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Fungal Xylanase production using agricultural and industrial wastes

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Abstract

Lignocellulosic biomass is the most plentiful of all the naturally occurring organic compounds. Conversion of cellulosic polymers into useful products by fermentation involves two stages: firstly conversion of cellulose to glucose by cellulase, and secondly microbial conversion of the resulting glucose to products. The experiments were conducted to obtain xylanase enzyme from the *Aspergillus niger* strain *AUMC 14230* by using corn cob (1%) as main component in fermentation media. The experiments were carried out in the laboratory of Soil, Water and Environmental Institute, Giza Egypt. To achieve this target. The best carbon source in this study is corn cob 1% concentration. The ideal nitrogen source is corn steep liquor (CSL) 3% concentration. The highest production of the enzyme was obtained when we were used modified medium (corn cob 1%, wheat bran1% and CSL 3%). The ideal environmental conditions to give highest production from both enzymes was found as: inoculum size 0.50% (v/ml), initial pH 4.5, aeration 1:5 (V_m:V_f), incubation temperature 50^{o}C, agitation rate 175 rpm and time course 72 hr.

KEYWORDS: Fungal Xylanase, agricultural and industrial wastes,

Aspergillus niger strain

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1. INTRODUCTION:

Enzymes are extensively used in various industries viz., food, feed, pharmaceutical, textile, paper and pulp bleaching etc. With increase in demand for constant innovation towards developing and adopting clean and green technologies, use of microbial enzymes assumes paramount significance. Enzymatic processes help in production of desired and specific products and thus the issues pertaining to undesired by products can be easily circumvented. Apart from being eco-friendly, enzymatic processes results in better product quality at reduced manufacturing cost with less waste generation and would require less per capita energy consumption. In contrast, conventional synthetic processes would result in large amounts of undesirable by product posing challenges in their separation or disposal. Microbial enzymatic activity can be tuned to suit any given process by careful manipulation of external variables such as enzyme dose, temperature etc. Enzymes being biocatalysts and specific in their action, the amount of enzyme used are relatively very less. Among various microbial enzymes xylanase is one which has wide variety of industrial applications including the degradation of polymeric xylan to biofuels and industrially important chemicals **(Pellerin et al., 1981).** Xylooligosaccharides obtained through the degradation of polymeric xylan by xylanases are used in food and feed applications **(Wang et al., 2014).** Furthermore, industries need low cost enzymes. Hence, increase of expression level and the competent production of xylanases are vital to bulk production at lower cost. Given this scenario, strain improvement using genetic engineering tools play a significant role in mass production of xylanases with preferred properties. Though xylanases constitute most of the commercially sold hemicellulases, it accounts only a small fraction of the total enzyme sale. With increase in attention to the potential use of these enzyme in different industrial processes, it is expected that sale of these enzymes will increase in future **(Sunna and Antranikian, 1997).** The less common and recognized application includes its use in brewing and detergent, preparation of coffee, antimicrobial agent production and antioxidant, rayon, cellulose ether and cellulose esters **(wong *et al.*, 1988, Katapodis *et al.*, 2002, Subramaniyan, S.; and Prema, P. 2002, Christakopoulos *et al.*, 2003, Qiu *et al.*, 2010 and , Gowdhaman and Ponnusami, 2019).**

The present work reports some prerequisites for production of high yields of xylanase from *Aspergillus niger* strain *AUMC 14230* and some factors affected on enzyme production are studied.

2. MATERIALS AND METHODS:

2.1. Raw materials

Raw materials used in this study were obtained locally. Corn steep liquor (CSL), protolan and corn gluten (by product of corn starch industry) were obtained from Egypt Starch and Glucose at Torah. Bagasse (by product of sugar industry) was obtained from sugar and Integrated Industries Company at Giza. Rice bran was supplied by the Experimental Station of Rice Breeding, Agriculture Res. Center at Giza. Corn Stalk and corn cobs were obtained from the area of

Fayoum Government. Raw waste materials were washed with tap water; air dried and used subsequently as a sole carbon and nitrogen sources.

2.2. 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.

2.3. Isolation, screening and identification

Cellulolytic fungi were isolated from a wide variety of sources from soil, organic matter and infected rice straw. Isolated fungi were inoculated on solubilized crystalline cellulose (CC) plates and CMC plates to cultivate for two weeks **(Deguchi *et al.*, 2007).** The microbes that could grow on CC and CMC were picked up corck poorer and inoculated onto malt extract agar (MEA). The plates were incubated at 30^{o}C for 3 days. The morphologically different colonies were inoculated into 50ml of the growth medium containing (gl^{-1}) peptone 8, yeast extract 2, K_2HPO_4 5 , $MgSO_4.7H_2O$ 3, and cellulose 20 (Sigma), and cultivated at 28^{o}C with agitation at 200 rpm for 5 days. Xylanase activity of the culture broth were analyzed using birchwood xylan (Sigma, St. Louis, MO) as substrate as described previously **(Ahmed *et al.*, 2016).**

2.4. Molecular identification of fungal isolate:

The fungal isolates cultured on Czapek`s yeast Extract agar (CYA) medium and incubated at 28°C for 5 days. It was sent to the Molecular Biology Research Unit, Assiut University for extraction of genomic DNA using Patho-gene-spin DNA/RNA extraction kit (Intron Biotechnology Company, Korea). The fungal DNA was then sent to SolGent Company, South Korea for PCR and gene sequencing using ITS1 (forward) and ITS4 (reverse) primers.

