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Full Length Research Paper

Physiological studies of cellulase complex enzymes of *Aspergillus oryzae* and characterization of carboxymethyl cellulase

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Investigation of the production of cellulase complex enzymes by the fungus *Aspergillus oryzae* on soluble bio-cellulosic waste like cheese whey was the goal of this work. The effects of incubation period, substrate concentration, initial pH and temperature on the production of endoglucanase, exoglucanase and cellulase (filter paper activity FPA) activities, were investigated. Seven days incubation period with an initial pH value of 6 and temperature of 40°C, and cheese whey concentration of 10% yielded the highest specific activities with 1.5 fold increases for endoglucanase and 1.4 fold increases for exoglucanase. Cellulase specific activity represented as FPA showed no fold increase at all. The optimum temperature of endoglucanase was observed at 55°C, and the enzyme activity has abroad pH range between 3.8 to 8.0 and 24.91% of the original activity was retained after heat treatment at 90°C for 30 min. Analysis of the partial purified enzyme preparation by SDS – PAGE revealed one protein band showing cellulolytic activity, The molecular weight of this band was estimated to be around 45.000 Daltons.

Key words: Cellulolytic enzymes, *Aspergillus oryzae,* cheese whey, dinitrosalicylic acid (DNS), fermentation variables.

INTRODUCTION

Cellulose is the most abundant polymer in the biosphere with its estimated synthesis rate of 10^{10} tons per year (Schlesinger, 1991; Singh and Hayashi, 1995; Lynd et al., 2002). Cellulose is an unbranched glucose polymer of 8000 to 12000 units, composed of anhydro- β-1,4-glucose linked units linked by β-1,4-glucosidic bonds, is the most abundant biopolymer on the earth polymer. Cellulose–rich plant biomass is one of the foreseeable and sustainable source of fuel, Figure 1 (US Department of Energy, 2009) illustrates the concept of carbon and energy cycle in the environment with an emphasis in biofuel, also the animal feed and feed stock for chemical synthesis (Bhat, 2000). The utilization of cellulosic biomass continues to be a subject of worldwide interesting in view of fast depletion of oil reserves and food shortages (Kuhad et al., 1997; Gong et al., 1999). The conversion of cellulosic mass to fermentable sugars has been suggested as a feasible process and offers a potential source to reduce the use of fossil fuels and environmental pollution (Dale, 1999; Lynd et al., 1999).

Cellulose is commonly degraded by an enzyme called cellulase. Cellulase(s) are important enzymes not only for their potent application in the different industries, like industries of food processing, animal food production, pulp and paper production, and in detergent and textile industry, but also for significant role in bio conversion of agriculture wastes into sugar, bioalcohols and other useful products, such as single cell protein, fuels and chemical feed stock (Ojumu et al., 2003). This enzyme is produced by several microorganisms, commonly by bacteria and fungi (Bahkali, 1996; Magnelli and Forchiassin, 1999; Shin et al., 2000; Immanuel et al., 2006).

Although a large number microorganisms are capable of degrading cellulose, only a few of these produce significant quantities of cell free enzyme capable of completely hydrolyzing crystalline cellulose *in vitro*. Several studies were carried out to produce cellulolytic enzymes from

Abbreviations: CMC, Carboxymethyl cellulose; **CMCase,** carboxymethyl-cellulase; **DNS,** dinitrosalicylic acid; **FPA,** filter paper activity.

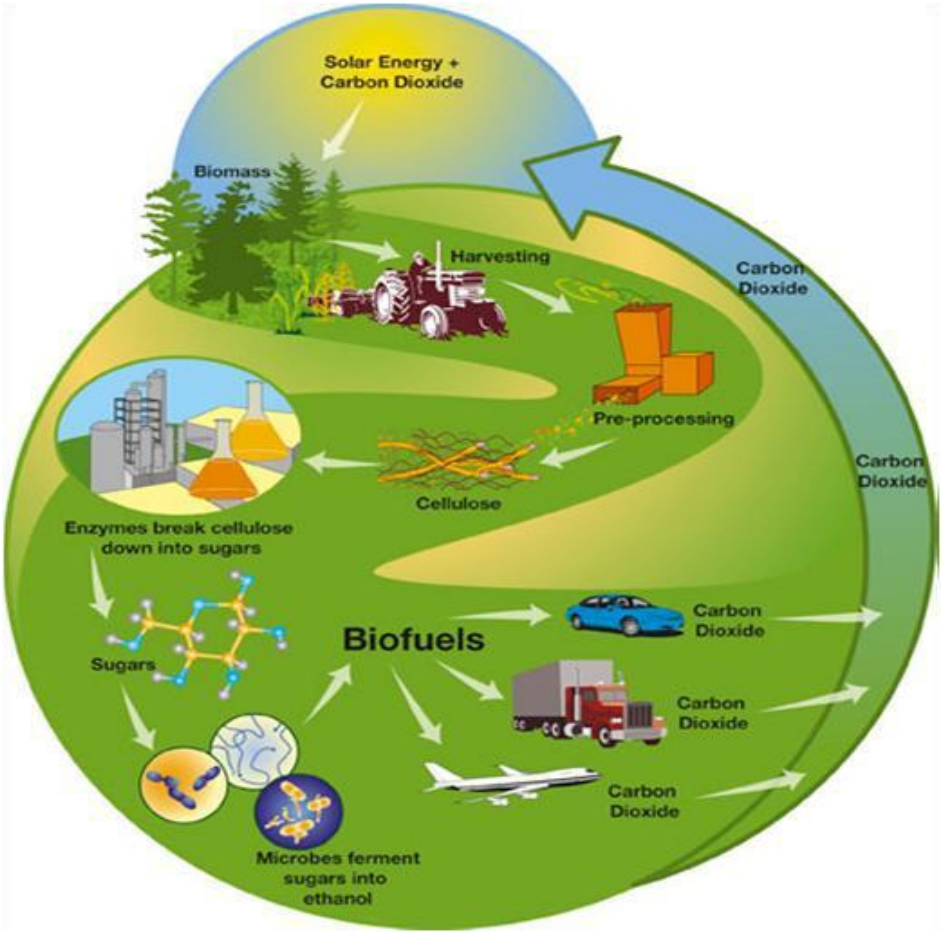

Figure 1. Carbon dioxide and biofuels in the energy cycle (US Department of Energy, 2009).

