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The ability of *Trichoderma harzianum* on cleavage of cellulose of date palm leaves

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Abstract

Trichoderma harzianum pour isolate was obtained from Al-Tahaddi insecticide , It was best growth on Malt extract agar medium show spores numbers 8066.6 spore /disc (0.3 cm) and colony diameter was 9 cm. It was best growth on cellulase production medium contained 100% substitution level from palm leaves, spores numbers was 3900 spore/disc (0.3 cm) and colony diameter was 6.33 cm. The cellulase production best was 100% substitution level from palm leaves on solid media growth ratio 376.7 μ/hour and halo diameter 7 cm. Cellulase enzyme complex activity was estimated on broth media with control sample (standard media), Higher production for β-gluconase was arrived (79.0 unit/ml) when 100% substitution level from palm leaves . Whereas 25% substitution level from palm leaves was give high production from Endoglucosidase and Exoglucosidase (6.5,5.2) unit/ml respectively comparison with other substitution levels. Higher production for Exoglucosidase was (5.8 unit/ml) in standard media comparison with other sampls.

Introduction

Cellulose is a natural substance that forms the cell walls of all plants and trees . It makes up 45% of palm trunk ,47% of fronds ,and 41% of on dry basis (Al-Baker,1972). Cellulose, a polymer of β-1,4 linked glucose molecules, is for man indigestible "roughage". Number of glucose units reaches thousand. Cellulose can be degraded into simple hexoses (D-glucose) by acidic hydrolysis (Nam,1979), or by cellulase activity .This activity a complex enzymatic system consists of three enzymes:

1- Endo -1,4-β –D – glucan 4-glucanohydrolase, (E.C.3.2.1.4)
2- Exo -1,4-β –D – glucan cellobiohydrolase, (E.C.3.2.1.91)
3- β- glucosidase (E.C.3.2.1.21)

The act of these enzymes on cellulose is co- operative and sometimes alternatively (Eveleigh, 1987). Cellulases are used for cellulose degradation because of the difficulties of acid hydrolysis method and the decomposition of other materials and the high cost of this process (Muniswaran and Charyulu, 1994) .

Many fungi were used for cellulase production. *Trichoderma* spp. was one of these fungi (Szakacs and Tengerdy, 1996; Vintilă *et al*.,2008 ; Folakemi *et al*.,2009).

El-Rayae *et al*. (1985) noticed that the ability of same fungi to hydrolyze cellulose was weak when they have been inoculated on cotton medium comparing to filter paper. Many studies was show cellulose activity by complex enzymatic system production from *Trichoderma* spp.(Sun et *al.,2009*; Ferreira et *al.,2009*) . Cellulase was produced by *Trichoderma reosei* by solid fermentation on optimum conditions : pH5 at 30C for 7 days and adding 0.3% of Tween-80 (Al-Aani and Sultan,1989). *Tri. harzianum* had the ability to produce cellulose in growth medium contains rice straw and sunflower stems, highest breakdown was observed at 2% carbon concentration (Ghazi *et al.*,2002).

The aim of this study was to produce cellulose from date palm leaves .

Material and Methods

1-Isolation of *Trichoderma harzianum*

Tri. harzianum was obtained from the Iraqi biological pesticide (Al-Tahaddi) produced by Ibaa center for Agricultural Research. One gram of pesticide was inoculated on Potato Carrot Agar (PCA) and incubated at 28C for 72 hours. It was purified for three times on the same media.

2-Best media growth

To choose the best medium for the *Trichoderma harzianum* growth , we have tested the following media: Czapek Solution Agar (CzSA),Malt Extracted Agar (MEA), Potato Carrot Agar (PCA), Potato Dextrose Agar (PDA) and Sabouroud Dextrose Agar(SDA).

