Factors Affecting Activity of Cellulase Enzymes Produced By Three Fungi

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ABSTRACT

Three fungi (*Trichoderma viride, Asperigillus niger* and *Penicillium digitatum*) were used in the present study for the production of the cellulase enzymes (carboxymethylcellulase and cellobiase). The effects of different metal ions on the activities of these enzymes were investigated using the reducing group method. The addition of Ca^{++}, Ba^{++}, K^{+} and Mn^{++}, caused a significantly higher increase in the activity of the carboxymethylcellulase enzyme of the three fungi, while a less increase was found by the addition of Mg^{++} and Zn^{++}. However, the addition of Na^{+}, Ag^{+}, Cd^{++}, Cu^{++}, and Fe^{++} caused a decrease in this enzyme activity. The activity of the cellobiase was increased with Ca^{++} and K^{+}, but it was decreased in the presence of Hg^{+} and Cd^{++}.The higher activity of the cellobiase enzyme produced by *A. niger* occurred was at the range of 200 – 900 ppm of K^{+}, while it occurred at 500 – 900 ppm for Mn^{++} and Ca^{++} , however, for Ba^{++}, it occurred at the range of 800 – 900 ppm. For the cellobiase of both *T. viride* and *P. digitatum*, no significant different was noticed with different concentrations of Ca^{++}.

On the other hand, Ba^{++} gave a lower activity for the cellobiase enzyme of both fungi at all concentrations. The optimum temperature for the carboxymethylcellulase enzyme activity was at 50°C for *A. niger*, while it was between 40 ^{0}C – 50 ^{0}C for both *T. viride* and *P. digitatum*. However, the optimum temperature for the cellobiase activity of the three fungi was at 40 ^{0}C. The optimum activity of the carboxymethylcellulase enzyme produced by *A. niger* was at pH 4, and for that of *T. viride* was at pH 6. Two peaks were detected for the same enzyme produced by *P. digitatum*, at pH 6 and pH 7. However, the optimum pH value for the cellobiase enzymes produced by the three fungi was at pH 4.0
only.

Key words: Fungi, Cellulase Enzymes.

INTRODUCTION

Microorganisms produce multiple cellulase enzymes that are either free or cell associated, and these enzymes degrade natural cellulosic materials (Bhat and Bhat, 1997; Lynd *et al.*, 2002). Certain ions were found absolutely necessary for the activity of some enzymes, while others are highly toxic to nearly all enzymes. Some may inhibit an enzyme at one concentration and be activators for the same enzyme at another concentration (Dixon and Webb, 1964). Ajaya *et al*. (2006) reported that Mg^{++}, Ca^{++}, Na^{+} and K^{+} were stimulatory to the cellulolytic enzyme activity, while small quantities of $HgCl_2$ and EDTA were inhibitory. Lin *et al.* (2010) reported that most metal ions such as Ca^{++}, Mg^{++}, Cd^{++} and Zn^{++} exhibited slight inhibition effect on the enzyme activities, whereas K^{+} and Mn^{++} enhanced the cellulases to a 10% extent. Among these metals and chemicals, Cu^{++} could bind the thiol groups and interact with the carboxyl groups of the amino acids.

Like most chemical reactions, the rate of an enzyme reaction increases as the temperature is raised. A 10 ^{0}C rise in temperature will increase the activity of most enzymes by 50- 100 %. The use of higher temperature causes an increase in the enzyme activity and a decrease in its stability, taking into account the period of time the enzyme is used.

However, few enzymes can be heated to above 100^{0}C and still retain activity; for instance, the denylatekinase enzyme can retain activity even after being maintained at a temperature of 100^{0}C. Storage of enzymes at 5^{0}C or below is generally the most suitable. However, some enzymes loose their activity when frozen (Bennett and Frieden, 1969).

Enzymes are affected by changes in pH, the most favorable value, is the point where the enzyme is most active, and is known as the optimum pH value. Extremely high or low pH values generally result in complete loss of activity for most enzymes (Holum, 1968).

