Purification and enzyme properties of an extracellular Endo- -1, 4- glucanase from *Paenibacillus amylolyticus MTCC 8084* isolated from sugarcane fields

P. ANURADHA, D. JHANSI RANI, S. ABID PASHA AND K.SRAVANI

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See end of the article for authors' affiliations

Correspondence to :
P. ANURADHA
Criya Biolabs,
TIRUPATI, (A.P.)
INDIA

SUMMARY

Endo- -1, 4 - glucanase is an extracellular key enzyme used by bacteria to decompose cellulose of sugarcane crop residue. It has been purified from *Paenibacillus amylolyticus MTCC 8084* isolated from sugarcane fields. The dialysed crude enzyme preparation was loaded on to a DEAE-Sepharose anion–exchange column. Active fractions were collected and loaded on to a Sephadex G-75 column for further purification. The purification fold was 11.7 with a recovery yield of 29.4 %. Specific activity of enzyme was 4.16 U/mg. The purified endo- -1, 4–glucanase gave a single protein band on polyacrylamide gel electrophoresis and molecular weight is approximately 93 kDa. The optimal temperature and pH were 50^{0}C and 5.0, respectively. Apparent kinetic parameters K_m and V_{max} were determined 8.2 mg/ml and 167 U/min/mg, respectively. Enzyme activity was stimulated by Cu^{+2} and inhibited by Hg^{+}, Ag^{+2}, Al^{+3} and EDTA.

Key words : Endo-β-1, 4–glucanase, *Paenibacillus* sp., Purification, Cellulose.

Bacterial degradation of cellulosic biomass of agricultural waste plays a vital role in carbon recycling. For the same reason, treatment of cellulose by cellulolytic enzymes for practical purposes has attracted the continuing interest of biotechnologists. Interest in transformation of biomass is both fundamental and applied (Clarke, 1997). In order to enhance the rate of saccharification, it has become necessary to search for highly efficient cellulolytic organisms with secretion of copious amount of cellulose.

Paenibacillus spp. produce several celluloses. Most of the enzyme is extracellular and a small amount is cell bound. Celluloses are characterised by a multiplicity of enzyme components whose exact number varies from one organism to another. Cellulose is the key enzyme produced by cellulolytic microorganisms for the degradation of cellulose. It is a complex enzyme comprising three major components, *viz*. Endoglucanase (E.C 3.2.1.4), Exoglucanase (E.C 3.2.1.91) and Cellobiase (E.C 3.2.1.21) which act synergistically and completely solubilize crystalline cellulose to glucose. Endoglucanases randomly hydrolyze internal glycosidic linkages, which results in rapid decrease in polymer length and a gradual increase in the reducing sugar concentration (Beguin and Aubert, 1994; Wood and Bhat, 1988). Exoglucanases hydrolyze cellulose chains by removing cellobiose either from the reducing ends or non reducing ends (Teeri, 1997). Glucose is produced primarly by the action of glucosidases on cellobiose.

Frequently, cellulolytic organisms also produce other polysaccharases, including xylanases, mannanases, galactosidases, which hydrolyze associated plant polysaccharides and thus facilitate cellulose access to the substrate. Besides the degradation of organic matter, celluloses are used in various industries like food, brewery, wine, textile, leather, paper, pulp and printing ink (Hamlyn, 2000) etc. This potential has stimulated the search for new microorganisms with better cellulolytic capabilities. The commercial possibility of using cellulose preparations to produce glucose, alcohol is under intensive study. A number of biomass conversion methods have been proposed and employed ranging from direct chemical methods like acid hydrolysis and pyrolysis to biological methods such as application of cellulose enzymes (Cooney *et al*., 1978).

Enzymatic hydrolysis of cellulosic wastes may give a relatively pure product with the consumption of less energy during the process (Fennington *et al.*, 1982). Substantial efforts have been made by enzyme suppliers and industrial users to improve existing enzymes (Brennan, 1996). In this report, the purification and partial characterization of extracellular endo-β-1, 4 - glucanase from *Paenibacillus*

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amylolyticus MTCC 8084 have been described.