3-Cellulase media production

It was prepared as described by Kassim and Ghazi (1987) in order to produce cellulase . It contains :KH_2PO_4 2gm ,$(NH_4)_2SO_4$ 1.4gm, Urea 0.3gm,$CaCl_2$ 0.3gm, $MgSO_4.7H_2O$ 0.3gm, $FeSO_4.7H_2O$ 5ppm, $MnSO_4$. H_2O 1.6ppm, $ZnSO_4.7H_2O$ 1.4ppm, $CoCl_2$ 2ppm, Peptone 0.8gm, Tween-80 2ml and the source of cellulose was Carboxyl Methyl Cellulose (CMC) or filter papers 8gm .Dissolved in 1 L distil water.

4-Cellulosic source

Cellulosic source in the medium was substituted by data palm leaves of Hellawi cultivar in basrah city .Dried leaves were ground by electrical mill. Substitution levels were 25,50,75 and 100% from source of cellulose. In addition to control sample with 100% CMC.

5-Cellulase production on solid media

The *Tri. harzianum* was grown on solid medium described previously and 20 gram of agar was added toughen the medium .A disc of fungus culture was taken at diameter of 5mm by sterile cork drill and was put in the middle of a petri-dish upside down and incubated at 30C for 7 days . *Tri. harzianum* growth rate was measured by micrometer units/ hour($\mu m.hr^{-1}$). Spores was counted by using Hemocytometer. Method of Yooh *et al*.(1985) was followed to detect the enzyme activity .It was done by using HCl-Iodine reagent (prepared by mixing 100 ml of HCl (0.1N) and 500 ml of Iodine solution which was prepared by dissolving 10 gm of Kl and 5gm of I_2 in 500 ml of distilled water), the reagent was added to the petri-dish and left for 5 minutes, poured and left to stand again for 10 minutes .Appearing of Light yellow halo indicate the enzymatic activity.

6-Cellulase assays in liquid media

Erlenmeyer flasks (250ml) containing 100 ml of the nutrient media were prepared for cellulose production at substitution levels 25,50,75 and 100% from source of cellulose with control sample of Carboxyl Methyl Cellulose (CMC) as a source of cellulose. Media were inoculated with three discs (5mm diameter) from *Tri. harzianum* growth to each flask and held in shaking incubator on 30C for 7days. The growth was then collected by centrifuge (4000 rpm for 5 minutes).The supernatant that contains enzyme complex was use to test enzymatic activity. B-glucosidase activity was assayed according to a method used by Kubicek (1982), while endogluconase, exogluconase activity were assayed to a method described by Mandels *et al*.(1976).

7- Statistical analysis

Experiments were done in two replicates and data subjected to analysis of correlation coefficient (r) under significant levels 0.05 and 0.01(Dean and Voss, 1999)

Results and Discussion

1-The growth on culture media:

Data of spores number and fungi colony diameter were indicated in (Table1) .It was clear that the MEA was the best medium for the fungus growth comparing with the others. Spores number and fungus colony diameter on MEA were 8066.6 microbe /disc and 9 cm respectively. Also, the data indicate that there was not any growth on (CzSA) medium .These results were not agreed with (EL-Katany *et al*.,2000) who pointed out the ability of this fungus to grow on (CzSA) and to degrade the carbon material of the medium (Fig:1). the results show a positive correlation

coefficient (r=0.978) between spores number per disc and colony diameter significant under significant level 0.05.

Table (1) Spores number and fungus colony diameter⋆

Medium	Spores Number per disc(0.3cm)	Colony Diameter(cm)
CzSA	0	0
MEA	8066.6	9
PDA	6133.3	6
PCA	4321.2	3
SDA	1933.3	1.5

