Production of Amylase from the fungus *Aspergillus niger* FA6 Isolated from Iraqi Soil.

Intesar Ali Mezeal
Noor Muafak Alwaan
Department of biology ,College of Science
University of AL- Mustansarya

Abstract

Filamentous fungi have been widely used to produce hydrolytic enzymes for many applications. Fungal enzymes amylase were produced using eight *Aspergillus niger* isolates from eight soil sample in Iraq, were screened for amylase production. *A. niger* FA6 (isolated from AL-Nasiriya soil) gave maximum amylase yield, The effect of varying pH, temperature, incubation period, carbon sources and nitrogen sources of the medium for the productivity of amylase from *A.niger*FA6 was investigated. The maximum activity of amylase was recorded after 7 days of submerged fermentation at pH 5 and temperature 30°C. from different nitrogen and carbon sources used peptone and sucrose respectively gave maximum amylase yield.

Keywords: Amylase, *Aspergillus niger*, Temperature, Soil .

Introduction

Amylases are enzymes which hydrolyze the starch molecules into polymers composed of glucose units , Amylases are among the most important enzymes and are of great significance for biotechnology, They can be obtained from several sources, such as plants ,animals and microorganisms(1). The amylases of microorganisms have a broad spectrum of industrial applications as they are more stable than when prepared with plant and animal amylases(2), amylase in industry amylase has been derived from several fungi, yeasts and bacteria. However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors (3).

Amylases have potential application in a wide number of industrial processes such as food, fermentation, textile paper, detergent, and pharmaceutical industries (3,4,5). Fungal sources are confined to terrestrial isolates, mostly *Aspergillus* and *Penicillium*(6). The *Aspergillus* species produce a large variety of extracellular enzymes and amylases are the

ones with most significant industrial importance(7). Filamentous fungi, such as *Aspergillus oryzae* and *Aspergillus niger*, produce considerable quantities of enzymes that are used extensively in the industry(8). *Aspergillus niger* has important hydrolytic capacities in amylase production and, due to its tolerance of acidity pH it allows the avoidance of bacterial contamination. yield of enzyme has always been a problem in the commercial production of amylases (9). Moreover, thermal stability is a desirable feature for economic viability of enzymatic processes ,Therefore, the present work was undertaken to screen various *Aspergillus* isolates for α amylase production, optimization of fermentation conditions for maximum yield and to stabilize the enzyme in liquid state us in various additives.

Materials and methods

isolation the Fungi from Soil

Fungal colonies were isolated form soil samples by serial dilution method , 50µl of soil samples diluted up to fife dilutions were spread on respective solidified Potato Dextrose Agar plates. The inoculated Petri plates were incubated at 28°C for 2 days. Eight *Aspergillus niger* different isolates differentiated on the basis of physical characteristics obtained after incubation were named as FA1,FA2, FA3……. and FA8. The isolates were further inoculated on sterile PDA plates by point inoculation and incubated at 28°C for 2 days in order to obtain pure fungal plates.

Screening of Fungal Isolates for Amylase Production

Eight *Aspergillus niger* isolates were screened for amylase production efficiency in starch agar media comprising the following in gm/1 L, yeast extract 1.5, peptone 0.5, sodium chloride 1.5, starch 10, agar 15 pH 5. All the isolates were streaked centrally on sterile solidified starch agar plates, a blank without inoculation was also maintained for comparison. Plates were incubated at 28°C for 2 days after that all the plates along with blank were flooded with iodine and observed for zone of hydrolysis, after that we measure the zone of hydrolysis(mm) .

Enzyme Production

Enzyme production was done by submerged fermentation ,*A.niger* FA6 was inoculated in potato dextrose broth and incubated at room temperature for 4 days. The medium was then centrifuged at 1000 rpm for 5 min and the supernatant was collected for separating extra cellular enzyme amylase The protein content and enzyme activity were recorded.

Extraction of Crude Enzyme

Crude enzyme was extracted by adding 100ml of 100mM Tris buffer agitating the flask in shaker at 180 rpm for one hour, the mixture was filtered through cheese cloth and centrifuged at 1000 rpm for 5 min. The supernatant was collected and treated as crude enzyme.

Protein Estimation in Crude Enzyme

Protein in crude enzyme was determined by Lowry's method (10) of protein estimation in which enzyme was reacted with the Lowry's reagents and the absorbance obtained was compared with a standard graph plotted by reacting a standard protein with known concentrations with the Lowry's reagents and plotting a graph between concentration of standard protein and absorbance at 660nm.

