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Optimum conditions for Inulinase production by *Aspergillus niger* using solid state fermentation

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Abstract:

Thirty local fungal isolates according to *Aspergillus niger* were screened for Inulinase production on synthetic solid medium depending on inulin hydrolysis appear as clear zone around fungal colony. Semi-quantitative screening was performed to select the most efficient isolate for inulinase production. the most efficient isolate was AN20. The optimum condition for enzyme production from *A. niger* isolate was determined by busing a medium composed of sugar cane moisten with corn steep liquor 5;5 (v/w) at initial pH 5.0 for 96 hours at 30 ^{0}C . Enzyme productivity was tested for each of the yeast *Kluyveromyces marxianus*, the fungus *A. niger* AN20 and for a mixed culture of *A. niger* and *K. marxianus*. The productivity of *A. niger* gave the highest specific activity of 153 U/mg, as compared with *K. marxianus* which gave 86 U/mg.

Key words: Inulinase , *Aspergillus niger*; *Kluyveromyces marxianus*

Introduction:

Inulinase are enzymes that degrade the β-(2,1) linkages of β-fructans, like inulin. Inulinase are classified into endo- and exo-Inulinases, depending on their mode of action. They act by using two mechanism: exo-inulinases (EC 3.2.1.80) sequentially split-off the terminal β-(2,1) fructofuranosidic bonds, while endo-inulinases hydrolyze (EC 3.2.1.7) the internal linkages in inulin and release inulooligosaccharides. Inulinases are produced from several fungal species [1]. The genus Aspergillus is one of the most important filamentous fungal genera. *Aspergillus species* are used in the fermentation industry, but they are also responsible of various plant and food secondary rot [2]. *Aspergillus niger* is a soil saprobe with a wide array of hydrolytic and oxidative enzymes involved in the breakdown of plant lignocellulose. A variety of these enzymes from *A. niger* is important in the biotechnology industry [3]. Production of inulinase by fermentation which is a method of generating enzymes for industrial purposes. Fermentation involves the use of microorganisms, like bacteria and yeast to produce the enzymes. There are two methods of fermentation used to produce enzymes. These are submerged fermentation and solid-state fermentation (SSF) [4]. The production of enzymes by SSF has gained much attention in biotechnology studies for production of lipases, inulinase, proteases, etc. The use of low cost residues, higher productivities, low energy requirements, lower waste water prouction, extended stability of products and low production costs are some of the main advantages of SSF inulinase from *Aspergillus niger* [5].

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Materials and Methods:

Chemicals: Potato-dextrose agar (PDA) was obtained from hi-medias, other chemicals were supplied by BDH Chemicals. Thirty local fungal isolates according to *Aspergillus. niger* were screened for inulinase production on synthetic solid medium depending on inulin hydrolysis appear as clear zone around fungal colony.

Fungal isolates:

Thirty fungal isolates according to *A. niger* were collected from different sources, 7 isolates from College of Science /Baghdad University, 3 isolates from College of Science for women / Baghdad University, 11 isolates from College of Science /Al-Mustansarya University, 4 isolates from Ibn-Alhaitham college/ Baghdad University, 3 isolates from Agriculture College/ Baghdad University and 2 isolates from College of Science / Babylon University. Three *Kluyveromyces* isolates were obtained from College of Science /Baghdad University, All isolates were sub cultured each two weeks on Potato Dextrose Agar (PDA) and incubated at 30 ^{0}C.

Semi-quantitative screening of inulinase producing isolates:

Isolates efficiency for inulinase production was screened according to the method described by Kumar *et al*. [6]. The screening was performed by culturing fragments of the collected isolates in the holes of the prepared medium, the holes were prepared by using 3 mm cork-borer after sterilization with alcohol and flame and transfer the fragment under sterilized condition by loop to the surface of the plates containing the synthetic medium containing gm/L (Agar :20, $(NH_4)_2SO_4$:0.5, KH_2PO_4 :3, $NaNO_3$:1.5, $MgSO_4.7H_2O$:0.01, Inulin:3). After 48 hours of incubation at 30 °C, a clear zone appeared around each colony as a sign of inulinase production, the clearance zone ratios were measured, and the colony with the higher ratio was selected as the most effective isolate in inulinase production.