biowaste degradation process by many microorganisms including fungi such as *Trichoderma, Penicillium, Aspergillus* sp. etc., (Mandels and Resse, 1985; Hoffman and wood, 1985; Lakshmikant and Mathur, 1990; Suto and Tomito, 2001), a number of work have been done on wastes like rice and wheat straw (Kocher et al., 2008; Singh et al., 2009) sugar cane bagasse (Ghada and Mahmoud, 2009) and orange waste (Omojasola and Jilani, 2008).

A single enzyme cannot accomplish the task of extensive cellulose degradation, multiple enzymes are required consequently, accordingly cellulose is roughly categorized into three classes (Figure 2) (Wikipedia, 2009) namely endoglucanase (CM cellulase, EC 3.2.1.4 endo-1,4-β glucanase), which cleaves internal glucosidic bonds, exoglucanase (Avicelase, EC 3.2.1.91--1,4-β-cellobiohydrolase), that cleaves cellobiosyl units from the ends of cellulose chains and β-glucosidase (EC 3.2.1.21), which cleaves glucose units from cello-oligosaccharides (Wood, 1989; Romero et al., 1989).

The maximum yield has been obtained when cellulose is used as substrate, but the economics of the cellulose bioconversion process still remain unattractive due to the high cost of enzyme production (Lange, 2007; Nystrom and Allen, 1976). Further the use of crystalline cellulose as insoluble substrate in a culture system may give rise to several problems (Cowling and Kirk, 1976). These problems could be overcome if the tested organism is grown on soluble sugar. The use of soluble carbon sources such as lactose (Ryu et al., 1979; Durand et al., 1988), and cellulose hydrolyzate (Allen and Roche, 1989), cellobiose (Vaheri et al., 1979) for cellulase production allows greater control of the fermentation and simplifies the operation of the process. Among soluble and cheaper carbon sources, cheese whey has been most widely used in cellulase production.

Hence, the present study was carried out to compare and optimize between cellulolytic enzyme activities, endoglucanase (carboxymethyl cellulase CMCase EC3.2.1.4), exoglucanase (avicelase EC3.2.1.1.91), and cellulase represented as filter paper activity (FPA EC 3.2.1) of a locally isolate of *Aspergillus oryzae* grown on cheese whey which is used as a cheap carbon and energy source. In the current study, some properties such as the optimum temperature, pH, heat stability, and molecular weight of endoglucanase (carboxymethyl cellulose CMCase) of

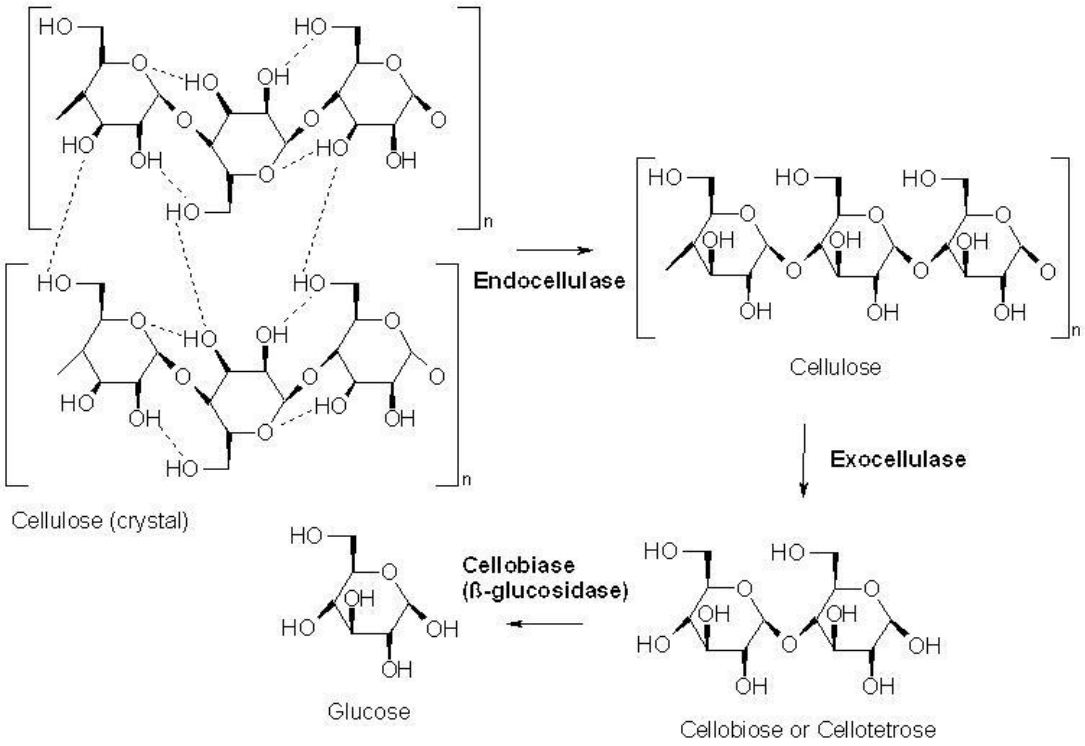

Figure 2. Three major classes of cellulase and their substrates (Wikipedia, 2009).