Enzyme assay in Crude Enzyme

Enzyme assay was carried out by DNS method of (11) , 0.5ml enzyme was reacted with 0.5ml of substrate (1% starch in 100mM Tris buffer) under standard reaction conditions , the reaction was stopped by adding 3,5-Dinitrosalicylic acid(DNS) reagent, amount of maltose released was determined by comparing the absorbance reading of the test enzyme at 540 nm with the standard graph plotted by reacting the known concentration of maltose ranging from 0.05mg/ml to 0.5mg/ml.

Effect of different condition on production of amylase

1-Effect of temperature

The effect of temperature on amylase production was investigated by incubating the fermentation medium at (20 °C, 30 °C, 35 °C,42°C) at pH 7 for 7days.

2-Effect of pH

The effect of pH on amylase production was investigated by incubating the fermentation medium at different pH, the pH of basal salt solutions to (4 ,5 ,6.5,7), then incubated for 7 days at 25°C.

3-Effect of incubation time

The effect of incubation time on amylase production was investigated by incubating the fermentation medium at different time (1,3,7,8) days of at pH 7 and at 25°C.

4- Effect of nitrogen source

The effect of nitrogen source on amylase production was studied by replacing the nitrogen source in basal salt solution, pH 7, with 2% of peptone, casein, yeast extract and malt extract, and incubated at 25°C for 7 days.

5- Effect of Carbone source

The effect of carbon sources on amylase production was investigated by supplementing the basal salt solution, pH 7, with 2% of different carbon sources such as glucose, maltose, lactose, and sucrose. The substrates were then incubated for 7 days at 25°C.

Results and Discussion

Screening of *A. niger* isolates for Amylase Production

Eight *A. niger* isolates differentiated on the basis of colony morphology were obtained after spreading, and were named tentatively as FA1, FA2, FA3 ……and FA8. All the isolates were subcultures by point inoculation and used for further studies ,All the isolates were subjected to screening procedure and after completion of incubation period plates were flooded with iodine solution and observed for zone of hydrolysis. The results of the same can be seen below in figure 1,*A.niger*FA6 was found to be the best amylase producer and hydrolysis zone of amylase produced by this strain was 8.2 mm in solid state fermentation. So, this potential strain was selected for further optimization of culture conditions. The results of this investigation showed that *A.niger* FA6 grew on a medium containing starch as sole carbon source producing amylase, capable of degrading glucosidic bonds of starch, hydrolytically Cultural conditions have an influence on enzyme production(12,13). Similar values of enzyme production, for *A. niger* isolates JGI24 and GCB – 34 have been reported (14).

