

Full Length Research Paper

Purification and characterization of cellulase from the wild-type and two improved mutants of *Pseudomonas fluorescens*

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Cellulases from the wild-type (WT) and two improved mutants (catabolite repression resistant mutant 4 and 24, abbreviated CRRmt₄ and CRRmt₂₄, respectively) of *Pseudomonas fluorescens* were purified to apparent homogeneity by ammonium sulphate precipitation, ion exchange chromatography on DEAE Sephadex A-50 and gel filtration on Sephadex G-100. Purification fold of about 5 was obtained for the WT and CRRmt₂₄ while purification fold of about 7 was achieved for CRRmt₄ by ammonium sulphate precipitation. Ion exchange chromatography gave purification fold of about 24, 22 and 25 for WT, CRRmt₄ and CRRmt₂₄, respectively. Gel filtration chromatography step yielded a homogeneous preparation with a specific activity of 6.8, 5.9 and 6.9 units/mg protein for the WT, CRRmt₄ and CRRmt₂₄, respectively. The purified cellulase gave a single protein band on polyacrylamide gel electrophoresis. The molecular weights of the three cellulases were estimated to be 36, 26 and 36 kDa for the wild-type, CRRmt₄ and CRRmt₂₄, respectively. Km values of 3.6, 3.1, and 5.3 mg/ml were obtained for the wild-type, CRRmt₄ and CRRmt₂₄, respectively. The optimum pH value for the purified cellulases was 6.5 – 7.0 and the enzymes were optimally active at temperature of 35 °C. The activities of the purified cellulases were stimulated by low concentrations (10-30 mM) of Na⁺ and Mg⁺⁺ while EDTA was found to inhibit enzyme activity at all concentrations.

Key words: *Pseudomonas fluorescens*, cellulase, mutants, purification, molecular weight, characterization.

INTRODUCTION

Cellulose is the most abundant renewable natural product in the biosphere (Whitaker, 1990, Solomon et al., 1997). Annual production of cellulose is estimated to be 4.0 x 10⁷ tons (Singh and Hayashi, 1995). The proportion of cellulose in plant tissues ranges from 20 to 45% of dry weight and to almost over 90% in cotton fibre (Stephens and Heichel, 1975). Much of the cellulose in nature

exists as waste paper (Crueger and Crueger, 1990).

The 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 industrial processes (Coughlam, 1990). The use of these materials especially animal wastes from feedlots is currently almost exclusively restricted to spreading on fields as fertilizer.

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 cellulase enzymes (Cooney et al., 1978). From the economic point of view,

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acid hydrolysis of cellulosic materials appears to be cheaper than cellulase hydrolysis but the former often requires high temperature and pressure; it is highly corrosive, and leads to the accumulation of objectionable by-products (Fennington et al., 1982). Enzymic hydrolysis does not have these disadvantages. The high moisture content of the biomass makes it to be less suitable for most chemical treatments and more suitable for biological processing. 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). Consequently, thermophiles are being aggressively pursued to provide new enzymes that are highly thermostable depending on the environment of the native organism (Brennan, 1996). Since biomass is abundant and reasonably inexpensive, the key to successful commercialization of process using biomass is the development of efficient and economical conversion methods such as enzyme hydrolysis. The objective of the present report was therefore to produce mutants of *Pseudomonas fluorescens* capable of producing cellulase, and to purify and characterize the induced cellulase.

MATERIALS AND METHODS

Materials

Folin-ciocalteu's (phenol) reagent, bovine serum albumin (BSA), D-glucose, Ethylenediaminetetraacetic acid (EDTA disodium salt), Ovalbumin, ChymotrypsinogenA, Potassium sodium tartrate and RibonucleaseA were obtained from Sigma Chemical Company, Limited, St. Louis, MO, U.S.A. DEAE Sephadex A-50 and Sephadex G-100 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Acrylamide, N,N'-methylene bis-acrylamide (TEMED) and ammonium persulphate were products of Feinbiochemica, Heidelberg, Germany. Coomassie brilliant Blue R250, sucrose, 2-mercaptoethanol, methanol, ammonium molybdate, glacial acetic acid, formaldehyde, ethanol, glycine and carboxymethyl cellulose (CMC) were obtained from BDH chemicals Limited, Poole, England. All other reagents were of analytical grade and were obtained from either Sigma Chemical Company or BDH. The organism, *Pseudomonas fluorescens* NCIB3756 for this study was supplied from the culture collection of the Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria. The culture was originally obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland. The stock culture was maintained on nutrient agar slants and stored at 4°C in the refrigerator. Subculturing of the organism into new agar slants was carried out every month in order to have fresh culture of the organism throughout the duration of the study.

