Enhanced production of xylanase enzyme by Fusariummoniliforme using submerged fermentation

Th. H. Shaltout[2] , Z. H. Kheiralla[1], N. Sh. El-Gendy[2], H. A. Ahmed[1], and M. M. D. Hussein[3]

[1]Botany Department,Faculty of Women for Arts, Science and Education, Ai Shams University.

[2]Petroleum Biotechnology lab, Department of Processes Design & Development,Egyptian Petroleum Research Institute, Nasr City, Cairo, Egypt.

[3] Chemistry of Natural and Microbial Products Department, National Research Center, Dokki, Giza, Egypt.

Abstract:

Hemicellulose degrading enzymes play an important role in bioconversion of agro and agro-industrial wastes. In this study, production of hemicellulase by six fungal isolates was determined under submerged culture using corn cobs xylan as a carbon source and enzyme inducer at different incubation periods. The results indicated that *Alternariatenuis*showed the lowest enzyme productivity (156.95 ± 2.07U/l)while the highest enzyme production (2,594.44 ± 62.25U/l) was found by *Fusariummoniliforme*. One-factor-at-a-time (OFAT) revealed maximum enzyme productivity of 10,950.11 ± 98.45 U/l at; corn cobs xylan (6g/l), yeast extract (4g/l), inorganic salts ($MgSO_4$, KH_2PO_4, $CaCl_2$, $FeSO_4$, and $MnSO_4$), initial pH (5), initial inoculum size (4%), 150 rpm and temperature (30℃).

Key words: Xylanase;*Fusariummoniliforme;*submerged fermentation;corn cob xylan

***Corresponding author**: Micro_1a@yahoo.com

1. Introduction:

Enzymes are used to produce a wide range of biotechnologicalproducts utilized by the food, chemical, and allied industries andhave been already recognized as valuable catalysts for productionof fine chemicals and pharmaceuticals and several organic transformations[**1].**Hemicellulose is a branched hetero-polymer consisting of pentose (d-xylose and d-arabinose) and hexose (d-mannose, d-glucoseand d-galactose) sugars where xylose is most abundant. Hemicellulose is the one of the most abundant second renewable biomassin nature after cellulose, which accounts for 25–35% of lignocellulosic biomass [**2]**. The most abundant hemicellulose in natureis xylan which contains mainly -d-xylopyranosyl residues linkedby β-1, 4-glycosidic bonds [**3, 4]**.

In plants, an overlying layer ofxylan and cellulose forms through hydrogen bonding which is further covalently linked with lignin leads to formation of an outsidesheath for the protection of the plant. Xylan accounts for 30–35% of total dry weight, however, the exact amount of the xylandifferefrom plants to plants which display a large variation in composition during extraction from different sources [**4, 5]**.

Hemicelluloses degradation needs the hemicellulytic enzymes. These enzymes have wide range of applications such as utilization of lignocellulosic substances to cellulosic bioethanol, clarification of juice and animal feed, in paper industry, etc. [**2]**. These enzymes are produced by a large number of microorganisms such as bacteria, actinomyces, and fungi, but fungi have a great interest because they excrete their enzymes extracellularly[**6]**.

Xylan, with its β-1, 4-linked polyxylose backbone branched with other pentoses, hexoses and uronic acids, is the main component of hemicellulose. **[7,8].** Among the xylanolytic enzymes, endo-1, 4-β-xylanases (play important roles during hydrolysis of xylan, as they can cleave β-1, 4-linked xylose backbone of xylan[**2]**.Filamentous fungi are particularlyremarkable producers of xylanases from an industrial point of view, owing to the fact that they secrete xylan-degrading enzymes into the medium, thus avoiding cell disruption. Furthermore, the xylanase levels obtained from fungal cultures are typically much higher than those obtained from yeast or bacteria. In addition, fungi typically produce several auxiliary enzymes that are necessary

for debranching of the substituted xylans**[9].**Mostly the production of xylanases has been studied in submerged liquid culture but there are few reports concerning the xylanase production in solid state fermentation using lignocellulosic wastes. Submerged fermentation process is mostly preferable because of more nutrients availability, sufficient oxygen supply and less timerequired for the fermentation than other fermentation techniques.

The present work aims to optimizexylanaseproductivity by*F.moniliforme* using submerged fermentation.