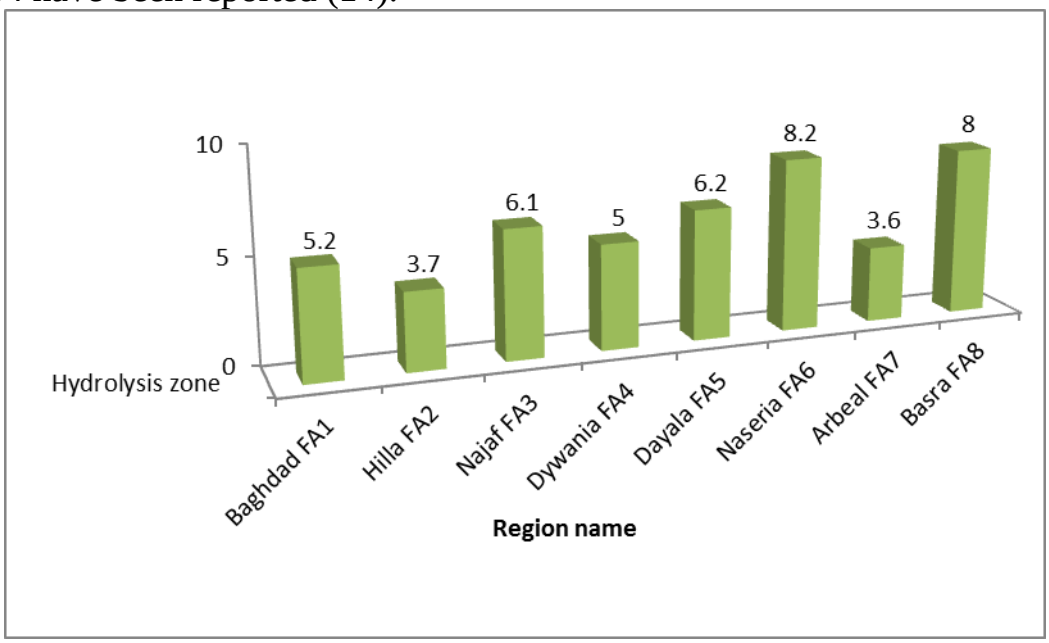


figure 1: Screening for Amylase Production

Effect of Different Condition on amylase activity

1- Effect of temperature

A.niger FA6 inoculated at different temperature seen from the figure 2 , that 30 °C is the temperature at which maximum production of amylase. Incubation temperature not only influences the growth of microorganisms but also their biological activities. The effect of temperature on amylase activity *A. niger* FA6 was studied by varying the temperature from 20°C to 42°C and the results have been depicted in the figure 2. It is clear from the results that a temperature of 30°C was found to be best suitable for amylase activity and maximum activity found was 9.8U\ml, it was observed that at 20°C enzyme activities were low 6.1U\ml and showed a gradual increase with the increase in temperature to 30°C. Further increase in temperature resulted in decrease in production of amylase that at 42°C enzyme activities were 6.5U\ml.

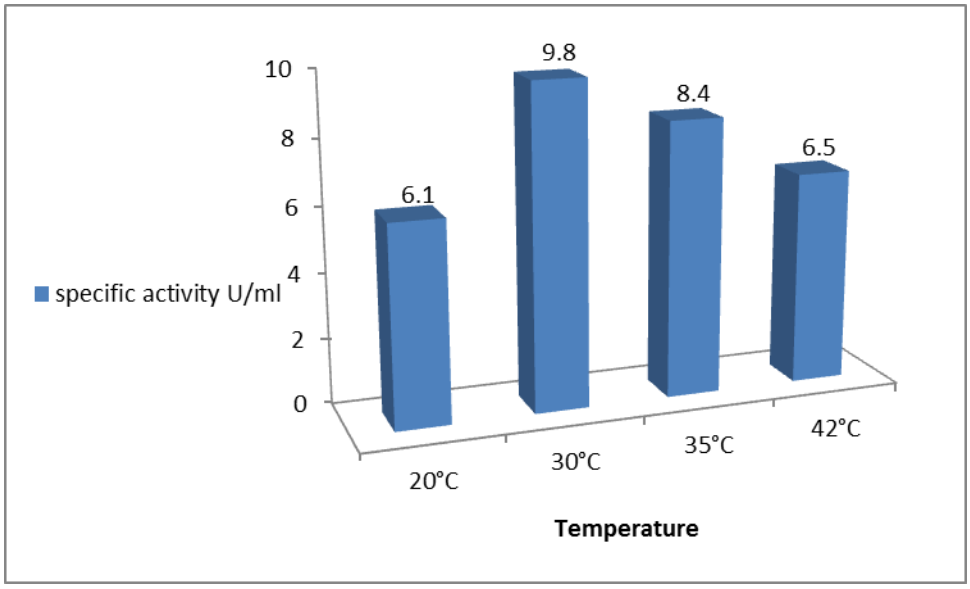


figure 2: Effect of Temperature

The influence of temperature on amylase activity of the crude enzyme showed that enzyme activity increased progressively with increase in temperature from 20°C reaching a maximum at 30, Above 35 C there was a reduction in the amylase activity ,It is reported that best enzyme production in *A. niger* at room temperature both in SmF and SSF and reported 30C to be the best for enzyme production by *Penicillium fellutanum*(14), However the optimum temperature for enzyme production was reported as 30C in many literatures(15,16).