Isolation of *P. fluorescens* mutants

The modified procedure of Shonukan and Nwafor (1989) was employed to mutagenize the cells of *P. fluorescens*. Ethylmethanesulphate (EMS) was used as the mutagenic agent.

Preparation of crude enzyme

All the mutants and the wild-type were cultured in basal medium containing 0.2% (w/v) CMC and incubated for 24 h on a rotary shaker (100 rev/min) at 37°C. Each of the cultures was centrifuged at 6,000 g for 15 min. The clear supernatants were collected aseptically as extracellular cellulase preparations.

Cellulase assay

Cellulase activity towards carboxymethyl cellulose was measured by the appearance of reducing end groups in solution of CMC using Nelson (1944) method. The cellulase activity was determined by incubating the enzyme (0.5 ml) and 0.2% (w/v) CMC (0.5 ml) in 0.1 M sodium phosphate buffer, pH 7.0 at 37°C for 1 h. The reaction was terminated by the addition of 1.0 ml of combined copper reagent and heated for 20 min in a boiling water bath. The tubes were cooled down to room temperature and 1.0 ml of arsenomolybdate reagent was added to all the tubes. The tubes were diluted with distilled water (7.0 ml) and the optical density (OD) was read at 540 nm against the blank. The amount of glucose released per ml was estimated from a standard curve prepared with known glucose concentration. One unit of cellulase activity was expressed as the amount of protein that liberated reducing sugar equivalent to 1 µg of glucose per minute under assay condition. The specific enzyme activity was expressed as the unit of enzyme activity per mg of protein.

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.

Enzyme purification

Ammonium sulphate precipitation: The proteins in the crude preparation were precipitated by the addition of solid ammonium sulphate to 90% saturation. The precipitate was allowed to form at 4°C for 24 h, and was collected by centrifugation at 4,000 g in a cold centrifuge at 4°C for 30 min. The precipitate was redissolved in 10 ml of 0.02 M sodium phosphate buffer, pH 7.0 containing 1 mM EDTA and 1 mM 2-mercaptoethanol.

Purification by ion exchange chromatography on DEAE-sephadex A-50: DEAE Sephadex A-50 column was packed into a vertically mounted column (1.5 x 40 cm) at a flow rate of 30 ml/h. The column was equilibrated with 3 bed volumes of 0.02 M sodium phosphate buffer, pH 7.0 containing 1 mM EDTA and 1 mM 2-mercaptoethanol. The enzyme concentrate obtained from ammonium sulphate precipitation was redissolved in minimal amount of buffer and dialysed for 24 h against 0.02 M sodium phosphate buffer, pH 7.0 containing 1 mM EDTA and 1 mM 2-mercaptoethanol with two changes of the buffer per hour. Undissolved precipitate was removed by centrifugation and the clear supernatant was layered on the prepared column. The column was washed to remove all unbound proteins and a linear gradient of 0 to 0.5 M NaCl in 0.02 M sodium phosphate buffer pH 7 was used to elute any bound proteins. Fractions (5 ml) were collected at a flow rate of 30 ml/hr. Fractions containing cellulase activities were pooled together and precipitated with ammonium sulphate. The precipitate was collected by centrifugation at 4000 g in a cold centrifuge at 4°C for 30 min, redissolved in 2.5 ml of 0.02 M sodium phosphate buffer, pH 7.0 and dialysed against the same buffer for 6 h.

Table 1. Summary of Purification of Cellulase from the improved mutants and wild-type of *Pseudomonas fluorescens*.