A. oryzae natural isolate are reported with a view to identify potential sources of industrial enzymes.

MATERIALS AND METHODS

Isolation and maintenance of cellulolytic fungi

Cellulolytic fungi were isolated from local garden soil by using Czapek- Dox medium (Samson et al., 1996) containing carboxymethyl - cellulose (1%) as a carbon source. The collected samples were sprinkled over the Dox agar plates and were incubated for 5 days at 30°C. The individual colonies were isolated and re-streaked on the same agar medium. The identification was conducted in the fungal center of Assuit University, Egypt. The most common fungal strain was identified as *A. oryzae.* The identified isolate was given the name *A. oryzae* AG1 to be stored among our laboratory culture collection. *A. oryzae* was maintained as stock culture in Czapek-Dox agar slants. The fungus was grown at 30°C for 5 days and stored at 4°C for regular sub culturing.

Inoculums' preparation

A seed culture (100 ml) was prepared for culturing using Czapek-Dox broth distributed in 250 ml flasks. The culture was kept in shaker (200 rpm) at 35°C for 24 h before it was used for the fermentation process.

Fermentation process

Cultivation was performed on minimal medium (%), NH_4NO_3, 0.1; Mg $SO_4.7H_2O$, 0.05; $FeSO_4$ $7H_2O$, 0.01; $CaCl_2$ $2H_2O$, 0.05; KH_2PO_4, 0.05; and supplemented with 10% liquid cheese whey (Department of Dairy Science, Faculty of Agriculture, Alexandria University), used as a soluble carbon and nitrogen source, pH was adjusted to 7and the medium was sterilized by autoclaving at 121°C for 15 min. A sterile 50 ml of the medium was distributed in sterile 250 ml Erlenmeyer flasks, which were inoculated with 2 ml of the seed cultures and incubated at 35°C on a rotary shaker (180 rpm) for 7 days.

Enzyme assay

In submerged fermentation the grown culture was filtered through filter paper (whatman No. 1), filtrate was centrifuged at 10,000 rpm for 5 min at 4°C and the supernatant was used as the crude enzyme. Endoglucanase activity, (carboxymethyl-cellulase; CMCase): was measured as Ghosh (1987) using a reaction mixture containing 1 ml of 2% carboxymethyl-cellulose (CMC) in 0.05 M acetate buffer (pH 4.8) and 1.0 ml of culture supernatant. The reaction mixture was incubated at 50°C for 10 min and the reducing sugar produced was determined by dinitrosalicylic acid- DNS method of Miller (1972) taking glucose as the standard. Blanks were prepared with inactivated enzymes. One unit of endoglucanase activity was defined as the amount of enzyme releasing 1 mg of reducing sugar per min. Exoglucanase (Avicelase) activity was determined as described for endoglucanase activity, but the incubation was carried out with 1 ml of 1% avicel (Sigma) suspension instead of carboxymethyl-cellulose. For filter paper activity (cellulase; FPA) measurement, it is a combined assay for endo and exo β -1;4 glucanase, according to Stephen et al. (2003). 1 ml of the culture supernatant as a enzyme source was added to whatman No. 1 filter paper strip (1 x 6 cm; 50 mg) immersed in 1 ml of 0.05 M sodium acetate buffer of pH 4.8.

After incubation at 50°C for 10 min, the reducing sugar released was estimated by DNS method. One unit of the filter paper (FPA) activity was defined as the amount of enzyme releasing 1 mg of reducing sugar from the filter paper per milliliter per minute of the reaction.

Partial purification of carboxymethyl cellulase (CMCase)

Enzyme source preparation

The fungal organism *A. oryzae* was grown in 100 ml of optimized media at 30°C for 24 h individually. Then after growth, the culture filtrates were collected separately by centrifugation process.

Alcohol precipitation of cellulose

To the culture filtrate (250 ml), ethanol previously chilled to -20°C was added drop wise at 4°C with continuous stirring to the final concentration of 75% and the solution was left at -20°C for 24 h. The resultant precipitate was collected by centrifugation and dissolved in 30 ml of phosphate buffer (50 mM, pH 5.0) and was dialyzed against of the same buffer over night at 4°C (Bhella and Altosaar, 1984).

Optimum temperature, pH and heat stability

In all the determinations, CMCase activity was measured using CMC as substrate. For the estimation of optimum temperature, pH and heat stability, activity was determined by carrying out the standard assay at several temperatures or pH values.

Sodiom dodecyl sulfate - polyaccylamide gel electrophoresis (SDS-PAGE)

For the determination of homogeneity and molecular weight, the enzyme preparations and protein markers were subjected to electrophoresis by the method of Bollag and Edelstein (1991) with the use of 10% acrylamide gel. CMC (0.2%) was incorporated into the separating gel prior to the addition of ammonium persulphate and polymerization. After electrophoresis, the gel was stained with coomassie Blue R dye in methanol- acetic acid–water solution (4:15, by volume) for 1 h and distained in the same solution without dye. For the activity staining of CMCase activity, SDS was removed by washing the gel at room temperature in solution A (sodium phosphate buffer, pH 7.2, containing isopropanol 40%) for 1 h and solution B (sodium phosphate buffer, pH 7.2) for 1 h respectively. Renaturation of the enzyme proteins was carried out by leaving the gel in solution C (sodium phosphate buffer, pH 7.2, containing 5 mM β- mercaptoethanol and 1 mM EDTA) at 4°C overnight.

The gel was then transferred on to a glass plate, sealed in a film, and incubated at 37°C for 5 h. The gel was stained in a solution of 1% Congo Red for 30 min, and distained in 1 M NaCl for 15 min. Clear bands indicated the presence of CMCase activity. Each sample was applied to a separate well in the slab gel along with a prestained SDS molecular weight marker (14-205 K Daltons).