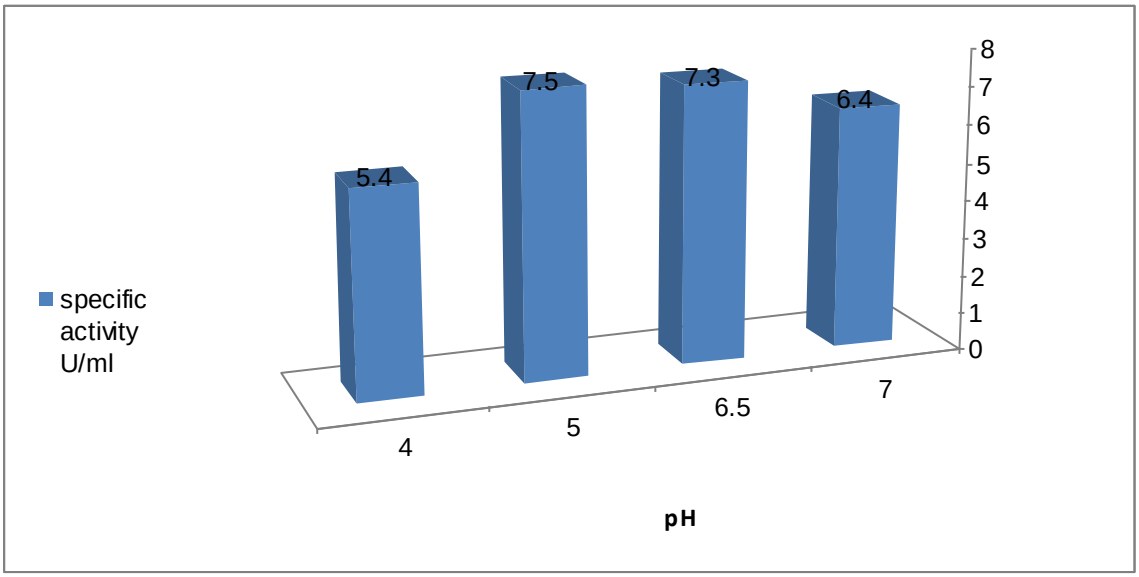
Temperature changes had an effect on amylase activity produced by *A. niger*. Optimum activity was at 35°C after which there was a decline in

activity The rate of enzyme catalyzed reactions increase with temperature. This occurs only within the temperature range at which an enzyme is stable and retains full activity (17).

2- Effect of pH

A. niger FA6 isolate when inoculated at different pH seen from the figur 3 below that pH 5.0 is the pH at which maximum production of enzyme was seen. The effect of pH on amylase activity of *Aspergillus niger* FA6was studied by varying pH from 4.0 to7.0, The results are depicted in figure 3. which indicate that with increase in pH value from 3 to 5, the activities of amylase enzyme reached to the maximum followed by a gradual decrease thereafter.

It is clear that pH of 5 was found to be best for amylase activity and maximum activity recorded was 7.5 U/ ml, amylase activity Among the physical parameters, the pH of medium plays an important role by inducing in enzyme secretion. According to(3) The synthesis of extra-cellular α-amylase is affected by the pH,



Figur3: Effect of pH

Under experimental conditions, a maximum enzyme activity was produced at pH 5 , 7.5U/ml and the lowest at pH 7 , 6.4U/ml Our findings are comparable to previous reports(16,18) with *Aspergillus* spp. and *A.niger* at pH varying between 5 and 6 . In contrast (19) reported pH 3.5 and pH 4.0 to be the best for the production of α-amylase by *B. amylolique faciens* and *A. awamori* .

Low and high pH values can also causes considerable de naturation and hence inactivation of the enzyme(17), It is reported that amylase production is high at pH 5 (15),and Amylase production in *A. ochraceus*

and A. niger UO 1 was optimum at pH 5(20). Maximum amylolytic activity of thermophilic fungi *Aspergillus fumigates* isolated from soil was observed pH 5 in mineral (21).

3- Effect of incubation pored

A. niger FA6 isolate when inoculated at different incubation pored it can be seen from the figure 4, Incubation period plays an important role in enzyme production ,The effect of incubation period was evaluated by checking enzyme activities .

