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Purification and some properties of fungal Xylanase

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Abstract

Partial purification of xylanase produced by *Aspergillus niger* strain *AUMC* 14230 is carried out with 80% saturation of ammonium sulfate under pH5.0 as first step. The second step in partial purification is Acetone 2.5/1 (v/v). The last step is gill filtrations which give the highest specific from both enzymes. Many factors affecting on purified enzymes. The best activity of xylanase was produced till 72 hrs and decreased by (8.8% & 10.9%), respectively after 72 hrs. Xylan (1mg) concentration was the best substrate to produce relative activity 100% for xylanase enzyme. We found that the glucose has an inhibitory effect on both enzymes activity. Optimum temperature for xylanase is 50^{o}C, increased temperature decreased productivity of both enzymes. The addition of Ca^{2+}, $Ba^{2+,}$ Mg^{2+} and EDTA strongly enhanced both xylanase activity, While, Co^{2+} and Ag^{+} decreased the productivity. On the other hand, the Fe^{2+}, Cu^{2+} and Mn^{2+} showed moderate inhibition.

KEYWORDS: Fungal Xylanase, agricultural and industrial wastes, *Aspergillus niger* strain

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Introduction

Xylan, the main component of hemicellulose consists of a β-1, 4-linked d-xylosyl residues backbone branched with other pentoses, hexoses and uronic acids. Xylanases and associated de branching enzymes produced by a variety of microorganisms, including bacteria, yeasts and filamentous fungi, bring about the hydrolysis of hemicelluloses **(Gilbert and Hazlewood, 1993, Maheshwari *et al.*, 2000 and Katapodis *et al.*, 2000 and 2002).** They also reported that Xylanase are also believed to be essential in improving the nutritive quality of animal feed. The abundance of xylan clearly indicates that xylanolytic enzymes can play an important role in bioconversion. Furthermore, it may also be possible to use xylanases in bio pulping processes for the preparation of cellulose pulps**.** Xylan is the major hemicellulose in hardwood from angiosperms, but is less abundant in softwood from gymnosperms; it accounts for approximately 15%–30% and 7%–12% of the total dry weight, respectively **(Whistler and Richards 1970; and Beg *et al.*, 2001).** Applications of xylanases can be found in the food, feed and pulp-paper industry. Filamentous fungi are particularly interesting producers of this enzyme from an industrial point of view, due to the fact that they excrete xylanases into the medium. Furthermore, xylanase levels from fungal cultures are generally much higher than those from yeasts or bacteria. In addition to xylanase, fungi typically produce several accessory xylanolytic enzymes, which are necessary for debranching substituted xylans **(Haltrich etal.,1996).**

The present study reports the purification of extracellular xylanase from *Aspergillus niger* strain *AUMC 14230*. Earlier we have reported the factors affecting on purified enzyme.

Materials and Methods

Fungi used

Strains isolated from corn cob collected from Egypt corn field in 2018 and also isolated from water, soil and air. This cultures were maintained on potato dextrose agar slants and held at 4°C and were renewed monthly.

Culture condition

Aspergillus niger was cultured on modified medium (Corn steep liquor (3%), Corn cob (1%) and Wheat bran (1%). The pH of the medium was adjusted to 4.5 befor autoclaving. Inoculum size 0.50% (v/ml), initial pH 4.5, aeration 1:5 (Vm:Vf), incubation temperature 50^{o}C, agitation rate 175 rpm and time course 72 hr and the supernatant assayed for enzymatic activities.

Enzyme assay

Xylanase activity was assayed by measuring the reducing sugars released from birchwood xylan. The reaction mixture containing 0.5 ml enzyme solution and 0.5ml of xylan solution 1% (w/v), in 0.05M acetate buffer (pH 5.0) was incubated at 50°C for 30 min. The reducing sugars released were determined as xylose by the method of **Somogyi (1952)**. One unit (U) of xylanase activity was defined as the amount of enzyme that produced 1m mole of xylose per min under assay conditions.

Enzyme purification

The xylanase was purified using three different methods:

1. By using ammonium sulfate

To the enzyme culture filtrate (12.5ml) at different pH values, solid ammonium sulfate was added up to 80% saturation (S). The mixture was

left over night at 4ºC and the precipitate was collected by centrifugation at 15,000 rpm for 30 min in a cooling centrifuge. The experiment was repeated using ammonium sulfate at different saturations being 10%, 20%, 30%, 40%, 50%, 60%, 70%,& 80%, (S).The precipitate was dissolved in 10ml of acetate buffer pH 4.5 then the enzyme activity and protein concentration were determined.