Purification Step	Strain	Total Volume (ml)	Total Activity	Unit Protein (mg/ml)	Spec. Activity Units/mg of Protein	Yield (%)	Purification fold
Crude Enzyme	WT	320	1987.2	46.64	0.13	100	1
	CRRmt ₄	320	2182.4	53.70	0.13	100	1
	CRRmt ₂₄	320	2758.4	45.3	0.19	100	1
(NH ₄) ₂ SO ₄ ppt	WT	45	1278.5	38.2	0.74	64	5.69
	CRRmt ₄	40	1370.4	36.4	0.94	63	7.23
	CRRmt ₂₄	41	1577.3	39.1	0.98	57	5.16
DEAE-Sephadex A-50	WT	14.0	881.0	20.0	3.15	44	24.20
	CRRmt ₄	16.5	753.9	16.0	2.86	35	22.00
	CRRmt ₂₄	14.0	951.0	14.2	4.78	35	25.16
Sephadex G-100	WT	5.6	371.8	9.8	6.77	19	52.10
	CRRmt ₄	4.0	220.7	9.3	5.92	10	45.50
	CRRmt ₂₄	4.0	294.5	10.7	6.88	11	36.2

Gel filtration on Sephadex G-100: Sephadex G-100 slurry was packed into a column (1.0 x 60.0 cm) and equilibrated with 0.02 M sodium phosphate buffer, pH 7.0. The peaks with highest cellulase activity from the ion exchange chromatography step were applied to the column. Fractions (3 ml) were collected at a flow rate of 10 ml/hr. The elution was monitored for protein concentration at 280 nm and was also assayed for enzyme activity. Fractions with high protein activity were pooled together and precipitated with solid ammonium sulphate. Again, the precipitate was collected by centrifugation at 4,000 g at 4°C for 30 min, redissolved in minimal amount of 0.02 M sodium sulphate buffer, pH 7.0 and then dialysed against the same buffer for 6 h. The purified enzyme thus obtained was stored at -22°C.

Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis was performed in the absence of SDS for the purpose of ascertaining the purity of the enzyme preparation. Polyacrylamide gel electrophoresis in the absence of SDS was performed on 7.5% separating gel and 4.6% stacking gel as described in the Pharmacia manual mentioned above. Electrophoresis was carried out in 0.5 M Tris-glycine buffer, pH 8.3.

Determination of apparent molecular weight

The apparent molecular weight of the purified cellulase from CRRmt₄, CRRmt₂₄ and the wild-type of *P. fluorescens* were estimated by gel filtration on a (1.0 x 60.0 cm) column of Sephadex G-100. The column was calibrated with bovine serum albumin (66 Kda), ovalbumin (45 Kda), chymotrypsinogen A (25 Kda) and ribonuclease A (13 Kda).

Determination of kinetic parameter

The apparent kinetic parameters (V_{max} and K_m) of the cellulase were determined by varying the concentration of carboxymethylcellulose from 0.0 to 0.7 mg/ml in 0.1 M sodium phosphate buffer, pH 7.0. The assays were performed with the enzyme, which had been diluted appropriately with 0.02 M sodium phosphate, pH 7.0. The apparent kinetic parameters were determined from double-reciprocal plots (Lineweaver and Burk, 1934).

Effect of pH on cellulase activity

In order to determine the optimum pH value for the enzyme from the wild-type and the two improved mutants, the activity of the enzyme was assayed between the pH values of 5.0 and 9.0.

Effect of temperature on cellulase activity

The wild-type and the two improved mutants' enzymes were incubated with the substrates at different temperatures ranging from 30°C to 60°C. The reaction mixtures were analysed for cellulase activity.

Effect of cations on cellulase activity

The effect of cations on cellulase activity was determined by using two cations; Na⁺ and Mg⁺⁺ at concentrations of 0, 10, 20, 30, 40, 50, 60 and 70 mM. The substrate-cation mixture was incubated at room temperature for 1 h before it was used in enzyme assay.

Effect of EDTA on cellulase activity

The effect of ethylenediamine tetraacetic acid (EDTA) at various molar concentrations on the activity of cellulase was determined. The following concentrations of EDTA (0, 10, 20, 30, 40, 50, 60 and 70 mM) were used. The substrate-chemical compound mixture was incubated at room temperature for 1 h before it was used in enzyme assay.

RESULTS

Enzyme purification

The results of the purification of the cellulase from WT, CRRmt₄ and CRRmt₂₄ of *P. fluorescens* are summarised in Table 1. Ammonium sulphate precipitation gave purification fold of about 5 for the WT and CRRmt₂₄ while purification fold of about 7 was achieved for CRRmt₄.

