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Properties of Endoglucanase of *Penicillium chrysogemum* PCL501

[1]S. Nwodo Chinedu, [1]C. Obinna Nwinyi, and [2]V.I. Okochi

[1]Department of Biological Sciences, College of Science and Technology, Covenant University, KM 10 Idiroko Road, Canaan Land, PMB 1023 Ota, Ogun State, Nigeria.
[2]Department of Biochemistry, College of Medicine, University of Lagos, PMB 12003 Idiaraba, Lagos, Nigeria.

Abstract: Crude extracellular enzyme from a 3-day culture of *Penicillium chrysogenum* (PCL 501), in basal medium containing cellulose as the sole carbon source, yielded 0.67 ± 0.03, 19.94 ± 1.30 and 8.50 ± 0.50 units mg protein^{-1} of 1, 4- β-endoglucanase, β-glucosidase and xylanase activity respectively. The crude enzyme was subjected to ammonium sulphate precipitation (80% saturation) and gel filtration. A purification-fold of 7.5 was achieved. Two active fractions of 1, 4 β-endoglucanase (EC 3. 2. 1. 4), which exhibited about the same activity towards carboxymethyl-cellulose (CMC), were obtained and pooled for the subsequent analyses. The endoglucanase gave a Vmax of 10.0 ± 0.4 µmol min^{-1} mg protein^{-1} and Km of 11.8 ± 0.4 gL^{-1} with CMC. The enzyme was most active at pH of 4.5 – 5.0 and temperature range of 40 – 50 ^{O}C. The optimum pH was 4.9 while the Optimum temperature was 48 ^{O}C. Divalent metal ions and EDTA affected the enzyme activity at 2.0 mM concentrations. Mn^{2+} and Fe^{2+} had stimulatory effects on the enzyme whereas Mg^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+} and EDTA inhibited the enzyme activity. The effect of Ca^{2+} was not significant. Over 3-fold increase in the enzyme activity was recorded with Mn^{2+}. Percentage inhibition of 65.9 and 79.7 respectively was obtained with Hg^{2+} and EDTA. The organism appears to produce two types of endoglucanase which differed in their molecular weight but not significantly in their activity. The enzyme activity was highly stimulated by manganese ion and inhibited by the metal-chelating agent, EDTA.

Key words: *Penicillium chrysogenum* (PCL 501), 1, 4 β-endoglucanase activity, kinetics, pH, Temperature, metal ions.

INTRODUCTION

Cellulase is the generic name for the group of enzymes which catalyze the hydrolysis of cellulose and related cellooligosaccharide derivatives. Synergistic action of three types of the enzyme, namely, endoglucanase (EC 3. 2. 1. 4), exoglucanase (EC 3. 2. 1. 91) and β-glucosidase (EC 3. 2. 1. 21), has been found to be vital for the complete enzymatic hydrolysis of native cellulose by fungi, such as *Penicillium pinophilum* (Wood and McCrae, 1986). Xylanase (EC 3. 2. 1. 8), has also been reported to be a requirement for the complete hydrolysis of native cellulose (Khan, 1980).

Cellulase action is generally initiated by the random acting endo-glucanases within the cellulose chain. Endoglucanase (1, 4 β-glucan glucanohydrolase; EC 3. 2. 1. 4), also called endo-1, 4-β-glucanase, endocellulase, or carboxymethyl-cellulase (CMCase), cleaves, at random, the β-1, 4-glucosidic bonds usually in the amorphous parts of the cellulose chain, thereby generating glucose and cellooligosaccharides of various lengths, and consequently new chain ends. The enzyme is adaptive in most fungi (Reese and Levinson, 1952) and also regulated by catabolite repression (Berry and Paterson, 1990); cellulose, sophorose, sawdust, and sugarcane pulp are among the substances found to stimulate its production (Mandels and Reese, 1956; Ryu and Mandels, 1980, Nwodo-chinedu *et al*., 2007 a, Nwodo-Chinedu *et al*., 2007 b).

