HIGH LEVEL PRODUCTION OF THERMOSTABLE β-XYLANASE, CMC-ASE AND β-GLUCOSIDASE BY AN *ASPERGILLUS FUMIGATUS (FRESENIUS) ALBINO* MUTANT STRAIN

Abstract

A local isolate of *Aspergillus fumigatus (Fresenius) albino* mutant obtained from rotten wood shavings, grew at 45°C on a solid medium containing cellulose as carbon source. This fungus can utilize a variety of lignocellulosic substrates, such as Kraft paper pulp, straw, microcrystalline cellulose and filter paper. The highest endoglucanase (CMC-ase), β-xylanase and β-glucosidase activities were obtained in shaken flasks at 45°C, with mainly oat straw as sole carbon source.

CMC-ase and β-xylanase activities showed the highest values (3.50 U/ml and 33 U/ml) at pH 4.0 and 5.0 respectively, and 70°C.

β-glucosidase activity was maximal at 65°C (43 U/ml), CMC-ase and β-xylanase enzymes were active in a large pH spectrum (3.0 to 8.0) and showed high thermostability at 60°C and above, especially for CMC-ase.

Keywords: *lignocellulosic materials, Carboxymethylcellulose, xylan, straw, cellulases, β-xylanases, thermophilic fungus, growth rate, submerged culture.*

Résumé

Une souche de moisissure thermophile Aspergillus *fumigatus (Fresenius)* ssp *albino*, a été isolée localement à +45°C sur milieu gélosé contenant de la cellulose microcristalline comme seule source de carbone.

Des cultures agitées en fioles d'Erlen ont été pratiquées pour la mise en évidence de la production d'enzymes lignocellulolytiques (CMC-ase, β-glucosidase et β-xylanase) avec différents substrats carbonés. Les enzymes produites dans le milieu extracellulaire sont thermostables et capables d'opérer dans une large gamme de pH.

Les activités β-glucosidase sont particulièrement importantes et ne semblent pas être affectées par les phénomènes de retroinhibition.

Mots clés: *Sous-produits lignocellulosiques, Carboxymethylcellulose (CMC), D-xylane, paille, pâte à papier, enzymes lignocellulolytiques, moisissure thermophile, culture submergée.*

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ملخص

تم إعزال *A. fumigatus* ssp *albino* في 1992 من خشب غابي في طريق الفساد. هذا الفطور الدقيق ينمو تحت 45°م في علب" البيتري" التي تحتوي على مادة السيليوز كمنبع وحيد لمادة الكاربون. هذا الفطور يتغذى من مواد السيليلوز المختلفة، كسيليلوز الإصطناعي (CMC) القزيلان (Xylan) تبن المرحي، ورق الكرافت،... ويحولها بفضل الإنزمات (Enzymes) المنتجة عنه، إلى مواد بسيطة كسكر القليكوز (D-Glucose) و القزيلوز (D-Xylose) و هذا في زجاجات الأرلينمير (Fioles d'Erlen) المحركة تحت 45°م .

كلمات الفتح: *مكونات الخشب، كربوكسيمتيل سيليلوز (CMC)، القزيلان، تبن مرحي، ورق الكرافت، نزيمات، فطور*

Various micro-organisms have been tested for their ability to degrade lignocellulosic materials to produce fermentable sugars (glucose, xylose,...) for biochemical processes [1-4] to treat fodder grain [5, 6] and for Single Cell Protein Production [7- 9].

Among these micro-organisms, fungi appear to excrete large amounts of cellulolytic and xylanolytic enzymes in active form into the culture medium. These enzymes have been the most extensively studied. *Trichoderma reesei* is frequently reported as the best known source of extracellular cellulases capable of hydrolysing crystalline cellulose [10-14]. Some other performant strains were also recognized for their potentialities to convert lignocellulosic wastes, especially thermophilic fungi such as *Thermoascus aurantiacus* [15], *Sporotrichum thermophile* [16], *Thermomyces lanuginosus* [17] and *Aspergillus fumigatus* [18, 19].

Some enzymes produced by these organisms have inherently superior temperature stability and higher saccharification capacity than enzymes excreted by mesophilic strains [20, 21]. Studies have also shown that unlike *Trichoderma* strains, which are poorer β-glucosidase producers, *Aspergillus* species are among the best producers of this enzyme [22-24]. β-glucosidase is responsible for the final step in cellulose degradation, namely the hydrolysis of cellulose-derived glucoside cellobiose to glucose [25, 22].

