JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2013. Vol. **7**(2), p. 987-996

Purification and Kinetic Studies of Exo-β-1, 4- glucanase and Endo- β -1,4-glucanase from a New Strain of *Clostridium papyrosolvens*

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(Received: 26 September 2012; accepted: 10 November 2012)

Anaerobic bacteria producing cellulases have not been extensively studied though they are the efficient producers of cellulase. *Clostridium papyrosolvens* CFR-1010, an anaerobic cellulolytic bacterial strain was identified as a potent cellulase producer and selected for the purification and kinetic studies of Exo-β-1,4-glucanase and Endo-β-1,4-glucanase. The molecular masses of the enzymes were 65 and 60 kDa, respectively. The enzymes showed maximum activities at pH 5.0 and at the temperature of 50 °C. The activities increased in the presence of $MnCl_2$, whereas, *N*-bromosuccinimide decreased enzyme activities by 68 and 75% respectively, thus suggesting the presence of tryptophan residues at the active sites of enzymes. Exoglucanase had a K_m of 20 mg/ml and V_{max} of 22 units/min/mg of protein whereas the endoglucanase exhibited a K_m of 6.66mg/ml and V_{max} of 11.76 units/min/mg of protein. Results of the present studies suggest the use of *C. papyrosolvens* for cellulase production in shorter periods of time and they also add significance for the exploration of this organism for industrial applications.

Key words: *Clostridium papyrosolvens*, Cellulase, Exo-β-1,4-glucanase, Endo-β-1,4-glucanase, Anaerobic, Cellulolytic.

Cellulose is the largest waste component, thus creating serious pollution problem. The biotechnological approach for conversion of cellulosic and lignocellulosic wastes will provide an alternate source of carbohydrates for the production of chemicals, food, feed and fuels. Further, this also helps in the reduction of waste accumulation in the environment. This involves the enzymatic degradation by fungi and bacteria. Enzymatic hydrolysis is simpler and more economical process than chemical and mechanical methods[1]. For the hydrolysis of the crystalline cellulose, three cellulase components, such as exo-β-1,4-glucanase, endo-β-1,4–glucanase and β-glucosidase are required[2,3]. There are certain limitations with the fungal cellulases such as low temperature stability and lower yield of exoglucanase. Some of the extensively studied fungi for cellulase production are *Trichoderma reesei*[4], *Trichoderma Koningi*[5], *Aspergillus niger*[6], *Aspergillus fumigates*[6] and *Aspergillus terreus*[7] which have a comparatively low exoglucanase production. In contrast to this, bacteria grow in shorter periods of time and produce all the components of cellulase and in particular, exoglucanase in higher titers[8].

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The low level of exoglucanase production in fungi results in the limited hydrolysis of cellulose owing to the accumulation of cellodextrins. Extensive studies have been made to enhance cellulase activity in fungi and reported but comparatively less number of reports are found on bacteria. The bacterial cellulases have shown higher activity against crystalline cellulose (cotton and avicel)[9] and also high thermostability[10-12]. Among bacteria, anaerobes are known to produce cellulase efficiently[13-15].

Earlier, we have isolated *Clostridium papyrosolvens* CFR 1010 strain that produced higher levels of exoglucanase and endoglucanase extracellularly under anaerobic conditions. The titers of the enzymes produced were higher in comparison with some of the fungal strains[16] and wild-type *C. papyrosolvens*[17]. From the biotechnological view, production of higher levels of extracellular exoglucanase and endoglucanase are of important and our current interest. Earlier, Sharmila *et.al.,* reported the purification and characterization of β-1,4–glucosidase from *Clostridium papyrosolvens*[18]. In addition, their studies have shown the influence of different carbon sources and regulation of cellulase production by trehalose in *Clostridium papyrosolvens*[19,20] and also the influence of anaerobic bacterial consortia on biogas production[21]. In the present investigation, the objective was focused on the purification and kinetic studies of exo-β-1,4-glucanase and endo-β-1,4-glucanase from *C. papyrosolvens* CFR-1010.