Many filamentous fungi secrete cellulases and other plant cell wall hydrolyzing enzymes into their culture media (Berry and Paterson, 1990). *Penicillium chrysogenum* PCL501, a cellulolytic microfungus, was isolated from a wood-waste dump in Lagos, Nigeria (Nwodo-Chinedu *et al*., 2005). The organism grows effectively in basal medium supplemented with sawdust or sugarcane as sole carbon sources (Nwodo-

Corresponding Author: Dr. Shalom Nwodo Chinedu, Department of Biological Sciences, College of Science and Technology, Covenant University, KM 10 Idioroko Road, Canaanland, PMB 1023 Ota, Ogun State, Nigeria.
E-mail: sncresearch@yahoo.com Tel: +234 (0)802 8626 605

Chinedu *et al*., 2007 a) and produce cellulases (Nwodo-Chinedu *et al*., 2005; Nwodo-Chinedu *et al*., 2007 b) and xylanases (Okafor *et al*., 2007) in media containing cellulose or agrowastes as sole carbon sources.
In the present study, the properties of partially purified endoglucanase (EC. 3. 1. 2. 4) of *penicillium chrysogemum* PCL501were investigated. Our data suggests that the organism produces two types of endoglucanase which differed in their molecular weight but not significantly in their activity. The enzyme activity was highly stimulated by manganese ion. Could the metal be a cofactor of the enzyme?

MATERIALS AND METHODS

Chemicals:

All chemicals and reagents were of analytical grade. Potato Dextrose agar and crystalline cellulose (Avicel) were obtained from Merck, Germany. Carboxymethyl-Cellulose (CM52) was obtained from Whatman Ltd, England. All other chemicals and reagents were obtained from Sigma Chemicals Co. Ltd, England.

Organism:

The strain of *Penicillium chrysogenum* (PCL501) was isolated from wood-wastes in Lagos, Nigeria and identified as described previously (Nwodo-Chinedu *et al*., 2005). The organism was maintained at 4 ^{O}C on Potato Dextrose Agar (PDA) slants.

Cultivation and Enzyme Production:

The extracellular enzymes were produced through submerged fermentation. The organism was grown on basal medium containing (per liter of distilled water): $NaNO_3$, 3.0 g; KCl, 0.5 g; KH_2PO_4, 1.0 g; $MnSO_4$. $7H_2O_4$, 0.5 g; $FeSO_4$. $7H_2O$, 0.01 g; and 10.0 g cellulose. One liter (1 L) of the media was supplemented with 1.0 mL of trace solution containing (per liter of distilled water) $ZnSO_4$, 1.0 g and $CuSO_4$. $5H_2O$, 0.5 g. The pH of each media was adjusted to 5.6. Conical flasks (250 mL) containing 100 mL of respective media were autoclaved at 121 ^{O}C for 15 minutes, cooled and inoculated with 1.0 mL of spore suspension in 0.1%Tween80 (2- 4 X 10^6 spores per mL) of the pure fungal isolate. The cultures were incubated for 72 hours with continuous agitation at 100 osi/ min using Griffin flask shaker. Cells were harvested by centrifugation at 6000 x g for 15 minutes at 4 ^{O}C using ultra centrifuge (Superspeed RC-B, USA). The cell-free culture supernatant was used as source of crude extracellular enzyme.

Protein Assay:

Protein content of the culture supernatant was determined by the folin ciocalteau method described by Lowry *et al*. (1951) using Bovine Serum Albumin (BSA) as standard. The protein concentration of the chromatographic fractions was determined by extinction at 280 nm wavelength with spectrophotometer (Thermospectronic Genesys 4001/1, USA).

β- Endoglucanase (EC 3. 2. 1. 4) assay:

A modification of the reducing sugar method described by Khan (1980) was used to for the assay of Endo-1, 4-β-Glucanase (EC 3. 2. 1. 4) activity. Carboxymethyl-cellulose (CMC) was used as enzyme substrate. The reaction mixture contained 2.0 mL of 0.1% (w/v) CMC in 0.1M sodium acetate buffer (pH 5.0) and 2.0 mL of cell-free culture supernatant (or 0.5 mL of partially purified enzyme). The mixture was incubated at 37^{o}C in water bath with shaking for 30 minutes. The reducing sugar released was measured using 3, 5-dinitrosalicylic acid and read at 540nm using a spectrophotometer (Miller, 1959). The released reducing sugar was expressed in glucose equivalent and expressed in Units mL^{-1}. A unit of activity was defined as amount of enzyme required to liberate 1µmol of Glucose per minute under the assay conditions.

β-Glucosidase (EC 3. 2. 1. 21) Activity:

β-Glucosidase (EC 3. 2. 1. 21) activity was determined using a modification of Hagerdel method as described by Workman and Day (1982) with p-nitrophenol-β-glucopyranoside (p-NPG) as substrate. A 2.0 mL portion of 10.0 mM p-NPG solution in 0.1 M sodium acetate buffer, pH 5.0 was heated to 50 ^{o}C. Then, 0.5 mL of cell-free culture supernatant was added to the substrate and the mixture was incubated at the same temperature (50 ^{o}C) for 15 minutes. After incubation, 3.0 mL of 1.0M Na_2CO_3 was added to stop the reaction and the absorbance was read at 400 nm. The amount of p-nitrophenol Liberated was obtained using a p-nitrophenol standard curve. β-Glucosidase activity was expressed as nanogram (ng) p-nitrophenol librated mg $Protein^{-1}min^{-1}$ or in Unit mg $Protein^{-1}$. A unit of activity was defined as amount of enzyme required to liberate 1 nanomole (nmol) of nitrophenol per minute under the assay conditions.