MATERIALS AND METHODS

The effect of cofactors (metal ions):

Different salts were used (Ca^{++}, Ba^{++}, Zn^{++}, Mg^{++}, Mn^{++}, Cu^{++} , Cd^{++}, Ag^{+}, Hg^{+} and Fe^{++}). 1.0 mg/ml of each salt was added to the reaction mixture containing I ml of each enzymes prepared from each of the three fungi. Immediately after adding the appropriate salt solution, the enzyme was assayed by the reducing group method(A Adel Wahab and Abdel-Rahim, 2013), using carboxymethylcellulose (CMC) as a substrate, and as was described by Abdel-Rahim (1980).However different concentrations were used for Mn^{++}, Ca^{++}, Ba^{++}.

Effect of temperature:

Reaction mixtures containing enzyme preparations were incubated in water baths, at temperatures ranging from 20 ^{0}C to 60 ^{0}C for different periods of time. The reactions temperatures were maintained, using different thermostatically controlled waterbaths.

Effect of pH:

Reaction mixtures in buffers of different pH values ranging from 4.0 to 10 and containing preparations of cellulases obtained from cultures of the three fungi, were assayed. The following buffers were used:
Acetic acid - sodium acetate (pH 4.0 – 5.0), phosphate buffer (pH 6.0 – 7.0), Tris-HCl (pH 8.0 – 9.0) and glycine-Na0H (pH 9.0 – 10.0).

RESULTS

Effect of different metal ions on the activities of both enzymes:

The effect of different metal ions (Ca^{++}, Ba^{++}, Mg^{++}, Mn^{++}, Cu^{++}, Cd^{++}, Ag^{+}, Hg^{+}, Zn^{++}, Fe^{++}, K^{+} and Na^{+}) on the cellulase activity of the three fungi (*A. niger*, *P. digitatum* and *T. viride*) were made using the reducing group method. The results on Table (1) showed that the addition of Ca^{++}, Ba^{++}, K^{+} and Mn^{++}, to the enzyme assay caused a significantly higher increase in the activity of the carboxymethylcellulase enzyme of the three fungi. However, less increase was found by the addition of Ag^{+}, Mg^{++} and Zn^{++}. On the other hand, the addition of Na^{+}, Hg^{+}, Cd^{++}, Cu^{++}, and Fe^{++} caused a decrease in the activity. However, the activity of the cellobiase was highly increased with Ca^{++} and K^{+}, less increased with Mg^{++}, Zn^{++} and Ba^{++}, but it was decreased in the presence of Hg^{+} and Cd^{++} (Table, 2).
The results on Table (3) show that the higher activity of the carboxymethylcellulase enzyme produced by *A. niger* occurred at the range 200 – 900 ppm of K^{+}, while it occurred at the 500 – 900 ppm in the case of Mn^{++}. The active concentration for Ca^{++} and Ba^{++} was recorded at the range 700 – 900 ppm. For the carboxymethylcellulase of both *T. viride* and *P. digitatum*, no significant different was noticed with the different concentrations of Ca^{++}. On the other hand, Ba^{++} gave a lower activity for the enzyme of both fungi at its all concentrations (Tables, 4 and 5).

Effect of temperature on the activities of both enzymes:

The filtrates of the three fungi (*A. niger*, *P. digitatum* and *T. viride*) were incubated in the presence of CMC or cellobiose as substrates for the carboxymethylcellulase and cellobiase enzymes, respectively, at temperatures varying from 10 ^{0}C to 60 ^{0}C, then the reaction mixtures were assayed by using the reducing group method. The results (Fig, 1) showed

that the activity of the carboxymethylcellulase enzyme of the fungus *A. niger* was increasing with increasing temperature with the maximum at 50 ^{0}C, then decreased.