Enzyme Production:

Five ml of corn steep liquor (pH 5) was added to 5 gm of carbon source powder in 250 erlenmyer flask, then the medium was sterilized by autoclaving at 121^{0}C for 15 Min. One ml of selected *A. niger* suspension 10^6 spore/ ml was inoculated into each flask and incubated at 30^{0}C for four days. After incubation time, 50 ml of 0.1 M sodium acetate pH 4.8 was added to each flask, and 150 rpm for enzyme extraction, then the extract was filtrated by No. 1 Whitman filter paper. The filtrate considered crude enzyme, then the enzyme activity and protein concentration were estimated.

Estimation of inulinase Activity and concentration: Inulinase activity was estimated in solutions resulted after extraction of the enzyme by sodium acetate pH 4.8, by the method described by Miller which depends on inulinase analysis (substrate concentration (inulin) 1% in sodium acetate) to the reduced sugars formed by enzyme activity. Unit of enzyme activity is defined as the amount of enzyme necessary to liberate 1mM of Fructose in one minute in standard conditions. Protein concentration was estimated according to the method described by Bradford depending on Bovine serum albumins standard curve and using of coomassiee blue G-250 and measured at 595 nm [7,8].

Optimization for Inulinase Production:

Many factors that influence inulinase production from selected *A. niger* had been studied, these factors included type of carbon source, moisturizing solution, moisture ratio, initial pH of the medium and the incubation temperature, period of incubation.

Optimum carbon source: Four carbon sources were tested to determine the optimum carbon source for inulinase production from selected isolate, these sources were leek, onion, garlic and sugar cane. All sources were washed with tab water then sliced to small pieces and dried. These dried parts were grinded until they became powder.

Moisturizing solution:

Three different moisturizing solutions with different nitrogen sources were examined to determine the best solution for inulinase production from selected *A. niger*, these solutions that described by Skowronek and Fidurek [9] are:

A. Yeast extract (0.0144 gm), $MnSO_4$ (0.166 gm), K_2HPO_4 (0.021 gm), and corn steep liquor.

B. NH_4NO_3 (0.0318 gm), $MnSO_4$ (0.130 gm), Soya been (0.303 gm), K_2HPO_4 (0.0262 gm) and Distilled water.

C. Corn steep liquor and Distilled water used as control treatment.

5 ml of each solution was added separately to 5 gm of sugar cane in 250 ml flask, and inoculated with *A. niger* 10^6 spore/ml, then incubated at 30°C for four days.

Moisture ratio:

Five gram of sugar cane was moisten with different volumes of corn steep liquor containing (Yeast extract, $MnSO_4$ and K_2HPO_4). Different moisture ratios were tested 5:5, 5:10, 5:15, 5:20 (w/v) to select the optimum moisture for inulinase production.

Optimum pH:

Production media was distributed into flasks, the pH of moisturizing solution was then adjusted to 4, 5, 6, 7, 8, 9 and then inoculated with *A. niger*, it was then incubated at 30 °C for 4 days. The inulinase activity was determined after incubation to determine the optimum pH for inulinase production.

Incubation temperature and period:

The culture which consists of the medium contained on sugar cane (5 gm), corn steep liquor (5 ml) and pH 5.0, inoculated with 10^6 spores/ml of selected *A. niger* was incubated in different temperature degrees (20, 25, 30, 35, 40 45 , 50) °C to find the optimum incubation temperature for enzyme production, then the inoculation has done with the medium sugar cane (5 gm), corn steep liquor (5 ml) and pH 5.0 with 10^6 spores/ml of selected *A. niger*, the culture was incubated at 30 °C and checked every day for 5 days to estimate enzyme activity, protein concentration and specific activity for inulinase.

Mixed culture of *A. niger* and *Kluyveromyces marxianus*: Inulinase production medium was inoculated with three type of single and mixed inoculum at optimum conditions according to the method described by Ongen-Baysal and Sukan [10], After incubation of 30 °C for 96 hours, inulinase activity and protein concentration were determined.

Results and Discussions:

Semi-quantitative screening of inulinase producing isolates:

In order to examine the most efficient isolate for inulinase production, all fungal isolates of *Aspergillus niger* were recultured on the medium described by Kumar *et al*.[8]. The culture was incubated for 4 days, at 30 °C. Results showed different efficiencies in inulinase production. Inulin hydrolysis appear as clear zone around fungal colony. The most efficient isolate produces the wider clearance zone as in table (1).