2.5. 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.

2.6. 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.

3. RESULTS AND DISCUSSION:

3.1. Isolation and screening of xylanolytic strains and the media used in isolation

In order to select the most efficient xylanolytic isolates, an experiment was run to compare xylanolytic activity between these ten local isolates (2, 3, 6, 10, 12, 26, 57, 60, 61 and 62) by the submerged technique using 3 different fermentation media. Incubation was done using rotary incubated shaker (150 rpm) at 30^{o}C for 6 days. The results obtained in Figure (1)

concluded that *Aspergillus niger* strain *AUMC 14230* gave the higher productivity of xylanase enzyme (98.5 Uml^{-1}). These results are in agreement with many researches which used *Aspergillus niger* to produce xylanase enzyme (**Abdul Wahab *et al.*, 2016, Bajaj and Abbass 2011, Bhardwaj *et al.*, 2019, Das and Ray 2016**, **Sorgatto *et al.*, 2012 and Yuan *et al.*, 2005**). In this connection **Pirota *et al.*, (2013)** reported that *Aspergillus oryza* is considered most promising fungi for xylanase production. In contrast, *Trichoderma reesei* was the most suitable fungi for produced xylanase according to **(Suh *et al.*, 1988; Merivuori *et al.*, 1990 and Haltrich *et al.*, 1992)**.

3.2. Identification of the strain isolate and Phylogenetic analysis:

As shown in Fig. (2 and 3) the fungal strain recovered in the current study (*Aspergillus niger* strain AUMC 14230) (FAY3) showed 100% similarity with the type strain of *Aspergillus niger* ATCC 57360 (GenBank accession No. NR3168) recorded in USA. It also showed 100% identity with several strains of *A. niger* isolated from mango fruit sugar syrup in India, cosmetic and Branches of *Malus sieversii* in China, soil in Egypt, 19th century art lamina in Costa Rica, Sponge *Cinachyrella sp* in Viet Nam clinical sample in Oman as well as from unspecified source in Nigeria, Netherlands and USA.

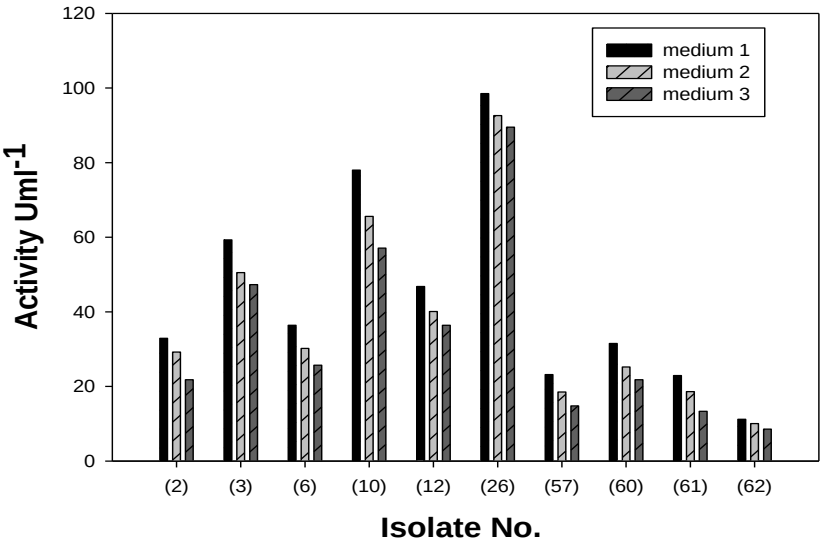

Figure (1) Selecting the most producible cellulolytic isolates on different media using shake flasks in a batch culture technique.

ITS sequences of *Aspergillus niger* (AUMC14230)

CTGCGGAAGGATCATTACCGAGTGCGGGTCCTTTGGGCCCAACCTCCCATC
CGTGTCTATTGTACCCTGTTGCTTCGGCGGGCCCGCCGCTTGTCGGCCGCC
GGGGGGGCGCCTCTGCCCCCCGGGCCCGTGCCCGCCGGAGACCCCAACAC
GAACACTGTCTGAAAGCGTGCAGTCTGAGTTGATTGAATGCAATCAGTTAA
AACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGA
AATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTG
AACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTC
ATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGTCGCCGTCCCCCTCTCCG

GGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCG
TATGGGGCTTTGTCACATGCTCTGTAGGATTGGCCGGCGCCTGCCGACGTT
TTCCAACCATTCTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTG
AACTTAAGCATATCAATAAGCGGAGG

Figure (2) Sequencing results for (FAY3)