Effect of pH on the activity of cellulose enzyme:

The effect of pH level was tested for the enzyme activities, using CMC as substrate for the carboxymethylcellulase assay and cellobiose for the cellobiase assay. Results obtained by using the reducing group method (Fig. 3), showed that the maximum activity of the carboxymethylcellulase produced by *A. niger* was detected at pH 4, and the maximum activity for the carboxymethylcellulase of *T. viride* was found at pH 6, while, the higher activity of carboxymethylcellulase produced by *P. digitatum* was detected at two peaks pH 6 and pH 7. Similar results were also obtained by using the viscometric method except that only one peak was detected for the carboxymethylcellulase of *P. digitatum* (Fig. 4). However, the optimum pH value for cellobiase enzymes produced by the three fungi was detected at pH 4.0 only *(*Fig. 5).

Table (1). Effect of different ions on carboxymethylcellulase activity (mg/ml) produced by the three fungi

Ions added	Carboxymethylcellulase activity as reducing sugars (mg/ml).		
	A. niger	*P. digitatum*	*T. viride*
Ba^{++}	92.8	77.8	99.5
Ca^{++}	104.5	99.5	106.1
Zn^{++}	34.5	32.8	34.5
Mg^{++}	36.1	34.5	31.2
Na^{+}	49.5	62.8	49.5
Mn^{++}	92.8	106.1	112.8
Cu^{++}	9.5	12.8	6.2
Cd^{++}	14.5	9.5	9.5
Ag^{++}	64.5	49.5	34.5
K^{+}	99.5	111	106.1
Hg^{+}	49.5	34.5	46
Fe^{++}	17.8	9.5	16.1

Table (2). Effect of different ions on cellobiase activity (mg/ml) Produced of the three fungi

Ions Organism	Cd^{+}	Mg^{++}	Ba^{++}	Zn^{++}	Hg^{+}	Ca^{++}	K^{+}
T . viride	25.6	51.6	42.8	46.6	10.8	76.1	75.6
A .niger	10.6	38.6	40.1	35.5	12.8	68.8	54.5
P. digitatum	6.6	5.8	27.8	39.5	6.5	44.6	34.8

Table (3). Effect of different concentrations of the highly effective ions on the carboxymethylcellulase activity (mg/ml) produced by *A. niger*

PPM	K^+	Mn^{++}	Ca^{++}	Ba^{++}
100	65.6	12.8	32.8	14.5
200	80.1	33	29.5	12.8
300	79.3	52.5	29.5	12.8
400	78.3	46.0	24.5	14.5
500	69.8	61.6	21.1	11.2
600	86.8	57.6	27.8	11.8
700	82.5	71.1	57.8	17.2
800	81.1	65.1	84.5	16.2
900	81.1	64.6	74.5	16.2

Table (4). Effect of different concentrations of the highly effective

PPM	K^+	Mn^{++}	Ca^{++}	Ba^{++}
100	75.2	86.6	92.8	44.1
200	74.8	84.5	93.6	41.1
300	73.6	86.5	92.6	41.8
400	73.6	87.8	91.0	43.1
500	75.8	78.9	108.6	59.5
600	75.0	84.0	109.5	57.8
700	74.8	84.0	108.8	69.6
800	75.3	84.0	108.1	69.5
900	75.6	85.8	108.1	56.8

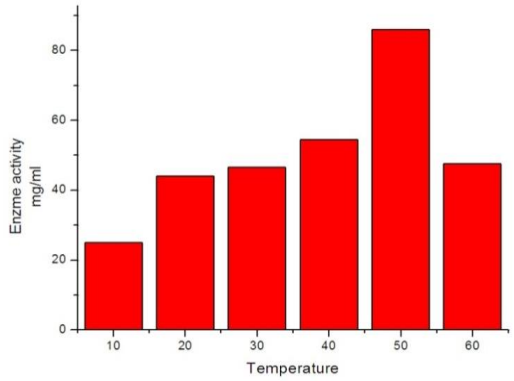
carboxymethylcellulase activity(mg/ml) produced by *T. viride*

ions on carboxymethylcellulase enzymes of both *T. viride* and *P. digitatum* were highly active between the temperatures 40 ^{0}C – 50 ^{0}C (Fig. 10). However the optimum temperature for cellobiase activity of the three fungi was found at 40 ^{0}C(Fig, 2). the activity decreased sharply. However, the Table (5). Effect of different concentrations of the highly effective ions on