2.By organic solvents

This experiment was conducted to find out the most suitable solvent for the highest recovery of enzyme. Acetone, ethanol and isopropanol 1/1, 1.5/1,2/1, 2.5/1, 3/1,3.5/1 and4/1 V/V were used for the precipitation of enzyme culture filtrate. Solvents, precooled to 20oC were added slowly **(Mill and Tuttobello, 1961).**

3.By gel filtration

The dialyzed sample was separated on non-denaturing electrophoresis slab gel (12% polyacrylamide) containing 10% sucrose. After electrophoresis the slab gel was laid on the agar sheet containing 0.5% (w/v) xylan and 20% (w/v) NaCl in above mentioned buffer as replica plate and left for 2 h at 50 ◦C, the agar overlay was removed and stained with 0.2% (w/v) aqueous. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a 12% polyacrylamide gel. After electrophoresis, the protein gel was stained with Coomassie Brilliant Blue R-250 **(Prakash et al., 2012).**

Determination of soluble protein

Protein in these experiments was determined by the method of **Lowry *et al*. (1951)** using bovine serum albumin as standard.

Effect of pH on the rate of catalyzed reaction

1.Optimum pH

Therefor the effect of substrate was estimated by using suffiently high concentration of substrate the enzyme at all pH used. The effect of pH on the activity of the partial purified xylanase of *Aspergillus niger* strain *AUMC 14230* was studied in various buffers over the pH ranges of (3.0-8.0). The buffer used was citrate buffer (3.0-6.0), and phosphate buffer (7.0-8.0**).**

2. pH stability

The pH value was adjusted to various levels between 3.0 to 8.0 using citrate phosphate buffer (3.0-7.0) and phosphate buffer (7.0-8.0) and adjustment by pH meter. All solution in the flasks adjusted to the desired pH were left at 4ºC for one or five days. The pH stability was investigated by measuring the residual activity on xylanase at 50ºC pH 5.0 for 10 min after 24h or 120h incubation at the same range of pH as before.

Effect of substrate concentration

To study the effect of substrate concentrations on the velocity of the partial purified xylanase from *Aspergillus niger* strain *AUMC 14230* the following experiment was run. The concentration of xylan varied from 0.03 to 0.45mM. The mixture was incubated for 10min at 50ºC and pH 5.0, while the concentration of cellobiose varied from 0.1 to 1.0mM.

Substrate specificity

One ml of enzyme solution was incubated with 1ml of each substrate in citrate-phosohate buffer (0.05M, pH 5.0) at the optimum temperature of each enzyme. The sugars obtained from carboxy methyl cellulose (CMC), xylan, filter paper, cellulose, Salicin, treated and untreated rice straw were determined (as glucose) by the method of **Smogyi, (1952)**. The glucose released from lactose, maltose and xylose determined by glucose-oxidase reagent.

Glucose inhibition

The inhibition of glucose on enzyme activity was measured by adding various concentrations of glucose (10 to 100 mM) and xylan concentration with (0.1, 0.2, 0.3 to 1.0 mM). Inhibition study by glucose was performed with xylan as the substrates.

Effect of temperature

1. Optimum temperature

In each experiment 0.5ml of xylanase were added to 0.5ml of sodium acetate and the reaction continued for 30 minutes. The experiment was then conducted at different temperature i.e. 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70 and 75^{o}C. The reaction velocity was determined and the optimum temperature was calculated by plotting enzyme activates against temperatures.

2. Thermal stability

An experiment was designed to study the effect of different temperatures and times stability of xylanase. The enzyme incubated at nine various temperatures ranged from 30^{o}C to 70^{o}C with 5^{o}C intervals. Samples were taken for potency determination at various times being 10, 20, 30, 40, 50, and 60 min, the residual activities in each case was determined and calculated as relative activity.

Effect of metal ion and some substances on cellobiase activity

The effect of metal ions and some substances on reaction velocity catalyzed by cellobiase was determined using Na^{+}, K^{+}, Co^{+2}, Ca^{+2}, Mg^{+2}, Mn^{+2}, Cu^{+2}, Zn^{+2},Ag^{+} and EDTA. All factors were kept constant then the xylanase activity was determined as affected by addition of metal ions and some substances.