2. Materials and methods

2.1. Fungal strains:

The hemicellulolytic activities of six fungal isolates were investigated throughtout the present work.*Alternariatenuis, F. moiliforme, F. oxysporum* were isolated from old deteriorated valuable manuscript(Al Sultan Malak, library No.1405)present in the Stores of General Egyptian Book Organization(G.E.B.O).Cairo, Egypt .*Mucormucedo ,RhizoctoniaSolani, Rhizopusoryza*were isolated from rhizosphere of local tomato plant . Isolation and identification of these fungi were achieved by Dr.A.F.Sahab, Plant protection Lab, Plant Pathology department, National Research Centre. The fungal strains were grown on potato dextrose agar medium (PDA).Slants were incubated at 30℃ for 7 days and stored in a refrigerator at 4℃ .

2.2. Inoculum preparation:

This was done by adding 10ml normal saline (0.9gNaCl/100ml) to each 7 days old cultureand scratched with sterile needle.This spore suspension was used as inoculums. The tube was shacked to make homogenous.

2.3. Fermentation technique:

Cultivation was carried out in 250 ml Erlenmeyer flasks, each containing 25 ml of the fermentation medium**[10]. ,** it containedKH_2PO_4(1.5g/l) $MgSo_4.7H_2O$(1.0g/l),$CaCl_2$(0.2g/l),$FeSO_4.7H_2O$(0.4g/l), MnSO4.5H2O (0.3g/l), yeastextract (2g/l). The pH was adjusted by adding 1N NaOH. Carbon source was corn cobs xylan and sterilized for 15 min at 121℃ (1.5 atm). The flasks were then inoculated with 1ml of fungal conidial suspension

under aseptic condition and incubated at 30℃ on a rotary shaker at 150rpm for several days. After incubation for different periods the culture broth from each flask was centrifuged in cooling centrifuge at 4000rpm for 10 minutes. The clear culture filtrate was taken in which the protein content and enzyme assays were determined. All experiments were run parallel in duplicate.

2.4.EstimationEnzymeactivity:

The diluted culture filtrate or enzyme solution (0.2ml) was added to 0.8ml of hemicellulosic suspension (2.5%) in 0.05 M citrate buffer (PH 4.8). The reaction mixture was then incubated at 50 ℃ for 30 min .The released reducing sugars were determined as xylose by Nelson's reagent according to **[11].** . Enzyme activities were determined in culture filtrate by measuring the released sugars from substrates. One unit of enzyme activities was defined as the amount of enzymes required to release 1µ mol of xylose per min under the assay conditions.

2.5. Determination of soluble protein:

Soluble protein content of the studied sample was determined by the method of **[12].**

2.6. Optimization of cultural conditions for maximum production of xylanaseby selected fungal isolates:

Various nutritional conditions like different concentration of carbon source (2-10 g/l), different nitrogen sources (yeast extract ,backer's yeast, soybean milk, corn steep liquor, urea, $NaNO_3$, $(NH4)_2SO_4$,NH4Cl), different concentration of yeast extract (1- 6 g/l), different inorganic salts($MgSO_4$,KH_2PO_4,$CaCl_2$,$FeSO_4$,$MnSO_4$)and some culture condition Such as intial pH (4.5-7) ,Inoculum size (2-10), different r.p.m. (0,150,180), different temperature (25,30,37) and some additives with different concentrations such as(Tween80(1-3ml/l), wheat bran(1-5g/l) and glucose(1-5g/l)).

2.7.Statistical analysis:

The differences in enzymes activities variables among the treatment effects were tested using F-test. In addition, After testing the data for normality, one way analysis of variance (ANOVA) was used to assess the significance of variations of enzymes activities among the

different treatment effects by using Duncan's multiple range tests at $P < 0.05$ according to SPSS software **[13].**.

3. Results

3.1. Screening of some fungal isolates for theirxylanaseactivity:

Table, 1 showed the results of the wide range of differences were observed in the hemicellulytic activities of the investigated fungal isolates. The highest hemicellulase activities 2594.44± 62.25 U/l recorded in the culture filtrate of *F. moniliforme*isolated from old deteriorated valuable manuscript.

Noteworthy, is that most of the studied fungal isolates showed their higher hemicellulytic activities (Table 1) after 7 days (other than 14 days) of incubation.

3.2. Optimization of cultural conditions for maximum production of xylanase by selected fungal isolates.

Based on the results of Table (1), the highest values were showed by each of *F.moniliforme.* Consequently, this isolate was selected to undertake the next parts of the present work.

Table (1): Table (1) xylanase activity of the tested fungi.