Determination of kinetic parameters

Michaelis constant (Km) and maximal velocity (Vmax) of the purified enzyme (for CMC) was determined as follows: 0.4 ml of CMC in 10 mM sodium phosphate buffer, pH 8.0 in concentrations from 1.0 to 5.0 mg/ml as substrate, add 0.1 ml diluted enzyme solution and incubated at 60°C for 20 min. The reducing sugar produced was measured calorimetrically with DNS reagent. The Vmax and Km were calculated from double-reciprocal plots according to the method of Lineweaver and Burk (1934).

RESULTS AND DISCUSSION

Effect of incubation period

In an attempt to investigate the multi-cellulolytic activities (endo-, exoglucanase and FPA cellulase), and the biomass of *A. oryzae* at different incubation periods, were carried out under shaking condition. Each flask (250 ml) contained 50 ml of the fermentation medium supplied with 10% of cheese whey as a carbon source, each flask was inoculated with 2 ml of the seed culture and incubated at different time intervals ranging from 3 to 14 days.

The specific activity U/mg biomass of the multi–cellulolytic enzymes was represented in (Figure 3). The activity was increased and reached its highest value at the end of the exponential phase. The highest values of endo-, exoglucanase and FPA cellulase specific activities (15.70, 12.63 and 1.68 U/mg biomass) were obtained after 7 days of incubation. At longer incubation periods, the activities decreased gradually and after 14 days of incubation the culture showed a low endoglucanase activity value representing 45.22% of the activity obtained at 7 days incubation. Similarly, Coral and Colak (2000) showed that *Aspergillus niger* produces glucoamylase enzyme with high concentration during exponential growth phase of the fungus.

Effect of substrate concentration

The effect of dose response of cheese whey supplementation within a range of 3 to 25% on cellulase production of *A. oryzae* was examined. Yields of fungal biomass and multi-cellulolytic enzyme activities after one week were measured and specific activity as U/mg biomass was represented in (Figure 4). Increase in substrate concentration beyond 10% level did not result in proportionate increase in specific activity. The highest specific activities (15.70, 12.63 and 1.68 U/mg biomass) for endo-, exoglucanase and FPA cellulase activities respectively, were recovered on the medium supplemented with 10% substrate concentration.

Cheese whey which is released during the manufacture of cheese is rich in carbon and nitrogen source. Cheese whey consists of approximately lactose, whey protein, salts, lactic acid and vitamins (Kim, 2004). Bioconversion of whey of value- added products such as: organic acids, ethanol, enzymes, PHB (poly-3 hydroxybutyrate), xanthan gum and biomass have been extensively studied by many investigators (Kawahara and Obata, 1998; Lee and Yun, 1999; Lee et al., 2000).

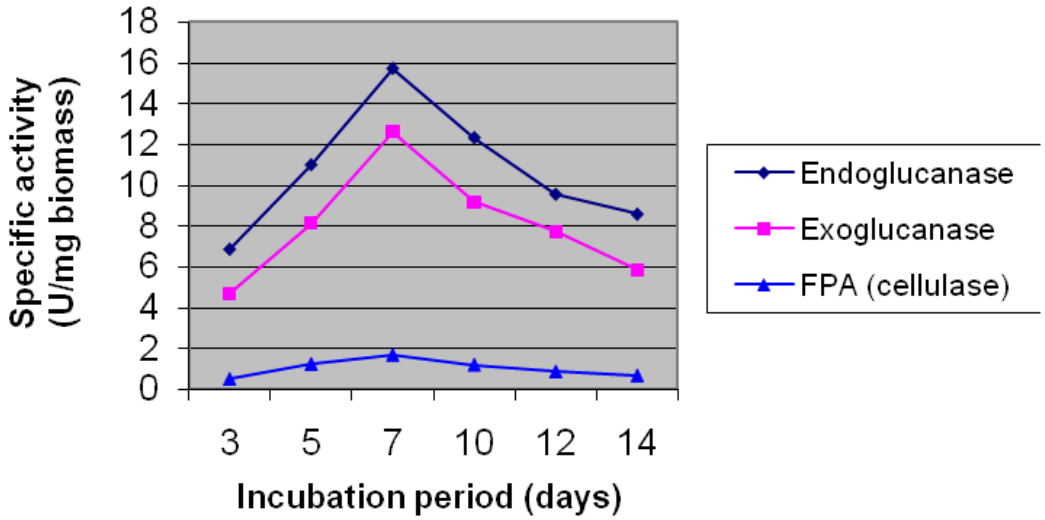

Figure 3. Cellulolytic activity of *A. oryzae* culture at different incubation periods.