Xylanase Assay:

Xylanase (3. 2. 1. 8) activity was assayed by the modification of the reducing sugar method described by Khan (1980) using β-D-xylan as enzyme substrate. The reaction mixture contained 2.0 mL of 0.1% (w/v) substrate in 0.1M sodium acetate buffer (pH 5.0) and 2.0 mL of cell-free culture supernatant (or 0.5ml of partially purified enzyme). The mixture was incubated at 37 °C in water bath with shaking for 30 minutes. The reducing sugar released was measured using 3, 5-dinitrosalicylic acid (Miller, 1952). The colour was developed by boiling in water bath for 5 minutes. Absorbance was read at 540nm using a spectrophotometer. The released reducing sugar was expressed in xylose equivalent and expressed as µg xylose released per mg Protein per minute (µg Glucose mg $Protein^{-1}min^{-1}$) or in Units mg $Protein^{-1}$. A unit of activity was defined as amount of enzyme required to liberate 1 µmol of Xylose per minute under the assay conditions.

Partial Purification of the Cellulase Enzyme:

Partial purification of the crude extracellular enzyme was achieved by the process of Freeze drying, Ammonium sulphate precipitation/Dialysis, and Gel chromatography.

Two hundred milliliters of the crude extracellular enzyme was freeze dried at - $4^{O}C$. The enzyme was redissolved in 20 mL acetate buffer (pH, 5.0) and precipitated with $(NH_4)_2SO_4$ at 80 % (w/v) saturation. This was centrifuged at 1000 X g for 15 minutes. The supernatant was carefully decanted and the precipitate was redissolved in the acetate buffer and dialyzed over-night at 4 ^{O}C against the buffer.

Gel chromatography (molecular sieving) was done using Sephadex G 25-300 (Sigma). Ten-grams of sephadex (Sigma) was suspended in 50 mL sodium acetate buffer (0.5M, pH5.0) and packed into a glass column (12 X 400mm) at room temperature. The gel in the column was further washed with the same buffer. Five-milliliter (mL) of the concentrated dialyzed enzyme was then introduced to the top of the sephadex-packed column. After the enzyme had been absorbed, the same buffer was passed through the column at a constant flow rate of 1 mL per minute. Five-milliliter (mL) fractions were collected and assayed for their protein and 1, 4-β-endoglucanase activity. Enzymatically active fraction were pooled and concentrated by freeze drying.

Properties of 1, 4-B-Endoglucanase:

pH:

The pH profile of the enzyme was determined by varying the pH of the reacting mixtures between 3.0 and 9.0. These substrates were prepared in two buffer solutions: 0.1 M acetate-NaOH (pH 3.0 to 7.0) and 0.1 M Tris-HCl buffer (pH 8.0 to 9.0).

Temperature:

The temperature profile of the enzyme was determined by incubating the enzyme with substrates for 30 minutes at various temperatures between 30-80 ^{O}C. The various enzyme activity values were determined.

Time Course:

The time course of the enzyme was determined by measuring the enzyme activity at different period of incubation under the above standard assay conditions of pH (5.0) and temperature (40 °C). Total reducing sugars (Glucose equivalent) released by the enzyme per time was monitored at 10 minutes interval for 60 minutes.

Effect of Substrate Concentration:

The effect of various substrate (CMC) concentrations (2.0 to 20 g/L) on the enzyme activity was studied under the above standard assay conditions of pH (5.0) and temperature (40 °C). The Michealis-Menten constant (Km) and maximum velocity (Vmax) of the enzyme were obtained using the reciprocal plot (Line-weaver–Burk plot) .

Effect of Metal ions and EDTA:

The effect of divalent cations and ethylene diamino tetraacetic acid (EDTA) on the enzyme activity was determined by incubating the standard enzyme-substrate (CMC) mixture containing 2.0 mM salts of the respective cations at the standard assay conditions for 30 minutes. The salts included $MgSO_4$, $MnSO_4$, $CuSO_4$, $CaSO_4$, $HgCl_2$, $FeSO_4 .7H_2O$. Effect of cation-chelator, EDTA, on the enzyme activity was also determined by including 10.0 mM of EDTA in enzyme-substrate mixture.