MATERIALS AND METHODS

Microorganisms and culture media:

Paenibacillus spp. was isolated from soil samples collected from sugarcane fields around Tirupati, Chittoor district, A.P. Soil sample was subjected to serial dilution and spread on to cellulose agar medium (pH 7.0) as follows (In g/L) KCl, $MgSO_4$, yeast extract, 0.5; CMC, $NaNO_3$, K_2HPO_4 , Glucose, 1; Agar, 17. These plates were incubated at 30°C for 5-7 days. Efficient bacterial isolates were selected on the basis of their growth and formation of clearing zones on cellulose agar medium using Carboxy methyl cellulose-blue (2 g/L) as chromogenic substrate (Lee and Lee, 1997)

Among six isolates obtained, one bacterial strain exhibited highest cellulolytic activity and identified as *Paenibacillus amylolyticus MTCC 8084* by IMTECH, Chandigarh. The isolated bacteria were aerobic, gram positive, motile, spore forming, rod shaped organisms and catalase positive. Acid was produced from D-glucose, D-xylose and D-mannitol.

Extraction of crude enzyme:

Basic liquid mineral medium (100 ml in 500 ml Erlenmeyer flasks) was inoculated with 1 ml of overnight culture and incubated at 37°C with vigorous aeration in a shaker at 150 rpm for 2 days. Composition of basic liquid mineral medium (pH 7.0) was as (In g/L) $FeCl_2$, 0.05; KCl, $MgSO_4$, yeast extract, 0.5; $NaNO_3$, K_2HPO_4, KH_2PO_4, CMC, Glucose 1. Cells were separated by centrifugation at 8,000 x g for 20 minutes at 4°C. The cell free culture filtrate was used as crude enzyme source.

The cell-free supernatant was precipitated with 40 to 80 % ammonium sulphate saturation and centrifuged. The pellet was suspended in a minimum volume of 0.05M citrate buffer (pH 5.0) and dialysed against four changes of the same buffer. The dialysed crude enzyme preparation was used for all subsequent studies.

Determination of protein concentration:

The protein concentration of the crude as well as that of the purified enzyme was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard.

Endo- -1, 4- glucanase assay:

Activity of endo-β-1, 4 - glucanase in the culture filtrate was assayed according to Ghosh (1987). The reaction mixture contained 1.0 ml of 1% CMC in 0.2ml of culture filtrate with appropriate dilution was added to the reaction mixture and incubated at 50^0C in water bath for 10 min. Appropriate controls devoid of substrate or enzyme were simultaneously run. The reducing sugar produced in the reaction mixture was determined by dinitro salicylic acid method (Miller, 1959). 2, 5 – DNS reagent was added to aliquots of the reaction mixture and the colour developed was read at 540 nm in a spectronic 20 – D – spectrophotometer. One unit of endoglucanase activity was defined as the amount of enzyme releasing 1μ mole of reducing sugar per minute.

Purification of Endo- -1, 4- glucanase enzyme:

A two step chromatographic procedure was employed to purify endoglucanase. The experiments were performed at room temperature.

Ion-exchange chromatography on DEAE – Sepharose:

Approximately 4 ml of clear crude extract was loaded onto a DEAE – Sepharose fast flow anion – exchange column (type XK26, Pharmacia, Piscataway, N. J.) previously equilibrated in 50 mM Tris-HCl buffer (pH 7.4). Endoglucanase was eluted in 50mM Tris-HCl buffer (pH 7.4) with a 0.1 to 0.5M NaCl gradient. Fractions of 1.5 ml were collected at a flow rate of 1 ml/min and tested for enzyme activity. Active fractions were pooled, concentrated and dialysed against the same buffer for 6 hours.

Gel filtration on Sephadex G – 75:

Sephadex G –75 slurry was packed into a column (2.5 x 50 cm, Pharmacia, Piscataway, N. J.) and equilibrated with 0.05M Sodium citrate buffer pH (5.0). The peaks with highest activity from the ion exchange chromatography step were applied to the Sephadex G-75 column. Fractions (1.5 ml) were collected at a flow rate of 1 ml/min. The elution was monitored for protein concentration at 280 nm and assayed for enzyme activity. Active fractions with high protein activity were pooled, concentrated and dialysed against the same buffer for 6 hr. The purified enzyme thus obtained was stored at -20°C.