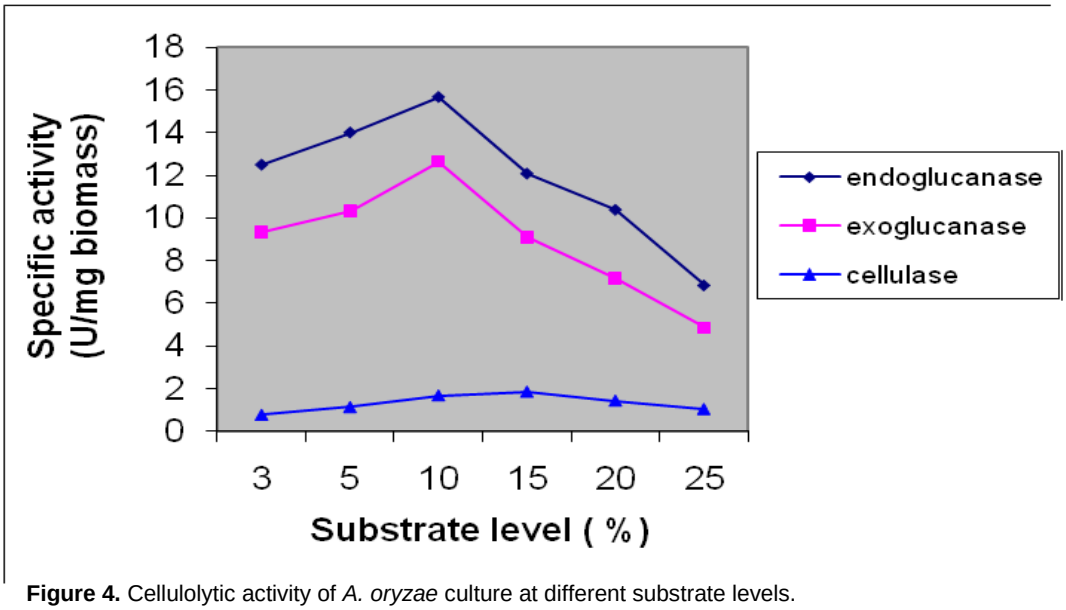

Figure 4. Cellulolytic activity of *A. oryzae* culture at different substrate levels.

At low concentration of whey (low conc. of lactose), the enzyme activities were higher, when a higher substrate concentration above 10% was used. This result could be explained on the basis of catabolic repression. A higher concentration of utilized whey, resulted in a higher utilization of lactose, which is metabolized into glucose. A decrease in the activity of certain catabolic enzymes in the presence of an easily metabolized substrate is called catabolic repression. Commonly this effect is caused by glucose (glucose repression). It has been reported previously that glucose addition greatly repressed enzyme activity, the repression was concentration-dependent (Bindu et al., 2006; Suzuki et al., 2008).

Effect of pH on the production of cellulolytic enzymes

The optimized media were prepared using 10% cheese whey concentration and the pH was set at different levels such as 3, 5, 6, 7, 8, 9, and 10 respectively (by adding 1% NaOH and concentrated HCl) before autoclaving. After inoculation, the flasks were incubated under shacked condition (150 rpm) at 35°C for 7 days. The three different cellulolytic enzyme activities were detected separately (Figure 5). The highest specific activities of endo-, exoglucanase (18.5, 14.8 U/mg biomass respectively) were obtained at an initial pH 6. However, cellulase showed the highest specific activity (1.68 U/mg

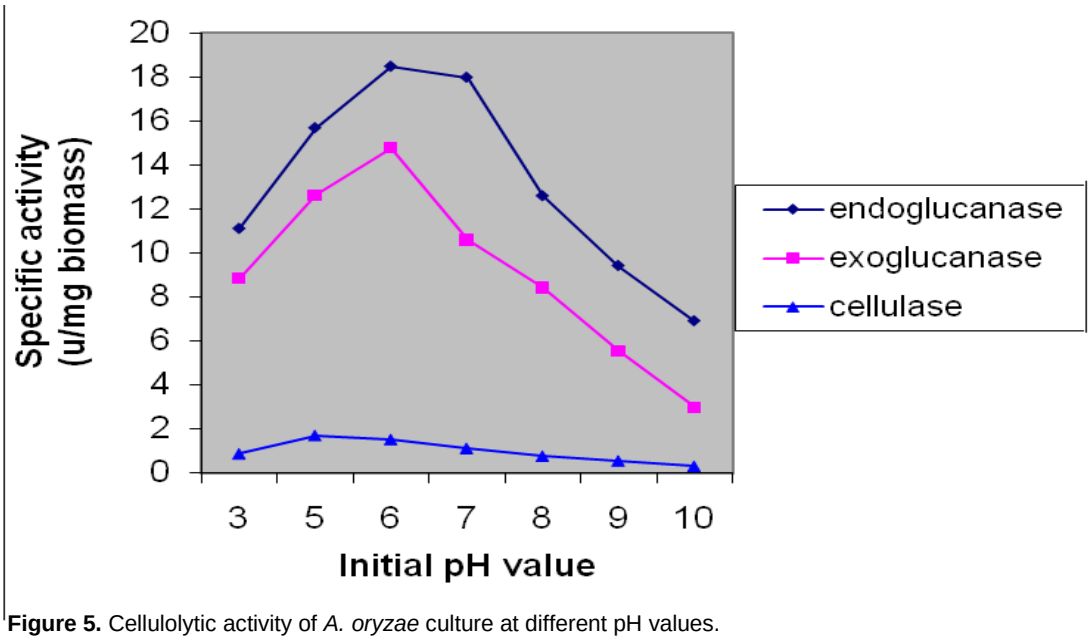

Figure 5. Cellulolytic activity of *A. oryzae* culture at different pH values.

biomass) at pH 5. Further increase in the pH level, resulted in a decrease in enzyme production. The in observation was comparable to *Rhizopus oryzae* that can thrive in a pH range, of 5.0 to 7.0 (Murashima et al., 2002).

It was observed that the cellulase enzyme activity has a broad pH range between 3.0 and 9.0 (Coral et al., 2002). The optimal pH for endoglucanase from *Aspergillus niger* was found to be 6.0 to 7.0 (Parry et al., 1983). Akiba et al. (1995) reported that cellulase production was high at pH 4.0 and pH 4.5 by *A. niger*. The pH requirement was determined at pH 5.0 in fungal species *Trichoderma harizianum* (Sidhu and Shadhu, 1984), and *Fusarium avenaceum* (Forbes, 1977).