RESULTS AND DISCUSSION

Results:

Partial purification of 1, 4-β-endoglucanase:

Table 1 shows the purification steps for the 1, 4-β-endoglucanases of *P. chrysogenum*. The enzyme was purified by 1.4 folds using Ammonium sulphate at 80% saturation and 7.5 folds through gel filtration with Sephadex G25-300. The elution profile of the 1, 4-β-endoglucanase is shown in Figure 1. Two different active enzyme peaks were obtained. There was no significant difference between the activities of the two enzyme fractions. The 1, 4-β-endoglucanase fractions A and B gave the activity of 0.028 ± 0.002 and 0.031 ± 0.002 Units/ mL respectively.

Table 1: Purification of 1, 4-β-endoglucanases of *P. chrysogenum*.

Purification Step	Total Activity (Unit)	Total Protein (Mg)	Specific Activity (Units/ mg Protein)	Enzyme Yield (%)	Purification Fold
Crude Enzyme	59.3 ± 1.9	88.5 ± 4.5	0.67± 0.02	100	1
$(NH_4)_2SO_4$	28.3 ± 0.8	30.9 ± 2.2	0.90 ± 0.03	47.8	1.4
Sephadex G25-300	19.6 ± 0.6	2.6 ± 0.1	5.18 ± 0.2	20.9	7.5

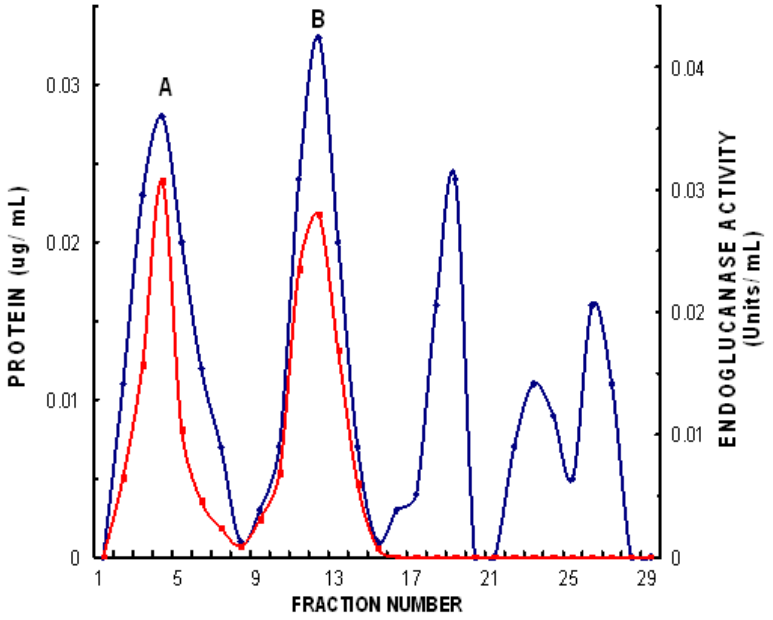

Fig. 1: Elution profile of 1, 4-β-endoglucanases of *P. chrysogenum* on Sephadex G25-300 showing protein content (♦) and enzyme activity (■), and the two active enzyme fractions, A and B. (0.1M Acetate buffer, pH 5.0; Flow rate, 1.0 mL/ minute).

Properties of the 1, 4-β-endoglucanase:

The effects of different pH, temperatures, metal ions and substrate concentrations on the partially purified 1, 4-β-endoglucanases of *P. chrysogenum* were determined.

Effect of pH:

Figure 2 shows the effect of different pH (3.0-9.0) on the activity of the 1, 4-β-endoglucanases of *P. chrysogenum*. A single peak at pH 4.9 was obtained for the 1, 4-β-endoglucanases of *P. chrysogenum*.

Temperature Profile: The effects of different temperatures (30-80 ^{O}C) on the 1, 4-β-endoglucanases of *P. chrysogenum* are shown in Figure 3. The Figure shows the relative 1, 4-β-endoglucanase activity (Percentage of maximum) incubated at 30-80 ^{O}C. The 1, 4-β-endoglucanase showed optimal activity at 48 ^{O}C with over 50% activity at 70 ^{O}C.