⋆ The data are the average of three frequencies

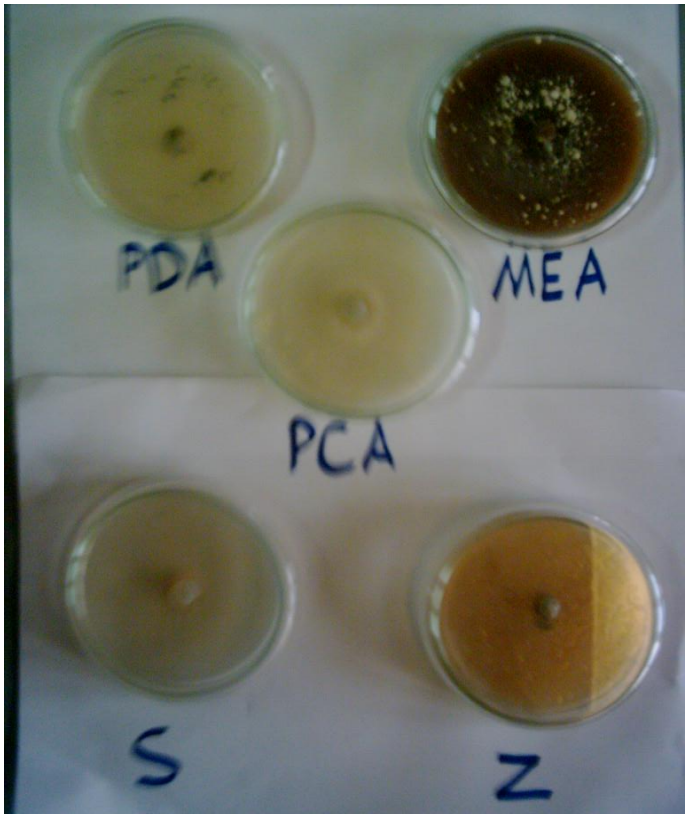

Figure (1) *Tri. harzianum* growth on media culture types

2-Fungal growth and cellulose hydrolysis on solid media.

Table (2) indicates that as substitution level increased the diameter colony and spores number were increased comparing with standard media substitution level of 100% was the best ratio for *Tri. harzianum* growth . These results agree with the results of Ghazi *et al*.(2002) ; Abd El-Zaher and Fadel (2010) which showed the ability of *Trichoderma* spp. to hydrolyze cellulosic rice straw under solid state fermentation and show cellulase activity .correlation coefficient (r=0.945) between spores number per disc and colony diameter significant under significant level 0.01.

Table (2) Effect of substitution level on spores number and fungus colony diameter⋆

Medium	Spores Number per disc(0.3cm)	Colony Diameter(cm)
Control	800	4.76
25% sub.	1400	5.30
50% sub.	2400	6.00
75% sub.	2700	6.16
100% sub.	3900	6.33

⋆ The data are the average of three frequencies

3-Cellulase activity and growth rate measuring:

The results have showed that the 100% substitution level was the best ratio to produce cellulase activity since the diameter of yellow halo has reached 7 cm , and the growth rate during 7 days was 376.7 units / hour (μm.hr^{-1}) (Table 3) (Fig:2). Also, the results show a positive correlation coefficient (r =0.963) between the growth and halo diameter. significant under significant level 0.05. The greater growth shows to the highest enzymatic activity. And consequently , the higher the substitution level ,the greater the activity.

Table (3) Fungal growth rate and halo diameter of the substitution on solid media⋆

Medium	Growth μm.hr^{-1}	Halo diameter (cm)
Control	283.33	4.5
25% sub.	315.40	5.2
50% sub.	352.10	5.7
75% sub.	366.6	6.3
100% sub.	376.7	7

⋆ The data are the average of triplecates

The results do not presuppose that the fungus has a complete system to hydrolyze cellulose though the results show high growth rates and positive detection of the enzymatic activity on solid media

. 4-Cellulase production on liquid media

Table (4) and fig. (3) show β-gluconase production at large amount in the substitution 100% comparing with control sample. The activities were 820 units / ml and 15 units / ml respectively. The importance of this enzyme is because of its ability as well as its susceptibility to break down cellobiose which is considered as a strong inhibitor to exoglucosidase and endoglucosidase (Bisset and Stemberg, 1978)