MATERIALS AND METHODS

Bacterial strain and cultural conditions

The anaerobic cellulolytic bacterium, *Clostridium papyrosolvens* was isolated from a consortium developed in the laboratory from the compost sample collected in the surroundings of Mysore, Karnataka, India. The strain was characterized by standard taxonomical methods from The Bergey's Manual of Systematic Bacteriology. The organism was grown in the modified CM3 medium[17] cultivated under anaerobic conditions, in the glove box (Don Whitley, U.K.) and it was named as *C. papyrosolvens* CFR – 1010.

Chemicals

Cellulose, cellobiose, carboxymethyl-cellulose (CMC), Avicel, Acryl amide, bisacrylamide and Di ethyl amino ethyl (DEAE) Sephadex A-25 were obtained from sigma chemicals (St. Louis, MO. U.S.A). Protein standards were obtained from Biorad (Richmond, CA, U.S.A). All other chemicals were procured from Hi media (Mumbai, India).

2.3 Preparation of the enzyme

In brief, for production of exo-β-1,4–glucanase and endo-β-1,4-glucanase, 300 ml of pre-reduced cultivation medium was inoculated with cells of a previously grown culture and incubated at 30°C in a rotary shaker for 96 h. The cultivation medium contained 1% (w/v) cellulose, 0.25% bactocasitone, 0.06% yeast extract, 0.4% sodium bicarbonate, 15 ml of solution A (consisting of 300 mg of KH_2PO_4 600 mg of $(NH_4)_2SO_4$, 600 mg of NaCl, 60 mg of $MgSO_4$, 60 mg of $CaCl_2$ anhydrous in 100 ml of distilled water), 15 ml of solution B (300 mg of K_2HPO_4 in 100 ml of distilled water), 10 ml of centrifuged rumen fluid, 0.05% cysteine hydrochloride and 0.1 ml of 0.1 % resazurin. The medium was sterilized at 121°C for 15 min. After cooling, oxygen was removed by flushing with gas mixture of $N_{2+}CO_2$ (80:20, v/v) until it turned from pink to colorless. The culture broth was centrifuged at 10,000 g for 30 min at 4°C and supernatant was taken for further studies.

Enzyme assays

Activities of exoglucanase (avicelase) and endoglucanase (CMCase) were determined by estimating the glucose units, released from the substrates avicel (microcrystalline cellulose, 10 mg/sample) and carboxymethyl cellulose (10 mg/sample) respectively. For both the samples, 1ml of appropriately diluted culture supernatant was added to the substrates in 0.2 M citrate phosphate buffer (pH 5.0). The reaction mixtures were incubated at 50 °C for 30 min. The reactions were stopped by the addition of 2 ml of Dinitrosalisilic acid (DNS) reagent, kept in boiling water bath for 5 min, followed by cooling and diluted with 20 ml of Double Distilled Water (DDW). In case of avicelase activity, residual substrate was removed through membrane filter (0.4-¼). The amount of reducing sugars produced was measured at 540 nm and estimated from a standard glucose curve. One unit (International unit -IU) of enzyme activity is defined as 1μmol of glucose released per minute per ml of the culture supernatant.

Total cellulase activity (Filterpaperase -

FPase) was estimated based on the reducing sugars released, from the filter paper (1x6 cm strip of whatman No.1, weighing 50 mg) strips. The incubation period of the reaction mixture was 60 min. The other part of the assay was same as explained above.

Purification of enzymes

All the purification steps were carried out at 4°C. The crude enzymes were collected by centrifugation of the culture broth and separating it from the cell debris. This was followed by precipitation, which was done separately by using ammonium sulphate, ethanol, isopropyl alcohol and acetone at 66% (v/v) final concentration. The mixture was stirred vigorously and then kept for 2 hrs, further centrifuged at 10,000 g for 15 min. The precipitate was dissolved in 10 mM ammonium acetate buffer (pH 5.0) separately. An aliquot of the dissolved precipitate obtained from a precipitant giving maximum recovery of the enzymes (mentioned in section 3.1) was applied onto the pre-equilibrated DEAE-Sephadex A-25 column (3 cm x 30 cm). The unbound proteins were eluted with 10 mM ammonium acetate buffer (pH 5.0). Elution was performed with an increasing gradient of the buffer (0-1 M, pH 5.0) with flow rate of 20 ml/h. Fractions were collected and examined for exoglucanase, endoglucanase and total cellulase activities.