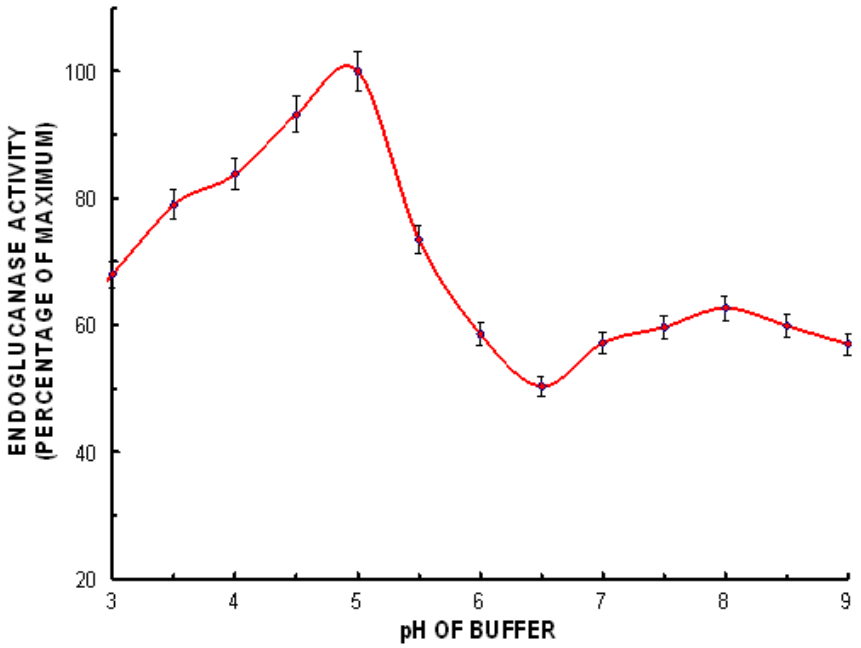

Fig. 2: Effect of different pH (3.0-9.0) on 1, 4-β-endoglucanases of *P. chrysogenum* (100 % = 5.18 ± 0.20 Unit mg Protein^{-1}).

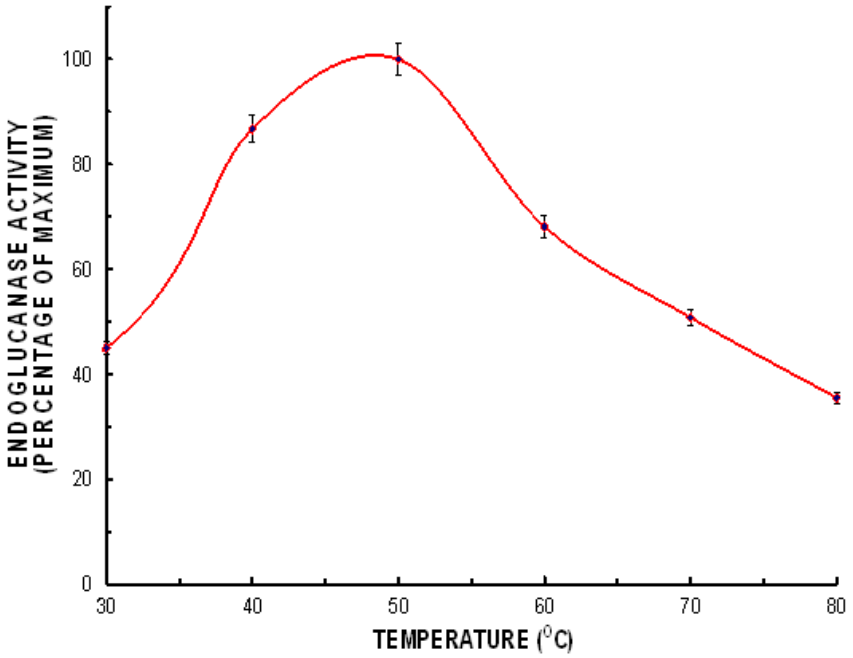

Fig. 3: Effect of different temperatures (30-80 ^{O}C) on the 1, 4-β-endoglucanases of P. chrysogenum (100 % = 5.18 ± 0.20 Unit mg Protein^{-1})

Time Course of the 1, 4-β-endoglucanases:

The graphs in Figure 4 show the total reducing sugars released by the 1, 4-β-endoglucanases of *P. chrysogenum* at the different time of incubation. Total reducing sugars released increased with incubation time, but at disproportionate rates. There was a very rapid release of glucose in the first 30 minutes followed by a decline thereafter up to 60 minutes. The rate of the release of glucose in the first 30 minutes was about 2.50 mmol glucose mg Protein^{-1} min^{-1}.