This paper reports the study of the production of CMC-ase, β-glucosidase and β-xylanase by a local isolate of a thermophilic fungus identified as *Aspergillus fumigatus (Fresenius) albino* mutant.

The conditions of enzymes production have been studied in submerged culture with ball milled oat straw as carbon source.

MATERIALS AND METHODS

Micro-organism

The strain used was isolated in 1992 from rotten wood shavings in Kabylia region (Tizi-Ouzou , Algeria) during the Summer season and was identified as *Aspergillus fumigatus (Fresenius) albino* mutant by Professor G.L. Hennebert (Laboratory of Systematic and Applied Mycology - Université Catholique de Louvin - Belgium).

Cultivation of the strain was carried out at + 45°C and storage on Potato Dextrose Agar (PDA) slants at +4°C.

Inoculum

A suspension of spores and mycelium was prepared from a strain on Malt Extract Agar (MEA) medium at 45°C, and was transferred aseptically into 200 ml of modified Mandels and Weber (MW) medium [14].

Culture conditions

Culture medium was prepared as following:

* 4 g of ball milled oat straw (sieved at 0.18 mm - 0.20 mm) or other substrates were transferred into 175 ml distilled water and autoclaved 50 min at 120°C.
* 20 ml of ten fold concentrated MW mineral medium was added aseptically in 500 ml flasks. This M W medium contained 2.0 g KH_2PO_4, 1.4 g $(NH_4)_2 SO_4$, 0.3 g $CaCl_2$, 5.0 mg $FeSO_4$ $7H_2O$, 1.6 mg $MnSO_4$ H_2O, 1.4 mg Zn SO_4, $7H_2O$, 2.0 mg $CoCl_2$ and 0.5 mg yeast extract per litre of distilled water. It was sterilized by membrane filtration.
* The initial pH of the MW medium was adjusted to 5.0.
* Cultures were incubated at 45°C ± 1°C, on a rotary shaker at 150 rpm. The supernatant after cultivation was used for enzyme activity determinations.

Enzyme assays

β-xylanase and endoglucanase (CMC-ase) were measured in a reaction mixture containing 1ml of 1% oat spelt xylan (Sigma) and carboxymethycellulose (medium viscosity, Sigma) respectively, in 0.05 M acetate buffer pH 4.8 and 1 ml of appropriate diluted enzyme solution.

The mixture was incubated at 50°C. After 15 min incubation, the reducing sugars formed were determined using the dinitrosalicylic acid (DNS) method of Miller [18]. The enzyme reaction was stopped by cooling in water ice.

One unit of each enzyme was defined as the amount of the enzyme which released 1µmol of reducing sugar per min.

β-glucosidase was measured in 1.2 ml of a reaction mixture containing 1ml of 0.15 % p-nitrophenyl-β-D-glucoside (PNPG, Sigma), in 0.1 M acetate buffer pH 4.8, and 0.2 ml of appropriately diluted enzyme solution.

The mixture was incubated at 50°C. After 30 min incubation, the reaction was stopped by adding 4ml of glycine-NaOH buffer pH 10.8.

The p-nitrophenol (PNP) ion formed was measured spectrophotometrically at 430 nm [27].

One unit of β-glucosidase was defined as the amount of the enzyme, which released 1µmol of PNP per min.

Dry matter content

The culture broth was filtred through glass fiber filter Whatman GF/A. The mycelial residues were rinsed twice and oven dried at 105°C to constant weight.

Determination of protein

Crude protein content was estimated on the mycelial residues by determining the Kjeldhal nitrogen content (Buchi apparatus), using the factor 6.25 [28].

Effect of temperature and pH

The effects of pH, temperature and incubation time on the cellulolytic and xylanolytic activities were evaluated in a culture sample taken at 144 hours of cultivation.

The pH optimum was determined with different buffers within a pH range of 1.5 to 10.4 at 50°C.

The buffers used were as following: 0.2 M acetate pH 3.0, 4.5, 5.0 and 6.0; 0.1 M citrate pH 3.0, 4.0, 4.5 and 5.0; glycine-NaOH pH 8.6, 9.0 and 10.0 ; 0.1 M phosphate pH 6.0, 7.0 and 8.0; 0.2 M KCl-HCl pH 1.5 and 2.8; 0.2 M Tris-HCl pH 7.3, 8.2, 9.2 and 10.4.