Fungal strain	incubation Period (day)	Final PH	Dry weight of cells and residual hemicelluloses (g/l)	Protein Content Of c.f. (µg/l)	Hemicellulase activitiy (U/l)
Rhizopusoryzae	7	7.5	2.56	38.02	296.10 ± 2.51 d
	14	7.5	1.78	66.54	555.56 ± 1.53 c
Rhizoctonia Solani	7	7	2.56	67.93	222.23 ± 2.51 e
	14	7.5	2.37	118.88	60.00 ± 1.53 g
Fusarium moiliforme	**7**	**7**	**3.44**	**90.10**	**2594.44 ± 62.25 a**
	14	7	2.82	56.55	1766.67 ± 32.64 b
Alternariatenuis	7	6.5	2.192	106.60	174.44 ± 3.11 f
	14	7	2.22	83.52	156.95 ± 2.07 f
Fusariumoxysporum	7	7	2.72	131.95	187.26± 0.38 f
	14	7	2.82	56.55	223.65± 0.34 ef
Mucormucedo	7	7	2.30	67.61	2268.93± 20.07a
	14	7	1.55	134.85	1032.58±37.67b

The mean values of the enzyme activities ± SD are presented. The different letters show significant difference (P < 0.05).

3.2.1. Effect of differentcarbon sources concentrations on hemicellulase production:

The results of Figure 1 revealed that the highest hemicellulase values 5618.5U/l were showed by *F.moniliforme* by using corn cobs xylan 6g/l. The statistical results showed thatthe concentrations 4 and10g/l were non-significant effect on xylanase activity by *F.moniliforme*. Total soluble protein was estimated with enzyme activity. According to the data represented in fig (1), enzyme activity affected on the total protein produced, parallel.

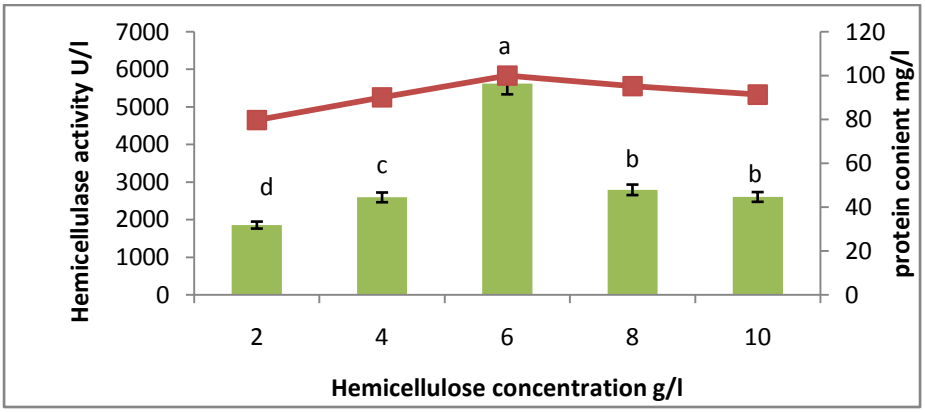

Fig. 1: Effect of different concentration of carbon source on hemicellulase of *Fusariummoniliforme.* The mean values of the enzyme activities ± SD are presented. The different letters show significant difference (P < 0.05).

3.2.2Effect of different nitrogen:

The result of fig.2 showed that the yeast extract (control N source) was selected as the most suitable N source to be used in the next parts of the present work. The statistical results showed that corn steep liquor and $NaNo_3$had non-significant effect xylanase activity by *Fusariummonilifrome.* The nature of nitrogen source has considerable effect on enzyme activity and total protein content. By using organic nitrogen sources, there was semi stability with fewer differences in enzymes activities. There were higher differences in total protein with using inorganic nitrogen ranged from (63.21 mg/l by using ammoniumchloride) to (81.60mg/l by using sodium nitrate).