Effect of incubation temperature

Influence of different incubation temperatures on the production of cellulolytic enzymes was examined. The inoculated flasks were incubated at different temperatures covering range from 20 to 60°C. The results revealed that the specific activity by *A. oryzae* reached a maximum of 24.2, 18.1 and 1.65 U/mg biomass at 40°C further the temperature increased. The specific activity was significantly decreased for endo-, exoglucanase and cellulase activity at 60°C respectively as shown in Figure 6. Most filamentous fungi are mesophilic requiring optimal temperatures between 25 and 35°C (Reid, 1998; Suresh et al., 1999). A temperature of 40°C was identified as optimum for metabolite production and sugar utilization by *A. niger* ATCC 10577 and *A. niger* V. Tiegham (Roukas, 2000; Fawole and Odunfa, 2003).

Both exo-, and endoglucanase were stable at 40 to 50°C in *A. terreus* (Emtiazi et al., 2001). Kitamoto et al. (1996), studied *A. oryzae* KBN616, in which, two endoglucanases Cel A and Cel B were identified, Cel A showed optimum temperature of 50°C and Cel B showed optimum temperature of 45°C. Two other exoglucanases were also identified Cel C and Cel D (Kitamoto et al., 1996). A high level of cellulase enzyme production was obtained at 40°C by *A. niger* and *fumigatus* (Immanuel et al., 2007). In this study, the locally identified *A. oryzae* showed an optimum temperature of 40°C for the three studied cellulolytic enzymes.

Seven days incubation period, at an initial pH of 6, a temperature of 40°C, and cheese whey concentration of 10% yielded the highest specific activities with 1.5 fold increases for endoglucanase and 1.4 fold increase for exoglucanase. Cellulase specific activity represented as FPA showed no fold increase at all. The stabilization of FPA could be explained on the basis of that the filter paper is composed of not only fibrous (cellulose) but also non fibrous material (Sahin and Arslan, 2008). The presence of impurities beside cellulose could result in hindering the increase of cellulase specific activity.

Characterization of partial purified endoglucanase (Carboxymethyl cellulase CMCase)

Temperature of the reaction mixture

Estimation of the optimum temperature of the CMCase activity of the partial purified enzyme, the activity was determined by carrying out the assay at several temperatures between 25 and 80°C (Figure 7), the optimum temperature was observed at 55°C. An increase

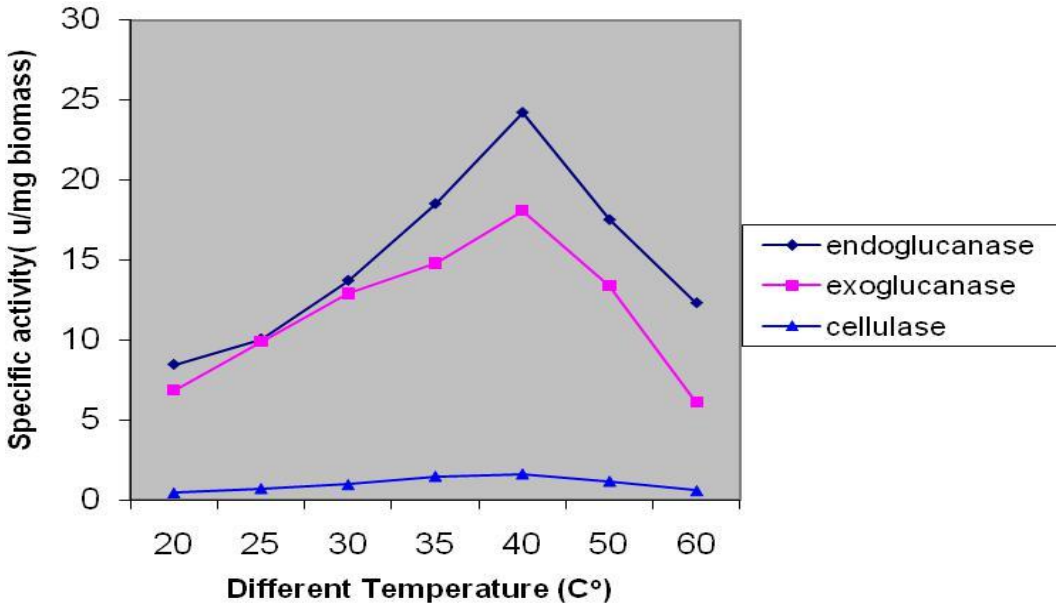

Figure 6. Cellulolytic activity of *A. oryzae* culture at different incubation temperatures.