The temperature optima for enzyme activities were measured within the range 30 to 90°C in acetate buffer pH 4.8. The determination of thermal stability was measured within the same temperature range for 1 hour incubation. The remaining activity was assayed at 50°C.

RESULTS AND DISCUSSIONS

Growth and production of enzymes

The strain *Aspergillus fumigatus (Fresenius) albino* mutant grew well at 45°C on solid medium. Growth rate at 25°C is 40-45 mm/ 2 weeks and superior to 65 mm/ 1 week at 45°C. This is a common trait for *Aspergillus fumigatus* species [29, 30].

In liquid MW medium the fungus grew under the form of spherical pellets of low diameter (1 - 2 mm). The strain was cultivated on different carbon sources and the evolution of three enzyme activities, β-xylanase, carboxymethylcellulase (CMC-ase) and β-glucosidase, during growth is presented in figure 1.

In general higher CMC-ase and xylanase activities were produced on straw. Kraft paper pulp or Sigma cellulose (microcrystalline cellulose) were poorer inducers of the enzymes.

β-xylanase and CMC-ase activities appeared during the first hours of culture, increased gradually and reached maximal values of 27 U/ml and 2.60 U/ml respectively at 168 hours. About 90% of these activities were reached after 96 hours of cultivation. The short time taken to reach a high enzyme titre may probably be attributed to the high rate of

		β-xylanase (U/ml)	
Hours	**Oat straw**	**Paper pulp**	**Sigma cell**
0	0	0	0
24	12	1,05	0
48	17,5	10	1,00
72	17	-	2,50
96	23	18,75	-
120	22,80	13,75	7,75
144	22,5	13	32,50
168	26,90	6,45	34
192	26,80	6,00	27,5

		CMC-ase (U/ml)	
Hours	**Oat straw**	**Paper pulp**	**Sigma cell**
0	0	0	0
24	0,25	0,10	0
48	1,75	0,20	0,25
72	2,00	-	0,30
96	2,40	0,85	-
120	2,10	0,70	1,20
144	2,25	0,45	1,50
168	2,60	0,40	1,75
192	2,20	0,40	1,45

		β-glucosidase (U/ml)	
Hours	**Oat straw**	**Paper pulp**	**Sigma cell**
0	0	0	0
24	3,00	0	0
48	9,00	0	0
72	30,00	0	0,20
96	60,00	0,50	-
120	80,00	1,80	2,00
144	86,00	1,75	2,85
168	130,5	2,85	2,35
192	140	4,00	2,00

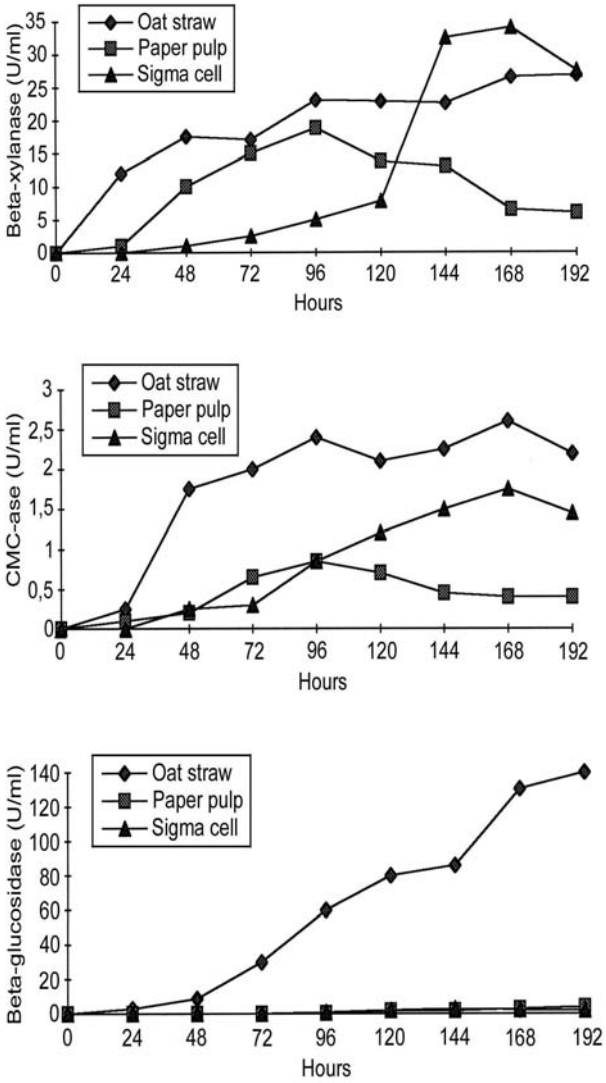

Figure 1: Effect of different lignocellulosic materials on enzyme production by the strain *Aspergillus fumigatus (Fresenius) albino* mutan. The strain was cultived on MW medium, containing 2% of oat straw, kraft paper pulp, or sigma cellulose, as carbon source.