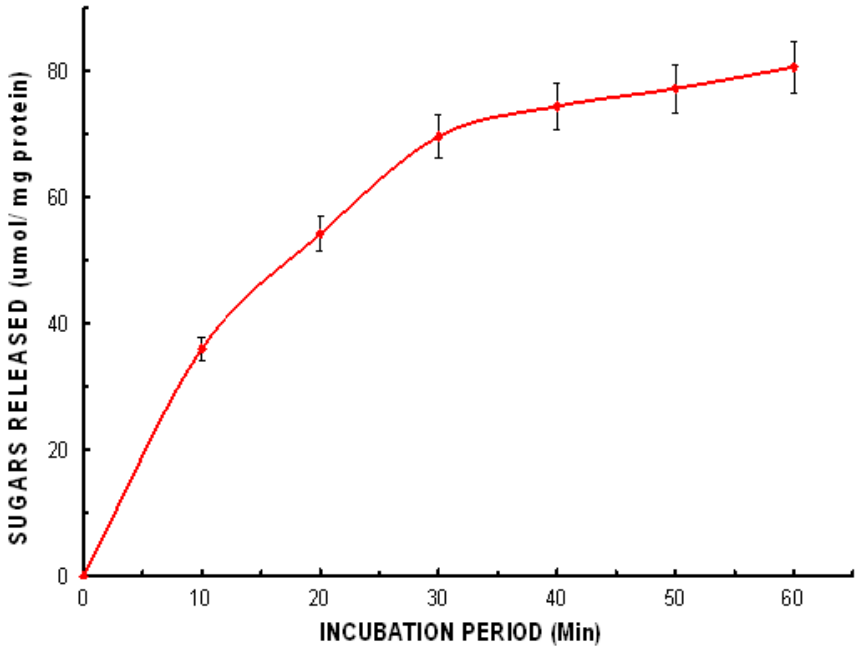

Fig. 4: Time Course of the 1, 4-β-endoglucanases of *P. chrysogenum*.

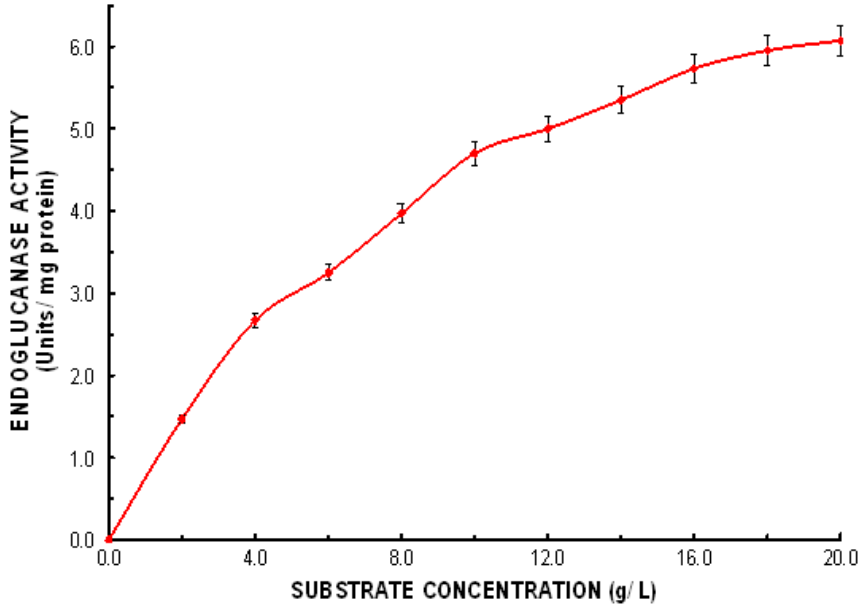

Fig. 5: Effect of substrate (CMC) concentration on the velocity (activity) of 1, 4-β-endoglucanase of *P. chrysogenum*.

Effect of Substrate Concentration on 1, 4-β-endoglucanases Activity:

Plots of endoglucanase activities versus substrate concentrations showed normal hyperbola curve (Figure 5). The cellulase activity increased rapidly as the substrate concentration increased between 2.0 – 10.0 gL^{-1}. Thereafter, subsequent increases in the substrate concentration had very small effect on the rate of enzyme activity. The Line Weaver – Burk plot (Figure 6) was used to determine the maximum velocities (Vmax) and Michaelis-Menten constants (Km) of the enzyme. Maximum velocity (Vmax) of 10.0 µmol min^{-1} mg $protein^{-1}$ was obtained for the endoglucanase *P. chrysogenum*. The Km value was 11.8 g L^{-1}.