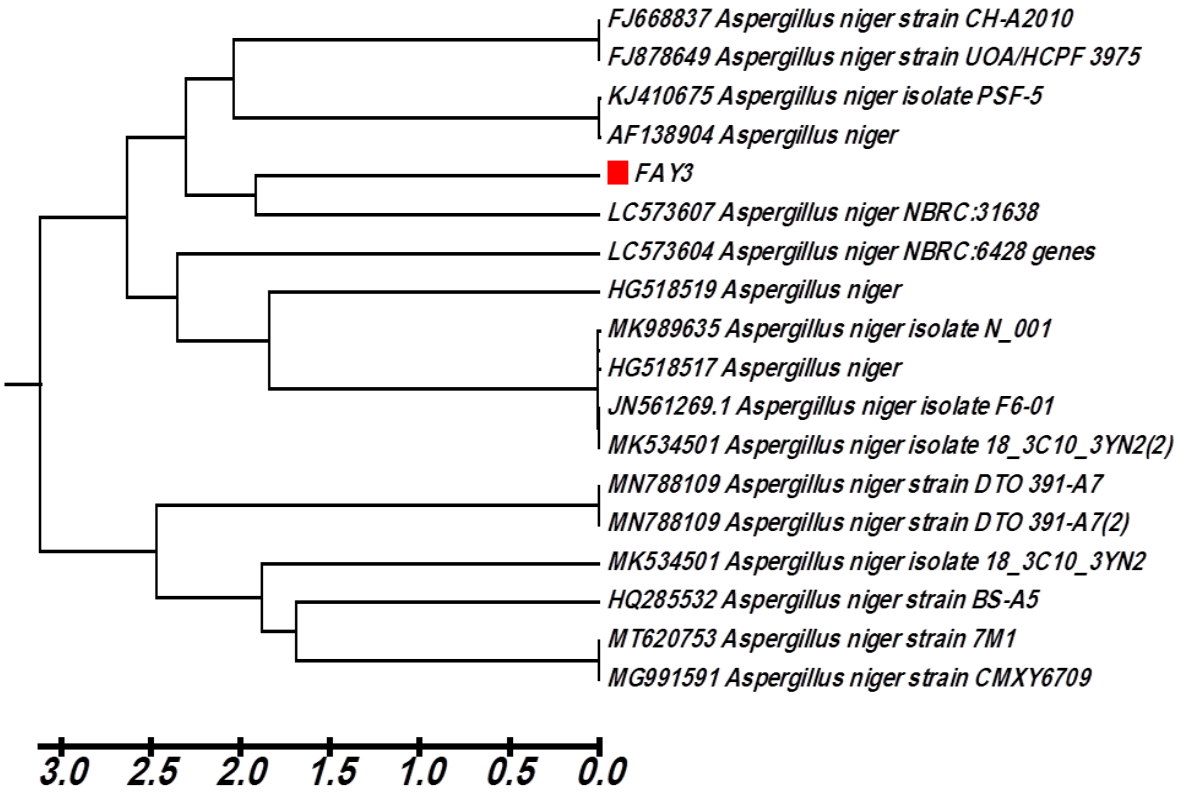

Figure (3): Phylogenetic tree based on ITS sequences of rDNA of Aspergillus niger (AUMC 14230) isolated in the present study aligned with closely related sequences accessed from the GenBank. Aspergillus fumigatus is included in the tree as an out-group strain. Strain No. and GenBank accession No. is indicated opposite to each fungal strain.

3.3. Comparing between physically treated and chemically treated raw materials on production of xylanase enzyme.

The results presented in Figure (4) revealed that among agriculture residues the imported one was physically treated corn cob which gave higher xylanase production with activity (100 Uml^{-1}) followed by physically treated wheat bran by activity (84 Uml^{-1}). While the treated residues gave lowest activity (87 Uml^{-1} and 72 Uml^{-1}) for corn cob and wheat bran respectively. The results obtained may be due to Chemical treatment remaining acid or alkali affects the activity of the enzyme, while grinding increases the surface area and decreasing crystallinity and thus enhancing the hydrolysis efficiency leads to the higher xylanase activity. Also, these results may be referred to saving the coast of chemical pretreatment step as an economic neutralization. Our results are agreement with **(Kadowaki *et al.*,**

1997, puchart *et al.*, 1999, and Ahmad *et al.*, 2016).

3.4. Effect of corn cob concentrations on xylanase production.

The results in Figure (5) concluded that the activity of xylanase enzyme from *Aspergillus niger* strain *AUMC 14230* decreased by increasing the concentration of corn cob. The highest activities were obtained at 1% corn cob (95 Uml-1) while the lowest activity was obtained at 5% (35 Uml^{-1}). These results reflected that the high concentration of corn cob depressed the synthesis of the enzyme with feedback inhibition. These results are in the same trend with those reported by **(Kadowaki *et al.*, 1997, Puchart *et al.*, 1999, sahare *et al.*, 2012, Su *et al.*, 2011, Ahmed *et al.*, 2011, Das and ray 2016 and Boonchuay *et al.*, 2016).**