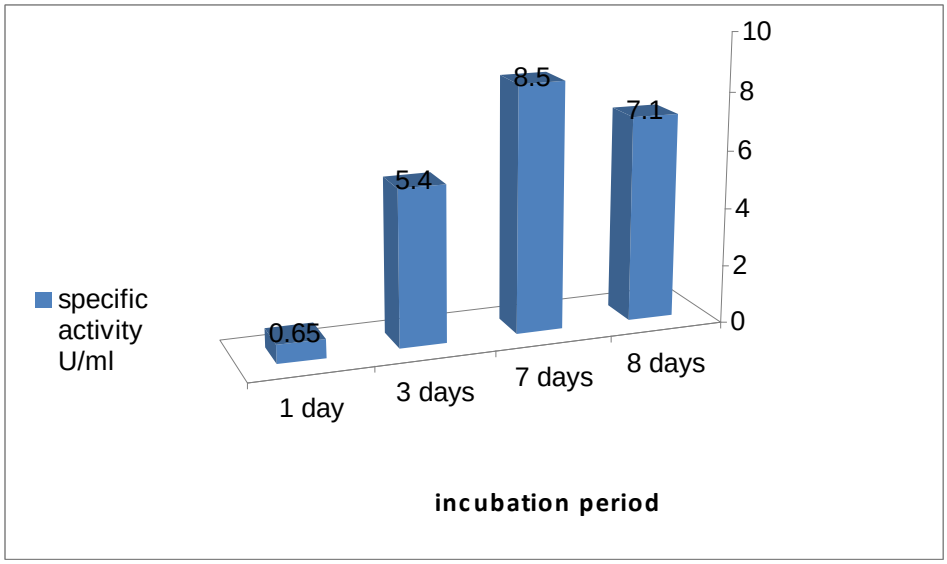


figure 4: Effect of incubation pored

The cultures were incubated for 1- 8 days, The enzyme was extracted and the specific activity of the amylase produced at different days of incubation was 5.4 U/ml in 3 days of incubation and The specific activity was 8.5 U/ml for the enzyme at 7 days of incubation ,while The specific activity was 7.1 U/ml for the enzyme at 8 days of incubation. The incubation period varies with production of enzyme and Short incubation period offers potential for inexpensive production of enzyme(22). Similar results were reported earlier that amylase activity was produced after two days of cultivation(23).The maximum amylase activity was recorded during the period of fungus autolysis and reported the maximum production of amylase enzyme at five days of incubation period (15),*A. niger* amylase increased with increase in days of incubation, α-amylase was recorded as 450 U/mg after 7 days of submerged fermentation (24).

4- Effect of nitrogen sources

A. niger FA6 isolate when inoculated at different nitrogen sources it can be seen from the figur5, below that peptone is the nitrogen source at which maximum yield.

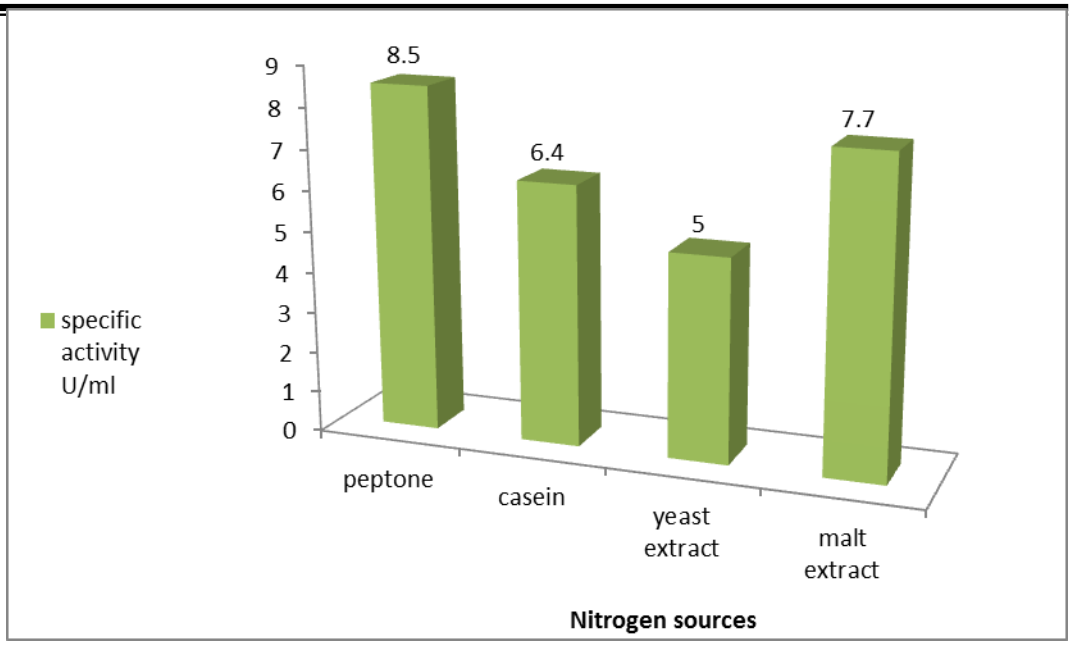


figure 5: Effect of nitrogen sources

Addition of organic sources such as peptone, casein, yeast extract and malt extract to the medium resulted in consider able increase in the production of amylase ,Media supplemented with peptone showed maximum amylase activity 8.5 U/ ml compared to casein ,malt extract and yeast extract were 6.4 ,5 and 7.7 respectively , Similarly the supplementation with nitrogen sources to amylase production by *A. niger* is done with success increase in the yield of enzyme in submerged fermentation(25).