Dialysis

Fractions exhibiting higher enzyme activities were pooled and dialyzed against DDW for exoglucanase and endoglucanase separately and the samples were freeze- dried and were used for electrophoretic analysis.

Electrophoresis

The freeze-dried samples were dissolved seperately in sample buffer (SDS 2%, Tris 60 mM, glycerol 15%, Bromophenol Blue 0.001%, pH 6.8) and electrophoresed. An 8 and 11% of polyacrylamide gel were used for native and Sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS- PAGE) respectively. Electrophoresis was performed for 4 h at a constant current of 20 mA. After that, gels were stained with 0.025% Coomassie Brilliant Blue R-250 prepared in 18% of methanol (in 7% acetic acid and 5% of trichloroacetic acid) and kept for overnight and destained by placing the gels in 500 ml of 0.5 M NaCI solution for 6 h.

RESULTS AND DISCUSSIONS

Purification of enzymes

The effect of selective precipitation on the recovery of different components of cellulase complex is given in Table 1. It is evident from the results that maximum recovery of the components of cellulase was obtained from ethanol precipitation that resulted in the recovery of 62% of FPase, 55% of avicelase and 60.77% of CMCase and they exhibited enzyme activities of 6.02, 4.28 and 10.04 IU, respectively.

The different peaks obtained from the gel filration chromatography of DEAE sephadex A-25 are shown in Fig.1 and Fig.2. The enzyme complex was resolved into five major protein peaks. The major exoglucanase activity was exhibited in peak 5 and endoglucanase activity in peak 3. First and second peaks exhibited mixtures of the enzyme

Table 1. Precipitation and recovery of Fpase, Avicelase and CMC ase of the crude enzyme of *C. papyrosolvens*

	Total cellulase (FPase)		Exoglucanase (Avicelase)		Endoglucanase (CMCase)	
	Units /ml	% Recovery	Units/Vol	%Recovery	Units /ml	%Recovery
Cell free culture supernatant	9.6	100	7.7	100	16.8	100
Ammonium Sulphate	4.5	46.9	3.0	39.2	7.9	47.0
Ethanol	6.0	62.0	4.3	55.0	10.0	60.8
Isopropyl alcohol	3.3	34.1	2.4	30.7	6.9	40.6
Acetone	3.0	29.5	2.5	33.0	5.0	29.6

66% concentration of precipitants and solvents were used in each case.
Values are taken as mean of 3 samples

activities whereas the fourth peak exhibited β-glucosidase activity.

The results of the purification of exoglucanase and endoglucanase are summarized in Tables 2 and 3 respectively. After the DEAE – Sephadex chromatography, purified protein content of the pooled fractions was decreased to 0.3% with a 153 fold increase in specific activity of exoglucanase compared to crude enzyme preparation (Table-2). The yield of the enzyme was found to be 51%. These values are higher as compared to that of earlier report for exoglucanase (Avicel II) of the anaerobic bacterium *Clostridium stercorarium*[9], exoglucanase extracted from the starchy endosperm of barley[22] and that of the aerobic fungus *Trichoderma viride*[23].

Similarly, in case of endoglucanase, the protein content was decreased to 0.2% and there was 202 fold increase in specific activity compared with crude enzyme (Table 3). These findings are consistent with the previous studies on *Thermotoga neapolitana* reported by Bok *et al*.,[10] and aerobic fungus *Talaromyces emersonii* CBS 814.70[25].