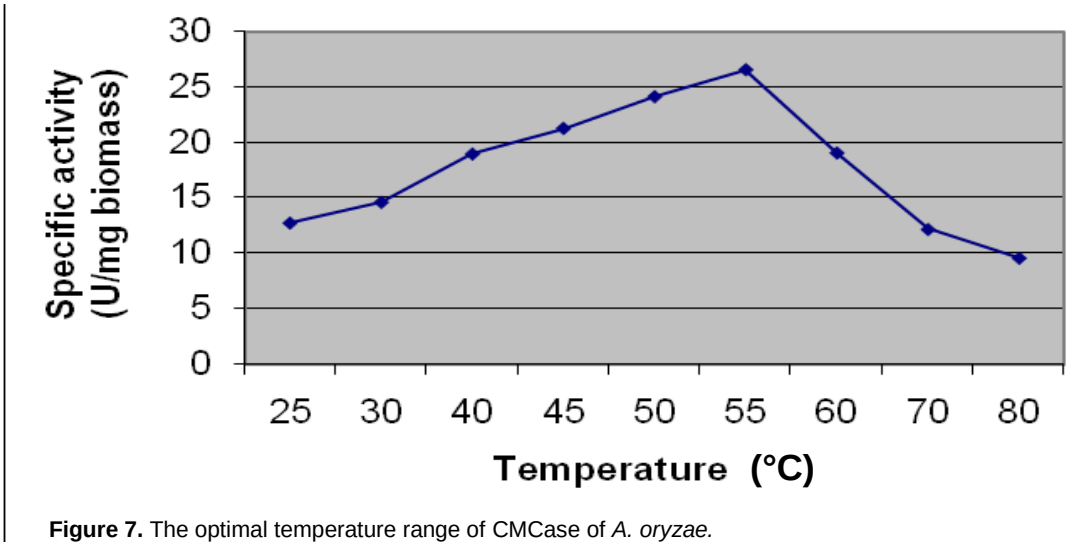

Figure 7. The optimal temperature range of CMCase of *A. oryzae.*

in the reaction mixture over 55°C leads to a decrease in enzyme activity and at 80°C , The activity decreased by 63.37% of that obtained at 55°C .Deerland cellulase 4000 is an enzyme preparation derived from *A. niger* and has high cellulase activity together with β-glucosidase and hemicellulase. The optimum temperature of this cellulase was reported to be 60°C.

pH of the reaction mixture

To study the effect of the pH value of the reaction, acetate buffer of different pH values ranging from 3.8 to 5.6 and phosphate buffer ranging from 6.0 to 8.0 were used (Figure 8). It was found that the CMCase activity of *A. oryzae* has abroad pH range between 3.8 and 8.0. The enzyme shows a major activity peak at pH 5.2. It was reported that the optimal pH for a CMCase from *A. niger* was found between 6.0 and 7.0 (Akiba et al., 1995) and another study showed that the optimal pH activity of *A. niger* CMCase was found to be between 4.0 and 4.8 (McCleary and Glennie-Holmes, 1985). Thus, it was seen that there was no agreement regards enzyme data results. Such different results may be due to the difference within the same genus. In addition, no comparative investigation has been published on the

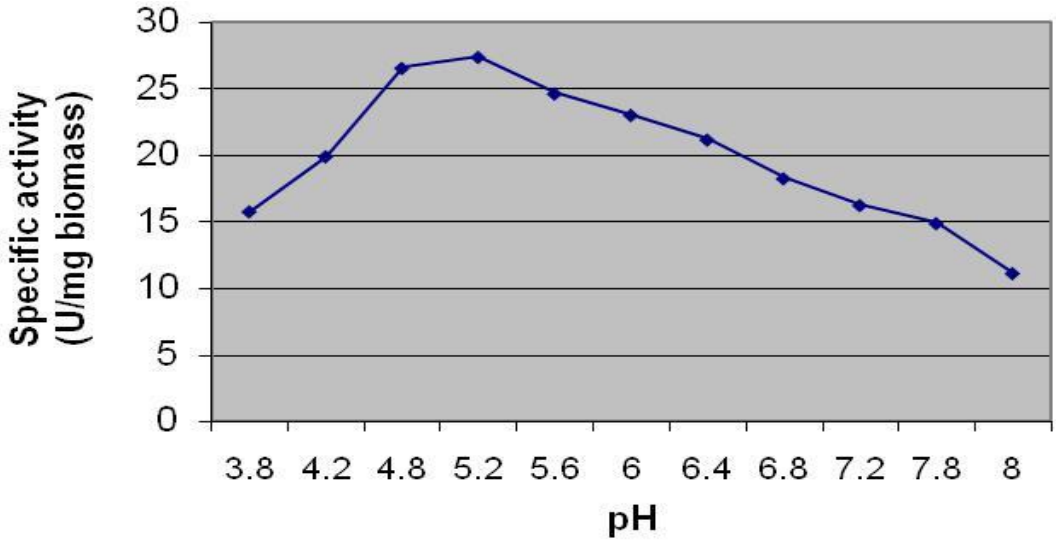

Figure 8. The optimal pH range of CMCase of *A. oryzae.*

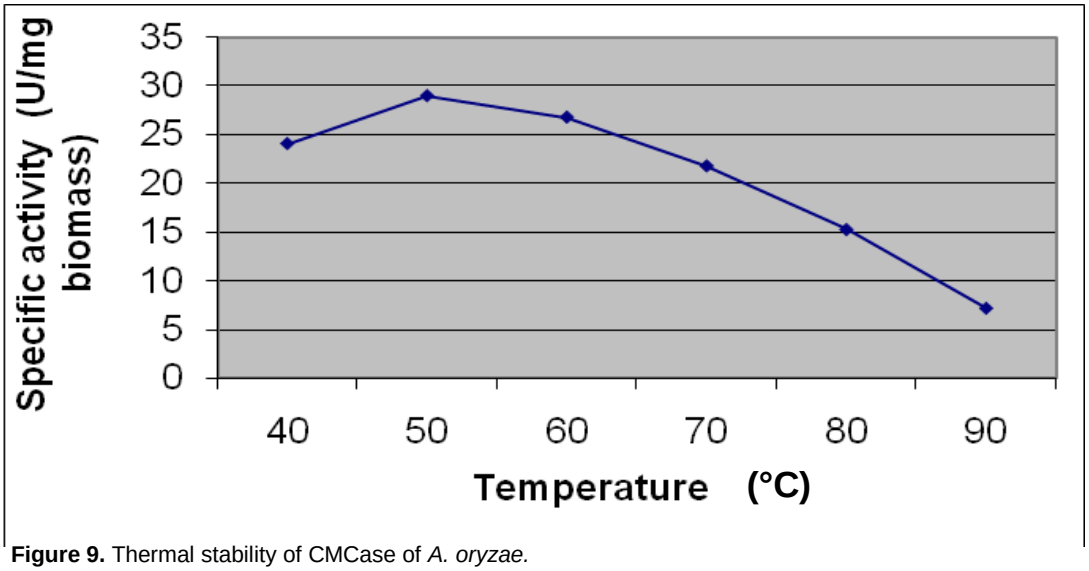

Figure 9. Thermal stability of CMCase of *A. oryzae.*

enzymes from these organisms, but differences appear to be as small as the differences in morphology between the species (Aunstrup, 1979).