Determination of purity and molecular weight :

Based on the Laemmli method (Laemmli, 1970) Sodiumdodecylsulfate (SDS) Polyacrylamide Gel Electrophoresis (PAGE) was carried out on 12.5% Polyacrylamide gels in the presence of â-mercaptoethanol with Bio-Rad apparatus. Electrophoresis was carried out in 0.5M Tris-Glycine buffer, pH 8.3. The Gels were stained with coomassie

blue R 250. Low-molecular weight protein markers were purchased from Bio-Rad, Richmond, Calif.

Determination of kinetic parameters:

The apparent kinetic parameters (K_m and V_{max}) of the endo-β-1, 4-glucanase were determined by varying the concentration of the substrate, Carboxy methyl cellulose (CMC) within a range of 1-5mM in citrate buffer (0.05 mM, pH 5.0) and incubated with a fixed amount (0.3 ml) of enzyme for desired length of time (10 min) at 40°C under standard assay conditions. The apparent kinetic parameters were determined from Line weaver and Burk double-reciprocal plots (Lineweaver and Burk, 1934).

Effect of pH on Endo- -1, 4- glucanase activity:

To determine the optimum pH for the enzyme activity, assay was conducted with different buffers (0.05 mM) adjusted to pH of 3.0 - 9.0. The buffers used were: Acetate, pH 3.0, Citrate, pH 5.0, Phosphate, pH 7.0, Tris buffer, pH 9.0.

Effect of temperature on Endo- -1, 4- glucanase activity:

Enzyme was incubated with the substrate at different temperatures ranging from 10°C to 80°C. The reaction mixtures were analysed to monitor enzyme activity.

Effect of metal ions and chelating agent (EDTA) on Endo- -1, 4- glucanase activity:

The effect of Hg^{+2}, Al^{+3}, Ag^{+}, Cu^{+2} and chelating agent EDTA of 1mM level on the activity was determined. Substrate-cation mixture was incubated at room temperature for 1 hour before the assay.

RESULTS AND DISCUSSION

Enzyme purification:

The crude enzyme was first precipitated with 40 to 80% ammonium sulfate saturation and a two-step chromatography was conducted to purify the endo-β-1, 4- glucanase enzyme. DEAE-Sepharose anion-exchange chromatography had purified the enzyme partially (Fig. 1).The final purification step *i.e.* Gel filtration chromatography (Sephadex G-75) yielded pure endoglucanase (Fig 2). The enzyme was purified 11.7 fold with yield of 29.4%. Its specific activity was 4.16 U/mg. The results of purification are summarized in the Table 1. Po-Jui Chen *et al.* (2004) purified CMCase from *Sinorhizobium fredii* which had a yield of 26.4% with specific activity 3.82 U/mg. CMCase purification from

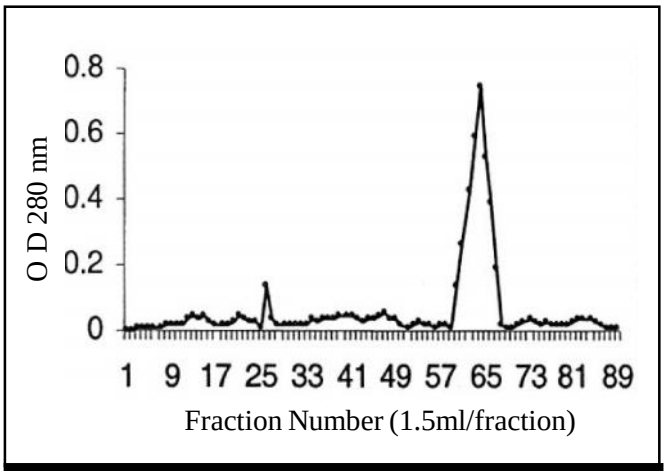

Fig. 1 : The elution profile of DEAE-Sepharose Fast Flow anion exchange chromatography. The dialyzed enzyme solultion collected from 40-80% saturation ammonium sulfate was loaded onto a DEAE-Sepharose Fast Flow column (1.5 x 40 cm) pre-equilibrated with 50mM Tris-HCl buffer at pH 7.4. Active fractions (59-70) were pooled, concentrated and dialyzed against the same buffer