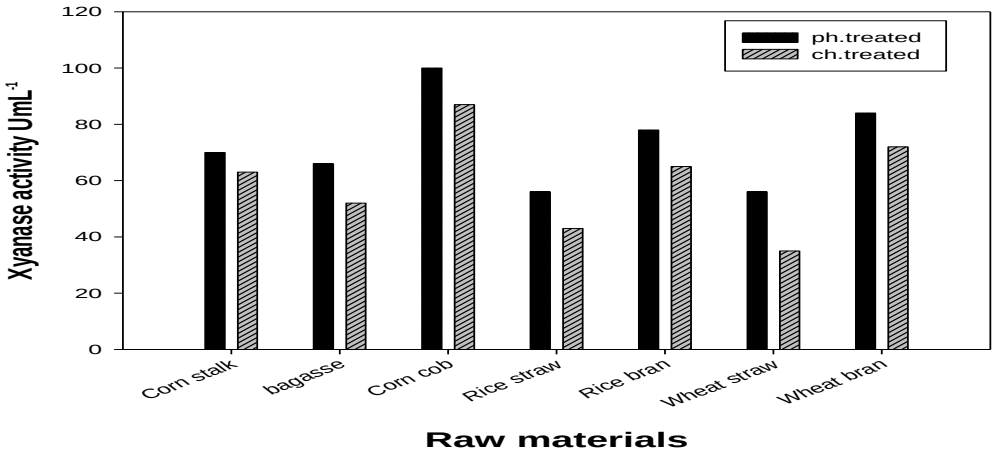

Figure (4) Comparing between physically and chemically treated raw materials on production of xylanase activity from *Aspergillus niger.*

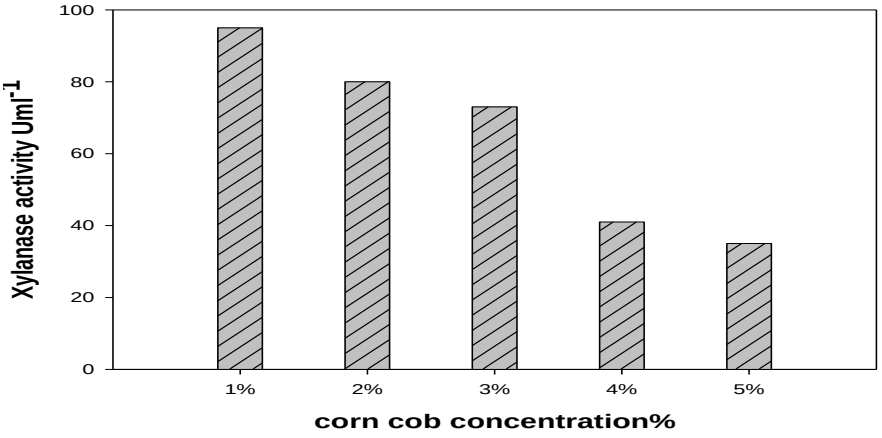

Figure (5) Effect of corn cob concentrations on xylanase production by *Aspergillus niger.*

3.5. Effect of nutritional requirements

3.5.1. Carbon sources

3.5.1.1 Effect of chemical carbon sources

The results in Fig. (6) referred that the chemical carbon sources used in the media have a very important concern and the production of xylanase varied to organism sp. So the quantity of this production depends on the carbon source used in the medium. It could be concluded that the disaccharide (lactose) as carbon source when added to the media produced the higher concentration of xylanase for *Aspergillus niger* strain *AUMC 14230* used, followed by the mono saccharide (glucose) whereas, sorbitol and manitol (Alcohols) gave lowest concentration of xylanase for this strain. In a previous study, in which xylan was the only carbon source in the production medium, approximately the same activity was observed at the end of day 13. When different carbon sources (sucrose, maltose and lactose) were then added separately to the media to determine their effects on xylanase production maximum activity was observed for media containing sucrose **(Seyis and Aksoz, 2003 and 2005)**. Whereas **Das and Ray 2016** reviewed that only xylan was capable of increasing xylanase production. Sorbitol causes a marginal increase in enzyme production. It can be inferred that since xylanase production is induced by the presence of xylan, it is an inducible type of enzyme. In contrast, **Irfan *et al* (2014)** had reported xylose to be the most suitable inducer.

3.5.1.2. Effect of raw materials as carbon sources

The results obtained revealed that many agriculture residues (1% concentration) may be used as an effective carbon source when added to the medium and gave high production activities for xylanase enzyme. Our results in Figure (7) indicate that adding corn cob as carbon source to the basal medium produced the highest value of xylanase activity for *Aspergillus niger strain AUMC 14230* followed by wheat bran and rice bran. But using corn stalk and rice straw decreased the activity for the same *Aspergillus* strain. These results may be due to the chemical composition, physiochemical properties, fats, vitamins and minerals of corn cob give it a very important role to produce high activity from xylanase as described at the research on corn cob by **Ashour *et al.*, 2013**. Our results are in agreement with the results of **Kadowaki, et al., (1997)** Who reported that the maximum production of xylanase (285-350 U/mL) was obtained when *Aspergillus tamarii* was grown on media containing 5-8% (w/v) corn cob after 5 d of incubation. as the nitrogen source. They observed maximum xylanase production when ammonium sulphate was used as the sole nitrogen source. On contrary, Sodium nitrate, one inexpensive nitrogen source, gave the best results and it was used in subsequent experiments (**Katapodis *et al.*, 2007**, **Boonrung *et al.*, 2014 and Ahmed *et al.*, 2016)**.