saccharification of lignocellulose exhibited by the strain at 45°C. The highest β-xylanase activity, 34.00 U/ml after 168 hours cultivation was obtained with Sigma cell, while CMC-ase activity reached a maximum level of 1.80 U/ml.

With Kraft paper pulp as a carbon source, the maximal activities were measured after 96 hours cultivation: 19 U/ml for β-xylanase and 0.9U/ml for CMC-ase.

The β-glucosidase activity of the shaken cultures on straw were the most important (140 U/ml) after 192 hours of growth time. Comparatively to other strains of *Aspergillus* the present isolate produced higher amounts of β-glucosidase and β-xylanase activities but lower CMC-ase. The activities measured after 192 hours cultivation of *Aspergillus fumigatus* (*Fresenius*) *albino* mutant were 4 fold lower for CMC-ase but 29 fold higher for β-glucosidase than for *Aspergillus niger* cultivated on bagasse [24]. In the case of *Aspergillus niger* 33/20, endoglucanase (CMC-ase) activity was 8 fold higher [31], but β-glucosidase was 96 fold lower as compared to the *A. Fumigatus albino* mutant.

Straw was found to be a suitable carbon source for enzyme production. As shown in table 1, a concentration of 3% of this substrate gave the highest CMC-ase, β-xylanase and especially β-glucosidase activities.

Straw content (%)	**CMC-ase (U/ml)**	**β-xylanase (U/ml)**	**β-glucosidase (U/ml)**
0,5	1,23	6,55	75,50
1,0	1,75	16,00	125,10
2,0	2,60	26,80	140,00
3,0	2,70	28,70	462,75
4,0	1,15	3,30	316,85

Tableau 1 : Effect of the concentration of straw on the level of lignocellulolytic enzymes activities produced by *Aspergillus fumigatus* (*Fresenius*) *albino* mutant after 8 days cultivation on MW medium.

For *Trichoderma reesei* F522, the optimal straw concentration was 1.5%, with higher CMC-ase activity but

very low β-glucosidase and β-xylanase avtivity compared to our strain [11]. Concentrations of straw higher than 3% caused a decrease in enzyme synthesis by *Aspergillus fumigatus (Fresenius) albino* mutant, especially for β-xylanase. Lower yields were observed with 4% straw in the medium (table 1). This could be due to the adsorption of the enzymes to straw, or to a decrease in pH, which becomes inhibitory for the growth and induction of the enzymes [18, 11].

For further studies, 3% of oat straw were used in the media.

Figure 2 shows the kinetic of enzyme synthesis during 8 days of shake culture on 3% straw. Little activities were detected during the 24 hours induction phase. CMC-ase activity increased slowly and reached a maximum of 3 U/ml after 120 hours. Subsequently, the apparent rate of enzyme synthesis remained nearly unchanged. Conversely β-xylanase activity increased very rapidly, reaching a final titre of 98 U/ml after 48 hours, then declined till 28 U/ ml at the end of cultivation.

Hours	CMC-ase (U/ml)	Mycelial proteins (% dry weight)	β-xylanase (U/ml)	β-glucosidase (U/ml)
0	0	0	0	0
24	0,30	26	25	25
48	1,85	38	97,90	30,5
72	2,25	36	95,00	46,00
96	2,50	35	90,00	90,00
120	3,00	37,5	72,00	100,00
144	2,85	45	26,00	98,00
168	2,75	15	26,50	165,00
192	2,65	18	28,00	462,75

