and 78% was observed with $HgCl_2$ and $CuSO_4$. The addition of $FeSO_4$, $Pb(NO_3)_2$, $NiCl_2$ and $CdCl_2$ in the concentrations of 1 mM and 10 mM showed gradual decrease in the enzyme activity. There was negligible reduction in enzyme activity after addition of $CaCl_2$, NaCl and $MnSO_4$ in the reaction mixture (Table 3). In an earlier

study[3], increase in endoglucanase activity has been reported in the presence of Mg^{2+} and Co^{2+}. Increase in endoglucanase activity by Zn^{2+} is also reported in *Bacillus circulans*, *Thermomonospora curvata* and *Thermomonospora palustris*[20]. Inactivation of endoglucanase by Cu^{2+} and Hg^{2+} probably indicate the presence of thiol and histidine as active site residues of the enzyme.

In conclusion, an extracellular endoglucanase having specific activity of 69 U/mg and molecular mass of 42 kDa was purified to homogeneity from *P. pinophilum* MS 20. The wide pH stability (4-7) and temperature stability up to 50°C of endoglucanase makes the enzyme suitable for use in cellulose saccharification at moderate temperature and pH.

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