Results and discussion

1. Purification studies

1.1. Effect of ammonium sulfate concentration

The results presented in Table (1) revealed that the xylanase activity by *Aspergillus niger* was started with low values when the low ammonium sulfate concentration were used (10, 20, and 30%). Then it increased as the ammonium sulfate increased to reach its maximum values (2000Uml^{-1}) at the high concentrations of ammonium sulfate (80, 90 and 100%) which gave the same xylanase activity. The same trend was found for protein where the high concentration of ammonium sulfate (80, 90 and 100%) gave the highest protein found in precipitation (14 $mgml^{-1}$). The most employed salt is ammonium sulfate, on account of its large solubility in water and absence of harmful effects on most enzymes **(Englard and Seifter, 1990)**. Isolation of xylanase from *Aspergillus niger* was carried out by different types of chromatography **(Prakash *et al.*, 2012)**. It has in fact a stabilizing action on many enzymes and it is usually not necessary to carry out the fractionations at a low temperature. Therefore, the experiments were carried out to study the effect of some factors on enzyme precipitation using ammonium sulfate. The filtrates were filtered through paper and concentrated using evaporator under vacuum equipped **(Chapla *et al.*, 2012)**. In this connection **Walia *et al.*, (2017)** reported that crude xylanase preparation was subjected to ammonium sulfate fractional precipitation and caused substantial concentration of proteins. The activity could be recovered from the ammonium sulfate fractional range of 30–80% with maximum at 60–80%. There were sufficient reports regarding the inclusion of ammonium sulfate fractionation in the purification procedures. The specific activity of the concentrated preparation was 10.75

U/mg proteins with a purification fold of 1.21 and the yield as 71.43%.

2.Effect of organic solvents

The obtained results in Table (2 and 3) indicate that using the organic solvents in the precipitation of xylanase considered a very important step in partial purification of both enzymes. Using acetone in the concentration of 2 /1 ml (crude enzyme) gave the highest values of enzyme activity, S. activity, degree of purification and E.recovery. Whereas, using ethanol at a concentration of 2/1 gave the maximum enzymes activity, S. activity, degree of purification and E. recovery. On the other hand, isopropanol 2/1 was highest in enzyme activity, protein concentration, S. activity, E. recovery and degree of purification. In our study we preferred to use acetone because of its strong hydration effect.

Table (1) Effect of ammonium sulfate concentration on xylanase enzyme precipitation from *Aspergillus niger* strain *AUMC 14230.*

Ammonium sulfate S % saturation		Protein mg mL^{-1}	Activity UmL^{-1}	Specific activity Umg	Degree purification fold	Enzyme recovery %
Crude enzyme		**34**	**2050**	**60**	**0.0**	**100**
10	**0.13**	5	25	5	0.08	1
20	**0.26**	7	265	37	0.61	12
30	**0.39**	8	450	56	0.93	21
40	**0.52**	9	705	78	1.31	34
50	**0.65**	10	986	98	1.62	48
60	**0.78**	11	1232	112	1.80	60
70	**0.91**	12	1575	131	2.18	77
80	**1.04**	14	2000	143	2.38	97
90	**1.16**	14	2000	143	2.38	97
100	**1.28**	14	2000	143	2.38	97

Table (2) Effect of organic solvents on xylanase precipitation from *Aspergillus niger.*

precipitation v/v	Activity UmL-1	protein Concentration mg%	S.activity (Umg-1)	Degree of purification	E.recovery %
Control	**3050**	**51**	**60**	**1**	**100**
1-Asetone					
1/1	**1674**	**17**	**98**	**1.60**	**55**
1.5/1	**2007**	**19**	**105**	**1.75**	**66**
2/1*	**2442**	**20**	**122**	**2.00**	**80**
2.5/1	**2025**	**19**	**106**	**1.76**	**66**
3/1	**1905**	**18**	**105**	**175**	**62**
3.5/1	**1720**	**17**	**101**	**1.68**	**56**
4/1	**1503**	**15**	**100**	**1.66**	**49**
2-Ethanol					
1/1	**1470**	**17**	**86**	**1.4**	**48**
1.5/1	**1960**	**18**	**108**	**1.8**	**64**
2/1	**2266**	**19**	**119**	**1.9**	**74**
2.5/1*	**2620**	**21**	**125**	**2.0**	**85**
3/1	**2150**	**20**	**107**	**1.7**	**70**
3.5/1	**1860**	**19**	**98**	**1.6**	**60**
4/1	**1360**	**18**	**75**	**1.3**	**44**
3-Isopropanol					
1/1	**1480**	**17**	**87**	**1.4**	**49**
1.5/1	**1806**	**19**	**95**	**1.6**	**59**
2/1*	**2107**	**20**	**109**	**1.8**	**69**
2.5/1	**1966**	**19**	**103**	**1.7**	**64**
3/1	**1745**	**18**	**97**	**1.6**	**57**
3.5/1	**1500**	**16**	**94**	**1.5**	**49**
4/1	**1160**	**15**	**77**	**1.3**	**38**