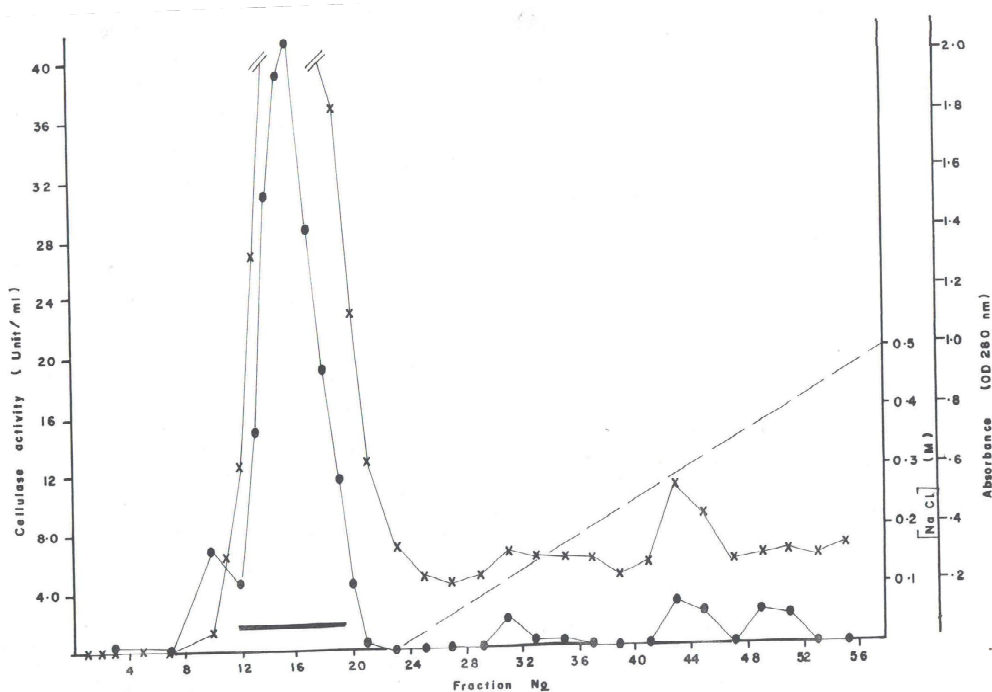


Figure 1. Elution profile of partially purified cellulase from the wild-type of *P. fluorescens* on DEAE-Sephadex A-50 column. The cellulase activity (O-O) was determined in each fraction by the appearance of reducing end group in solution of CMC (Nelson, 1944). The growth (X-X) was monitored as described in the text. A linear NaCl gradient (---) was used to elute proteins bound on the column. Similar patterns were obtained for the catabolite repression resistant mutant 4 and 24 (CRRmt₄ and CRRmt₂₄).

Dialysed enzymes from the solid ammonium sulphate precipitation steps were subjected to ion exchange chromatography on DEAE Sephadex A-50 (Figure 1). The enzyme did not bind to the ion exchanger when NaCl ion gradient was applied. The ion exchange chromatography yielded a single peak of cellulase activity. The percentage recovery of the enzymes were 44, 35 and 35% for WT, CRRmt₄ and CRRmt₂₄, respectively, while the purification fold of about 24, 22 and 25 were obtained for WT, CRRmt₄ and CRRmt₂₄, respectively (Table 1).

The elution profiles of cellulase from the three strains gave a single peak of cellulase activity. The WT, CRRmt₄ and CRRmt₂₄ were purified 52, 45 and 36 fold compared to the crude enzyme. The overall level of recovery of cellulase activities from the crude enzyme were 19, 10 and 11% for WT, CRRmt₄ and CRRmt₂₄, respectively. The purification procedure yielded a homogenous cellulase with a specific activity of 6.8 5.9 and 6.9 units/mg of protein for the WT, CRRmt₄ and CRRmt₂₄ respectively (Table1).