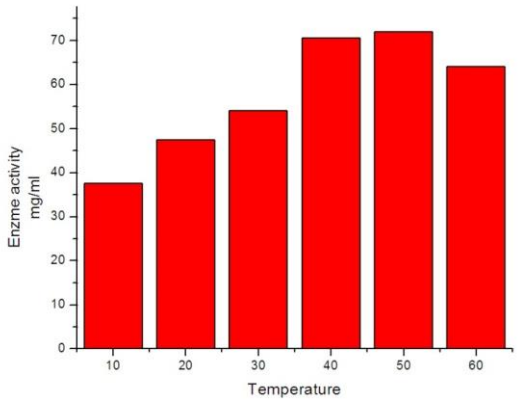
PPM	K^+	Mn^{++}	Ca^{++}	Ba^{++}
100	71.6	74.1	80.5	42.8
200	69.8	74.5	82.8	40.3
300	71.6	74.6	82.8	43.5
400	71.1	74.5	78.9	43.6
500	71.8	74.6	81.6	42.8
600	87.1	80.5	90.8	55.1
700	88.8	74.6	99.8	55.6
800	86.8	76.6	98.8	40.8

	900	87.6	80.0	100.6	41.6

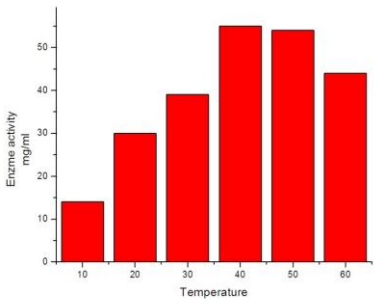
carboxymethylcellulase activity (mg/ml) produced by *P. digitatum*



A



B



C

Fig (1): Effect of temperature on carboxymethylcellulase activity (mg/ml) produced by the three fungi: (A) *A. niger*, (B) *P. digitatum*, and (C) *T. viride.*

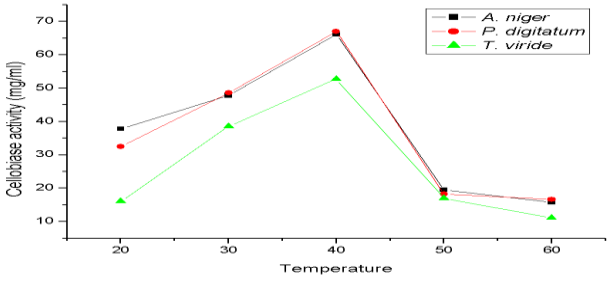


Fig (2): Effect of temperature on lcellobiase activity (mg/ml) produced by the three fungi: (A) *A. niger*, (B) *P. digitatum*, and (C) *T. viride.*

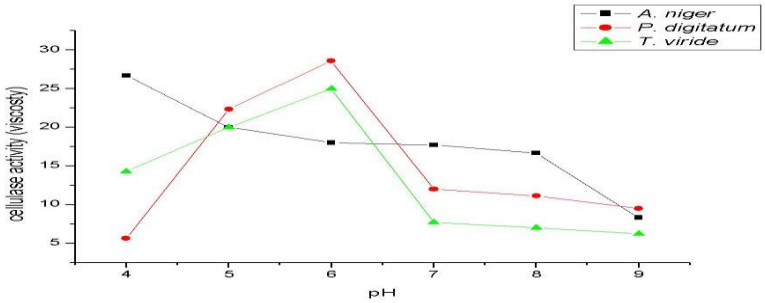


Fig. (3). Effect of different pH levels on the carboxymethylcellulase activity produced by the three fungi using the reducing group *m*ethod.