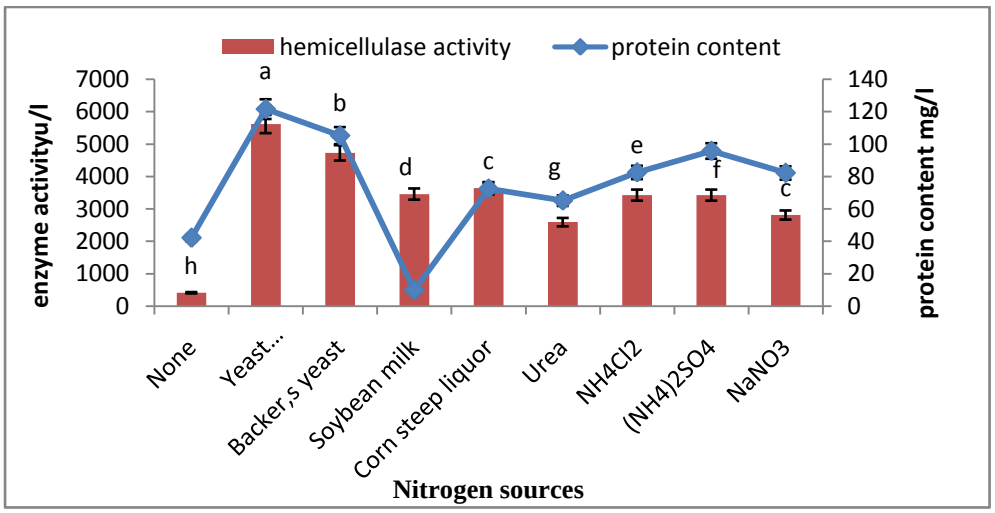

Fig. 2: Effect of different nitrogen sources on hemicellulase activity of *Fusariummoniliforme.* The mean values of the enzyme activities ± SD are presented. The different letters show significant difference (P < 0.05).

3.2.3. Effect of different yeast extract concentrations:

The results of Fig.3 indicated that on using 4g/L yeast extract, *F.moniliforme*exhibited their highest hemicellulase activity 8074.04U/l, respectively. Thus, yeast extract (at 4g/l) was chosen to be used (as N source) in the next experiments during this study.

The statistical results showed that allThe concentrations 1 and2g/l yeast extract were non-significant effect on xylanase activity by *F.moniliforme*. Soluble protein increased with increasing of yeast extract concentration. The total protein content affected parallel with enzyme activity.

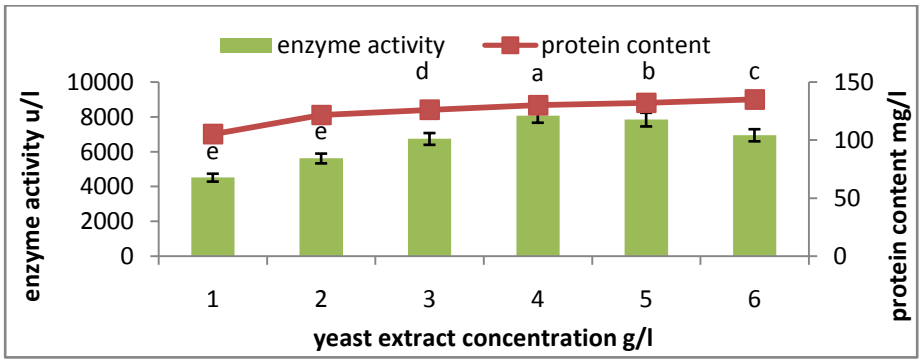

Fig.3: Effect of different yeast extract concentrations on hemicellulase activity of *Fusariummoniliforme*. The mean values of the enzyme activities ± SD are presented. The different letters show significant difference (P < 0.05).

3.2.4. Effect of inorganic salts:

The results of Fig.4 revealed that *F.moniliforme* was needed for all of the tested inorganic salts to exhibit its own higher activity. The statistical results showed that all Elimination of $MgSo_4$and$CaCl_2$ had non-significant effect on xylanase activity by *F.moniliforme.* The total protein content affected parallel with enzyme activity.

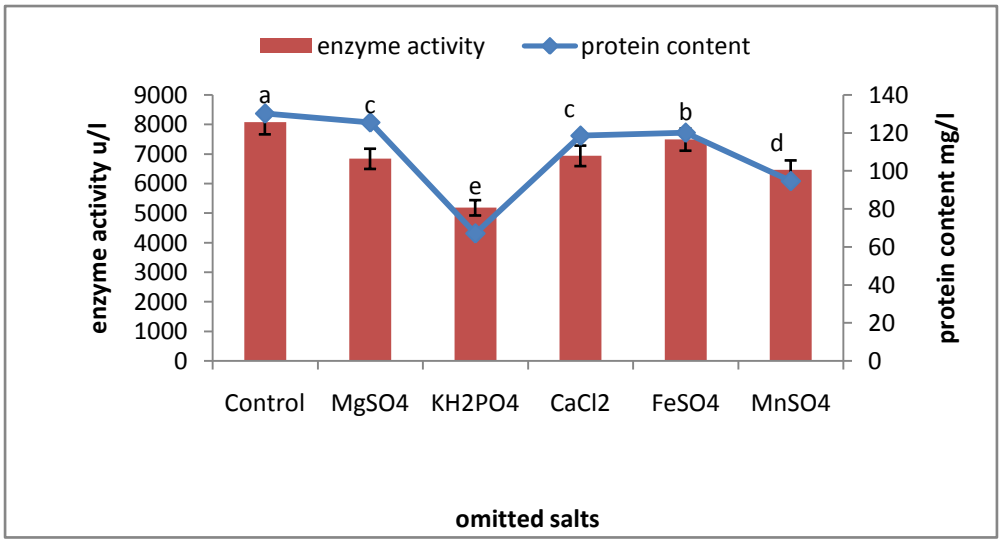

Fig.4: Effect of inorganic salts used in studied media on hemicellulase activity of *Fusariummoniliforme.* The mean values of the enzyme activities ± SD are presented. The different letters show significant difference (P < 0.05).