Molecular weight and purity determination

The purified enzymes have molecular weights of 36, 26 and 36 kDa for the WT, CRRmt₄ and CRRmt₂₄,

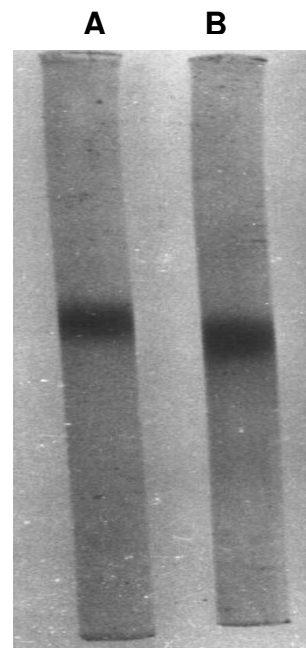


Figure 2. Polyacrylamide gel electrophoresis of the purified cellulase from *Pseudomonas fluorescens*. Electrophoresis was performed as described in the text. Lane **A** is purified cellulase from the wild-type type while lane **B** is purified cellulase from the CRRmt₂₄.

respectively as determined by gel filtration on Sephadex G-100. Polyacrylamide gel electrophoresis of the purified cellulase in the absence of sodium dodecyl sulphate showed a single band of protein indicating a homogeneous preparation (Figure 2).

Determination of kinetic parameters

The Michaelis-Menten constants, K_m and V_{max} , of the purified cellulase for carboxymethylcellulose were estimated from the double reciprocal plot of the data obtained for *P. fluorescens* cellulase at varying substrate concentrations. The K_m and V_{max} obtained for the purified cellulases are 3.6 mg/ml and 8.3 units/ml; 3.1 mg/ml and 3.3 units/ml; and 5.3 mg/ml and 10.0 units/ml for WT, CRRmt₂₄ and CRRmt₂₄, respectively.

Effect of pH on cellulase activity

The optimum pH for the wild-type and the two improved mutants of *P. fluorescens* is shown in Figure 2. The pH optima for CRRmt₂₄ was 6.5 while the wild-type and CRRmt₄ have optima pH of 7.0.

Effect of temperature on cellulase activity

The effect of temperature on the activity of the enzymes is shown in Figure 3. The enzymes were activated at 30° to 35°C after which the activity began to drop. The optimum temperature for the three strains was 35°C.

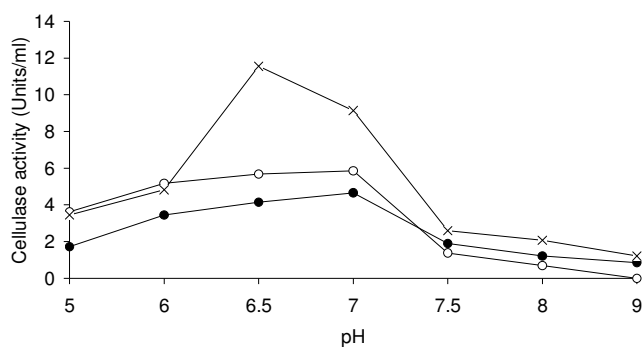


Figure 3. Effect of pH on the activity of cellulase synthesized by wild type, WT, (●-●); catabolite repression resistant mutant 4, CRRmt₄, (O-O) and catabolite repression resistant mutant 24, CRRmt₂₄, (X-X) of *P. fluorescens*. Values shown in the graph are means of at least three determinations.

Effect of cations on cellulase activity

Addition of some cations to the reaction mixture at various concentrations affected the cellulase activity

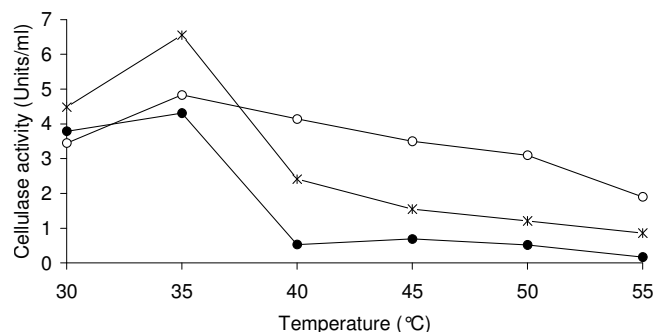
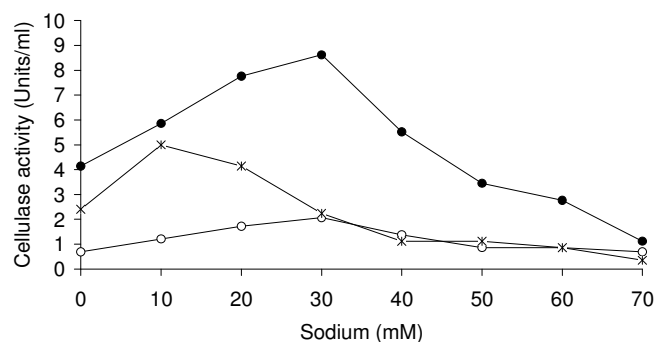