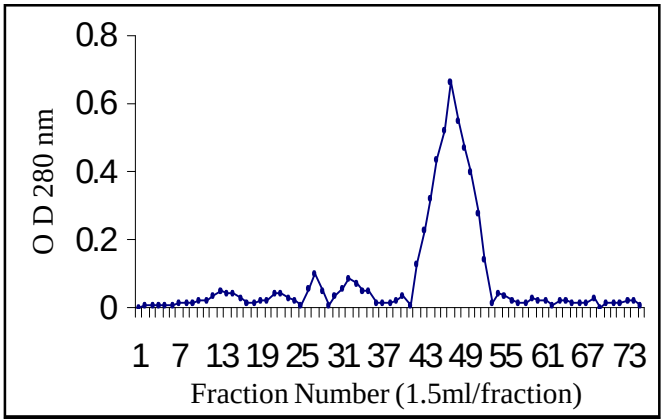

Fig. 1 : The elution profile of Sephadex G-75 gel filtration chromatography. The dialyzed enzyme solution collected from anion exchange chromatography was applied to a Sephadex G-75 column (2.5 x 50 cm) pre-equilibrated with 0.05 M citrate buffer at pH 5.0. The elution was monitored for protein concentration at 280 nm and was also assayed for enzyme activity. Fractions with high protein activity (41-54) were pooled, concentrated and dialysed against the same buffer for 6 h. The purified enzyme thus obtained was stored at 20^{0}C

the alkaline *Bacillus* spp. strain HC-1 at pH 9.5 had a yield at 10.6% and specific activity 5.7 U/mg (Khyami-Horani, 1991). The purified endoglucanase appeared to be a monomer, when compared to the low molecular weight standards of Bio-Rad electrophoresis within SDS revealed a single band of a molecular weight of approximately 93 kDa. No other endoglucanase isozyme was detected under

Table 1 : Summary of purification of Endo-β-1, 4- glucanase enzyme

Purification step	Total volume (ml)	Total protein (mg)	Activity (n mole/min)	Specific activity (U/mg)	Yield (100%)	Purification fold
Crude enzyme	10	2.4	0.85	0.354	100	1
Ammonium sulfate preceipitation	8	0.64	0.4	0.625	47.05	1.7
DEAE -Sepharose	4	0.56	0.36	0.642	42.3	1.8
Sephadex G-75	1	0.06	0.25	4.166	29.4	11.7

Note : Values represented in the table are means of three separately conducted experiments

these conditions.

Determination of kinetic parameters:

The Michaelis – Menten constants, K_m and V_{max} of the purified endo-β-1, 4- glucanase for carboxy methyl cellulose was estimated from the double reciprocal plot of the data obtained for *Paenibacillus amylolyticus* of varying substrate concentrations. The K_m and V_{max} obtained for the purified endo-β-1, 4 - glucanase were 8.2 mg/ml and 167 U/min/mg (Fig. 3). This is in correlation with that of the endoglucanase was 8.7 mg/ml from *Sclerotorium* (Waksman, 1991). However, these values were higher than 0.5 mg/ml obtained for cellulose of *Myrothecium verucaria* (Halliwell, 1965).

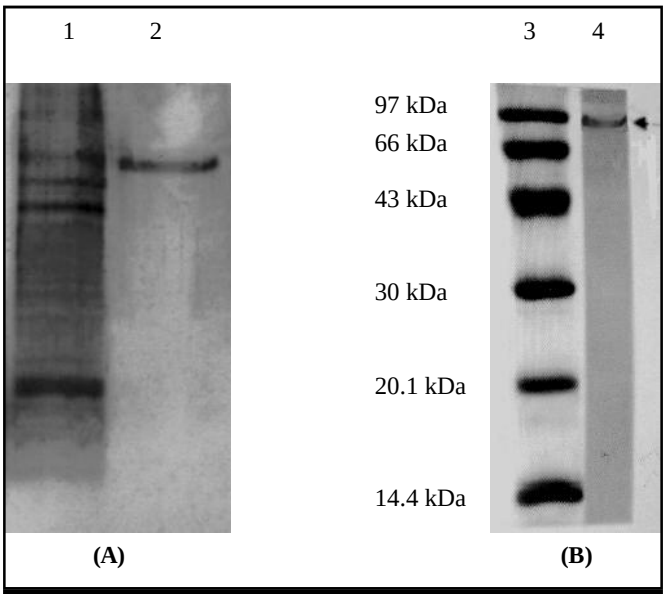

Fig. 3 : 12.5% SDS-PAGE of endoglucanase (a) Lane 1, Crude enzyme Lane 2, Purified enzyme (b) Lane 3, low-molecular weight Bio-Rad standard markers (Richmond, Cali.), Lane 4, Molecular weight of purified enzyme