Table (3) Summary of partial purification of xylanase secreted by *A.niger.*

Purification steps	Total activity Uml -1	Total protein mg ml -1	Specific activity Umg protein	Purification factor (fold)	Recovery %
Crude extract	**265000**	**6755**	**39**	**1**	**100**
Ammonium sulfate	**152000**	**330**	**460**	**12**	**57**
Acetone	**110000**	**200**	**550**	**14**	**46**

3. Electrophoresis of the SDS-PAGE

It is clearly shown in Figure (1) that after migrated xylanase enzyme on SDS-PAGE there is 3 bands at 130, 52, and 6.5 Kda.

Factors affecting partial purified enzyme reaction

Optimum pH and pH stability

Results in Figure (2 and 3) revealed that both enzyme activities and relative activity increased by increasing pH till it reached the highest level between 4.5 and 5.5 with 100% relative activity at 5.0. The results are in agreement with those obtained by **Kaushik *et al.*, (2014)** who found that the enzyme was more stable at acidic pH ranges. Good stabilities were seen at the pH range of 4-7 even after 3 h of incubation. Enzyme was most stable at pH 5, retaining more than 98% activity even after 3 h of incubation. Even at pH 8 and 9, more than 85% and 75% enzyme stability, respectively, was observed after 3 h of incubation. Also, **Das and Ray (2016)** reported that the pH stability of the enzyme was studied at the pH range of 3.6 to 8.0 using 0.1 (M) acetate buffer for pH 3.6 to 5.0 and 0.1 (M) phosphate buffer for pH 5.4 to 8.0. It was done by incubating the enzyme at this pH for 1h at 30^{o}C and then determining the residual xylanase activity under standard assay conditions. These results are similar to those obtained by **(Chipeta *et al.*, 2008, Bakri *et al.*, 2011, Walia *et al.*, 2015 and Bedade *et al.*, 2017**).