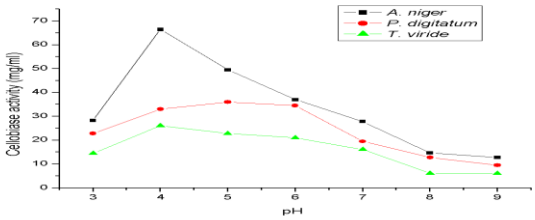


Fig. (4). Effect of different pH levels on the carboxymethylcellulase activity produced by the three fungi using the viscometric method.

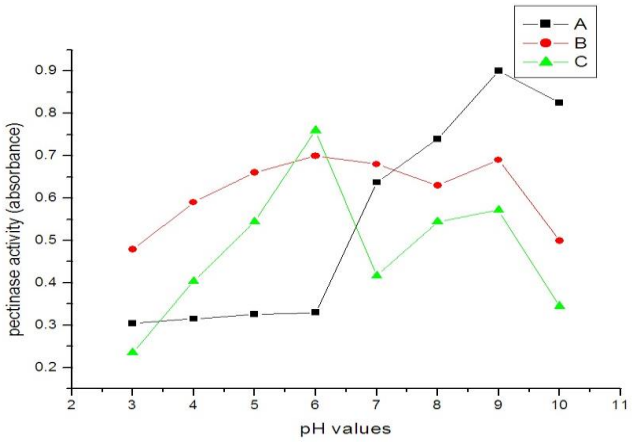


Fig. (5). Effect of different pH levels on cellobiase activity (mg/ml) produced by the three fungi (using the reducing group method)

DICUSSION

It was found that the addition of Ca^{++}, Ba^{++}, K^{+} and Mn^{++} to the enzyme assay caused a higher increase in the activity of the cellulase enzymes (carboxymethylcellulase and cellobiase) of the three fungi. On the other hand, the addition of Ag^{+}, Cd^{++},Cu^{++} and Fe^{++} caused a decrease in the

enzyme activity. These results are in agreement with Dong *et al*. (2001), who reported that Mn^{++}, Ba^{++} and Ca^{++} caused an increase in the total reducing sugars, while, a significant decrease in total reducing sugars was observed with Hg^{+}, Cu^{++} and Pb^{++}. Also Ali (2006) reported that cellulase enzymes activities increased in the presence of Na^{+}, K^{+}, Ca^{++}, Ba^{++} and Mn^{++} and decreased in the presence of Fe^{++}, Pb^{++}, Ag+, Zn^{++} and Co^{++} salts. While, Lin *et al.* (2010) reported that the purified cellulase of *Bacillus subtilis* was activated by Mn^{++} and Co^{++}, but inhibited by Hg^{+}, Cd^{++}, Fe^{++} and Fe^{+++}. Bakarr *et al*. (2005) also reported that the activities of the purified cellulases of *Pseudomonas fluorescens* were stimulated by low concentrations (10-30 mM) of Na^{+} and Mg^{++}, while EDTA was found to inhibit the cellulase enzyme activity at all of its concentrations. Ponnuswamy and Prakash (2012) also reported that the cellulase of *Bacillus* sp. was highly active in the presence of Mn^{++}, and strongly inhibited by Hg^{++}.

The optimum temperature of the carboxymethylcellulase activity produced by *A. niger* was found at 50 ^{0}C. This value is higher than that obtained by Gokhan *et al*. (2001) who found it around 40 ^{0}C. However, the optimum temperature to the same organism was reported by Christina and Sunil (2012) to be 45 ^{0}C. On the other hand, the results showed that the optimum activity of the cellulases produced by both fungi *T. viride* and *P. digitatum* was found to be between 40–50 ^{0}C, although the carboxymethylcellulase from *A. niger* isolate No. RD-2231 was found to be stable at temperatures below 40 ^{0}C, however, its activity at 65 ^{0}C it was 28 times higher than its value at 30 ^{0}C (Demerdash and Attia, 1992). Cellulase enzymes from different microbes showed highest activities at different temperatures. The optimum temperature for cellulase of *Bacillus* sp., was

shown at 50 ^{0}C (Kim *et al*., 2009), while the optimum temperature of cellulase produced by *P. chrysogenum* was obtained at 48 ^{0C} (Nwodo *et al*., 2005). However, the cellulase enzymes of termites have an optimum temperature in the range of 45 – 50 ^{0}C (Purwadaria *et al*., 2003).

