

Original Research Article

PURIFICATION AND CHARACTERIZATION OF ENDOGLUCANASE FROM SORGHUM (*S. bi-color*) AND MILLET (*Pennisetum typhoides* & *Digitaria exilis*) MALTS

ABSTRACT

Endoglucanase (EC3.2.1.4) from sorghum (*S. bi-color*) and millet (*Pennisetum typhoides* & *Digitaria exilis*) malts were purified to homogeneity through the methods of ammonium sulphate precipitation and gel filtration. Molecular mass of 35KDa and 41KDa were determined by SDS-PAGE. The purified enzymes catalyzed the hydrolysis of carboxy-methylcellulose with optimum pH range of 4.5-6.5 and optimum temperature of 60-70°C, retaining more than 90% activity for *S. bi-color* and *Pennisetum typhoides* and 55% activity for *Digitaria exilis* after 1.0 hour pre-incubation at 60°C. K_m values of 0.11, 0.09, 0.20mM and V_{max} 17.53, 15.0 and 11.10U/mg/min were obtained for *S. bi-color*, *Pennisetum typhoides* and *Digitaria exilis* respectively. Co^{2+} inhibited endoglucanase activity whereas Ca^{2+} , Ba^{2+} , and Zn^{2+} stimulated enzyme activity. The enzyme was inactivated by glucose, a major end product of cellulose hydrolysis. Results indicate that endoglucanase of *S. bi-color* and *Pennisetum typhoides* are suitable for malting and a blend of the two will produce high quality malt.

Key words: endoglucanase, sorghum, millet, malting, purification, characterization

INTRODUCTION

Endoglucanases is one of the cell wall degrading enzymes (cellulase complex- endoglucanases, exoglucanases and glucosidases) activated during the germination period of the malting process [1]. Endoglucanases (EC.3.2.1.4) preferentially cleave the internal glycosidic bonds of cellulosic chains resulting in a rapid decrease in polymer length and gradual increase in the reducing sugar concentration [2] [3]. By decomposing the cell wall cellulosic biomass, other hydrolytic enzymes from the aleurone cells are able to have access to the starch reserve in the endosperm. Endoglucanases play key roles in increasing the yield of fruit juices, beer filtration and oil extraction, in improving the nutritive quality of bakery products and animal feed [4].

Endoglucanases have been purified mainly from bacterial and fungal sources by procedures which include ammonium sulphate precipitation, gel filtration, anion-exchange chromatography and SDS-polyacrylamide gel electrophoresis (PAGE) [5] [6] [7] [8] [9] [10]. Endoglucanase is generally reported to be thermostable, having profound activity within a broad pH range of 4.0 – 11.0 with considerable stability at pH 4.0 – 7.0. Divalent cations such as Ca^{2+} and Mg^{2+} are noted to stimulate endoglucanase activity [6]. The major end products of cellulose hydrolysis, cellobiose and glucose have been found to inhibit endoglucanases in some cases [5] [11] [12] while other reports indicate insensitivity of the enzyme to the end-products [13]. Molecular weight in the range of 30 – 60kDa [13] [4] [14] [10] and as high as 85.1KDa are recorded for endoglucanases obtained from microorganisms.

Purification and characterization of endoglucanase in plants, even barley is still scanty, inspite its great importance in extract development of these plants. Sorghum and millet malts are used in Africa in the production of local beer and alcohols like Kaffir beer, 'Burukutu' and Opaque beer which contain undegraded starch thereby reducing the product yield and nutritive quality[15] (Singh *et al.*, 1988). Study by [16]Nzelibe *et al* on the malting and brewing characteristics of some sorghum and millet varieties indicates that some of the varieties have high brewing potentials. Since plant cell wall is a major barrier to the modification of endosperm during processing, a good understanding of the properties of the cell-wall degrading enzymes of sorghum and millet varieties will open doors for improved grain modification. It is against this background that partial purification and characterization of endoglucanase in malted sorghum and millet species were carried out.

MATERIALS AND METHODS

Materials

Dried grains of *Sorghum bicolor* (yellow variety - 'kaura') and *Pennisetum typhoides* ('dauro') were purchased from Samaru market, Zaria while *Digitaria exilis* (fonio-bhull) were purchased from Chori in Kaduna State. [The name 'bhull' is based on the source while the others in brackets are their Nigerian local names]. Materials were identified using breeder's characteristics.