3.5.2.2 Effect of a raw materials as nitrogen sources

Our results presented in Figure (9 and 10) show that the raw materials used as nitrogen source for producing xylanase by *Aspergillus niger* strain *AUMC 14230* were zea gluten, protolan, protovene and corn steep liquor were

compared with ammonium nitrate as control. It could be revealed that the corn steep liquor (CSL) as nitrogen source applied to the media produced the highest activities of both enzymes by the two fungi strains when compared with ammonium nitrate followed by zea gluten. These compounds are belonged to organic compounds and each nitrogen source was added separately to the medium instead of ammonium nitrate.

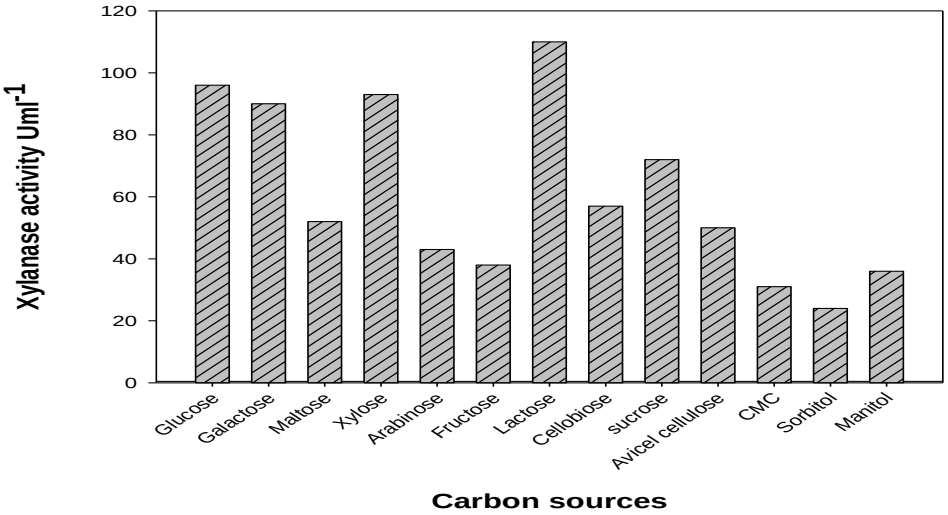

Figure (6) Production of xylanase using different agriculture residues as carbon sources by *Aspergillus niger*.

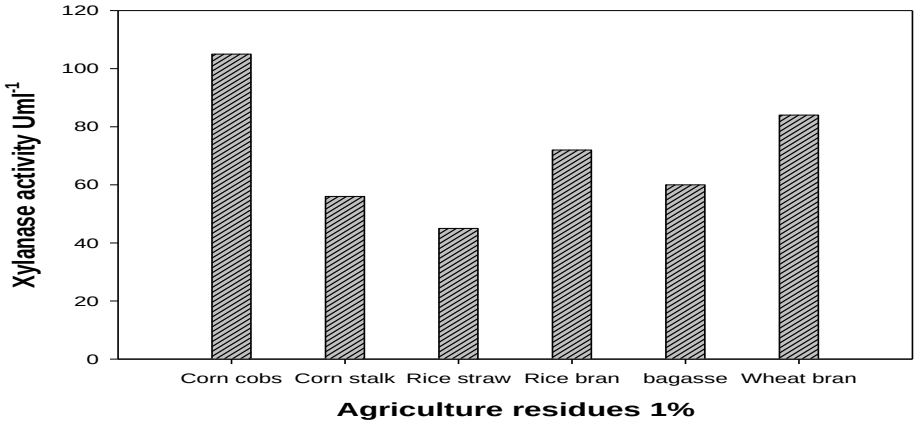

Figure (7) Production of xylanase using different agriculture residues as carbon sources by *Aspergillus niger*.

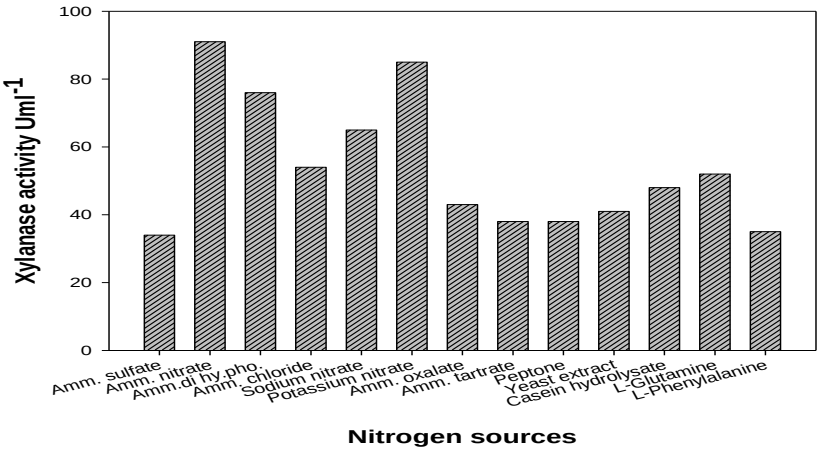

Figure (8) Effect of different chemical Nitrogen sources on xylanase enzyme production by *Aspergillus niger*.