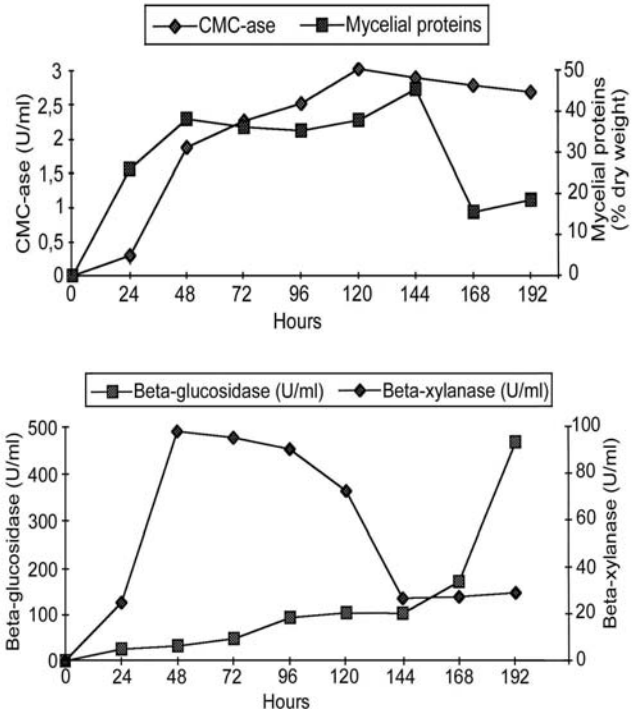

Figure 2 : Enzyme production and mycelial protein of the strain *Aspergillus fumigatus (fresenius) albino* mutant, cultived on MW medium containing 3% ball milled oat straw.

β-glucosidase behaved differently, started to increase slightly during the first 24 hours and then increasing rapidly till a maximum titre of 460 U/ml at the end of cultivation. No inhibition or plateau phases were observed for this enzyme.

The same kinetics aspect was obtained with *Trichoderma hamatum USD B 0008* but with lower activities [32]. Our strain produced apparently a large amount of mycelial proteins on 3 % straw after 144 hours cultivation (fig. 2).

The maximum protein level obtained was 40 % of the dry weight.

Characterization of the enzymes

The strain *Aspergillus fumigatus (Fresenius*) *albino* mutant produced CMC-ase and β-xylanase active between pH 3 to 8 with respective optima of 4.0 and 5.0 (fig. 3). This was observed for other thermophilic fungi such as *Sporotrichum thermophile* and *Thermoascus aurantiacus* [21].

pH	CMC-ase (U/ml)	β-glucosidase (U/ml)
0	0	0
1,5	0	1,0
2,8	0,6	3,75
3,0	2,50	6,37
4,0	3,50	25
4,5	3,37	32
5,0	3,25	32,5
6,0	2,80	31,25
7,0	1,75	22,50
8,0	0,75	19,00
9,0	0,05	1,50
10,0	0,05	0,50

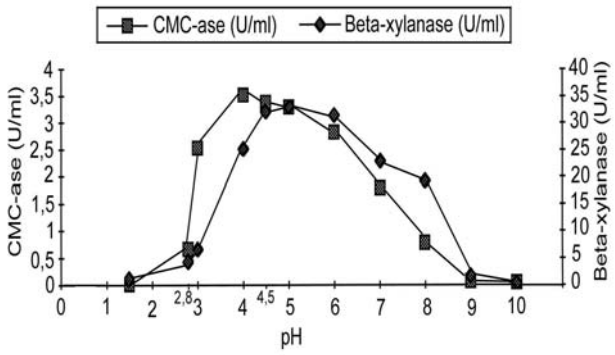

Figure 3 : Effect of pH on the Endoglucanase (CMC-ase) and Beta –xylanase activities of the strain *Aspergillus fumigatus (fresenius) albino* mutant.

β-xylanase and CMC-ase showed a temperature optimum for activity at 70°C. At higher temperatures, the activities were also important: as at 90°C, 93% of the β-xylanase activity was preserved and only 60% of the CMC–ase activity were lost (fig. 4).

β-glucosidase showed a temperature optimum of 65°C with about 67% of the activity at 80°C but no activity at 90°C. The optimal temperature for this enzyme was similar to that from *Aspergillus fumigatus* [33], which was also stable up to 60°C for 20 min.