Chemicals and Reagents

Ammonium sulphate, acrylamide/bis, ammonium persulphate (APS), tetramethylene diamine (TEMED), ovalbumin, trypsinogen, coomassie, acetic acid, citric acid, sodium acetate (anhydrous), sodium hydroxide, sodium dihydrogen phosphate, disodium hydrogen phosphate, 3,5 dinitrosalicylic acid (DNS), Rochelle salt (Sodium potassium tartrate), standard reducing sugar (glucose) and protein (Bovine Serum Albumin), Biuret reagent, carboxymethyl cellulose (CMC). All the chemicals and reagents were of analytical grade and were obtained from the following: British Drug House (BDH) Ltd, Poole England; Sigma chemical company US; May and Baker (M & B) England and Hopkins and Williams.

METHODS

Preparation of the raw grains

About 1.50kg of each of the grains were weighed and cleaned to remove dirt, stones and broken kernels. About 50grams of each sample was milled into powdered form using a Thomas Wiley Laboratory mill and then stored safely prior to analysis.

Malting procedure

The malting procedure of [17] was employed with few modifications. Steeping was at room temperature (28-30°C). Each variety was divided into two groups. A group of the samples were treated with 8ml of 2.5µg/l gibberellic acid (GA₃) after steep out. Samples were withdrawn from the treated and non-treated groups at 0, 1, 2, 3, 4 and 6 day of the malting and then kilned (between 16-24hrs) at 50°C until the rootlets could be removed by hand. The grains were then ground to powder and packed in air tight containers for analyses.

Preparation of extracts

About 0.5g of malted grain powders per ml of 0.05M citrate buffer at pH 4.8 was thoroughly shaken (150 oscillations per minutes) at 4°C for one hour and the slurry centrifuged twice at 3,000g for 10mins and then filtered through whatman number one filter paper. The crude extract was dialyzed against 3 volumes of the same buffer for 48hours with three changes.

Enzyme purification

The method by [18] was adopted for ammonium sulphate precipitation. Protein extracts were differentially precipitated up to 80%. The precipitates were collected and immersed in 3mls of citrate buffer then dialyzed against the same buffer with three changes for 2 days. The protein extracts were loaded into a sephadex G-75 column (1.5 × 50cm) and pre-equilibrated with 50mM citrate buffer pH 4.8. The column was eluted with the same buffer at a flow rate of 1ml per 6minutes. The active fractions were collected and assayed for protein activity.

Protein determination

Protein content was measured by the method of [19], with BSA as standard. Protein was also detected by measuring absorbance at 280nm.

Enzyme assay procedure

Dinitro-salicylic acid (DNS) method was employed for the enzyme assay in accordance to the procedure of [20]. Exactly 1.0ml of enzyme solution was added to 1.0ml of 1% substrate carboxymethyl-cellulose (CMC) and incubated for 30 minutes at 50°C. DNS reagent (3.0ml) was then added to stop the reaction. The tubes were placed in boiling water for 5 minutes and cooled thereafter. The reducing sugar was determined spectrophotometrically as glucose at 540nm. Blanks of buffer enzyme without substrate and substrate without enzyme were used. All assays were in triplicates. One unit of enzyme activity is defined as the amount of the

enzyme that liberates one micromole of glucose per minute ($1 \mu\text{mol}$ of glucose = 0.180mg/ml) from CMC under the specified assay conditions [21]. Specific activity is the number of units per milligram protein.

Influence of pH and Temperature

The activity of endoglucanase enzyme was measured at different pH values (3.0–9.0) using citrate, phosphate and Tris-HCl buffers at 50°C by the DNS method.

Enzyme solutions were incubated in citrate buffer, pH 4.8 at different temperatures (30°C – 90°C) for 1.0 hour before the addition of 1% CMC.

Molecular weight determination

SDS/PAGE was performed according to the procedure of [22]. Molecular weights were calculated as described by [23].