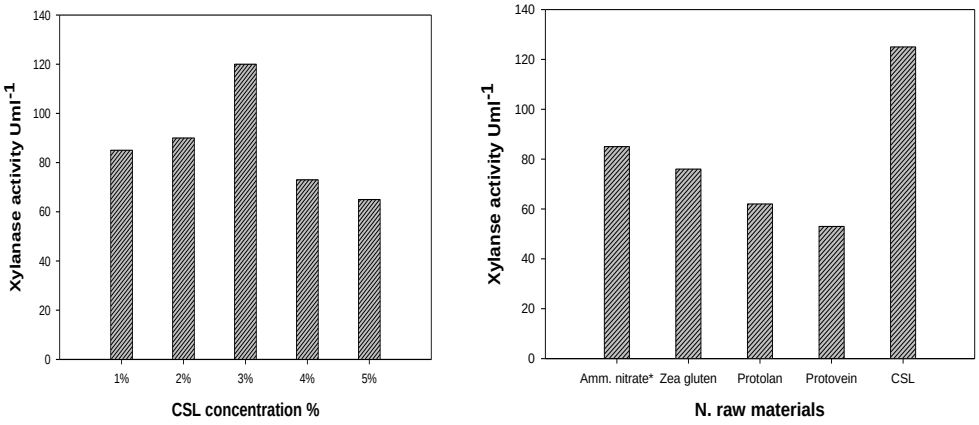

Figure (9) Effect of Corn steep liquor (CSL) concentrations, Figure (10) Effect of different raw materials as a nitrogen on xylanase enzyme production by Aspergillus niger source on xylanase enzyme production.

3.6. Environmental conditions

3.6.1. Effect of inoculum size

The results of our study in Figure (11) revealed that the preferable inoculum size for the high production of xylanase enzyme for *Aspergillus* strain tested was 1.0 ml (95 Uml^{-1}). While an inoculum volume beyond 5% decreased xylanase production. **Irfan *et al.*, (2014)** pointed that 10% inoculums volume as optimum with *Trichoderma viride – IR05*. But **Sanghi *et al.*, in 2008** reported that use of 15 % inoculum was the optimum with *B. subtilis ASH* using solid state fermentation.

3.6.2. Effect of initial pH

Figure (12) showed that control of pH value is an important factor in xylanase production. Previous studies reported that greatest enzymes production is achieved when the pH value drops from an initial value of about 4.5 to a more or less constant value of 3.5 in the

course of the fermentation. Our results were agreement with those founded by (**Smith and Wood, 1991, Polizeli *et al.*, 2005, Katapodis *et al.*, 2007, Sorgatto *et al.* 2012, Kaushik *et al.*, 2014 Das and Ray 2016 and Ahmed *et al.*, 2016)**.

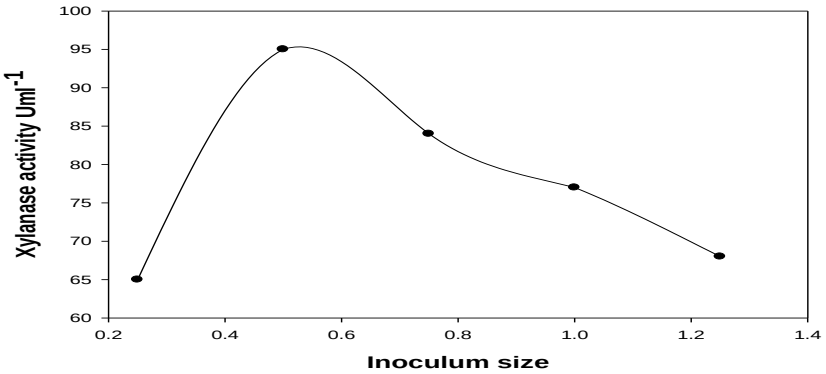

Figure (11) Effect of inoculum size on biosynthesis of enzymes by *Aspergillus niger*.

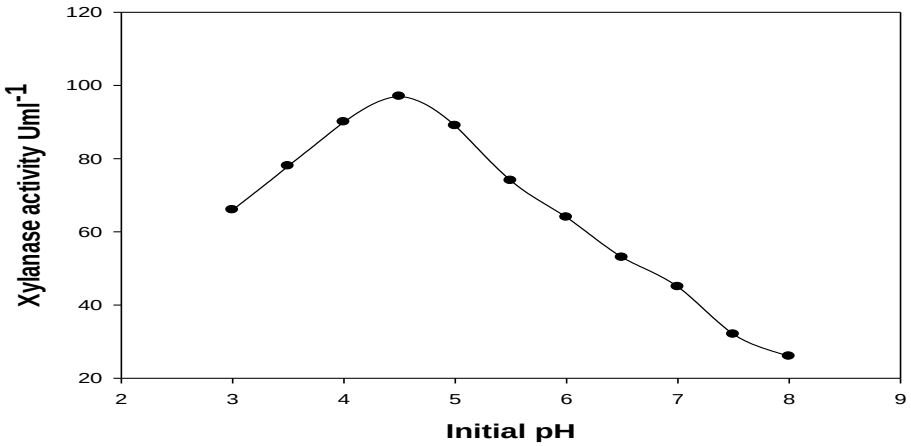

Figure (12) Effect of initial pH on xylanase enzyme production by *Aspergillus niger*