Electrophoretic characterization of purified enzyme

Electrophoretic analysis of different enzyme fractions is shown in Fig. 3. The crude enzyme showed numerous protein bands (Fig.3-lane f). In contrast, DEAE – Sephadex fractions showed only a single major band of both exoglucanase and endoglucanase enzymes indicating the purity of the isolated enzymes (Fig 4- lanes f and d respectively). The apparent molecular masses of the exoglucanase and endoglucanase were 63 kda and 60 kda respectively. These results are comparable with that of *Streptromyces flavogriseus* and *Trichoderma viride*[21,23]. The molecular mass of endoglucanase is well comparable with those of *Trichoderma viride*[23].

The SDS-PAGE analysis of the purified fractions from *C. papyrosolvens* resolved into single bands, that show the purity of exo-β-1,4-glucanase and endo-β-1,4-glucanase.

Activity of xylanase and protease

Xylanase and protease activities of all the five peaks were conducted. One unit of xylanase activity is one µmol of xylose released /ml/min. One unit of protease activity is one mg of tyrosine released by one ml of the enzyme /min. The activities of both the enzymes were exhibited by the peaks I (0.52 units of xylanase and 32 units of protease) and II (0.85 units of xylanase and 36 units

Table 2. Summary of the purification of exoglucanase from *C. papyrosolvens* CFR-1010.

Steps	Protein (mg/ml)	Total activity recovered (units /ml)	Specific activity (units /mg of protein)	Yield (%)	Purification (fold)
Crude extract	112.0	7.7	0.1	100.0	1.0
Ethanol precipitation	16.5	4.3	0.1	55.0	1.2
DEAE- Sephadex –A- 25.Peak No. 5.	0.4	4.0	10.7	51.4	153.6

Values are taken as mean of 3 samples

Table 3. Summary of the purification of endoglucanase from *C. papyrosolvens* CFR-1010

Steps	Total protein (mg/ml)	Total activity recovered (units /ml)	Specific activity (units /mg of protein)	Yield (%)	Purification (fold)
Crude extract	112.0	16.8	0.2	100.0	1.0
Ethanol precipitation	16.5	10.0	0.6	60.8	4.0
DEAE- Sephadex –A- 25.Peak No. 3	0.3	8.5	30.3	50.5	202.3

Values are taken as mean of 3 samples

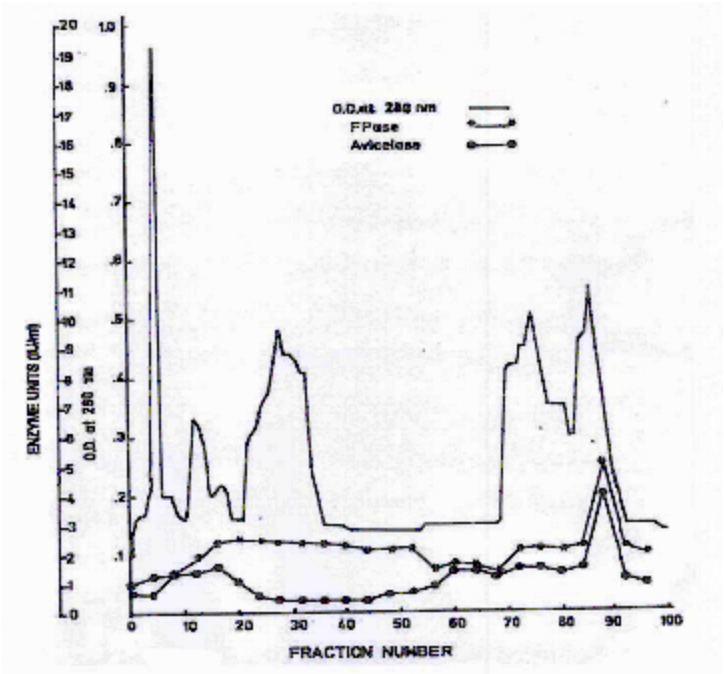

Fig. 1. Fractionation of the cellulase complex from precipitated enzymes of *Clostridium papyrosolvens*by DEAE-Sephadex A-25 ion – exchange chromatography

