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Phospholipase activitv in mycelia of *ASPergillus flavus*

M. A. Aouda
Chemistry Department
College of science
University of Thi-Qar

Abstract

A strain of *Aspergillus jlavus* was taken and cultivated in Sabouraud Deatrose agar, after that it transfered to another media contains Nutrient agar with egg Yolk which contain the lecithin as a phospholipid substrate.

For known enzyme activity, this pathogenic fungi transfered to liquid media for growth on it. Enzyme activity by using technique of (TLC) to know the producing fatty acids by using standard material from this acids was studied.

Introduction

It is generally known that pathogenic fungi secrete various hydrolytic enzymes such as protase and phospholipase into the growth medium and in mycelia [1]. Extracellular secreted hydrolytic enzymes may play a part in infection by causing damage to host cells [2] . The occurrence of phospholipase, lysophospholipase and lipase activities associated with the mycelia of various strains of filamentous fungi.

The destructive effect of secretory phospholipases found in snake venomes and arthropod and bacterial toxins. [3] The presence of phospholipase activity in pathogenic fungi and yeast was first detected by growing the fungus or yeast on media containing egg yolk [4,5] and lecithin [6]. Pagh & Cawson [7] demonstrated the localization of phospholipase activities in cells and culturemediumof C. albicans by acytochemical method. Recently, the quantitative detection of phospholipase activity in Candida albicans by aplate assay was decribed by price et al [8] .Who showed that large variations in phospholipase activity were found in Aspergillus .However Its ability to produce phospholipase is concidered to be an important phatogenic feature where the phospholipase is presente in several mammalian tissues including brain, kidney, liver, intestinal mucosa, fetal membranes and uterine decidua. macrophages, fibroblasts and platelets [9]. Extracellular phospholipases are produced by several bacteria and fungi and have been implicated in their pathogenicity by causing damage to host cell membranes [10].

Materials and Methods

Cultures were obtained from Department of Biology, college of Science, University of Basrah. The organism was maintained on (SDA): 30 g/L glucose; 10 g/L peptone; 10 g/L yeast extract and (2%) agar +5 mm disk of fungi [11,12] by transfer every 2 weeks at 25° C.

Phospholipase activity in solid media

Adescribed method [8] was applied with aminor modification, The test medium comprised of nutriet agar (20 g/L) , supplemented with ,1M sodium chlorid ; 0.05 M calcium chlorid and 8% Sterile egg Yolk ; powder of egg Yolk was prepared from fresh eggs to be used

as a sbstrate for enzyme test as follows; The Yolk was removed by separation of the egg albumin in a sterile container , then dried in an oven at 40° C for 1 h , The dried material was blended in sterile distilled water in morter and centerfuged at 500 g for 15 min. The supernatant was diluted with sterile distilled water up to 100 ml , then added to the sterile basal medium at pH 5.0 Fungal cultures were incubated at 23° C for 2 weeks. The precipitate zone was examined and measured as follow:

$$\text{Phospholipase activity (Pz value)} = \frac{\text{Diameter of the colony}}{\text{Diameter of the colony + precipitation zone}} \quad (1)$$

For growth of mycelium, we used a chemically defined medium containing per 1000 ml of distilled water: Glucose l6.5 gm; KH_2PO_4 1.7 gm; KCI 430 mg; $MnSO_4$. $4H_2O$ 2.5 mg ; Biotin 6 µg ; Methionine 1 gm ; $MgSO_4$ .7 H_2O 130 mg ; $FeCl_3$. 6 H_2O 2.5 mg ; Na_2HPO_4 4.5 g.

To this medium added com-steep liquor to a final concentration of 50 g/L and the pH of the medium was adjusted to 4.5 where indicated; Olive oil at a concentration of 10 g/L was incorporated into the medium in addition to or in place of glucose. 500 ml of culture medium in one liter conical flask were inoculated by addition of suspension containing 5 x 107 spores and incubated at 25° C for normally 72 h .