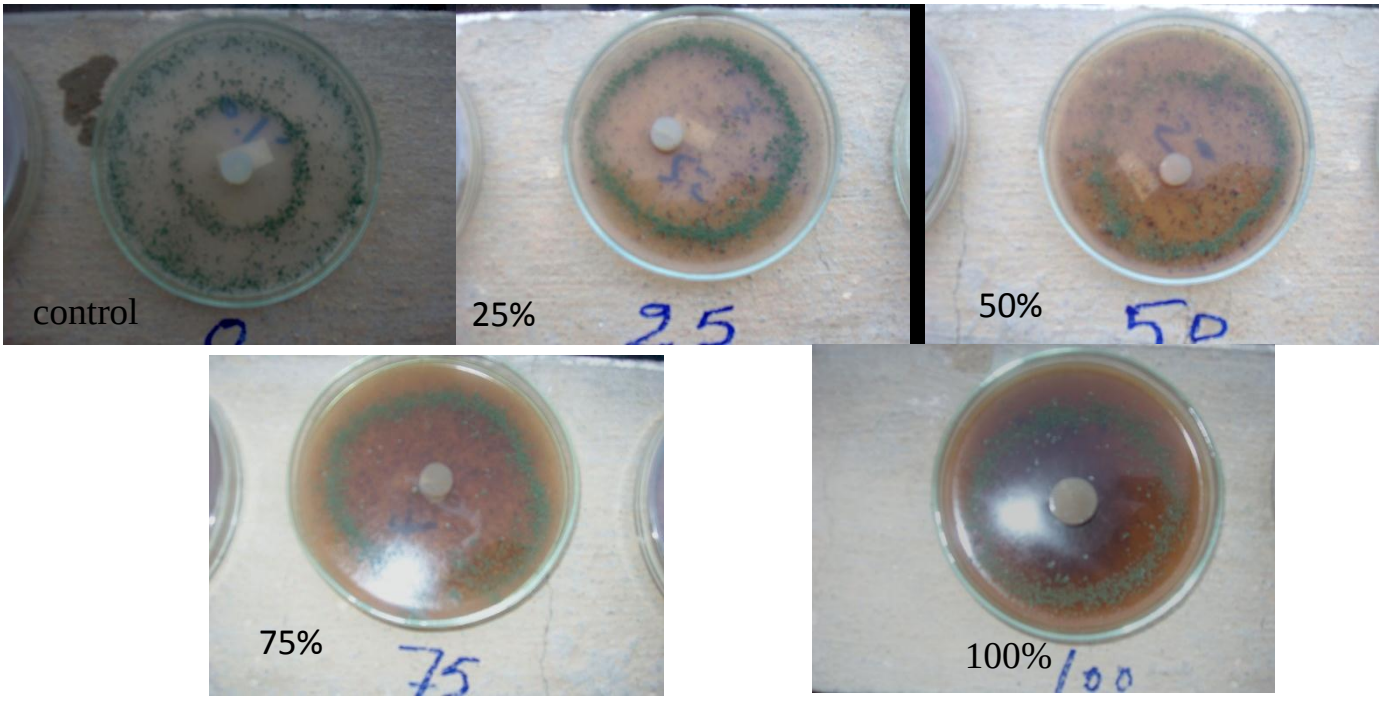

Figure (2) Cellulase production and cellulase activity on standard media substitution level palm by *Tri. harzianum*

Table (4) Production of Cellulase complex enzymatic system

Substitution level in media	β-gluconase units/ml	Endoglucosidase units/ml	Exoglucosidase units/ml
Control	15	1	5.8
25% sub.	72.5	5.6	5.2
50% sub.	77.5	5.1	4
75% sub.	79.0	4.6	3.4
100% sub.	82.0	4.4	1.5