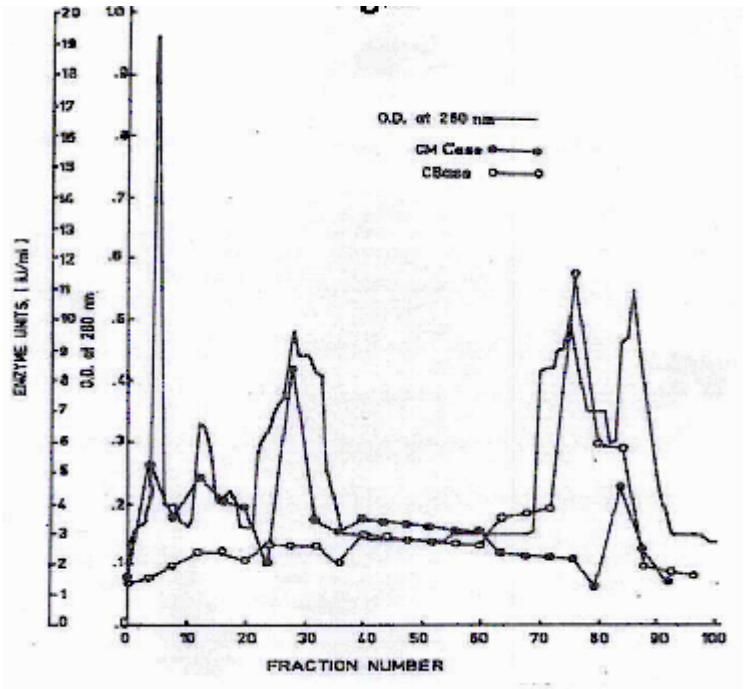

Fig. 2. Fractionation of the cellulase complex from precipitated enzymes of *Clostridium papyrosolvens* by DEAE-Sephadex A-25 ion – exchange chromatography

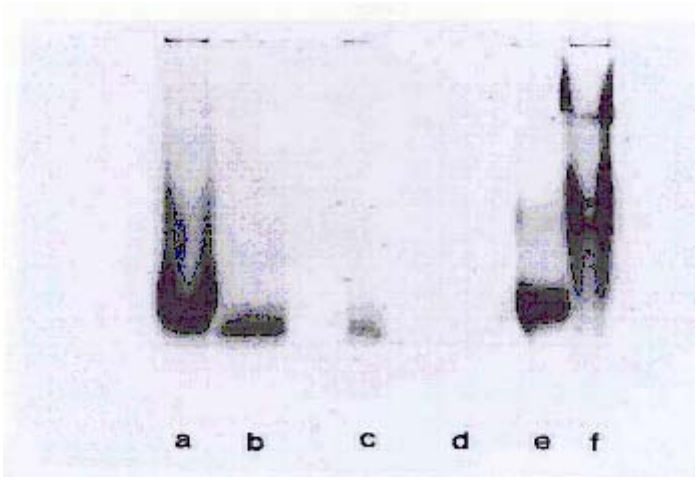

Fig. 3 : Native PAGE of cellulase complex: a) Peak I, b) Peak II,
c) Peak III, d. Peak IV, e. Peak V, f) Crude enzyme (10 µg of protein was loaded in each lane)

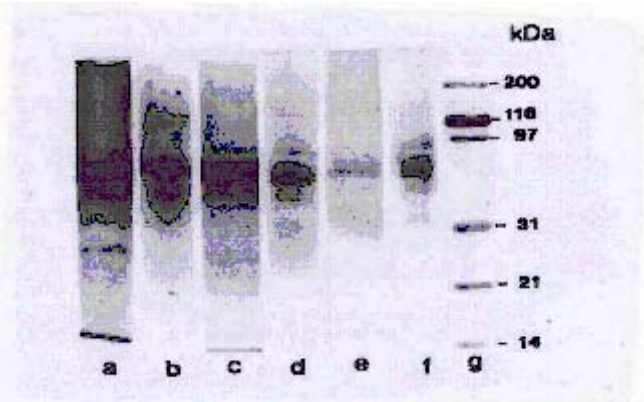

Fig. 4. SDS-PAGE of cellulase complex: a)Crude enzyme, b) Peak I, c) Peak II, d) eak III,
e) Peak IV, f) Peak V, g) Standard Molecular weight markers :Mysoline (200 kDa), β- galactosidase (116 kDa)
Phosphorylase b (97 kDa), Carbonic anhydrase (31 kDa), Soybean trypsin inhibitor (21 kDa)
And Lysozyme (14 kDa)