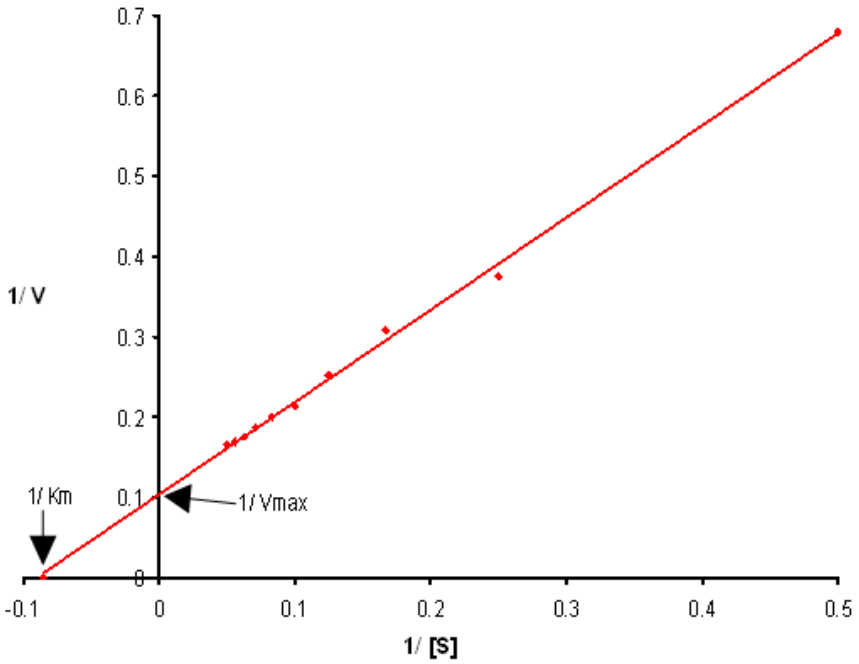

Fig. 6: Lineweaver-Burk plot (1/ V versus 1/ [S]) of the 1, 4-β-endoglucanases of *P. chrysogenum*.

Effects of Metal ions and EDTA on the 1, 4-β-endoglucanases Activity:

The effects of the metal ions and EDTA on the 1, 4-β-endoglucanase activity is shown in Table 2. Manganese ion (Mn^{2+}) exerted over 3-fold increase on the enzyme activity. Fe^{2+} increased the enzyme activity by 61.4%. Ca^{2+} has no significant effect on the enzyme activity. All the other metal ions and EDTA inhibited the enzyme activity. The percentage inhibition of Mg^{2+}, Cu^{2+}, and Zn^{2+}, Hg^{2+} and EDTA on the enzyme activity were 32.7%, 36.9%, 41.1%, 65.9% and 79.7% respectively.

Table 2: Effects of metal ions and Ethylene Diamino Tetraacetic acid (EDTA) on the activity of 1, 4-β-endoglucanases of *P. chrysogenum*.

Salts	Metal Ions	Concentration (mM)	% Activity	% Inhibition	% Stimulation
$MgSO_4 7H_2O$	Mg^{2+}	2.0	67.3	32.7	-
$MnSO_4.5H_2O$	Mn^{2+}	2.0	319.6	-	219.6
$FeSO_4.7H_2O$	Fe^{2+}	2.0	161.4	-	61.4
$CaCl_2$	Ca^{2+}	2.0	92.2	7.8	-
$CuSO_4.\ 7H_2O$	Cu^{2+}	2.0	63.1	36.9	-
$ZnSO_4$	Zn^{2+}	2.0	58.9	41.1	-
$HgCl_2$	Hg^{2+}	2.0	43.1	65.9	-
EDTA	-	10.0	20.3	79.7	-

Discussion:

Cellulase is recognized as a potential tool for the industrial saccharification of cellulosic biomass; its production is regarded as crucial for the successful utilization of cellulosic materials (Solomon *et al*., 1999; Wu and Lee, 1997). Several filamentous fungi are known to produce extracellular cellulases capable of hydrolyzing cellulose. *Trichoderma* and *Aspergillus species* are the main sources of available commercial cellulases (Berry and Paterson, 1990). The specific cellulase activity of 0.67 units/ mg protein (120.6 µg glucose/ min/ mg protein) obtained for the crude extracellular enzyme preparation of *P. chrysogenum* (PCL 501) compared favorably with the activity of 109.0 µg glucose/ min/ mg protein (0.61 units/ mg protein) reported for a commercial cellulase preparation from a strain of *A. niger* (Khan, 1980). The fact that the

organism produces endoglucanases as well as β-glucosidases and xylanases accentuates its suitability as a potential source of enzymes for industrial saccharification of cellulosic biomass into simple sugars. Xylanase is also required for the hydrolysis of native cellulose (Khan, 1980). The good yield of celulolytic enzymes (1, 4-β-endoglucanases, β-glucosidases and xylanases) by *Penicillium chrysogenum* (PCL 501) also validates the potential use of *Penicillium chrysogenum* mycelium from penicillin manufacture as additives in animal feeds to increase the content of proteins, sugars and mineral salts (Nuero and Reyes, 2002).