Statistical Analysis

The data were expressed as mean $\pm$ standard deviation (SD) and statistical analysis were carried out by one-way ANOVA. Data were considered significant for $P < 0.05$ at 95% confidence level. SPSS software was used.

RESULTS AND DISCUSSION

Purification of endoglucanase

The results of the enzyme purification are summarized in table 1.0 below. The ammonium sulphate precipitation of 'kaura', 'dauro' and 'fonio-bhull' gave purification folds of 2.5, 1.3 and 1.6 and recovery yield of 63, 41 and 38% respectively. The overall recovery of endoglucanase activities from crude enzyme after gel filtration were 44, 36 and 34% for 'kaura', 'dauro' and 'fonio' respectively. The purification procedure yielded endoglucanase with specific activity of 8.62, 6.09 and $5.09 \mu\text{mol/min/mg}$ protein and total purification fold of 5.39, 5.25 and 4.07 for 'kaura', 'dauro' and 'fonio' respectively. The elution profiles of endoglucanase from the three samples gave a single peak of enzyme activity as shown in figs 1.0 - 3.0. Fractions 14 - 16 of 'kaura' and 24 and 25 of 'dauro' and 17 - 18 of 'fonio-bhull' were the most active while the other fractions showed little or no activity. A single peak of enzyme activity in the elution profiles of endoglucanase from the three samples is an indication that the purification procedure yielded a homogenous endoglucanase. The recovery yield of 63, 41 and 38% for 'kaura', 'dauro' and fonio-bhull respectively after ammonium sulphate precipitation is consistent with 64, 63 and 44% obtained in cellulase of three strains of *Pseudomonas fluorescens* by [24].

Influence of pH on enzyme activity

Enzymatically active fractions dialyzed against 50mM citrate buffer had optimum activity at pH of 4.8, 5.0 and 6.0 for fonio, 'kaura' and 'dauro' respectively. Figure 4.0 shows that enzyme activities were stable over a broad range of pH, declining to low values below pH of 4.5 and above 7.0. The optimum pH of endoglucanase from fonio, 'kaura' and 'dauro' are similar to that of endoglucanases from *Thermoascus aurantiacus* with optimum pH of 4.4 [4], *Clostridium thermocellum* pH 5.5[11] and that from *Thermotoga neopolitana* of pH 6.0 [12].

Influence of temperature on enzyme activity

Fig 5.0 shows the result of temperature effect on the initial velocity of CM-cellulose hydrolysis using an incubation period of 30 minutes. Enzyme activity gradually increased up to 60°C in 'kaura' and fonio and 70°C in 'dauro'. The study result shows that temperature effect was significant on 'kaura' and 'dauro' than fonio millet. About 78%, 97% and 58% activities remained at 80°C in 'kaura', 'dauro' and fonio respectively showing that endoglucanases of 'dauro' is more thermostable followed by that of 'kaura'. The decrease in enzyme activity after the optimum temperature is due to denaturation which set in gradually in 'kaura' and fonio but sharply in 'dauro'. The optimum temperatures obtained in this study are similar to optimum temperature of 50 – 60°C reported for endoglucanase from *Clostridium thermocellum* [13] and exactly 70°C for *Thermoascus aurantiacus* [4].

Based on the results of temperature effect on enzyme activity, Arrhenius plots of activation energy (E_{act}) and inactivation energy (E_{inact}) were made. The E_{act} of 11.9, 10.9 and 23.9KJ/mole (figure not shown) and E_{inact} of 20.9, 55.4 and 65.4KJ/mole (fig 7.0) for 'kaura', 'dauro' and 'fonio' respectively were obtained. Activation energy is the minimum energy required to get the chemical reaction started, thus the lower the energy the more the reaction. The results indicate that endoglucanase of 'dauro' reacts faster considering the low activation energy. 'Dauro' also has a broad range of energy for reactive molecules before inactivation at 55.4KJ/mole. Endoglucanase of 'kaura' has a narrow range of energy (11.9 – 20.9KJ/mole) therefore requires careful regulation. Higher activation energy in fonio shows that increase in temperature decreases the forward reaction and this might be the reason for its poor enzyme activity under the study conditions.