Effect of pH on Endo- -1, 4- glucanase activity:

The optimum pH for the enzyme activity of *Paenibacillus amylolyticus* was recorded at pH 5.0 and is shown in Fig 4. Catriona *et al*. (1994) reported that the pH range over which the celluloses were highly active was fairly broad (pH 5.0-7.0). The pH optima of endogluconase in *C. thermocellum* (Ng and Zeikus, 1981; Petre *et al*.,1981) were found to be 5.2. Bok *et al*. (1998) also reported a pH range of 6.0 to 6.6 for two thermostable endocelluloses from *Thermotoga neapolitana*. Masatake Akita *et al*. (2005) reported the pH optimum between 6 and 8 in *Bacillus halodurans C-125.*

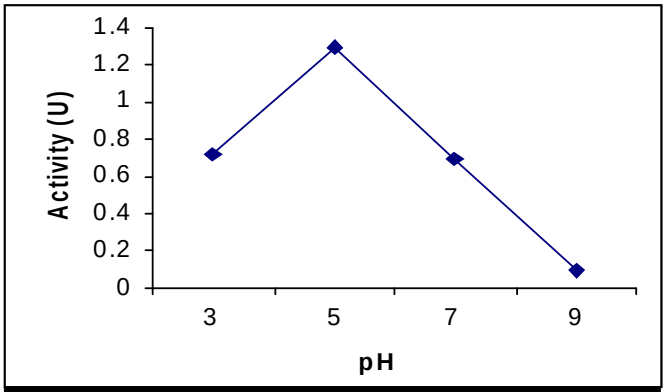

Fig. 4 : Effect of pH on endo-1- , 4-glucanase activity

Effect of temperature on Endo- -1, 4-glucanase activity:

Enzyme activity was maximum at temperature 50°C and low at extremes of temperature at 10°C and 80°C. At higher temperature than the optimum, enzyme activity decreases due to denaturation (Fig. 5). Kye Man Cho*et al*. (2006) recently reported the optimum temperature for glycosyl hydrolase (cellulose) was 50°C from *Paenibacillus polymyxa GS0I.* Maximum activity was achieved at 45°C in case of *Paenibacillus* spp. *BP-23* (Marta M. Sánchez *et al.*, 2003).

Effect of metal ions and chelating agent on Endo- -1, 4- glucanase activity:

Cu^{+2} stimulated the enzyme activity by 20% over control whereas Hg^{+2}, Ag^{+} and Al^{3+} and EDTA inhibited the enzyme activity by 10- 40% (Table 2). Celluloses are inhibited by silver, chromium, lead, mercury and zinc salts

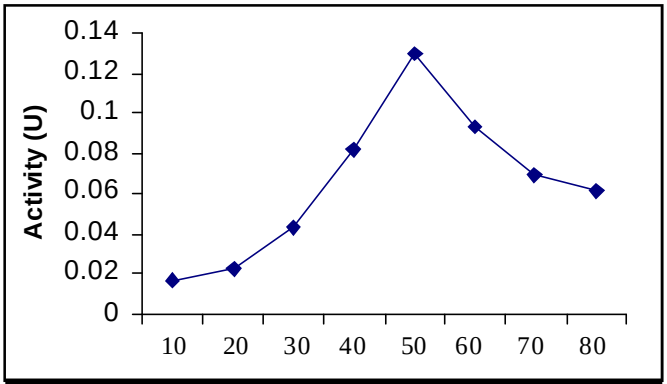

Fig. 5 : Effect of temperature on endo-1- , 4-glucanase activity

at about 10^{-3} concentration (Mandels and Reese, 1963). Inhibition by heavy salts is due to non specific salt formation (Verma *et al.*, 1963).

Table 2 : Effect of metal ions and chelating agent on enzyme activity

Heavy metal	Relative activity (%)
Control	100
$CuSO_4$	120
$A1_2O_3$	45
$AgNO_3$	69
$HgCl_2$	90
EDTA	67

Note : Activity measured in absence of metals and chelating agent was considered 100%. Activity in the presence of metals was relatively in terms of the activity of control.

Authors' affiliations

D. JHANSI RANI, S. ABID PASHA AND K.SRAVANI, Criya Biolabs, TIRUPATI, (A.P.) INDIA

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