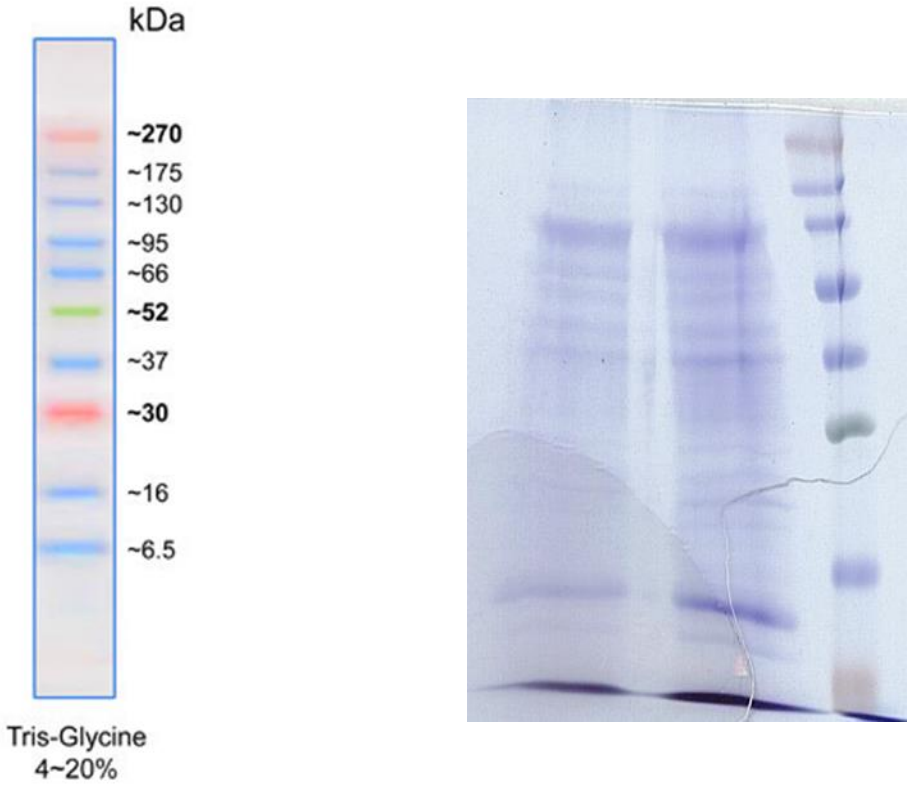

Figure (1)
GeneDireX **BLUltra** Prestained Protein Ladder (500ul) /PM001-0500

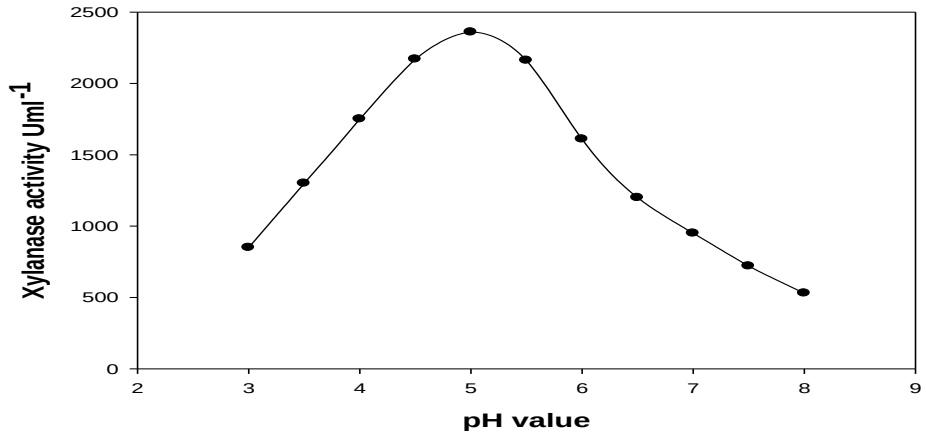

Figure (2) Effect of pH on the activity of partial purified xylanase from *A.niger*

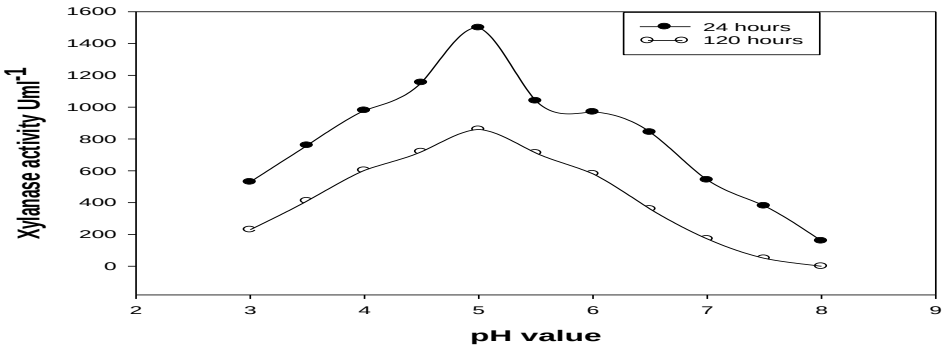

Figure (3) Effect of pH stability during storage of partial purified xylanase from *A.niger.*

Effect of substrate specificity

Among the substrate tested as shown in Figure (4) the xylan was the best substrate for the xylanase activity produced by *A. niger* followed by sucrose substrate. On the other hand, **Ping *et al.*, (2017)** revealed that *Tricoderma aurantiacus M-2* xylanase had high activity on xylan from beech wood and no activity on MCC, soluble starch, CMC-Na, 1,3 glucan and 1,6 glucan**.** These results were in harmony with those obtained by (**Seyis and Aksoz, 2003, Singh *et al.*, 2003, Simair *et al.*, 2010, and , Das and Ray, 2016 and Bedade *et al.*, 2017**).

Effect of substrate concentration

The results recorded in Figure (5) recorded that for xylanase activity a concentration of 1.0 from xylan was the concentration for producing the higher activity of β-xylanase from the *Aspergilus* strain. The results may be attributed to the saturation of the enzyme obtained from substrate used and the enzymes showed affinity to the xylan. Also, purified xylanase had a narrow substrate specifity and could hydrolyze only xylan. Our results are in the same trend with those reported by **(Bailey *et al.*, 1992, Khanna and Gauri 1993, Puchart *et al.*, 1999, Adsul *et al.*, 2004, Okafor *et al.*, 2007, Juturu and Wu, 2012, Prakash *et al.*, 2012, Kaushik *et al.*, 2014, Thomas *et al.*, 2015 and Ping *et al.*, 2017).**