Temperature (°C)	β-xylanase (U/ml)	CMC-ase (U/ml)	β-glucosidase (U/ml)
20			
30	6,00	1,30	15
40	12,5	1,37	25
50	16,25	1,75	42,5
55	25	2,37	42,5
65	26,5	2,38	43
70	28,75	2,50	40
75	26,25	1,60	33,5
80	26,25	1,50	30
90	27	1,00	0

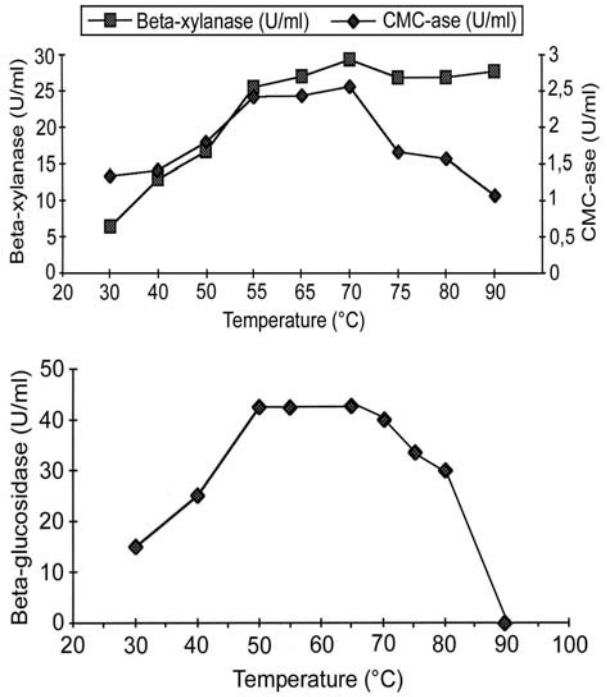

Figure 4 : Effect of temperature on the activity of Beta-xylanase, Endoglucanase and Beta-glucosidase of *Aspergillus fumigatus (Fresenius) albino* mutant. Crude enzyme preparations were incubated at different temperatures at pH 4.8 as indicated in "Material and methods".

The enzymes secreted by *Aspergillus fumigatus (Fresenius) albino* mutant showed high thermal stability: β-xylanase retained about 50% of activity at 60°C and 70°C. On the other hand, CMC-ase activity was more thermostable (fig.5). It was little affected by heating at 60°C for 1 hour. At 80 and 90°C, about 40% of activity was still retained. In contrast, the endoglucanase activity form *Aspergillus fumigatus (Fresenius)* IMI 246651 was rapidly lost at 60°C and above [34].

Other enzymes of thermophilic fungi were reported to have thermal stability between 60 and 65°C [21, 35] but mesophilic species have in general lower optimal and stability temperatures [36, 37].

CONCLUSION

The occurrence of *Aspergillus Fumigatus (Fresenius) albino* mutant strains have been already documented [29] but this type of strain might produce high amounts of lignocellulolytic enzymes operating at high temperature and are able to use a variety of lignocellulosic materials (straw, paper pulp, microcrystalline cellulose,…).

It is important to notice the high level of β-glucosidase production on straw. A potential application of β-glucosidase enzyme is the utilization in association with fungi producing low level of this enzyme such as *Trichoderma reesei* in order to saccharify cellulosics. High level of β-glucosidase can be produced by cultivating this selected *Aspergillus albino* strain at high temperature using cheap biomass residues, e.g. straw. β-xylanase showed excellent properties, such as activity over a broad range of pH and very good thermostability. Therefore, it is interesting for a potential application in bleaching of paper pulp or producing D–xylose.

Temperature (°C)	β-xylanase (U/ml)	CMC-ase (U/ml)
30	32,5	2,40
40	33	2,40
50	38	3,25
60	21,5	2,70
70	17,5	1,80
80	1,00	1,35
90	0	1,25

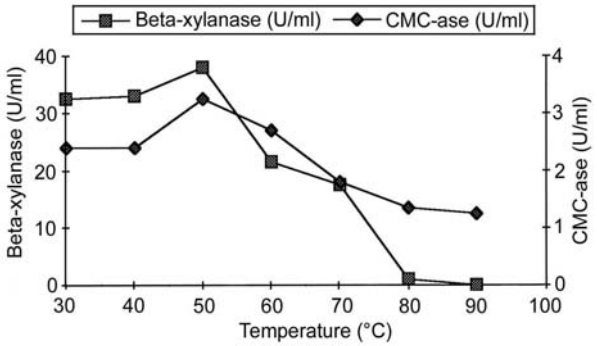

Figure 5 : Thermal stability of Endoglucanase (CMC-ase) and Beta-xylanase, produced by *Aspergillus fumigatus (Fresenius)* albino mutant. Crude enzymes were incubated at pH 4.8 for 1 hour. Residual activity was determined as described in "Material and methods".

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