3.2.5. Effect of initial pH-values:

The results of Fig. 5indicated that*F.moniliforme*led to production of the highest hemicellulase activities were 10950.11U/l. Based on these data the initial pH-value was adjusted at pH5 for growing the selected fungal isolates in the next experiments.

The statistical results showed that pH (4.5and 6) had non-significant effect on xylanase activity byand *F. moniliforme* .The total protein content affected parallel with enzyme activity.

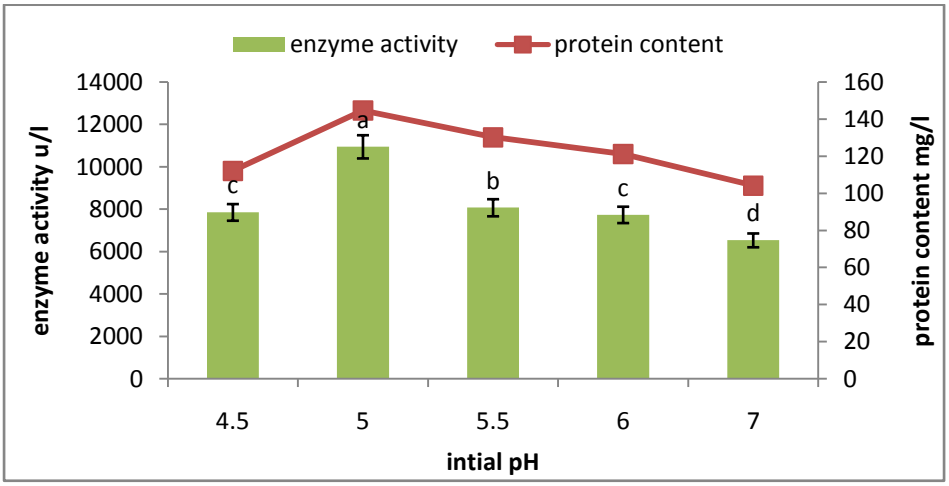

Fig.5: Effect of initial pH on hemicellulase activity of *Fusariummoniliforme.* The mean values of the enzyme activities ± SD are presented. The different letters show significant difference (P < 0.05).

3.2.6. Effect of inoculums size:

The results of Fig.6 revealed that the control inoculum size (4%) was found optimal for production of highest hemicellulase activities10950.11U/l of *F. moniliforme*. Thus, the inoculum size 4% was applied to grow the selected fungal isolates in the next experiments.

According to statistical results.inoculum sizes 2 and 10 % had non-significant effect on xylanase activity on xylanase activity by *F. moniliforme.* The total protein content affected parallel with enzyme activity.

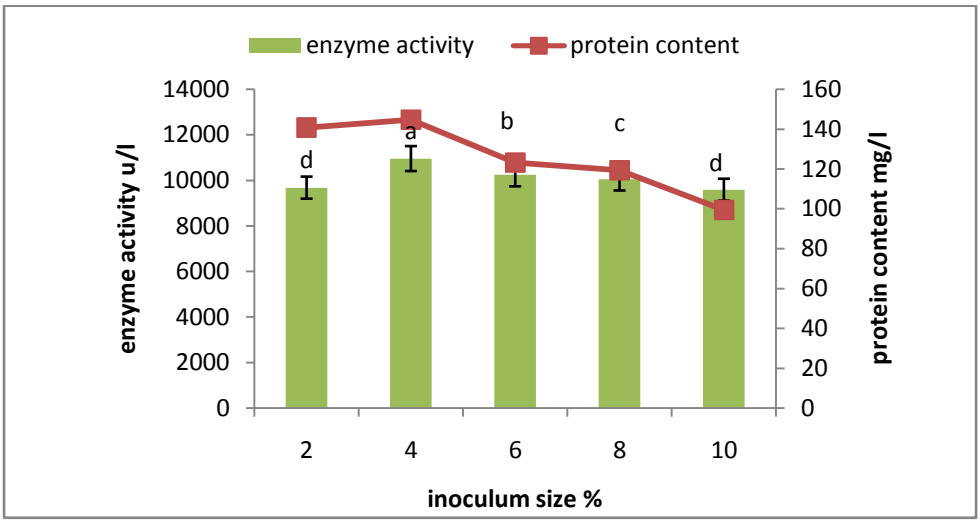

Fig. 6: Effect of different inoculum size on hemicellulases activity by *Fusariummoniliforme*. The mean values of the enzyme activities ± SD are presented. The different letters show significant difference (P < 0.05).