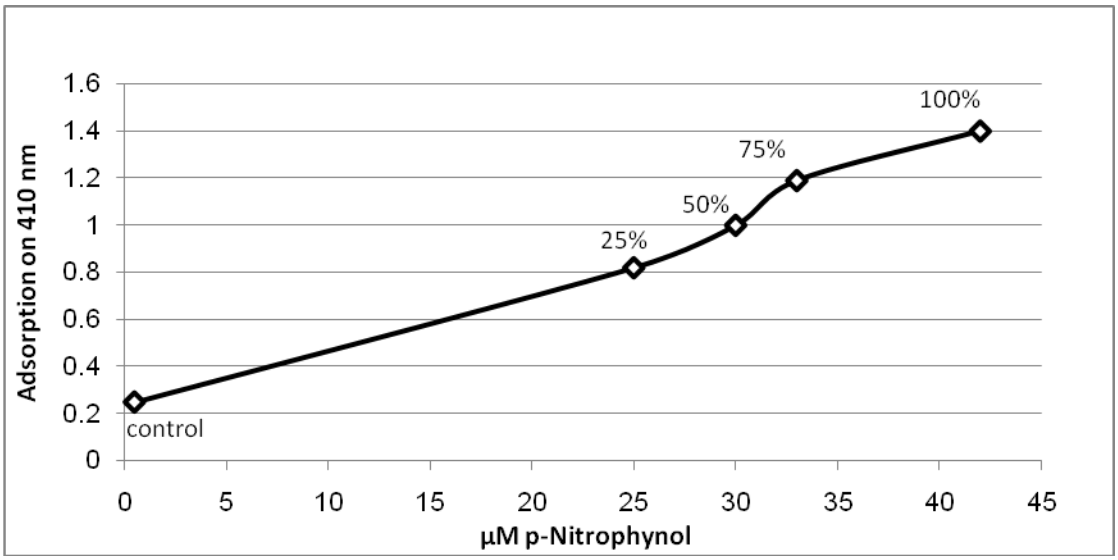

Fig. (3) Production of p-Nitrophynol by β-glucosidase from *Tri. harzianum*

Fig. (4) and table (4) indicate , that high productivity to endogluconase is at substitution level 25% which reaches 5.8 unit / ml. The productivity low with increasing of substitution level which reach 4.4 unit / ml at 100% level where as it reach 1 unit /ml on control medium and it introduces the lowest from all levels. These result don't agree with Ghazi *et al*. (2002) who showed that the higher.

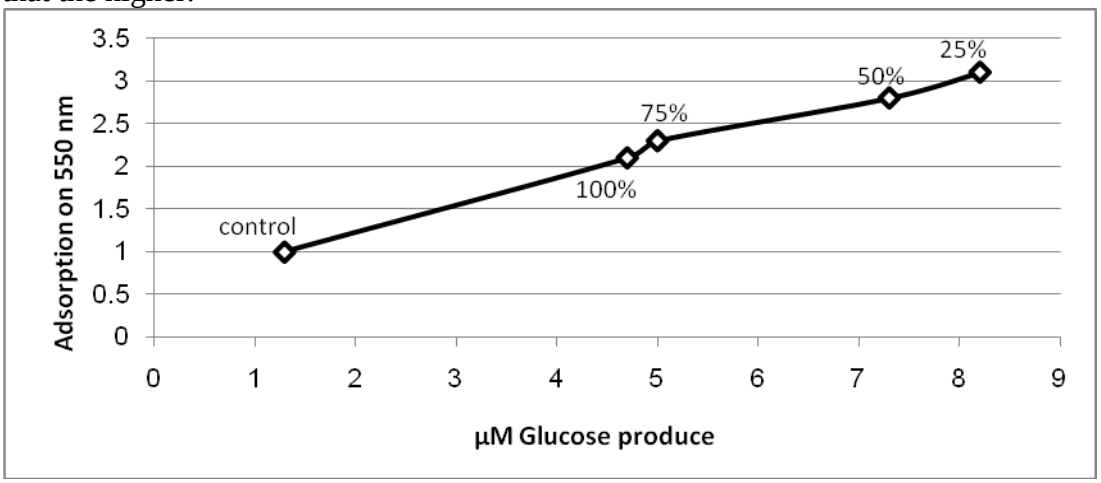

Fig. (4) Production of Glucose by endogluconase from *Tri. harzianum*

Productivity to this enzyme from *Tri. harzianum* was at the control medium 2.3 unit / ml and as substituted level increased enzyme production was increased but not reaching the productivity on control medium. The higher Productivity of exoglucosidase was at control medium which reached 5.8 unit / ml and it decreased as substitution level increased units it reached 1.5 unit / ml at 100% level (Table 4 and Fig.5)