Percentage residual activity of endoglucanase

Table 2.0 shows the result of enzyme solutions incubated in citrate buffer, pH 4.8 at different temperatures (30°C – 90°C) for 1.0 hour before the addition of 1% CMC. Endoglucanases of 'kaura' and 'dauro' were stable over a wide range of temperature retaining high activity of 94% and 90% respectively at 60°C. Fonio had considerable low values. However, at 80°C reasonable activity remained in the selected cereals. Above 80°C, the activity rapidly declined indicating denaturation of the enzyme. Exactly 60°C most stable temperature is reported for endoglucanase of *Bacillus licheniformis* [25], while 83% residual activity after 3.0 hours at 70°C is reported for *Caldibacillus cellulovarans* [14].

Effect of Substrate Concentrations

With fixed enzyme concentration, an increase in the concentration of substrate (0.8 – 2.0% CMC) resulted in increase in enzyme activity until a saturation point of 1.6% was reached, beyond which enzyme activity decreased. Based on Lineweaver-Burk plot, the partially purified enzymes gave a K_m value of 0.09, 0.11 and 0.20mM and V_{max} of 17.53, 15.0 and 11.10U/mg/min for 'kaura', 'dauro' and fonio respectively (results not shown). 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 [24].

The Michealis constant K_m measures inversely the strength of binding between enzyme and substrate. From the results 'kaura' with the lowest K_m of 0.09mM has a greater affinity towards carboxymethyl cellulose. High K_m in fonio shows that its endoglucanase is saturated only at very high concentrations of CMC. V_{max} which is the maximum rate of reaction indicates the amount of enzyme that will convert the substrate to product at a given time. Hence fonio endoglucanase is the most effective in the catalysis of CMC. K_m (0.09, 0.11 and 0.20mM) and V_{max} (17.53, 15.0 and 11.10U/mg/min) values for 'kaura', 'dauro' and fonio respectively are close to 0.30mM and 18.4U/mg/min of thermostable cellulase from *Thermotoga neapolitana* [12].

Effect of Divalent Cations

The activity of endoglucanase assayed in the presence of 1mM Zn^{2+} , Ba^{2+} and Ca^{2+} during the hydrolysis of CMC caused an increase yield in the reducing sugar as shown in Figure 6.0. On the other hand, a significant decrease yield of reducing sugar was observed with 1mM Co^{2+} indicating a strong inhibitory effect (fig 6.0).

The yield increase in the reducing sugar obtained during the hydrolysis of CMC by the addition of 1mM, Zn^{2+} , Ba^{2+} and Ca^{2+} means that the degradation of cellulose was stimulated by these metal ions. The values of Zn^{2+} , Ba^{2+} and Ca^{2+} for fonio were higher by 37%, 27 % and 11% respectively than 'kaura' which had an increment of 33%, 20% and 8% and 'dauro' with increments of 21%, 18% and 11% for Zn^{2+} , Ba^{2+} and Ca^{2+} respectively. This indicates that endoglucanase of fonio is more sensitive to these metal ions. In the three selected cereals, Zn^{2+} had the highest effect on endoglucanase hydrolysis of CM-cellulose; therefore it is the most stimulatory metal ion. Ca^{2+} activation on endoglucanase of *C. thermocellum* is reported by [6] while Zn^{2+} is reported to have significant inhibition on CMCase from *Caldibacillus cellulovarans* [14]. The increase by the metal ions would be as a result of proper orientation of substrate within the active site. Divalent cations bind to two or more ligands and induce changes in enzyme-substrate complex conformation. Co^{2+} inhibition may be due to complex-formation with, or catalysis of oxidation of, specific residues by thiol groups or the result of non-specific salt formation [11] [6].

Effect of glucose on enzyme activity

10mM glucose added to different concentrations (0.8 – 2.0%) of the substrate inactivated enzyme activity of the three samples investigated. Lineweaver-Burk plot of the data obtained from the enzyme assay gave a competitive inhibition pattern for ‘kaura’ while non-competitive pattern was obtained for ‘dauro’ and fonio (Results not shown). The inhibitory constant (K_i) values of 10.25mM, 12.54mM and 12.72mM were obtained for ‘kaura’, ‘dauro’ and fonio respectively.