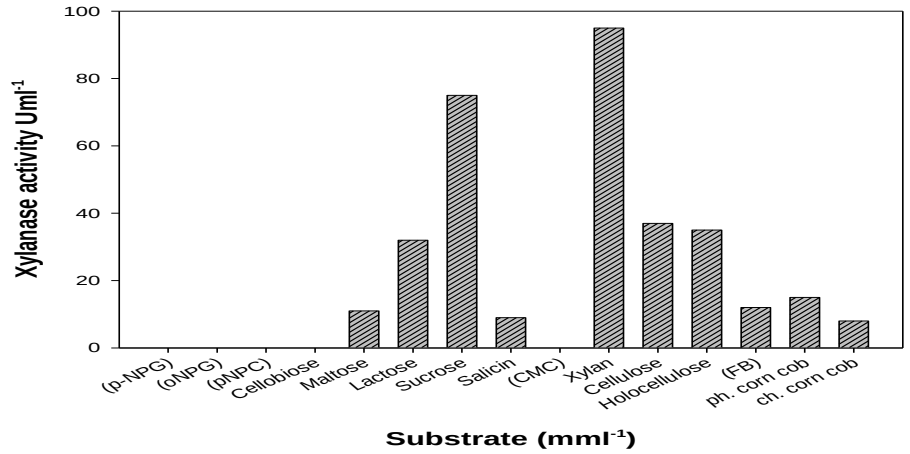

Figure (4) Substrate specificity of partial purified extracellular xylanase from *A.niger*

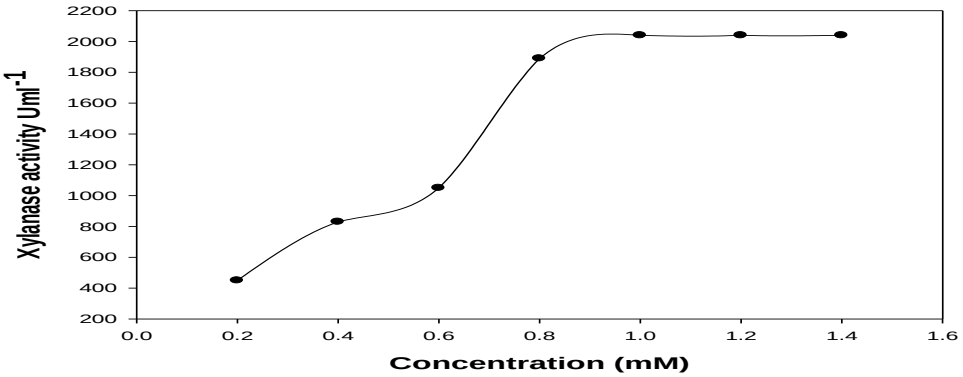

Figure (5) Effect of substrate concentration on partial purified xylanase activity of *A.niger*.

Thermodynamic of enzyme catalyze reaction

1. Optimum temperature

Results presents in Figure (6) revealed that the optimal temperature for thermodynamic of *A. niger* enzyme catalyzer reaction in this study was found to be 50^{o}C then decreased at 70^{o}C to be inactivated at 75 and 80^{o}C. These results may be attributed to that after 50^{o}C the enzymes are subjected to denaturation. Similar results were also obtained by **(Prakash *et al.*, 2012, Sarkar *et al.*, 2013, Pirota *et al.*, 2013 and Kaushik *et al.*, 2014). 2.Thermal stability of xylanase production from *A. niger.***

Results recorded in Figure (7) showed that the thermal stability of xylanase enzyme from *A.niger* was 50^{o}C with relative activity 100% after 10min, whereas, increasing the time to 20, 30, 40, 50 and 60min gradually decreased the enzyme activity to 71% for *A. niger* at 50^{o}C. Similar results were obtained by (**Prakash *et al.*, 2012, Sarkar *et al.*, 2013, Pirota *et al.*, 2013 ,Boonrung *etal.*, 2014, Kaushik *et al.*, 2014, Das and Ray 2016, Abdul Wahab *et al.*, 2016**, **Ahmed *et al.*, 2016, Boonchuay *et al.*, 2016**, **Bedade *et al.*, 2017, Ping *et al.*, 2017, and Mehnati-Najafabadi *et al.*, 2018).**