3.2.7. Effect of aeration rate:

The data of Fig. 6 indicated that shaking at 150 r.p.m. (control condition) afforded the optimal aeration rate for secretion of hemicellulase enzymes by the chosen fungal isolates. According to statistical results, all trials had significant effect on enzymes activities. The total protein content affected parallel with enzyme activity.

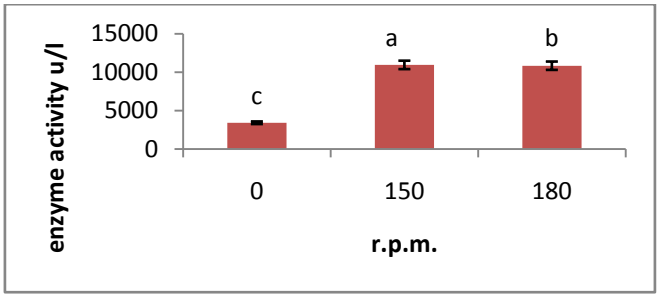

Fig. 7: Effect of different (r. p.m) on hemicellulases activity by *Fusariummoniliforme.* The mean values of the enzyme activities ± SD are presented. The different letters show significant difference (P < 0.05).

3.2.8. Effect of Incubation Temperature:

The data recorded in Fig. 8 showed that the control temperature, i.e., 30°C, was found the optimal one .Hence, the incubation of selected fungal isolates was carried out (in the next experiments) at 30°C.According to statistical results, all trials had significant effect on enzymes activities. The total protein content affected parallel with enzyme activity

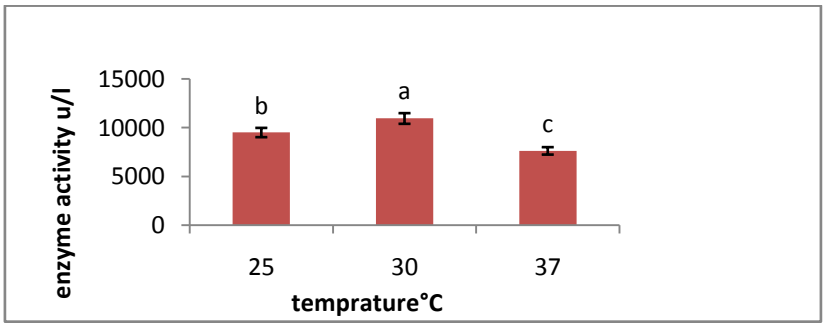

Fig. 8: Effect of different temperature on hemicellulase activityby*Fusariummoniliforme.* The mean values of the enzyme activities ± SD are presented.
The different letters show significant difference (P < 0.05).

3.2.9. Effect some additives:

The data of Fig.9 revealed that the supplementation with any of the aforementioned additives did not enhance the hemicellulase activities of *F.moniliforme*. Thus, no additives were used in the next experiment.

Statistical results showed that Wheat bran (5g/l) and glucose (3ml) had non-significant effect xylanase and Wheat bran (1g/l) and glucose (1g/l) had non-significant effect xylanase activity by *F.moniliforme.* The nature of additives affected on total protein content. Tween 80 (1,2 ml/l) has semi stability in protein content ,but (3ml/l) has increased the total protein with less increase in CMCase activity and less decrease in FPaseactivity.there was higher differences in protein content by using wheat bran as additives ranged from (84.50 to107.52 mg/l). By using glucose protein content ranged from (80.77 to 89.55).