The mycelia were harvested by filtaration , Washed several times with distilled water and lyophilized. The Freeze dried mycelia were defatted using diethylether in (Suxulet Apparatus) and stored in (referegrator) Unitil required

Chloroform: Acetone	: Methanol: Acetic acid	: Water
9 ml : 12 ml	3ml 3ml	: 2 ml

Substrate and assay procedures

The substrate used was (lecithin extracted from egg Yolk). Standard reaction conditions were as follows: 100 mg Freeze - dried, defatted and powdered mycelia were incubated at 37° C with 100µl of water and 1.5 ml of egg lecithin solution in disopropylether (1.5 ml of 0.1 M sodium citrate buffer (pH= 4) (The control tube contain same component but heating in 100 ° C)

In both assays, the reactions were stopped by adding 2 ml of a chloroform - methanol solution (2: 1 volume: volume) and the reaction products were extracted by the method of Bligh and Dyer [13] evaporated and redissolved in a small volume of chloroform methanol (6: 1 v: v) and applied to aplate (silica Gel 60: Merck, Darmstade , Germany) the plate was developed with

Results & Discussions

Most works had established that the highest yields of fungal lipolytic enzymes were obtained by use of a mineral salt medium supplemented with Com - Steep liquor to which has been added glucose and / or olive oil as carbon source.

The solid media used in this study allowed to a convenient survey for exocellular enzyme production by fungal isolates. It is known that the major role of enzyme produced by pathogenic fungi is the break - down of the fat. This result is agrees with that mentions in [4].

Figure (1) illusterates the activity of phospholipase from *Aspergillus* in different time, that activity is following by (plate assay) which illusterates in [9]. More researches about this subject are depending on this manner. The temperature of test is (25° C) and (pH = 4) but in changing the value of (pH) with constant temperature, we notice there are Fixing value in (Pz) for first four days, but after fourth day we notice there are sudderfClecreasing in that value until sixth day it stays stable to seventh day and starts decreasing gradually to tenth day, this means enzymic secreted continues (According to Fig.(6)) but not parallel.

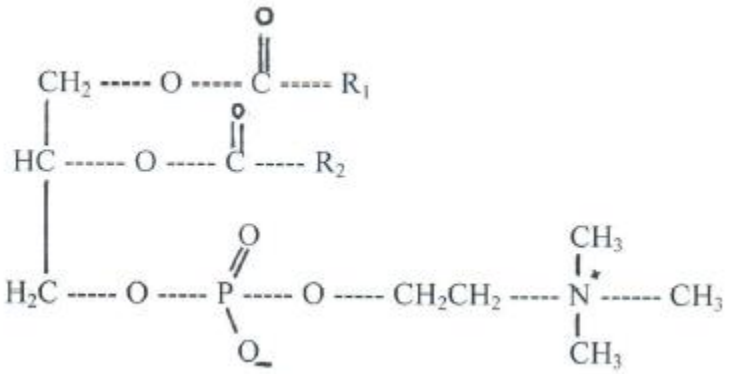
There are differences in conditions that should be ideal for growth of the fungi. Thus figure (3) illusterats more secreted may be opproaching to straight line and that means the ideal good conditions for growth that type of fungi is 30° C and (pH = 4) , that doesn't agree with the result to which reaches by (A-memmon 1983), the value of pH for growth is 4.5 and temperature 37° C.

(2)

The Figure number (4) illusterates there are gradual decreasing in (Pz) value isn't slopping for first four days, it is between (0.9 →1) , but there are large slopping in value of (Pz) after fifth day that refers to the activity in top value in this period of time and this exactly applies with result that mentions in [10] about studying phospholipase that is produced from *Cryptococcus neoformans.*

Figure (5) illustrates influence of essential substrate on enzyme activity we notice in using egg Yolk as a carbonic source, to value of (Pz) severs little decreasing about less than (1) more than 0.7 but in using lecithin as substrate found that large decreasing in (Pz) value from first day to tenth day, that proves that lecithin is the suitable nutritive source that paves the way for increasing enzyme activity because this lecithin is the phospholipid targeted which finds in the brain cells and never fabrics in human body.

The last step of this research is Fig.(7) illustrates using technic of (TLC) for testing enzyme activity with lecithin as a substrate after incubatting mixture of dry mycelia with lecithin in 37° C after extracting production by (Bligh & Dyer) manner by using silica gel plate, the comparism of the result with standard fatty acids find that extracted products contain the same value of (Rt) for oliec and linolinic acids that and that agrees with the previous figure for activity side , the result of analysis lecithin is known fatty acids that have it in carbon atom number (1) or (2) that illstereted below:



The structure of lecithin

(3)