Table (1): The clearance zone ratios of *A. niger* isolates.

Isolates	Clearance zone ratios	Isolates	Clearance zone ratios
AN1	1.20	AN16	1.05
AN2	1.10	AN17	1.30
AN3	1.20	AN18	1.40
AN4	1.60	AN19	1.70
AN5	1.40	AN20	1.95
AN6	1.20	AN21	1.80
AN7	1.50	AN22	1.23
AN8	1.20	AN23	1.10
AN9	1.10	AN24	1.40
AN10	1.30	AN25	1.50
AN11	1.50	AN26	1.20
AN12	1.17	AN27	1.21
AN13	1.20	AN28	1.26
AN14	0.90	AN29	1.30
AN15	1.60	AN30	1.20

Optimization of inulinase production:

Optimum carbon source: Four carbon sources were tested for their efficiency in inulinase production. These sources were leek, onion, garlic and sugar cane (figure 1). The highest activity is shown in sugar cane with specific activity 112 U/mg, while leek, garlic and onion showed specific activities as follows 24.15 U/mg, 86.1 U/mg and 87.35 U/mg respectively. This indicates that sugar cane is the most efficient source for inulinase production from *A. niger* AN2O. These results were similar to the results of Ettalibi and Baratti [11] who proved that selection of efficient media for inulinase production from *Aspergillus ficuam* depends on changing the media components, while Mazutii *et al*.,[12] found that the best medium for inulinase production from *Kluyveromyces marixianus* was sugar cane bagasse with corn steep liquor. Saber and El-Nagger [13] compared inulinase productivity from cultivation of *Aspergillus taamarii* different carbon sources included pure sources (glucose, maltose and inulin) and natural sources (Jerusalem artichoke, beetroot and chicory). They found that natural sources gave the highest activity among pure carbon sources where chicory gave 240.8 U/mg, while pure inulin gave 220.2 U/mg.

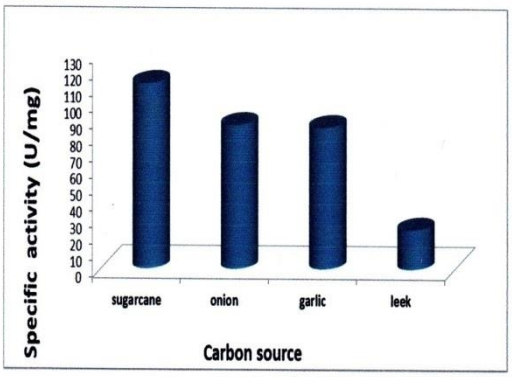

Fig.(1): The Effect of carbon source on inulinase production from local isolate *A. niger* AN20, incubation for 4 days at 30°C.

Moisturizing solution: To determine the best moisturizing solution for inulinase production, four different solutions A (Yeast extract, $MnSO_4$, K_2HPO_4 and corn steep liquor), B (NH_4NO_3, $MnSO_4$, Soya been, K_2HPO_4 and Distilled water), C (corn steep liquor) and D (Distilled water as a control treatment) were tested. Solution A gave the highest specific activity 124 U/mg, while B and C gave 93.7 U/mg and 61 .8 U/mg respectively. Distilled water gave very low specific activity 33.4 U/mg. These results prove that the highest specific activity of inulinase produced from *A. niger* AN2O, was obtained from the solution A (figure 2). Viswanathan and Kulkarni [14] found that the best moisturizing solution is corn steep liquor in presence of kuth root powder as source for inulinase production from *A. niger* with 92.4 U/mg specific activity. Kango [15] found that the use of yeast extract as nitrogen source with presence of dandelion root extract as carbon source was the best source for inulinase production from *A. niger*.

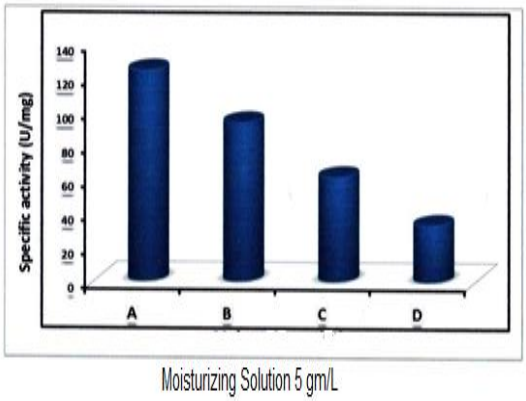

Fig. (2): The Effect of different moisturizing solutions on inulinase production from local isolate *A. niger* AN20,using sugar cane, incubation for 4 days at 30°C, pH 5. A.Yeast extract, $MnSO_4$, K_2HPO_4 and corn steep liquor. B. NH_4NO_3, $MnSO_4$, Soya been, K_2HPO_4 and Distilled water. C. Corn steep liquor. D. Distilled water.