Figure 4. Effect of temperature on the activity of cellulase synthesized by wild type, WT, (●-●), CRRmt₄ (O-O) and CRRmt₂₄, (X-X) of *P. fluorescens*.

(Figures 4a and b). The presence of these cations increased the enzyme activity of the wild-type and the two improved mutants. Optimum cellulase activity was observed at 20 mM of Mg⁺⁺ for the wild-type and CRRmt₄ while the optimum cellulase activity was observed at 30 mM of Mg⁺⁺ for CRRmt₂₄ (Figure 4b). For Na⁺ (Figure 5a), the optimum cellulase activity was observed at 30 mM for WT and CRRmt₄ while optimum cellulase activity was observed at 10 mM for CRRmt₂₄.

A.



B.

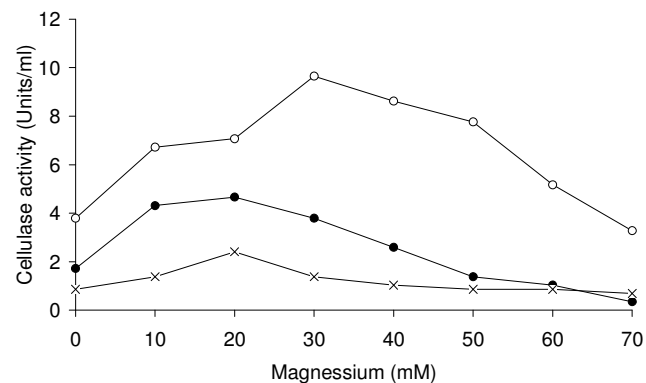


Figure 5. Effect of sodium (A) and magnesium (B) on the activity of cellulase synthesized by WT (●-●), CRRmt₄ (O-O) and CRRmt₂₄ (X-X) of *P. fluorescens*.

Effect of EDTA on cellulase activity

The effect of ethylenediaminetetra-acetic acid on the purified enzymes of the wild-type and the two improved mutants is shown in Figure 5. The activities of the three enzymes were inhibited by EDTA. It was also observed that the cellulase activity decreases rapidly with increasing concentrations of EDTA.

DISCUSSION

The culture filtrates of the wild-type of *P. fluorescens* and the mutants expressed cellulase which was subsequently purified to homogeneity. The gel filtration yielded only one peak with specific activities of 6.77, 5.92 and 6.88 units/mg protein for WT, CRRmt₄ and CRRmt₂₄, respectively. These values were greater than the values obtained for endoglucanase from some fungi such as *Thermoascus aurantiacus* (11.02×10^{-3} units/mg), *Aspergillus niger* (4.2×10^{-3} units/mg) (Tong et al., 1980; Hurst et al., 1977).

Molecular weights of 36,000dal, 26,000dal and 36000dal were estimated for the purified enzymes of the wild-type, CRRmt₄ and CRRmt₂₄ respectively. These values fall within the range (20 – 50 kDa) estimated for the organism by Suzuki et al. (1969) and 20 to 60 kDa for cellulase isolated from *Amitermes evuncifer* bacteria (Cole, 1980). The Km values of 3.6, 3.1, and 5.3 mg/ml were obtained for the wild-type, CRRmt₄ and CRRmt₂₄ respectively. These values were lower than those obtained for the enzyme from *Aspergillus niger* (Hurst et al. 3.1 mg/ml and 3.3 units/ml; and 5.3 mg/ml and 10.0 units/ml for WT, CRRm₄ and CRRmt₂₄, respectively).