Among the organic and inorganic nitrogen sources, inorganic sources showed maximum yield in which ammonium nitrate showed highest amylase activity of 475 U/mg at the same pH and temperature(24). peptone best nitrogen source for amylase production by *A. niger* 8.2 U/ml followed by sodium nitrate 7 U/ml, while Ammonium chloride and ammonium sulfate were poor nitrogen sources for amylase production (22), According to (26), nitrogen sources have been reported to have an inducing effect on the production of various enzymes including α-amylase. The results presented by (20,27) show that the highest amylase activity was obtained with meat extract, i.e., 6.47 g/L followed by urea, i.e., 5.70 g/L peptone, i.e., 5.08 g/L and ammonium phosphate ei.e., 4.66 g/L. The lowest biomasses were obtained with ammonium nitrate, ammonium sulphate and ammonium carbonate, i.e., 3.22, 3.40 and 3.45 g/L respectively ,Similar results were recorded by other researchers with *A. niger*, and *C.guilliermo ndii* , However, in contrast some studies shows

that the peptone was the best nitrogen source with *Trichoderma lanuginosus and Penecillium fellutanum* (28,29).

5- Effect of Carbon sources

A. niger FA6 isolate when inoculated at different carbon sources it can be seen from the figure 6, below that sucrose is the carbon source at which maximum yield was seen.

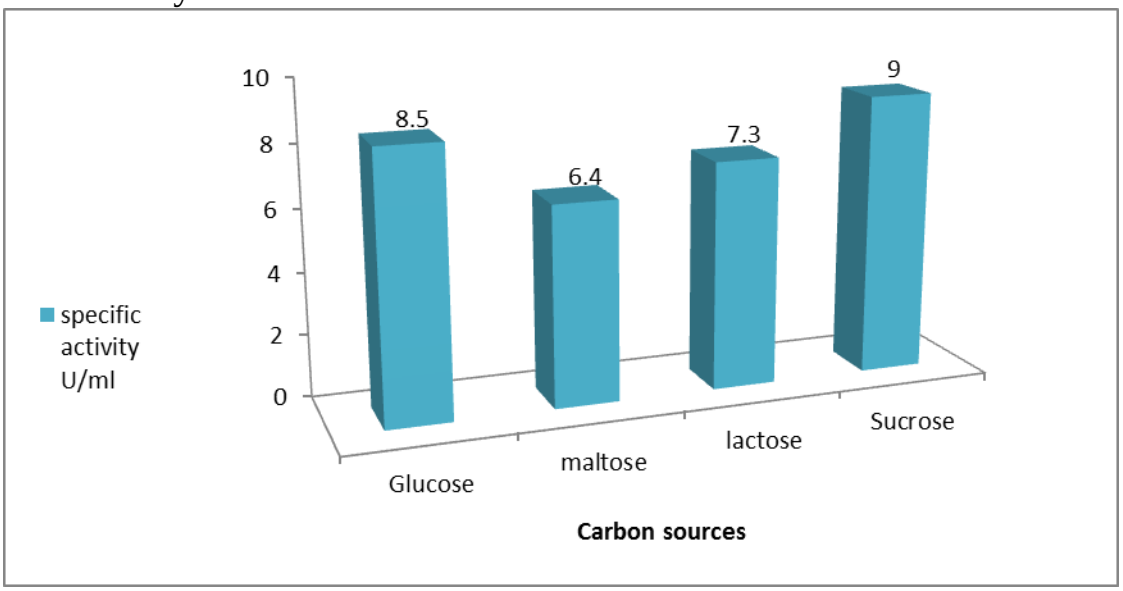


figure 6: Effect of carbon sources

Addition of different carbon source like glucose, maltose, lactose, and sucrose show change in the yield. Sucrose showed a better yield specific activity was 9 U/ml, while glucose maltose and lactose showed specific activity were 8.5,6.4 and 7.3 respectively .similar result get by(14). Similar results of catabolite repression of enzyme production by glucose, for *A. niger* CFTRI 1105 and for *Aspergillus* sp. JGI 12(16,30), optimum amylase activity was expressed on the seventh day as 0.47 Units, with ammonium chloride as nitrogen source and maltose as carbon source of growth, in this investigation, incorporated into the growth medium, induced amylase production by *A. niger*, starch and maltose, sucrose, lactose, glucose and galactose as carbon sources with potassium nitrate as nitrogen source supported growth and α-amylase production by *Lasiodiplodia theobromae* (31).