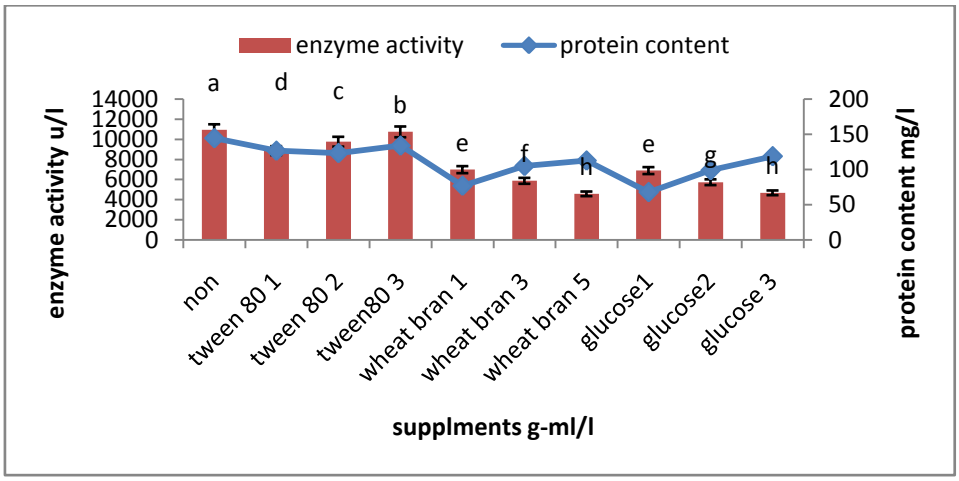

Fig.9: Effect of some supplements addition with different concentrations on hemicellulase activityby *Fusariummoniliforme.* The mean values of the enzyme activities ± SD are presented. The different letters show significant difference (P < 0.05).

5. Disscusion

Recently, xylanase has become an essential option for environmental friendly industrial biotechnological applications **[14.15].** The xylanase enzyme can be produced by a number of microorganisms, however,

filamentous fungi are considered as more potent producers of xylanases**[16, 17].** Six fungal isolates were selected for the xylanase production based on their ability to hydrolyze xylan in the medium. The maximum enzyme production was observed in *Fusariummoniliforme*(2594.44 ± 62.25U/l).

The optimization of medium composition is done to maintain a balance between the various medium components in production media which is done for commercial practice. Optimization helps minimizing the amount of unutilised components at the end of fermentation. Research efforts have been paying attention mainly towards evaluating the effect of various carbon, nitrogenous nutrients, metal ion, phosphate and salt as cost-effective substrates on the yield of enzymes **[18].**xylanases are produced worldwide through SmF which allows better control environment during aerobic fermentation process. It has been found that SmF is normally preferred when the preparations require more purified enzymes **[19]**

The effect of carbon and nitrogen sources on secondary metabolism is trained by several factors including the type of metabolic pathway, the producing organism, the type and concentration of the sources and whether cultures are stationary or submerged. In SmF, purified xylan is frequently used in bioprocesses for xylanase production. Most microorganisms can utilize both inorganic and organic forms of nitrogen which are required to produce amino acids, nucleic acids, proteins and other cell wall components. Recently, **[20]** isolated*A. niger* from garden soil and investigated the effect of different parameters for maximum xylanase production in shake flask. It has been observed that maximum xylanase production was found in presence of xylan as carbon source and yeast extract as nitrogen source, these resulte agreed with the result in the present work.

Fungi require a unique combination of several unusual nutrient conditions, that is, hydrogen ions, dissolved oxygen certain trace metals, or phosphate for sufficient growth or xylanase production. Fe^{2+}, $Mg^{2+,}$ Mn^{2} have positive effect on xylanaseactiviry**[21].**

The cultivation pH exerts a major influence on the overall fermentation efficiency. This is largely because the pH of the medium changes in response to the metabolic activities taking place. For example, the secretion of organic acids such as citric, acetic, and lactic acids will cause the pH to decrease,

while the uptake of organic acids present in some nutrient media can lead to an increase in pH. Enzymatic activity is usually strongly influenced by pH, because the active sites of enzymes often depend on the presence of ionic species to maintain conformations that enable efficient binding to the substrate**. [22]**reported that Optimum pH of xylanase production by fungi ranged from 4.5 to6 which agree with the result in the present work.

Highest enzyme activity appeared by inoculum size 4% and decreased by higher inoculum sizes .These results indicated that Increased level of inoculum mostlyreduced xylanase production in industrial fermentation process **[23].** This may be due to the depletion ofnutrients from the fermentation medium which resulteddecline in enzyme synthesis.