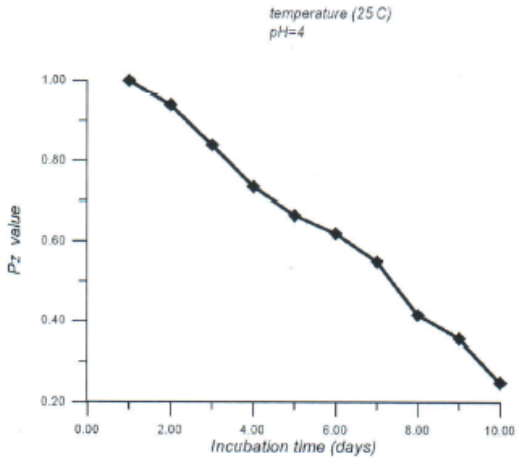
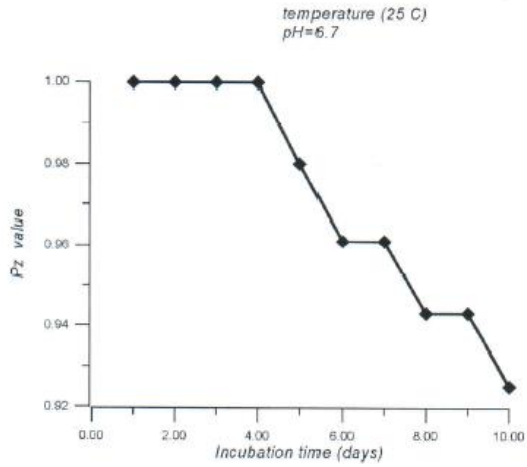


Fig.(f) Phospholipase production from Aspergillus fIavus at different time



Flg.(2) Phospholipase production from *Aspergillus f/avus***at** different time

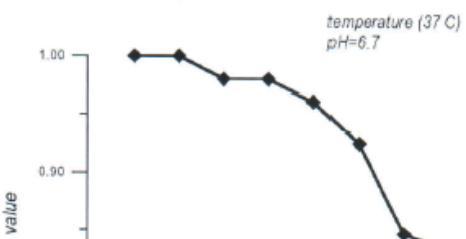


Fig.(3) Phospholipase production from Aspergillus ffavus at different time

Fig. (4) Phospholipase production from *Aspergillus flavus* at different time

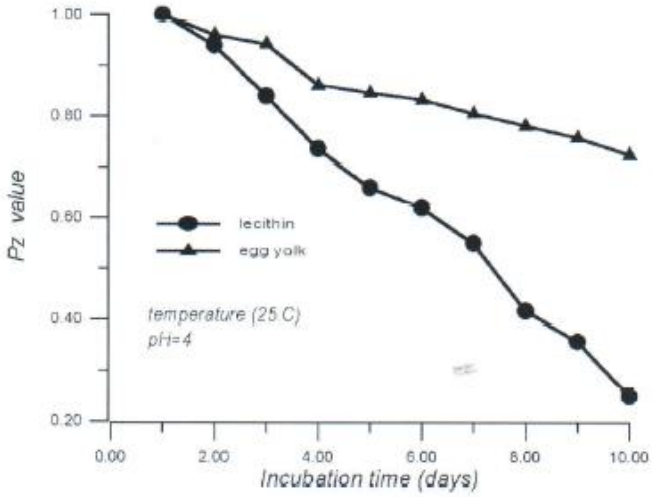


Fig.(5) Phospholipase production from *Aspergillus flavus* at different time

Fig. (6) : Phospholipase activity of *Aspergillus jlavus* in solid media

(5)

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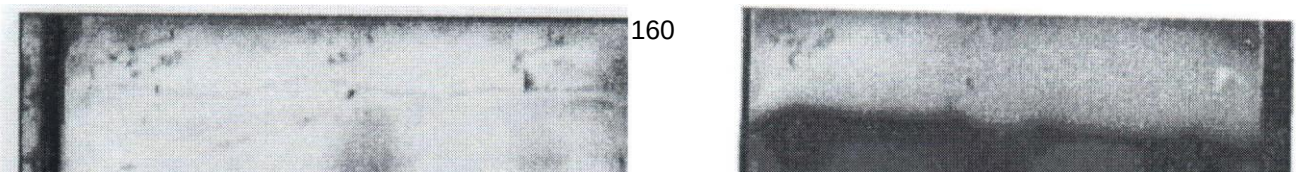

Fig. (7): TLC techinque of phospholipase activity by use standard fatty acids: (A) standards fatty acids (palmetic, linolinic, lenolic) . (B) In the middle, enzyme solution with lecithin.