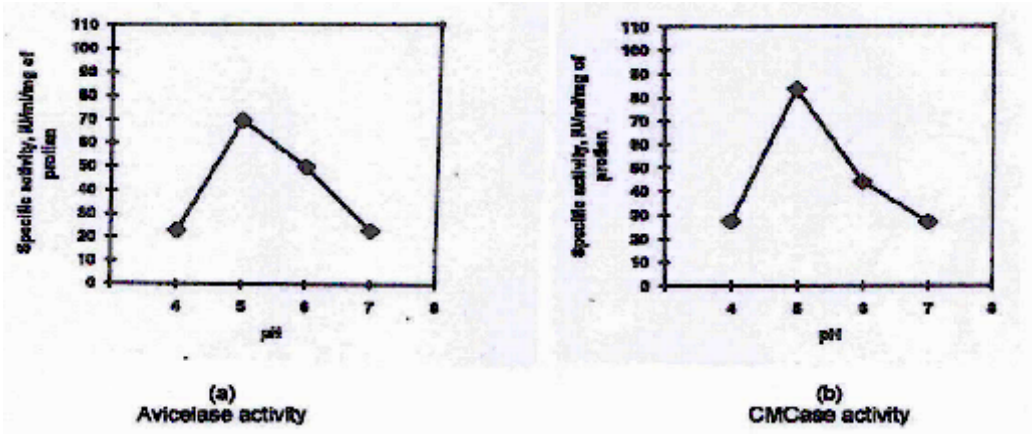

Fig. 5. pH optima for Avicelase and CMCase enzymes

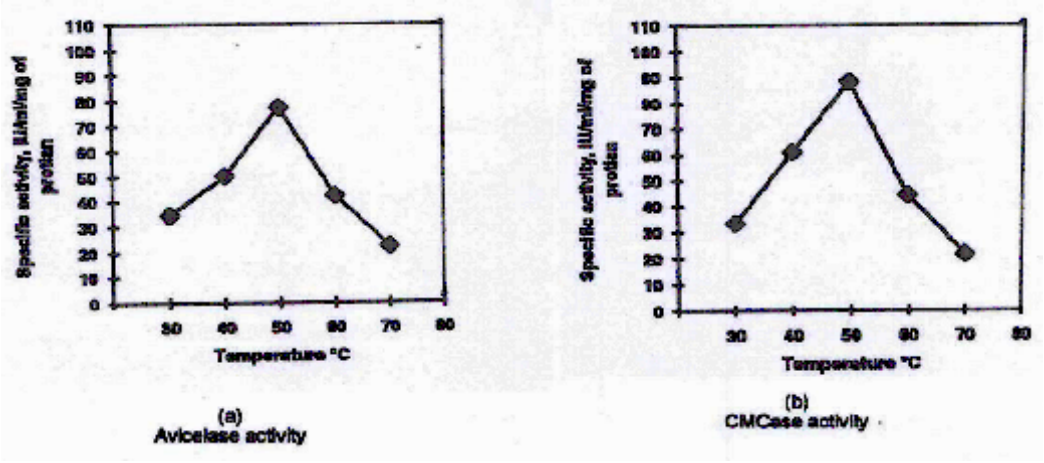

Fig. 6. Temperature optima for Avicelase and CMCase enzymes

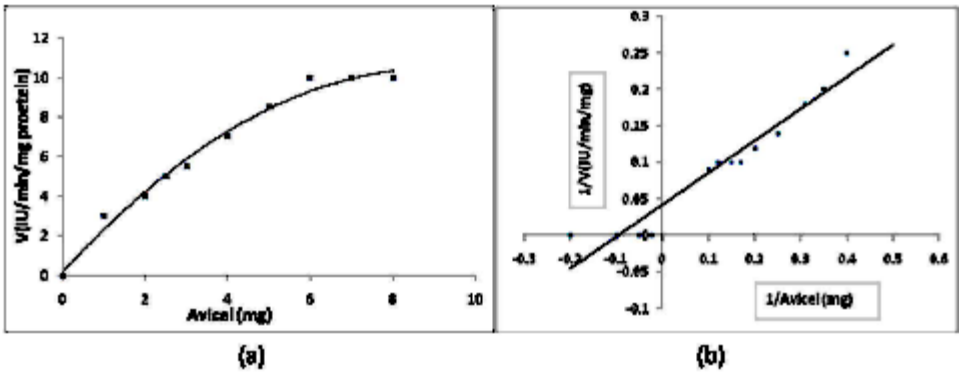

Fig. 7. (a) Michaelis-Menten plot of exo-β-1,4-glucanase activity with avicel as substrate
(b) Lineweaver-Burk plot

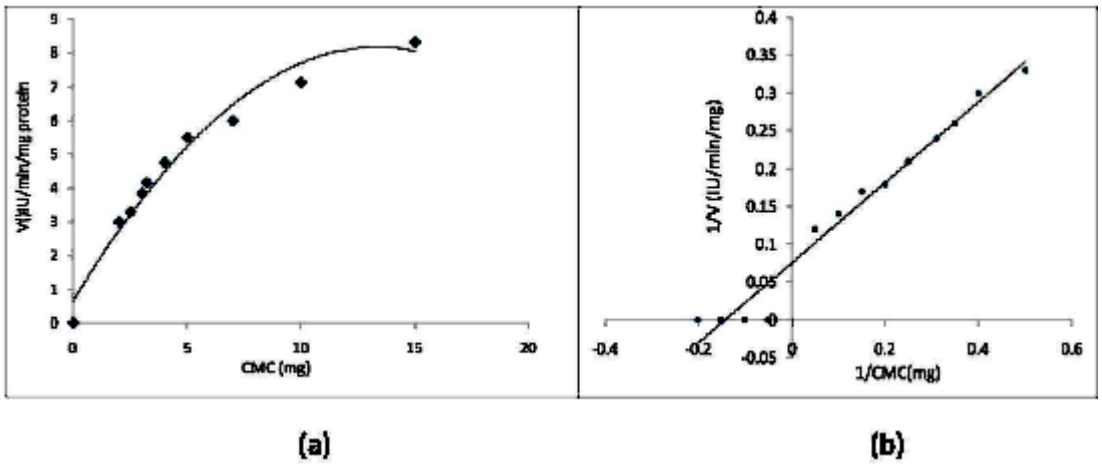

Fig. 8 : (a) Michaelis-Menten plot of endo-β-1,4-glucanase activity with CMC as substrate
(b) Lineweaver-Burk plot

of protease) whereas peak III exhibited low activity of protease (9 units) but no xylanase activity. Peaks IV and V did not show any of the activities. From this, it is evidenced that the peaks exhibiting majorly exoglucanase and endoglucanase activities (Pk III & IV) did not exhibit the other enzyme activities.

Factors affecting the activity of purified enzymes

The activities of the purified exoglucanase and endoglucanase were maximum at pH 5 (Figure 5a & b) and at the incubation temperature 50 °C (Fig. 6a & b). These findings are varied for endoglucanase when compared with that of *Thermoascus aurantiacus*[26] (pH 4.0 to 4.4), *Thermotoga neapolitana*[10] (6.0 to 6.6) and *Bacillus polymyxa*[27] (temp. 55-60 °C). But for exoglucanase, these findings were comparable with the previous reports[9, 22,23].

The effect of metal ions

The influence of various metal ions on the activity of exoglucanase and endoglucanase of *C. papyrosolvens* was carried out. It is evident that, exoglucanase enzyme activity was increased by 148% over the control with $Mncl_2$. A 68% inhibition of enzyme activity was observed when treated with N-bromosuccinimide, thus indicating the possible presence of tryptophan residues at the active sites of the enzymes.