The function of aeration is to maintain aerobic conditions, remove the carbon dioxide generated, and regulate the temperature and moisture level of the substrate.In aerobic SmF cultivations, the oxygen supply is often the limiting factor for growth, due to low solubility of oxygen in water. In the present work 150 r.p.m. was the optimum aeration rate.**[22]**

Incubation temperature is also a critical factor in the growth of fungus. Different experiments were performed on variousincubation temperatures ranging from 20 to 50 ^{o}C. Results ofthe study indicated that maximum enzyme production wasnoted at 30^{o} C**.** this results agree with the result of the present work. The incubation temperature was further increased decrease in enzyme production was also observed. Due to increasing the enzyme activity, some supplements were added to the optimized medium.Unfortunately, all supplemented tested have negative effect on enzyme activity. This result disagreed with **[23]**

Conculsion:

The fungal strain*F.moniliforme*expresses good xylanaseactivity, using the agro-industrial wastes; corn cobs xylan, as an individual carbon source and inducer(2,594.44 ± 62.25U/l). Upon optimization the enzyme productivity was approximately increased by four times, recording maximum enzyme productivity10,950.11 ± 98.45 U/l, under optimum operating conditions of; corn cobs xylan (6g/l), yeast extract (4g/l), inorganic salts ($MgSO_4$, $KHPO_4$, $CaCl_2$, $FeSO_4$, and $MnSO_4$), initial pH (5), initial inoculum size (4%), 150

rpm and temperature (30℃). Thus, optimization of process parameters is a prerequisite to enhance the enzyme yield and activity, which is very helpful in large-scale production.

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الملخص باللغة العربية

دراسه النشاط الانزيمى لبعض السلالات الفطريه

ثناء شلتوت ، زينب خيرالله[1]، نورالجندى[2]، هاله عبد المنعم[1، 2]، محمد مجد الدين درويش[3]

[1] قسم النبات –كليه البنات- جامعه عين شمس – القاهره- مصر.
[2]معمل البيتكنولوجى- قسم تطوير العمليات- معهد بحوث البترول- القاهره-مصر.
[3]قسم كيمياء المنتجات الطبيعيه والميكروبيه- شعبه الصناعات الصيدليه والدوائيه- المركز القومى للبحوث- الجيزه- مصر

يهدف العمل البحتى الى دراسه النشاط الانزيمى لبعض الفطريات. تم دراسة قدره سته فطريات على انتاج انزيمات , الزيلانيز xylanase وهى احد الانزيمات المهمه فى مجموعه انزيمات الهيمى سليولاز hemicellulase.اتضح من خلال الدراسه ان فطر*Fusariummoniliforme* والمعزول من الملفات القديمه وهو الاعلى انتاجيه لانزيم الزيلانيز خلال فتره سبعه ايام من التحضين وذلك باستخدام الزيلان المستخلص من اكواز الذره كمصدر كربونى corn cobs xylanبانتاجيه تقدر ب 2594.44وحده نشاط انزيميه/لتر . استخدم الفطر لاستنباط افضل الظروف الملائمه لتحسين انتاجيه الانزيم. اولا: تم دراسه تاثير تركيزات مختلفه من corn cobs xylanوكانت النتيجه ان تركيز 6جرام/لتر هو افضل تركيز لاعلى انتاجيه لانزيم ب 5618.5 وحده نشاط انزيميه/لتر.ثانيا:تمت دراسه مصدار مختلفه من النيتروجين وكان مستخلص الخميره yeast extractهو احسن مصدر نيتروجينى استخدمه الفطر لاظهار اعلى انتاجيه لانزيم السيلانيز .ثالثا:تم دراسه تركيزات مختلفه من مستخلص الخميره وكان 4 جرام /لتر هو التركيز المفضل للفطر لاظهار اعلى نشاط انزيمى وصل الى 8074.04 وحده نشاط انزيميه/لتر.رابعا:تم دراسه تاثير الاملاح غير عضويه وكانت النتيجه ان الفطر يحتاج جميع الاملاح التى تمت دراستها. خامسا:تم دراسه تاثير مجموعه مختلفه من الاس الهيدروجينى وكان الاس الهيدروجينى 5 هو الافضل بانتاجيه وصلت الى 10950.11وحده نشاط انزيميه/لتر.سادسا: تمت دراسه احجام مختلفه للحقنه الفطريه وكان 4 جرام /لتر هو الاعلى فى النشاط الانزيمى.سابعا: تم دراسه تاثير عدد اللفات لكل دقيقه على انتاجيه الانزيم وكان 150 لفه لكل دقيقه هو الاعلى انتاجيه.ثامنا: تمت دراسه درجات تحضين مختلفه وكانت اعلى انتاجيه للانزيم عند درجه حراره 30درجه مئويه.تاسعا:تم دراسه تاثير بعض المواد المضافه ولم يكن لاى منها تاثير ايجابى فى زياده انتاجيه الانزيم.

الكلمات الداله :

انزيم الزيلانيز- زيلان-فطر*Fusariummoniliforme*