The inhibitory effect suggests that endoglucanases of the samples are sensitive to the end-product (glucose) as reported by [12], but in contrast to the result of [6] which shows that 200mM glucose had no effect on endoglucanase from *C. thermocellum*. Competitive inhibition pattern obtained in ‘kaura’ is similar to that of *Thermotoga neapolitana* [12]. The inhibitory constant (K_i) measures the extent of binding between the enzyme and the inhibitor at given concentration of the inhibitor. ‘kaura’ with the lowest K_i value at 10mM glucose shows that glucose has greater degree of inhibition on its endoglucanase than ‘dauro’ and ‘fonio’. Higher K_i compared to K_m of the three samples is a clear indication that the enzyme is more inclined to the substrate CMC than glucose.

SDS-PAGE Result and Molecular Weight

The enzyme preparations migrated as a single band on SDS PAGE (plate 1). Molecular weight of 35KDa for ‘kaura’ and ‘dauro’ and 41KDa for ‘fonio’ were obtained based on [23] formula. The molecular weight of 35KDa for ‘kaura’ and ‘dauro’ and 41KDa for ‘fonio’ are close to molecular weight 34KDa of CMCase from *T. aurantiascus* and 50KDa of *Populus elba* determined by SDS PAGE [4] [16]. They are within the range 20 to 60 kDa estimated for cellulase isolated from *Amitermes evuncifer* bacteria [24] but lower than 94KDa of CMCase from *Sinorhizobium fredii* [26] and 76KDa and 83KDa of endoglucanase from *Clostridium thermocellum* [11] [6]. This result indicates that endoglucanase of ‘kaura’, ‘dauro’ and ‘fonio’ are low molecular weight endoglucanase.

CONCLUSION

The results of purification and characterization procedures obtained in this study indicate that endoglucanase of ‘kaura’, ‘dauro’ and ‘fonio’ are active within a wide range of pH and temperature but that of ‘kaura’ and ‘dauro’ were found to be more thermostable. Endoglucanase from these cereals were also found to be metallo-proteins of low molecular weight. Useful properties of endoglucanase from ‘kaura’, ‘dauro’ and ‘fonio’ indicate that ‘kaura’, and ‘dauro’ are more suitable for malting process. A blend of sorghum and pearl millet malt will produce malt with high profile.