Concerning to the pH effect, the maximum activity of the cellulases produced by *A. niger* was detected at pH 4. This is in agreement with Akiba *et al*. (1995) who reported that the highest activity for the cellulases of *A. niger* occurred at pH 4-4.5. In contrast Azzaz *et al*. (2012) found that the highest value of the cellulases production by *A. niger* was detected at pH 6. For *T. viride* the maximum activity of cellulase was found at pH 6. while, the higher activity of cellulase produced by *P. digitatum* was detected at two peaks pH 6 and 7. Baig *et al*. (2004) have reported pH 6 as an optimum
value for the maximum cellulase activity produced by *T. lignorum* using banana waste. Ahmed *et al*. (2009) observed the optimum pH for cellulase of *T. harizianum* at 5.5.

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العوامل التي تؤثر على نشاط انزيمات السليولوز المنتجة بواسطة ثلاثة فطريات

الملخص

ثلاثة من الفطريات تم استخدامها في هذه الدراسة لانتاج انزيمات السليولوز (انزيم الكاربوكسي مثيل السليولوز وانزيم السلوبايوز وشملت تلك الفطريات ما يلي(*Trichoderma viride*, *Asperigillus niger* and *Penicillum digitatum*) وتم استخدام طريقتي المجموعات المختزلة واللزوجة لقياس نشاط تلك الانزيمات. وقد اثبتت الدراسة أن اضافة أيونات الكاسيوم والبيريوم والبوتاسيوم والمنجنيز قد ادى الي زيادة كبيرة في نشاط انزيم الكاربوكسي مثيل السليولوز المنتج من الثلاثة فطريات، وفي حين اعطت ايونات الفضة والزيئبق والصوديوم والمغنسيوم والزنك زيادة أقل. ومع ذلك فقد ادت اضافة أيونات الكادميوم والنحاس والحديد الى انخفاض نشاط تلك الانزيمات. وبخصوص نشاط انزيم السلوبايوز فقد ارتفع كثيراً عند اضافة ايونات الكالسيوم والبوتاسيوم بينما ارتفع قليلاً مع اضافة ايونات المغنسيوم والزنك والبيريوم في حين انخفض نشاطه في وجود ايونات الزيئبق و الكادميوم. دراسة تأثير تركيزات مختلفة من ايونات المعادن الفاعلة اوضحت أن انزيم الكاربوكسي مثيل السليولوز المنتج من الفطر *A. niger* قد زاد نشاطه في حدود 200 – 900 جزء في المليون من ايونات البوتاسيوم وفي – 900 500من ايونات المنجنيز في حين أنه كان في حدود 700 – 900 بالنسبة لايونات الكالسيوم والبيريوم. هذا ولم تكن كنالك اي فروقات بين التركيزات المختلفة لايونات المعادن المذكورة ونشاط انزيم الكاربوكسي مثيل السليولوز المنتج من الفطريات *T. viride* و *P. digitatum*. واوضحت النتائج كذلك أن ازنيم الكاربوكسي مثيل السليولوز المنتج من الثلاثة فطريات كانت له درجة حرارة مثلى واحدة $40\ ^0C$. اما بخصوص درجات الأس الهيدروجيني (pH) فقد كانت الدرجة المثلى لانزيم الكاربوكسي مثيل السليولوز المنتج من الفطر *A. niger* هي pH 4.0 وكانت لتلك المنتجة من الفطر *T. viride* pH 6.0 وفي حين كانت كنلك درجتان للانزيم المنتج بواسطة الفطر *P. digitatum*. ومن الناحية اخري كانت درجة الأس الهيدروجيني المثلى للانزيم سلوبايوز المنتج من الثلاثة فطريات درجة واحدة pH 4.0.