Moisture ratio:

Five gm of sugar cane was moisten with different volumes of corn steep liquor containing yeast extract, $MnSO_4$ and K_2HPO_4. These treatments were tested to select the optimum moisture ratio for inulinase production. The best moisture ratio was 5:5 (w/v) which gave specific activity 152.6 U/mg while 5:10, 5:15 and 5:20 ratios gave 121.8, 95.8 and 93.3 respectively (Figure 3). Mazutii *et al*. [12] found that the optimum moisture ratio for inulinase production from *K. marixianus* was 65 %.

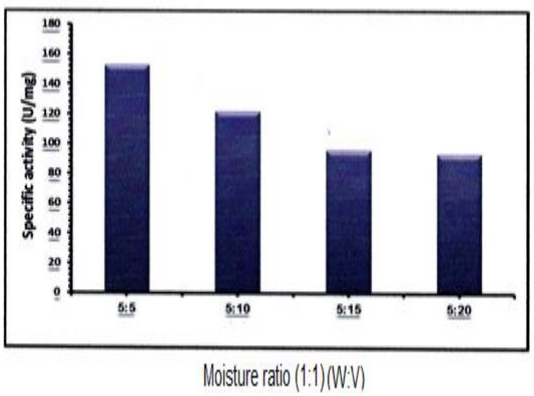

Fig. (3): The Effect of moisture ratio on inulinase production from local isolate *A. niger* AN20,using sugar cane, incubation for 4 days at 30°C, pH 5.

Most of solid substrates used in solid state fermentation are insoluble in water, therefore water will have to be absorbed onto the substrate particles, which can be used by the microorganisms for growth and metabolic activity (Pandey) [16]. Thus, it is concluded that the degree of hydration of the substrate plays an important role in the growth of the fungi and subsequently the enzyme production. Water causes the swelling of the substrate and facilitates good utilization of substrates by the microorganisms. Increasing moisture level is believed to have reduced the porosity of substrate, thus limiting the oxygen transfer into the substrate (Raimbault and Alazard, 1980 ; Moat *et al*.,) [17, 18]. Likewise, a lower moisture ratio leads to reduced solubility of the nutrients of the solid substrate, lower degree of swelling and a higher water tension (Ikasari and Mitchell) [19].

Initial pH of production media: The specific activity for inulinase was estimated after incubation to determine the optimum pH and the results were illustrated in figure (4), the optimum pH for enzyme activity was 5.0 because gave high specific activity 154.9 U/mg , while pH 4, 6, 7, 8, 9

gave 134.6, 130.1, 104.0, 88.6 and 74.3 U/mg respectively. The results of Ertan and Ekinci [20] proved that the optimum pH for inulinase productivity from *A. niger* is 5.0. Gouda [21] found the optimum pH for inulinase productivity from *A. fumigatus* was 6 which gave activity 35.56 U/mg. Fungi generally prefer slightly acid conditions and therefore tend to dominate bacteria when these prevail. The reason for the growth rate falling away either side of the optimum value is again due to alterations in three-dimensional protein structure (Moat *et al*.,) [18]. The pH affects in enzyme production because of its role in the solubility of medium substrates and its effect on the ionization of the substrate and it's availability for the fungal growth. Moreover the pH affects the productivity and enzyme stability.

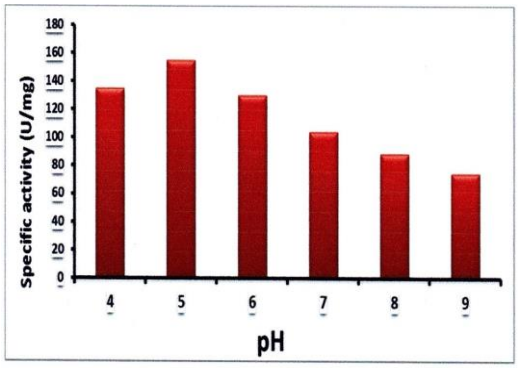

Fig. (4): The Effect of pH on inulinase production from local isolate *A. niger* AN20,using sugar cane, with 5:5 moisture, incubation for 4 days at 30°C.