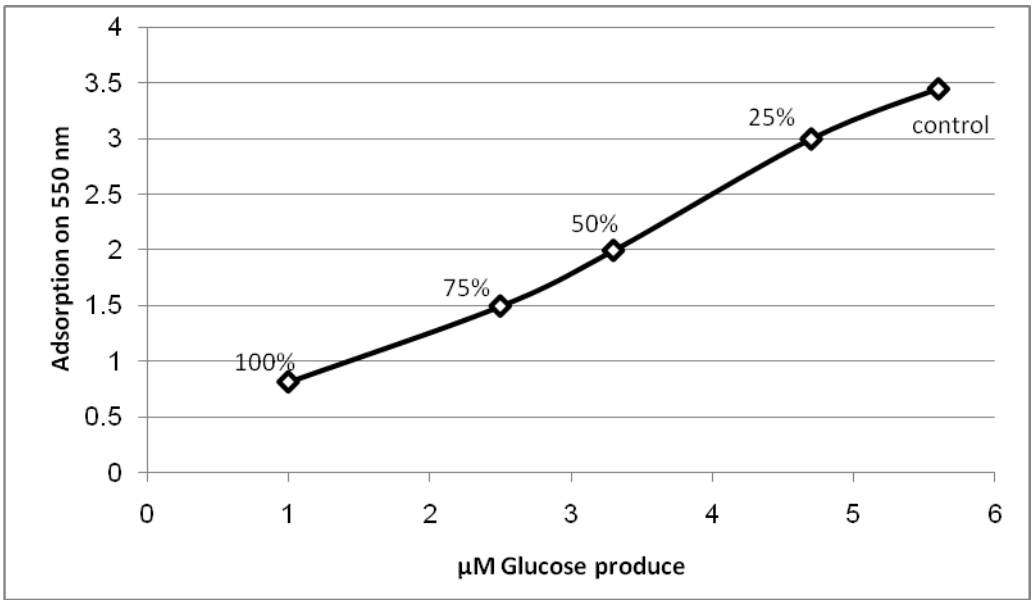

Fig. (5) Production of Glucose by exoglucosidase from *Tri. harzianum*

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قابلية عفن *Trichoderma harzianum* على تحليل سليلوز أوراق النخيل (السعف)

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الخلاصة

تم الحصول على عزلة نقية للفطر *Trichoderma harzianum* من المبيد الحيوي Al-tahaddi واظهر أفضل نمو على الوسط Malt Extract Agar إذ بلغ عدد السبورات 8066.6 سبور/ قرص (0.3 سم) وكان قطر المستعمرة النامية 9 سم ، وأعطى الفطر نمو جيد على وسط إنتاج السليليز المحتوي على نسبة استبدال 100% من أوراق النخيل وكان عدد السبورات 3900 سبور/قرص (0.3سم) وقطر مستعمرته كان 6.33 سم .

أظهرت نسبة استبدال 100% من ورق النخيل أفضل إنتاج للسليليز على الوسط الصلب لإنتاج الأنزيم فكان معدل النمو 376.7 مايكرومتر / ساعة وقطر هالة النمو 7 سم . قدرت الأنزيمات المكونة لمعقد أنزيم السليليز على الأوساط السائلة وبوجود الوسط القياسي ، فكان أعلى أنتاج لأنزيم β-gluconase إذ بلغ 79.0(وحدة/مل) عند نسبة استبدال 100% .بينما أظهرت نسبة 25 %أعلى إنتاج من أنزيم Endoglucosidase إذ كانت 6.5 (وحدة/مل) بينما أعطى الوسط القياسي أعلى أنتاج من أنزيم Exoglucosidase بلغ أنتاجه 5.8 (وحدة/مل) وجاءت نسبة استبدال 25 % أعلى من بقية النسب الأخرى في أنتاج هذا الأنزيم فكانت 5.2 (وحدة/مل).