In the case of endoglucanase, increased enzyme activity of 162% with $Mncl_2$ over the control and inhibition of 75% with N-bromosuccinimide were observed. Exoglucanase is being inhibited by many of the metal ions (Al^{3-} $^{3-}$, Ca^{2+}, Co^{2+}, Cl^{2-}, Cu^{2+}, Na^{+}, Mg^{2+}, and Fe^{2+}) thus indicating its sensitivity for them. Whereas endoglucanase is inhibited by very few metal ions (Ca^{2+}, Co^{2+}, Cl^{2-}, Zn^{2+}, Cu^{2+}), thus indicating its stability towards the same. In addition, EDTA and iodoacetamide inhibited the enzyme activity and nonidet P40 did not show any inhibition for exoglucanase. In contrast to exoglucanase, endoglucanase was inhibited by EDTA, iodoacetamide as well as by nonidet P40.

These findings are consistent with that of the previous reports on exoglucanases extracted from endosperm of barley[22]. But, endoglucanase activity of the fungus *Aspergillus terreus*[8] was reported to be positively modulated in the presence of Cu^{2+}, Na^{+}, Mg^{2+}, Ca^{2+}, DTT and mercaptoethanol.

Kinetic studies for Exoglucanase and Endoglucanase

The graph of substrate concentration verses enzyme activity (Michaelis Menten plot) was drawn for exo -β -1,4-glucanase (Fig.7 (a)) and it has shown the increased enzyme activity with substrate concentration up to 10 mg, above this range enzyme activity was inhibited. The K_m and V_{max} values of the exoglucanase for avicel, at pH 5.0 and 50°C were determined by plotting Lineweaver-Burk graph (Fig.7 (b)). The K_m value of enzyme is 20 mg/ml and V_{max} of 22 units/min/mg of protein. These values are comparatively higher as compared with the previous report on anaerobic bacterium, *Ruminococcus flavefaciens* [30] which exhibited the K_m of 3.08 mM and a V_{max} of 0.298 units/min/mg of protein respectively.

Similarly, the Michaelis Menten plot for endo-β -1,4-glucanase was drawn (Fig.8 (a)) and found that the enzyme activity increased with substrate concentration of 12 mg, above which the enzyme was inhibited. The K_m and V_{max} values of the endoglucanase for the substrate carboxymethyl cellulose at pH 5.0 and 50°c were determined by plotting Lineweaver-Burk graph (Fig.8 (b)). The enzyme had a K_m of 6.66 mg/ml V_{max} of 11.76 units/min/mg of protein. These values are comparable with the previous study[31].

CONCLUSIONS

Most exoglucanases are associated with bacteria and fungi. Higher titers of this enzyme are found to be produced in anaerobic bacteria[2-4] compared to fungal strains. Here, a strain of *C. papyrosolvens* isolated locally produced higher titers of extracellular exo-β-1,4-glucanase in the fermentation broth. The properties of the enzyme were however similar to those of other cell bound exoglucanases reported for other *Clostridium* species.

Exoglucanase was found to be the major component of *C. papyrosolvens* CFR-1010, whereas endoglucanase was the component next to it. Exoglucanase was purified to 153 fold and endoglucanase to 202 fold by ethanol fractionation, dialysis and DEAE-Sephadex A-25 chromatography. Analysis by gel electrophoresis exhibited a single major band of both exoglucanase and endoglucanase enzymes which indicate their

purity. Both enzymes were characterized by a high level of activity (high V_{max} value and low apparent K_m value) with avicel and carboxy methyl cellulose respectively. The exo 2-1,4-glucanse and endo-β-1,4-glucanase from *C. papyrosolvens* CFR-1010 are applicable for the biodegradation of cellulosic wastes which can be used in the biogas digesters and for the commercial production of glucose or various other fermentation products.

ACKNOWLEDGMENTS

T. Sharmila acknowledges the grant of SRF by the CSIR, New Delhi, India. Authors also acknowledge Dr. G. Sreeramulu for his help rendered in this work and Dr. R. Lakshminarayana for his critical reading of the manuscript and his comments.

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