3.6.3. Effect of aeration

Our results at Figure (13) concluded that the maximum production of xylanase was obtained at the aeration rate 1:5 (20ml) for the tested fungi. A positive relationship between the aeration and enzymes activity was observed. These results are in agreement with those obtained by **(Robison, 1984, Purkarthofer *et al.*, 1993, Hoq *et al.* 1994 , Haltrich *et al.*, 1996, Khasin *et al.*, 1993, Adsul *et al.*, 2004, Yuan *et al.*, 2005, Okafor *et al.*, 2007, Uday *et al.*, 2017 and Walia *et al.*, 2017)** who used aeration rate 1:5, while these results are disagreement with those obtained by **Khasin *et al.*, (1993)** who found that the xylanase production by different *Aspergillus* strains using Erlenmyer flasks (125ml) containing 50ml of culture medium at rate $V_m : V_f$ (1:2.5).

3.6.2. Effect of initial pH

Figure (12) showed that control of pH value is an important factor in xylanase

production. Previous studies reported that greatest enzymes production is achieved when the pH value drops from an initial value of about 4.5 to a more or less constant value of 3.5 in the course of the fermentation. Our results were agreement with those founded by (**Smith and Wood, 1991, Polizeli *et al.*, 2005, Katapodis *et al.*, 2007, Sorgatto *et al.* 2012, Kaushik *et al.*, 2014 Das and Ray 2016 and Ahmed *et al.*, 2016)**.

3.6.4. Effect of incubation temperature

Results of Fig (14) indicated that the optimum temperature for incubation the *A. niger* to produce the highest activities of xylanase was 50^{o}C. Increasing incubation temperature above 60^{o}C resulted in a sharp reduction in the xylanase produced by the same strain. Also, the temperature less than 30^{o}C gave reduction activities for the enzyme. It could be said that the decrease of xylanase activity at 20^{o}C may be due to the weak growth of *A. niger* at this temperature, while the decrease in enzyme activity at 80^{o}C may be attributed to the denaturation effect on protin at this temperature. These results are in agreement with those obtained by **(Katapodis *et al.*, 2007, Santiago-Herna´ndez *et al.*, 2007, Subramaniyan *et al.*, 2012, Sorgatto *et al.*, 2012, Kaushik *et al.*, 2014, Abdul Wahab *et al.*, 2016**, **Boonchuay *et al.*, 2016**, **Das and Ray 2016, Walia *et al.*, 2017**, **Bedade *et al.*, 2017 and Mehnati-Najafabadi *et al.*, 2018**).

3.6.5. Effect of agitation rate

Agitation is an important process for producing maximum yield of the enzymes at ideal conditions. It can be noticed at Figure (15) that as the agitation rate increased from 0.750 to 200 rpm the xylanase enzyme activities increased to the maximum values for the *Aspergillus* strain at 175rpm rate. Our study showed that no differences were obtained between 125rpm and 200rpm in enzyme production. These results mean that submerged fermentation increased the biosynthesis on these enzymes with much more than static culture up to 200rpm. 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**).

3.6.6. Effect of time course

It can be noticed from the results in Figure (16) that increasing time course from 24hrs to 120hrs gave pronounced increase in xylanase enzyme resulted from *A. niger* .Whereas, increasing time course than 120hrs gave small decrease in the production of enzyme by the same fungal strain. From the stand point of economic courses we can use the time course 120hrs for producing the higher amounts of xylanase enzyme from *A. niger*. The decrement of enzyme activities after 120hrs may be due to catabolic repression by glucose **Alani *et al.*, (2008)**. This decline can be caused by the presence of accumulated hydrolysis products resulting in catabolite repression of enzyme production at higher substrate concentration (**Kadowaki, *et al.*, 1997).** The similar nature catabolic repression was observed in various fungi **Yuan *et al.*, (2005).**Our results are in accordance with those obtained by **Katapodis *et al.*, (2007)**.

Conclusion:

With regard to optimization of xylanase production by both Aspergillus niger strain, agro-industerial waste corn cob, corn steep liquor, which are inexpensive and abundant, were found

the best. Economically, corn cob is for xylanase production. cheaper than pure xylan as a substrate