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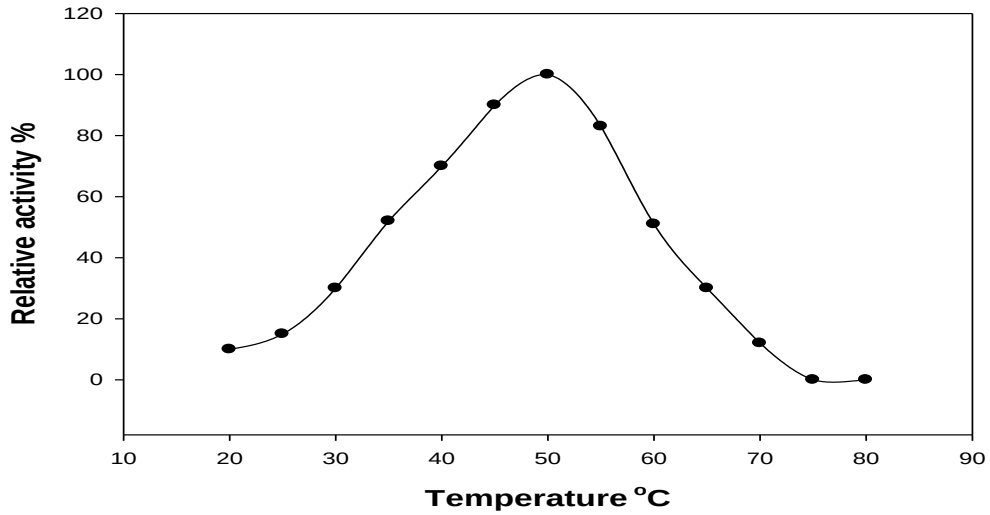

Figure (6) Effect of temperature on the activity of partial purified xylanase from *Aspergillus niger*.

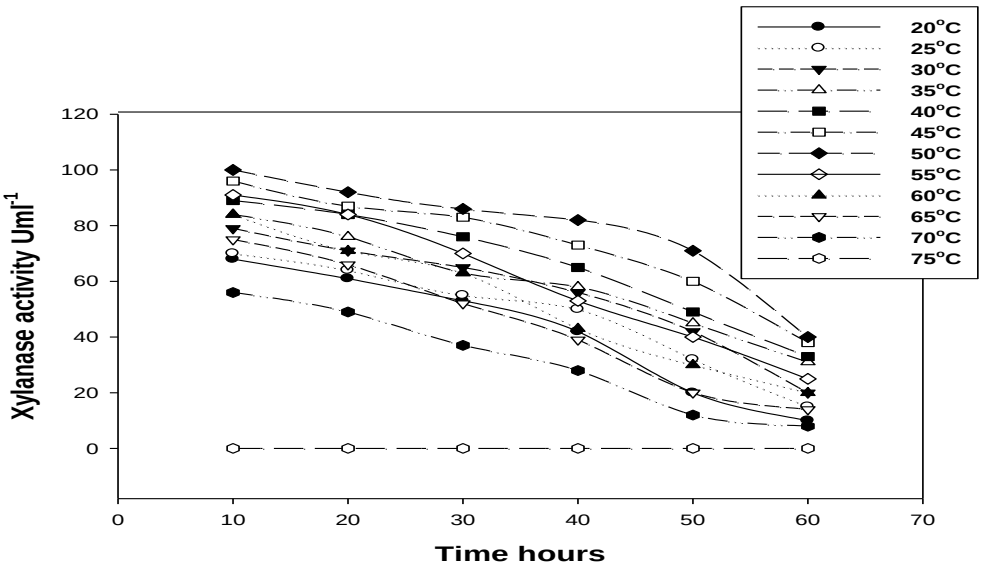

Figure (7) Thermal stability of partial purified xylanase from *A. niger.*

Effect of metal ions and chemical reagents on xylanase activity from A. niger.

Xylanase was tested in the presence of different metal ions and chemical reagents as show in Table (4). In our study $CaCl_2$, NaCl, $MgSo_4$ and EDTA strongly xylanase activity. But, was inhibited by $CoCl_2$. KCl, $FeSo_4$ and $CuSo_4$ and hydroxyl quinolone. Addition of K^+ and Mn^{2+} also caused inhibitory effect on xylanase activity. xylanase was activated by the presence of $CaCl_2$, NaCl, $MgSo_4$, NaCl and EDTA. Also, in is connection **Das and Ray (2016)** concluded that K^+ and Cu^{2+} have stimulatory effect on enzyme substrate reaction whereas Mn^{2+}, Pb^{2+} and Ni^{2+}were found to inhibit enzyme activity. Heavy metals like Hg^{2+} and Ag^+ have complete inhibitory effect on enzyme substrate reaction even at 1.0 mM concentration. Hg^{2+} may interact with the sulphite residues present on the enzyme, thus inhibiting the enzyme activity. The other metal ions have variable inhibitory effect on the activity at higher concentration. This could be due to metal catalyzed oxidation reduction of the active sites of enzyme.