References:

1- Reddy, N. S.; Nimmagadda, A. and Sambasiva Rao, K. R. S. (2003). An overview of the microbial -amylase family. Afr. J. Biotechnol. 2, 645-248.

2- Tanyildizi, M. S.; Ozer, D.and Elibol, M. (2005). Optimization of –amylase production by Bacillus sp. using response surface methodology Process Biochem 40, 2291–2296.

3- Gupta, R.; Gigras, P.; Mohapatra, H.; Goswami, V. K. and hauhan B. (2003). Microbial-amylases: a biotechnological perspective. Process Biochem 38, 1599 – 1616. .

4- Kandra, L. (2003). α-Amylases of medical and industrial mportanceJournal of Molecular Structure : Theochem,487, 666–667.

5- Pandey, A.; Nigam, P.; Soccol, C. R.; Soccol, V. T.; Singh, D. and Mohan, (2000). Advances in microbial amylases. Biotechnol App. 31,135-152.

6- Kathiresan, K. and Manivannan, S. (2006).Amylase production by Penicillium fellutanum isolated from mangrove rhizosphere soil. Afr.J .Biotechnol. 5, 829-832.

7- Hernández, M. S.; Rodríguez, M. R.; Guerra, N. P. and Rosés, R. P. (2006). Amylase production by Aspergillus niger in submerged cultivation ontwo wastes from food industries. J. Food Process Eng.73,93–100. .

8- Kammoun, R.; Naili, B. and Bejar, S. (2008). Application of a statistical design to the optimization of parameters and culture medium for alpha amylase production by *Aspergillus oryzae* CBS 819.72 grown on gruel)wheat grinding by-product). Bioresour Technol . 99, 5602-5609.

9- Djekrif-Dakhmouche, S.; Gheribi-Aoulmi, Z.; Meraihi, Z. and Bennamoun, L. (2006). Application of a statistical design to the optimization of culture medium for α -amylase production by Aspergillus niger ATCC 16404 grown on orange waste powder. J Food Process Eng.73, 190–197. .

10- Lowry , O. H; Rosebrough, A. L. and Farr, R. J. (1951) . Protein measurement with the Folin phenol reagent. J. Biol. Chem.193: 256–276. J. Biol. Chem,,193-256.

11- Miller, G. L.(1959). Use of dinitro salicylic acid reagent for determination of reducing sugar nalytical Chemistry, 31:426-429.

12- Dixon,M. and Webb, E. C. (1971).Enzymes.Longmas,London.pp:950.

13- Adejuwon, A. O. (2010). Synthetic production of amylase from Aspergillus niger isolated from citrus fruit. African Journal of Basic and Applied Sciences 2(5-6): 158-160.

14- Varalakshmi, K. N; Kumudini, B. S; Nandini, B.N; Solomon, J; Suhas, R; Mahesh, B; and Kavitha, A. P.(2009). Production and characterization of α amylase from *Aspergillus niger* JGI 24 isolated in Bangalore. Polish Journal of Microbiology; 58: 29-36.

15- Gupta, A; Gupta, V. K; Modi, D.R. and Yadava, L. P. (2008). Production and characterization of α-amylase from Aspergillus niger. Biotechnology(7):551-556.

16- Alva, S; Anupama, J; Salva, J; Chiu, Y. Y; Vyshali, P; Shruthi, M; Ogeetha, B.S; Bhavya., D; Purvi, J; Ruchi, K; Kumudini, B.S. and Varalakshmi ,K.N.(2007). Production and characterization of fungal amylase enzyme isolated from Aspergillus sp. JGI 12 in solid state culture African journal of technology; 6: 576 – 581.

17- Take,M. and Rajhans,G. (2012). Production and characterization off α-amylases from Aspergillus niger by solid state fermentation . Research Journal of Biotechnology. 7 (4). .

18- Renato, P. R. and Nelson, P. G.(2009). optimization of amylase production by Aspergillus niger in solid state fermentation using sugarcane bagasse as

solid support material. World Journal of Microbiology and Biotechnology 25 (11): 1929-1939.