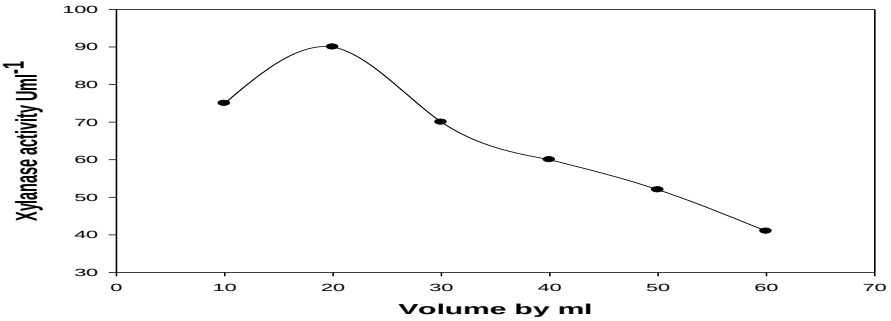

Figure (13) Effect of aeration on xylanase production by *Aspergillus niger*

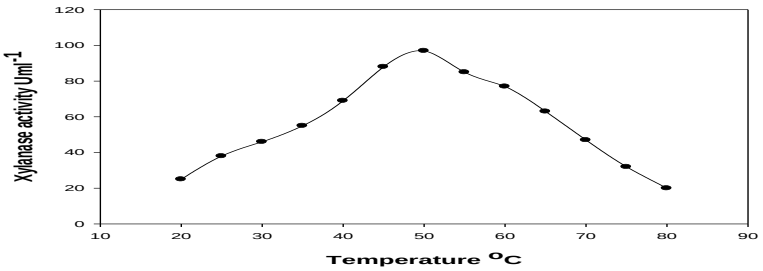

Figure (14) Effect of incubation temperature on xylanase production.

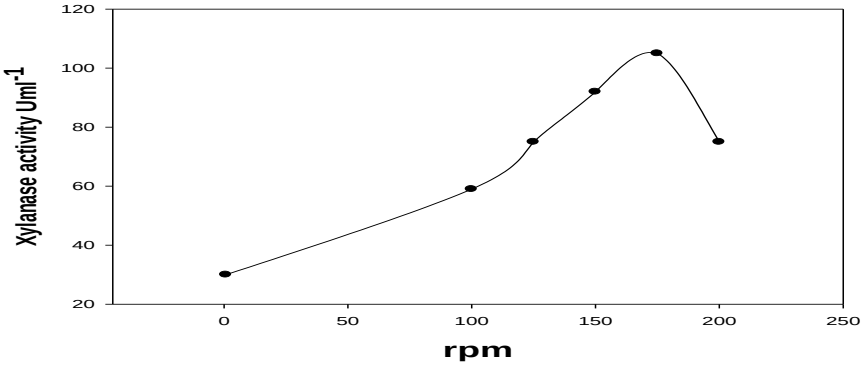

Figure (15) Effect of agitation rate on xylanase production.

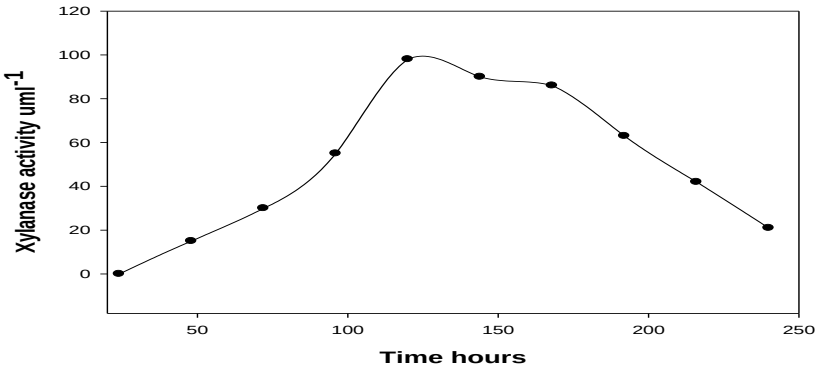

Figure (16) Time course of synthesis of xylanase from *Aspergillus niger.*

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الملخص العربي

استخدام المخلفات الزراعية في انتاج انزيم الزيلانيز بواسطة الفطريات

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المخلفات الزراعيه تعتبر كتله سليولوزية مهمه جدا. لتحويل هذه المركبات الى مركبات بسيطة يتطلب ذللك المرور بمرحلتين: اولا: تحويل الزيلان والسليولوز الى جلوكوز عن طريق انزيم الزيلانيز. ثانيا: التحويل الميكروبي لانتاج منتجات صناعية من الجلوكوز.
في هذه الدراسه تم انتاج انزيم الزيلانيز من سلالة الاسبرجيلس نيجر ، باستخدام مطحون اكواز الذرة اساسي للبيئه.
وهذه التجارب تمت في معمل الميكروبيولوجيا الزراعية بمعهد الاراضي والمياه والبيئه بمركز البحوث الزراعيه بالجيزة.
وقد اوضحت الدراسه النتائج التالية:
الطحن الناعم لاكواز الذرة. من خلال (١٠) عزلات فطريه تم اختيار العزله رقم (٢٦) لانتاجها اعلى نشاط انزيمي بالاضافة الى العزله الاجنبيه الموجوده الاسبرجيلس نيجر.احسن مصدر كربوني هو مطحون اكواز الذرة. وجد أن مطحون اكواز الذرة بنسبة ١%ادى الى الحصول على كمية عالية من الأنزيم. الكورن استيب ليكر هو احسن مصدر نيتروجيني في البيئة لانتاج الانزيم.اعلى انتاج انزيمي تم في بيئة تتكون من ١% جنين القمح ، و ٣% كورن استيب ليكر ، و ٢% مطحون اكواز الذرة ، وهذه هي البيئة الأمثل. العوامل البيئية المثلى لانتاج الانزيم وجدت كالتالي : حجم اللقاح ٠,٥٠% - الاس الهيدروجيني ٤,٥ – درجة حرارة التحضين ٥٠ درجة مئوية – درجة التهوية ٢٠ – عدد اللفات ١٧٥ أر.بي.أم.