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Tables

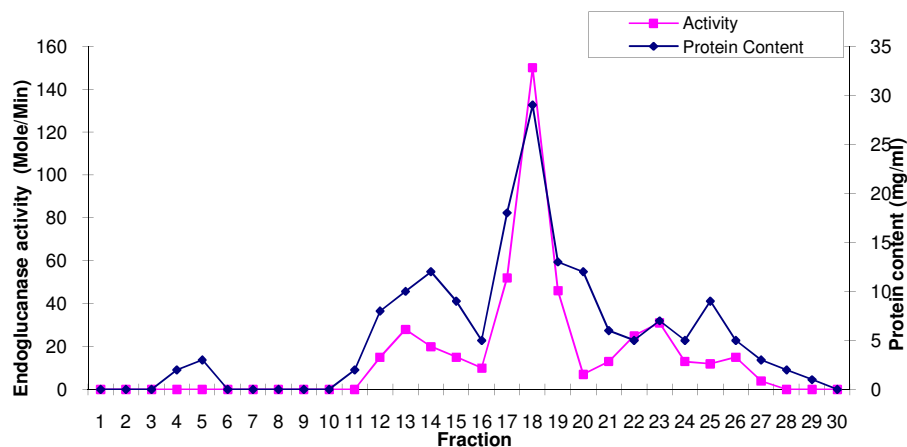
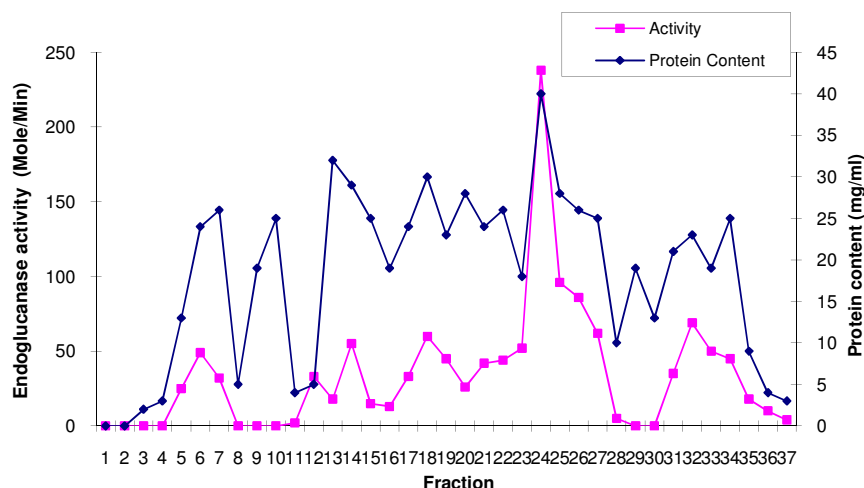
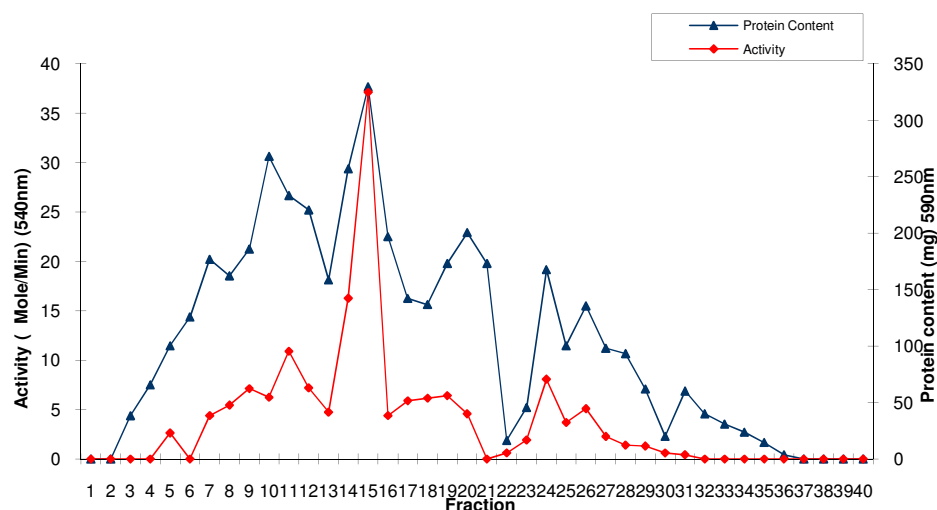
Stages	Total Vol. (ml)	Protein Concentration		(μmole /min/ml)	Enzyme Activity		Recovery Yield (%)	Purification (fold)
		mg/ml	Total Protein (mg)		Total Activity (μmole/min)	Specific Activity (μmole/ min/mg)		
Crude ‘Kaura’ ‘Dauro’ fonio	12	77.40 95.14 58.13	929.20 1141.70 697.50	124.0 110.0 72.4	1487.20 1324.80 869.20	1.60 1.16 1.25	100	1
(NH₄)₂SO₄ Precipitation ‘Kaura’ ‘Dauro’ Fonio	3	77.71 120.96 55.30	233.13 362.90 166.00	310.5 180.5 108.9	931.6 541.5 326.6	4.00 1.49 1.97	63.00 40.87 37.57	2.50 1.28 1.58
Gel Filtration ‘Kaura’ ‘Dauro’ fonio	2	37.66 39.45 28.74	75.31 78.90 57.48	324.6 240.5 145.3	649.2 481.0 292.6	8.62 6.09 5.09	43.65 36.30 33.66	5.39 5.25 4.07

Table 1.0: Purification table of endoglucanase from ‘kaura’, ‘dauro’ and ‘fonio-bhull’

Table 2.0: Percentage residual activity of endoglucanase after one hour of pre-incubation at different temperatures

	30 ⁰ C	40 ⁰ C	50 ⁰ C	60 ⁰ C	70 ⁰ C	80 ⁰ C	90 ⁰ C
Kaura (%)	88	90	93	94	90	88	75
Dauro (%)	73	79	88	90	84	80	64
Fonio (%)	48	54	68	73	65	50	27