Incubation temperature: The culture which consist of the medium(sugar cane, corn steep liquor), with pH 5.0, inoculated with 106 spores of *A. niger* AN20 was incubated in different temperature degrees (20, 25, 30, 35, 40, 45, 50) °C to find the optimum incubation temperature for enzyme productivity. The results in Figure (5) show that the optimum incubation temperature is 30 °C which gave the specific activity of 121.5 U/mg. Lower and higher temperatures decreases the specific activities because of the thermal effects of these temperatures on the microorganism growth and on the enzymatic reaction rate inside the cells which reflects on the vital creation of the enzyme. Darija *et al*. [22] found that 30-33 °C is the optimum temperatures for inulinase production from *A. ficuum*, while Nandogobal and Kumari [23] referred to the preference of the high incubation temperature because it has advantages in increasing the solubility of inulin in addition to prevention of microbial contamination in culture medium, where they found that 50 °C is the optimum temperature for inulinase production from *A. niveus* and *Penicillium purpurogenum*.

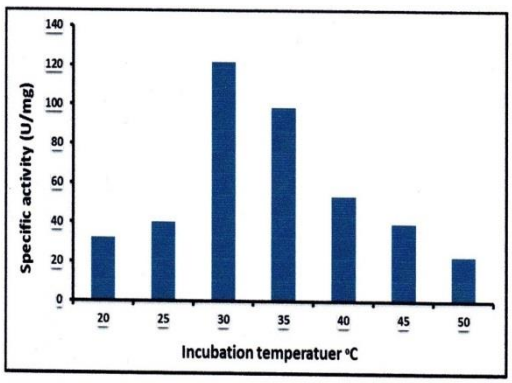

Fig.(5): The Effect of incubation temperature on inulinase production from local isolate *A. niger* AN20,using sugar cane pH 5, with 5:5 moisture, incubation for 4 days.

Temperature is one of the important parameters that determine the success of SSF system. Kheng and Omar [24] results indicated that the enzyme production corresponded closely to the growth of the fungus, and the optimum temperature for inulinase production from *A. niger* is similar to the optimum temperature for the growth of the fungus. This observation was in agreement with those reported by Sudgen and Bhat, and Biswas *et al*.

[25, 26], who showed that the highest inulinase activities were obtained at temperatures that were optimum for the growth of the fungi in solid-state fermentation.

Incubation period:

The results in figure (6) show the effect of incubation period 24-120 hrs. on inulinase production from *A. niger* AN20. The highest specific activity was at 4 days of incubation 140.5 U/mg and then became 76.3 U/mg after 5 days of incubation. These results corresponds to the studies of Kango, and Gouda [15, 21] who found that 4 days is the optimum incubation period for inulinase production from *A. niger* and *A. fumigatus*.

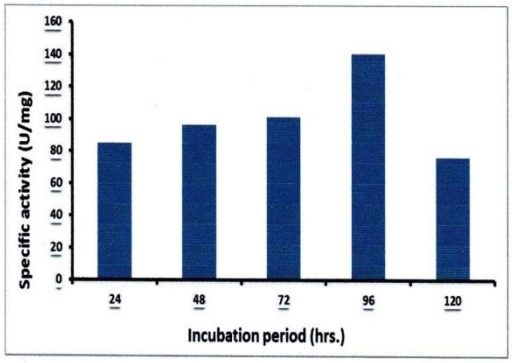

Fig. (6): The Effect of incubation period on inulinase production from local isolate *A. niger* AN20,using sugar cane pH 5, with 5:5 moisture, incubation at 30°C.

The specific activity of enzyme was decreased after 96 hrs. of incubation (figure 6).

The enzyme production decrease after 96 hrs. of incubation is due to The production of reducing sugar such as glucose and fructose in culture medium which may lead to repression of inulinase production because these sugars are more readily carbon source than inulin (Vandamme and Derycke,) [27]. This decrease in enzyme production occurred as a result of the reduce in nutrients of the medium and as a result of accumulation the catabolic repression of enzyme (Kheng and Omar,)[24].