19- Pavezzi, F. C; Gomes, E. and Roberto-Da-Silva, R.(2008). Production and characterization of glucoamylase from fungus Aspergillus awamori expressed in yeast Saccharomyces cerevisiae using different carbon sources. Brazilian Journal of Microbiology 39 (1):127-135.

20- Hernandez, M. S; Marilu, R. R; Nelson, P. G. and Renato, P. R.(2006). Amylase production by Aspergillus niger in submerged cultivation on two wastes from food industries. J. Food Eng. 73: 93-100.

21- Nwagu, T. N. and Okolo, B. N. (2010). Growth profile and amylase hydrolytic activity of a thermophilic fungi Aspergillusfumigatus isolated from soil. Asian.JBiotechnol. 3(1):46-57.

22- Sonjoy, S; Bill, B. and Houston , K.H. (1995). Cellular activity of Trichodermaressei (RUT-C30) on municipal solid waste. Appl. BiochemBiotechnol. 15: 145-153.

23- Ely, N. and Mirela, M. (2002) Control of amylase production and growth characteristics of Aspergillusochraceus. 44: 4-10.

24- Sundar, R. Rajila, L. and Suganyadevi, P. (2012). Amylase production by Aspergillus niger unduer submerged fermentation using Ipomoea batatas . International Journal of Applied Biology and Pharmaceuticalechnology3(2):175-182.

25-Pandey, A; Webb, C; Soccol, C. R. and LArroche, C. (2005). Rice bran as a substrate for proteolytic enzyme production. Enzyme technology, New Delhi: Asiatech Publishers, Inc.197 .

26-Kaur, P., Grewal, H. S. and Kocher, G. S. (2003). Production a-amylase by Aspergillus niger using wheat branin submerged and solid state fermentations. Indian Journal of Microbiology 43: 143-145.

27-Lagzouli, M; Charouf, R; El-Yachioui, O; Berny, M. E .H. and Jadal, M. (2007). Optimization croissanceet production de glucoamylase extra cellulaire par Candida guilliermondii, Bulletin de la Société Pharmacie 146: 251-70.

28-Kunameni, A., Permaul, K. and Singh, S. (2005). Amylase production in solid state fermentation by the thermophilic fungus Thermomycesla nuginosus Journal of Bioscience and Bioengineering 100: 168-171.

29- Kathiresan, K. and Manivannan, S. (2006). Amylase production by Penicillium fellutanum isolated fro mmangrove rhizosphere soil. African Journal of Biotechnology 5 (10): 829-832 .

30- Mukerjee, S. K. and Majumdar, S. K. (1993). Fermentative production of alpha amylase by Aspergillus flavus. Indian J. Exp. Biol. 11: 436-438.

31-Adejuwon, A. O. (2011). Nutritional factors affecting the production of amylasein Lasiodiplodia theobromae Pat. BiotechnologyBioinformatics and Bioengineering 1(1): 131-135.

انتاج الاملیز من الفطر *Aspergillus niger* FA6 المعزول من التربة العراقية

انتصار علي مزعل

نور موفق علوان

قسم علوم الحياة ، كلية العلوم ، الجامعة المستنصرية

الخلاصة :

تعد الفطريات الخيطية ذات استعمال واسع في مجال تطبيقات انتاج الانزيمات المحللة , انتج انزيم الاميليز الفطري من ثمانية عزلات فطرية شخصت بانها تعود للفطر *Aspergillus niger* وقد عزلت من ثمانية عينات من الترب العراقية (ومن ثمانية محافظات مختلفة) ، شخصت بأن العزله *A. niger* FA6 والمعزولة من تربة اخذت من محافظة الناصرية باعلى انتاج لانزيم الاميليز . وقد اختير تاثير عدد من العوامل الفيزيائية المختلفة على فعالية انزيم الاملیز للعزلة الاكبر انتاجا للانزيم (*A. niger* FA6) , فقد تم دراسة تاثيرات كلا من درجة الحرارة و الرقم الهيدروجيني و فترة الحضن و مصدر النايتروجين ومصدر الكاربون على فعالية انزيم الاملیز ، وقد وجد بان اكثر فعالية للاملیز كانت تحت درجة حرارة بلغت ٣٠ م ورقم هايدروجيني بلغ ٥ عندما حضنت العزلة لمدة ٧ ايام , بينما كان كلا من الببتون والسكروز مصدري النايتروجين والكاربون الافضل في زيادة فعالية انزيم الاملیز.

الكلمات المفتاحية :– الاملیز ، اسبرجلس نايكر ، درجة الحرارة ، التربة