Figures



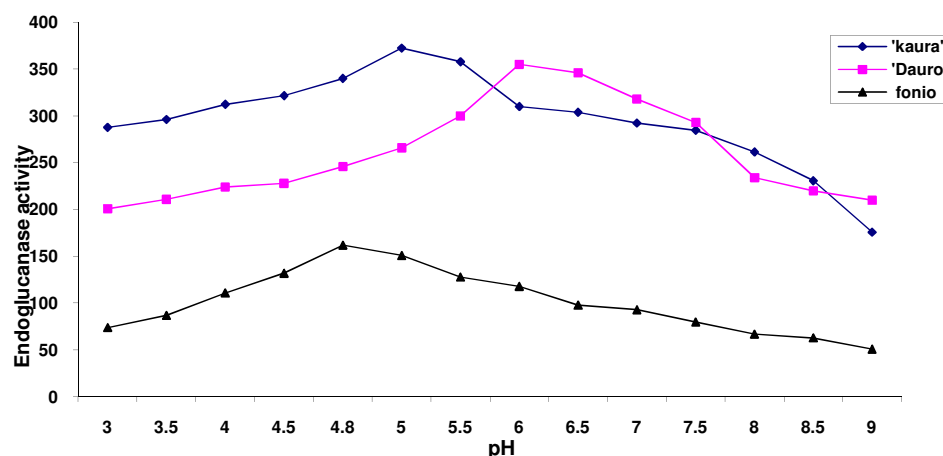


Fig.4.0: Effect of pH on Endoglucanase activity for 30 min at 50°C with 1% CMC in 50mM citrate buffer pH 4.8

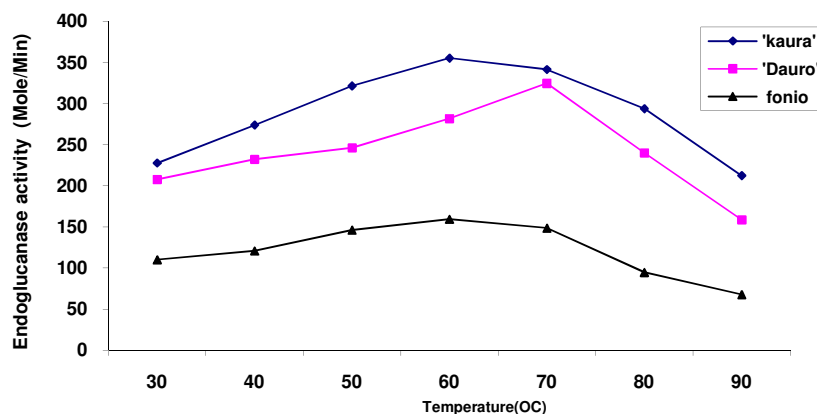


Fig. 5.0: Effect of temperature on Endoglucanase activity for 30 min with 1% CMC in 50mM citrate buffer pH 4.8

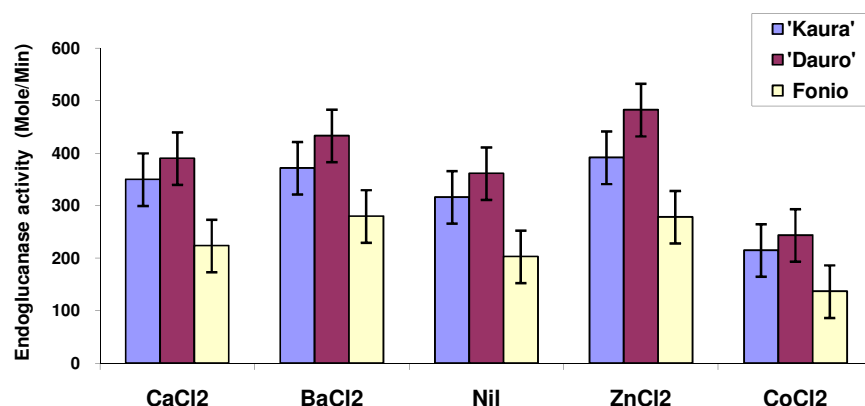


Fig. 6.0: Effect of divalent cations on Endoglucanase activity
The enzyme was assayed for 30 min at 50°C in reaction medium final concentration 1mM divalent cations with 1% CMC in 50mM citrate buffer pH 4.8