Some metal ions and reagents significantly affect xylanase activities. A common trend has been recognized in many cases for enzyme activity negatively affected by heavy metals like Hg^{+2}, Fe^{+2}, Co^{+2}, Mn^{+2}, Ag^{+2}, Cu^{+2}, and Pb^{+2} and reagents like urea and EDTA as inhibitors, whereas Ca^{+2} and Mg^{+2} have been reported to be enzyme activators **(Yinan *et al.*, 2008 and Zhou *et al.*, 2009).** Heavy metals like Ag^{+2}, Hg^{+2} and reagents like EDTA were shown to inhibit the enzyme activity by 80–90%. Bivalent alkaline elements Ca^{+2} and Mg^{+2} enhanced enzyme activity by 10–15%. Surprisingly, EDTA enhanced the activity of endoxylanase from *A. usamii*. It is predicted that Ca^{+2} and Mg^{+2} ions help stabilize the enzyme–substrate complex thereby elevating enzyme activity. On the contrary, EDTA is a chelating agent which removes ions from the enzymes thereby inhibiting their activity **(Juturu and Wu 2014)**.

Conclusion

Partial purification of xylanase enzyme is very important to prepare enzyme to use in applied.

Table (4) Effect of metal ions and chemical reagents on xylanase activity from *A.niger*.

Chemical reagents	Final molarity (m M)	Relative activity %	
None control	---------	100	
Ca^{2+}	**1**	111	+11
	5	104	+4
Co^{2+}	**1**	20	-80
	5	60	-40
Zn^{2+}	**1**	0.0	-100
	5	0.0	-100
Na^{+}	**1**	110	+10
	5	101	+1
K^{+}	**1**	90	-10
	5	85	-15
Fe^{2+}	**1**	50	- 50
	5	33	- 67
Cu^{2+}	**1**	5	-95
	5	17	-24
Ag No_3	**1**	140	+40
	5	103	+3
Mn^{2+}	**1**	13	-87
	5	15	-85
Mg^{2+}	**1**	140	+40
	5	135	+35
NaN_3	**1**	116	+16
	5	101	+1
$LiCo_3$	**1**	102	+2
	5	97	-3
EDAT* **	**1**	130	+30
	5	120	+20
8-hydroxy quinoline	**1**	90	-10
	5	85	-15

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الملخص العربي

تنقية انزيم الزيلانيز الفطري وبعض خصائصه

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في هذه الدراسه تم انتاج انزيم الزيلانيز من سلالة الاسبرجيلس نيجر ، باستخدام مطحون اكواز الذرة اساسي للبيئه. اوضحت الدراسه انه تم استخدام سلالة الاسبرجيلس نيجر في انتاج كميه كبيره من انزيم الزيلانيز باستخدام بعض المخلفات الزراعيه والصناعيه. وتم اجراء التنقية الجزيئية المثلى للانزيم وجدت باستخدام تركيز ٨٠% من كبريتات الأمونيوم لأفضل ترسيب.تحت (0.5) pH و الخطوه الاولى في التنقيه الجزئيه هي الأسيتون بنسبة ٢,٥ : ١ حجم : حجم هي الخطوة التالية لكبريتات الأمونيوم. الخطوة الأخيرة في التنقية هوالالكترود والتمرير على الجيل .كثير من العوامل أثرت في الأنزيم ، منها مدة التحضين ، حيث وجد أن كمية الأنزيم تزيد حتى ٧٢ ساعة ، وبعد ذلك تقل بدرجة ملحوظة.التركيز الأفضل للزيلان هو ١ ملي جرام .وجد أن للجلوكوز أثر تثبيطي على إنتاج الانزيم. درجة الحرارة المثلى للانزيم هي ٥٠ درجة مئوية. وجد أن من العوامل المحفزة لانتاج الانزيم الكالسيوم ، الباريوم ، الماغنسيوم ، والايدتا ، أما المثبطة فكانت الكوبالت ، والفضة ، وكان تأثير كلا من الحديدوز ، والنحاس ، والمنجنيز معتدل.