The endoglucanase of *P. chrysogenum* showed two different activity peaks on Sephadex G 25-300 gel chromatography. Gel chromatography separates proteins according to their molecular weights. The two fractions therefore imply the organism produces two different types of endoglucanases which differ in their molecular weights. Most cellulolytic fungi are known to produce isoenzymic forms of the endoglucanase having different molecular weights (Berry and Paterson, 1990; Beldman *et al.,* 1985). For instance, the *T. reesei* cellulase mixture consists of many catalytically active proteins; at least five endoglucanases (EG 1–5), two cellobiohydrolases (CBH 1-2), β-glucosidases, and hemicellulases have been identified by 2-dimentional electrophoresis (Beldman *et al*., 1985). The two enzyme fractions obtained in this study may therefore represent two isoenzymic forms of endoglucanase produced by the *P. chrysogenum* (PCL 501).

There was no significant difference between the activities of the two enzyme fractions. The endoglucanases have an optimum temperature of 48 ^{O}C and retain over 50% of their activity at 70 ^{O}C under the assay condition. It may be an adaptive property to the hot-humid climate from where the organisms were obtained. Optimal temperature of around 40 ^{O}C was obtained for the carboxymethyl-cellulase enzyme of a wild-type strain of *Aspergillus niger* Z10 (Coral *et al*., 2002). The higher optimal temperature obtained for the endoglucanase of the organism implies a better heat stability. This is desirable, especially in industrial processes where thermal treatment may be necessary. The enzyme showed an optimum pH of 4.9 and yielded at least 80% activity between pH 4.0 and 5.0. The implication is that the cellulase enzyme is well suited for the acidic environment of most fermentation processes, especially for simultaneous saccharification and fermentation (SSF) of lignocelluloses. Simultaneous saccharification and fermentation of sawdust and sugarcane pulp using the organism and a strain of *Saccharomyces cerevisae* yielded appreciable amounts of ethanol (Unpublished data).

A hyperbolic curve was obtained for the activities of the cellulase enzyme. The Line Weaver-Burk plot was used to obtain the maximum velocity (Vmax) as well as the Michaelis-Menten constant (Km) of the endoglucanases. With the Vmax of 10.0 μmolmin^{-1} mg protein^{-1} and Km value of 11.8 g/L, the cellulases of *P. chrysogenum* has better activity than that of most cellulolytic organisms. In a comparative work done in our laboratory, the cellulase enzyme of Aspergillus niger (ANL 301) gave a Vmax of 4.4 μmolmin^{-1} mg protein^{-1} and Km value of 12.5 g/L (unpublished data).

Metal ions and EDTA were found to have profound effects on the activity of the endoglucanases of the organism. The stimulatory effect of manganese ions on the cellulase activity is quite phenomenal. The metal ion exhibited over 200% stimulation of the activity of cellulase enzymes of the organism. This implies a significant increase in the yield of hydrolytic products. Fukumoto and Kishi (1952) reported a phenomenal increase of cellulase activity by manganese stimulation, but their finding was largely ignored because, as at the time, no cellulase enzyme had been shown to have any prosthetic group or coenzyme, or to require any metal ions for its activity (Mandels and Reese, 1956). In an earlier work, Fahraeus (1947) observed that *Cytophaga* can not grow on cotton wool unless calcium and manganese were added to the medium. This work clearly indicates that manganese certainly has stimulatory effect on the activity of the cellulases. Calcium on the other hand had no significant effect on the enzyme activity. There is the possibility that the metal ion may indeed be a requirement for the enzyme activity and might even be an integral component of the enzyme complex. The potent inhibitory effect of EDTA, a chelating agent of divalent metal ions, on the enzyme activity may be connected to the removal of the manganese ions from the enzyme mixture. There is the need to further examine and establish the mechanism of manganese stimulation of the cellulase enzymes. Mercury ions have a strong inhibitory effect on the cellulase activity. This is normal since heavy metals are known to inhibit the activity of most enzymes (Lehninger, 1982).

In conclusion, the activity of crude enzyme preparation from *P. chrysogenum* (PCL 501) was found to compare favorably with that of commercially available cellulase preparation. The endoglucanase (EC 3. 2. 1. 4) of the organism appear to occur in two isoenzymic forms with different molecular weights. Manganese ion has been found to be an effective activator of the endoglucanases of *P. chrysogenum*, with over 3-fold stimulatory effect on the hydrolytic activity of the enzyme.

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