The Effect of mixed culture *A. niger* AN20 and *K. marxianus* on inulinase production:

To examine the inulinase productivity of *A. niger* AN20 and *K. marxianus*, they were mixed together according to the method described by Ongen-Baysal and Sukan [10]. The results are illustrated in figure [7], and shows that *niger* AN20 culture gave the highest specific activity of 153U/mg, while mixed culture gave 141 U/mg, and *K. marxianus* culture gives 86 U/mg. This result of *A. niger* culture agrees with Ertan *et al* [1].

It is known that the synthesis of inulinase are controlled by the same mechanism in both organisms but the specific growth rate of organisms are found to be different with *K. marxianus* (0.53 hr^{-1}) and *A. niger* (0.11 hr^{-1}). Thus both organisms compete for the same carbon source. Therefore inoculums size and time of addition *K. marxianus* might be critical factor in the optimization of inulinase production by mixed culture (Ongen-Baysal and Sukan,)[10]. Sukan *et al* [28] found in his study on hydrolysis of *jerusalim artichoke* extract by inulinase from monocultures and mixed cultures of *A. niger* AN20 and *K. marxianus that A. niger* alone gave specific activity 122 U/mg, *K. marxianus 50* U/mg, while mixed culture gave 83 U/mg.

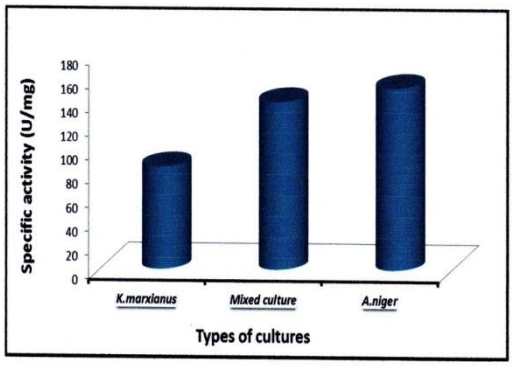

Fig. (7): Inulinase productivity from *A. niger* AN20, *K. marxianus* and mixed culture *A. niger* AN20 + *K. marxianus*, using sugar cane, with 5:5 moisture, incubation for 4 days at 30°C at pH 5.

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تحديد الظروف المثلى لأنتاج أنزيم الأنيولينيز من فطر *Aspergillus niger* بوساطة تخمرات الحالة الصلبة

زينب وليد عبد الأمير **بهاء نظام عيسى*** **غازي منعم عزيز**

قسم التقنيات الأحيائية- كلية العلوم/ جامعة بغداد
*قسم الشؤون العلمية/ رئاسة جامعة بغداد

الخلاصة:
غربلت قابلية ثلاثون عزلة محلية من الفطر *Aspergillus niger* على انتاج انزيم الأنيولينيز بأستعمال الوسط التركيبي الصلب اعتمادا على تحلل الأنيولين وظهور المناطق الشفافة حول المستعمرات الفطرية قيد الدراسة. نشطت جميع العزلات بأستمرار كل اسبوعين وحفظت في 30 ^{5}م، غربلت هذه العزلات بطريقة شبه كمية لأختبار العزلة الأكثر كفاءة في انتاج أنزيم الأنيولينيز، أختبرت العزلة الأكثر أنتاجية للأنزيم وأعطيت الرمز *A. niger* AN20. حددت الظروف المثلى لأنتاج الأنزيم من العزلة *A. niger* AN20 بأستعمال الوسط الغذائي المتكون من قصب السكر المرطب بمستخلص نقيع الذرة بنسبة 5:5 (V/W) برقم هيدروجيني 5 عند درجة 30 ^{5}م لمدة 96 ساعة. أختبرت انتاجية الأنزيم لكل من الخميرة *Kluyveromyces marxianus* والعفن *A. niger* AN20 والمزارع المختلطة من الخميرة والعفن، فوجد تفوق العزلة *A. niger* AN20 في انتاج الأنزيم بفعالية نوعية مقدارها 153 وحدة/ملغم بروتين مقارنة مع استعمال الخميرة حيث أعطت 86 وحدة/ملغم بروتين.

الكلمات المفتاحية: الأنيولينيز ، *Aspergillus niger*. *Kluyveromyces marxianus*