Thermal stability

The thermal stability of the crude enzyme preparation was studied at pH 5.2 at different temperatures 40, 50, 60, 70, 80 and 90°C for 30 min. and the reaction tubes were placed rapidly in ice bath. The activity was assayed by DNS methods under the standard conditions. Our enzyme preparation was thermo stable up to 70°C (Figure 9) for 30 min and lost only 27.04% of its original activity. A maximum decrease of the enzyme activity was recorded when heating the enzyme at 90°C for 30 min. Endoglucanase from *A. fumigatus* was reported to be stable at 50°C (Parry et al., 1983*).* This comparison shows that the heat stability of CMCase from *A. oryzae* is higher than that of the enzyme mention previously. Both exo-, and endoglucanase of *A. terreus* were stable at 40 to 50°C (Emtiaziet et al., 2001).

Kinetic parameters

The substrate concentration effect on the hydrolase rate for CMC was determined. The Michaelis constant (Km)

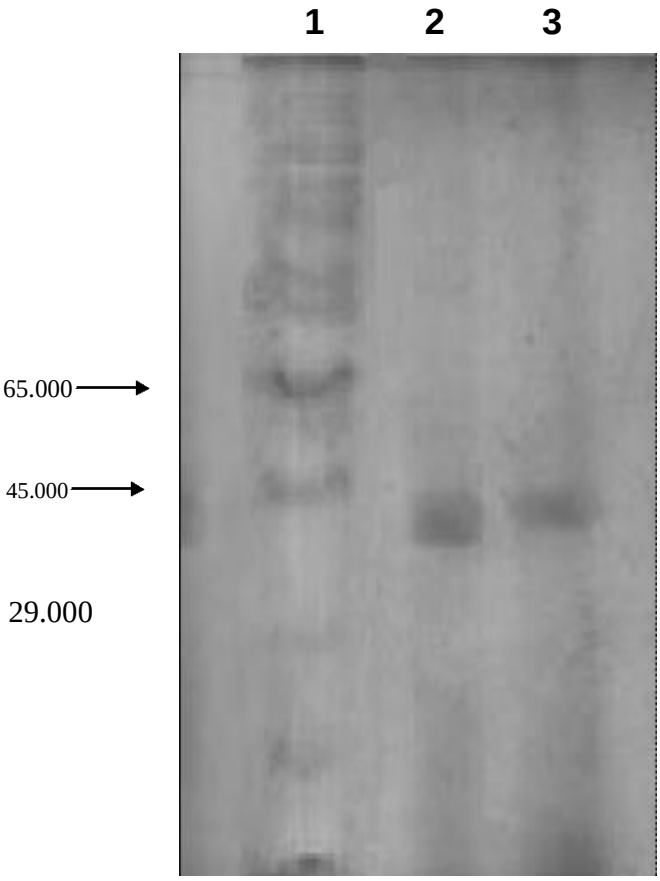

Figure 10. gel is of partially purified Figure (10): SDSgel electrophoresisofpartially purified carboxymethyl cellulase from A. oryzae carboxymethyl cellulase from A. oryzae (lane 2) and of the crude enzyme (lane 3). Lane one represents molecular weight marker protein. KDa: Molecular weight of marker proteins used (kilo Dalton) and maximum velocity (Vmax) were calculated from Lineweaver–Burk plots. The enzyme had a Km of 3 mg/ml and a Vmax of 4.5 µmol of glucose/min/ml for CMC hydrolysis. Km value may serve only to denote the amount of substrate needed to achieve half the maximal initial reaction velocity (Tong et al., 1980). Km is thus a measure of the apparent affinity of an enzyme for its substrate. The km value, (3 mg/ml) was obtained from A. oryzae isolate and this value was lower than those obtained for the enzyme from A. niger (Hurst et al., 1977) which was 52 to 80 mg/ml. While cellulase obtained from the wild-type, CRRmt4 and CRRmt24 of Pseudomonas fluorescens has Km values 3.6, 3.1, and 5.3 mg/ml respectively (Bakare et al., 2005) while Km of endoglucanase from Sclerotinia sclerotorium (Waksman 1991) was 8.7 mg/ml. It is not an easy task to define the Km values of insoluble substrates such as cellulose and Vmax value of 3.4 unit/min was obtained for A. oryzae isolate, this value was differ than those obtained for the enzyme from P. fluorescens strains, wild-type, CRRmt4 and CRRmt24, which were 8.3, 3.3 and 10, respectively (Bakare et al., 2005).

Determination of apparent molecular weight

Molecular weight of the partial purified enzyme was determined by SDS- PAGE (12%) as described previously in the materials and methods. Analysis of the enzyme by SDS- PAGE revealed the appearance of one band with molecular weight of 45.000 Daltons (calculated from the relation between the maker and the relative mobility of the calculated molecular weight), Figure (10).

$$R_m = \frac{\text{Distance traveled by protein}}{\text{Distance traveled by font dye}}$$

Similar results were mentioned, hence, a celluolytic enzyme from *A. niger* was separated as a homogeneous entity in SDS- PAGE, but showed one major and two minor bands in disc, gel electrophoresis, these bands (proteins) may be isoenzymes or the different subunits of the same enzyme protein on electrophoresis gel (Coral et al., 2002).

According to Lee et al. (1988) research, two endoglucanase containing fractions were separated from *A. niger*. In comparison to the low-molecular-weight standards of Bio-Rad electrophoresis within SDS revealed a single band at a molecular weight of 90 KDa of thermophilic Actinomycetes (Aboul-Enein et al., 2010).

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