Effect of pH on cellulase activity

The optimum pH for the wild-type and the two improved mutants of *P. fluorescens* is shown in Figure 2. The pH optima for CRRmt₂₄ was 6.5 while the wild-type and CRRmt₄ have optima pH of 7.0.

Effect of temperature on cellulase activity

The effect of temperature on the activity of the enzymes is shown in Figure 3. The enzymes were activated at 30° to 35°C after which the activity began to drop. The optimum temperature for the three strains was 35°C.

Effect of cations on cellulase activity

Addition of some cations to the reaction mixture at various concentrations affected the cellulase activity (Figures 4a and b). The presence of these cations

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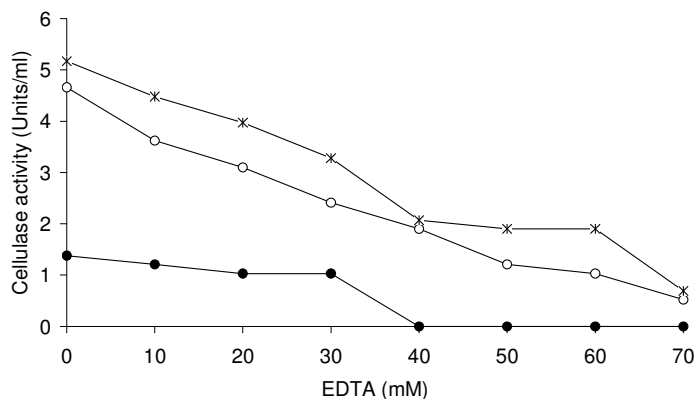


Figure 6. Effect of ethylenediaminetetraacetic acid (EDTA) on the activity of cellulase synthesized by WT (●-●), CRRmt₄ (O-O) and CRRmt₂₄, (X-X) of *P. fluorescens*.

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were obtained for the wild-type, CRRmt₄ and CRRmt₂₄ respectively. These values were lower than those obtained for the enzyme from *Aspergillus niger* (Hurst et al., 1977) which was 52-80 mg/ml, and endoglucanase from *Sclerotinia sclerotiorum* (Waksman, 1991) which was 8.7 mg/ml. However, these values were higher than 0.5 mg/ml obtained for cellulase of *Myrothecium verrucaria* (Halliwell, 1965). It is not an easy task to define the Km values of insoluble substrates such as cellulose and Km value may serve only to denote the amount of substrate needed to achieve half the maximal initial reaction velocity (Tong et al., 1980). K_m is thus a measure of the apparent affinity of an enzyme for its substrate. The V_{max} values of 8.3, 3.3 and 10 unit/min were obtained for wild-type, CRRmt₄ and CRRmt₂₄ respectively. The activities of cellulase from this organism were greatly influenced by the concentration of the substrate. With fixed enzyme concentration, an increase in the concentration of substrate results in increase in enzyme activity until a saturation point is reached beyond which enzyme activity decreases. This was probably because at high substrate concentration, ineffective complexes were formed between enzyme and substrate. Also, since the substrate molecules were too many around the enzyme molecules, they may be bound to regions on the enzyme, which are not the active site or alternatively, may crowd the active site (Dixon and Webb, 1971).

The optimum temperature for cellulase activity in this study was 35°C for the wild-type and the two improved mutants. Yamane et al. (1970) obtained a similar result for cellulase produced by *P. fluorescens var cellulosa*. This information suggests that the mutation by EMS has not affected the structural gene in this organism. At higher temperature than the optimum, enzyme activity decreases because of denaturation. The optimum pH values for cellulase in this study were 6.5 for CRRmt₂₄ and 7.0 for wild-type and CRRmt₄. This is similar to the result of Yamane et al. (1970). These results showed that near neutral pH value was more favourable to cellulase activity in this organism. Catriona et al. (1994) reported that the pH range over which the cellulases were highly active is fairly broad (pH 5.0 – 7.0). Bok et al. (1998) also reported a pH range of 6.0 to 6.6 for two thermostable endocellulases from *Thermotoga neapolitana*.

EDTA was inhibitory to the activities of the cellulase from the wild-type, CRRmt₄ and CRRmt₂₄. EDTA is a metal chelating agent and inhibition of the enzymes by EDTA suggests that the enzyme activities probably depend on chemical activities and that they may contain inorganic groups, which formed inactive complexes with EDTA.

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