References

1) M. Takahashi, Y. Banno and Y. Nozawa Secreted *Candida albicans* phospholipase; purification and characterization of two forms of lysophospholipase - transacylase .Journal of medical veternany Mycology (1991) 29: 193-204.

2) Casson , A., DE bernardis , F. , Mondello, F. Ceddia. T. & Agatensi, L. 1987. (Evidence for a correlation between proteinace secretion and valvovaginal candidosis , TournaI of Infection Disease, 156: 777-783 .

3) Rosemary T. Santangelo M. Hussein Nouri - Sorkhabi, Tania C. sorrell, Michelle cagneg , Sharon C.A. Chen, Philip W. Kuchel and Lesley C. Wright. ((Bioclemical and functional characterization of secreted phospholipase activities from *Cryptococcus neoformance* in their naturally occurring state)) J. Medial Microbiology vol. 48 (1999) 731 - 740 .the pathological society of Great Britain and Ireland lssn 0022 - 2615 .

4) Costa, A.L., Missefari, A. & Amafo, A. 1967 a. Enzymatic activities ofmycetes. 1. Enzymatic activity of *Candida albicans* egg Yolk - containing media Atti XIV congresso Nazional di Microbiologia, Messina Taormina.

5) Werner,H. 1966 . Untersuchungen uber die lipaseaktivitat bei hefen and hefeahnlichen pilzen. Zentralblatt fur Bakteriologie, I, Ab. Orgininale. 200: 113 -124.

6) Costa, A.L. , Missefari A. 1. Amoto, A. 1967 b . Enzymatic activities of mycetes .II phosphatidase activity in *Candida albicans* growing in media containing lecithin. Atti XIV congresso Nazionale di Microbiologia, Messina Taormina.

7) Pagh, D. & Cawson, R. A. 1975. The cytochemical localization of phospholipase A and lysophospholipase in *Candida albicans,* Sabouraudia, 13 : 110-115 .

8) Price, M. F. , Wilkinson, 1. D. & Gentry, L. O. 1982 plate method for detection of phospholipase activity in *Candida albicans* . Sabouraudia, 15 : 179-185 .

9) K. M. Mohamed Shakir, Cuthbert 0, simpkins, Susan L. Gartner, Douglas O. Sabel, Taffy J. wilIams ((phospholipase C activity in human polymorphonuclear Lenkocytes : partial characterization and Effect ofIndomethacin)) Enzyme 1989 ; 42 : 197 - 208 .

10) Sharon C. A. Chen, Michael Muller, Jin zhong zhou, Lesley C. wright and Tania C. Sorrell ((Phospholipase Activity in *Cryptococcus neoformance* : Anew viralence Factor?)) The Journal ofInfections Diseases 1997; 175: 414 - 420.

11) A. Memon, J.D.E. Patterson, C.E.L. Shaw and J.A. Blain (Phospholipase B activity in mycelia of *Aspergillus niger* (FEMs Microbiology letters 18 (1983) 15-18 .

12) Y. Banno, T. Yamada and Y. Nozawa ((Secreted phospholipases of the dimorphic fungus, *Candida albicans* ; separation of three Enzymes and some biological properties . Sabouraudia: Journal of Medical and veterinary Mycology (1985) 23: 47 - 54.

13) Bligh EC, Dyer WJ. A. rapid method of total lipid extraction and purification, can JBiochem plysiol1959 : 37 911 - 917 .

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فعالية انزيم الفوسفولايبيز في الخيوط الفطرية للفطر Aspergillus flavus

محمد عجة عودة
قسم الكيمياء
كلية العلوم
جامعة ذي قار

الخلاصة :
اخذت عزلة من فطر Aspergillus flavus ونميت على سطح الزرعي الصلب (SDA)،بعد ذلك نقلت الى وسط زرعي اخر خاص لقياس فعالية انزيم الفسفولايبيز يحتوي على الاكار المغذي وصفار البيض كمصدر الى مادة اللسثين التي تعد من الدهون المفسفرة المستهدفة من قبل الانزيم. لمعرفة فعالية الانزيم نقلت هذه العزلة الى وسط زرعي سائل ، استخدمت تقنيسة كروماتوغرافيا الطبقة الرقمية (TLC) لمعرفة الاحماض الدهنية الناتجة بالمقارنة مع احماض دهنية قياسية.