Production of lipase from *Aspergillus oryzae* (T4) isolate by solid-state fermentation

إنتاج إنزيم اللايبيزمن العزلة *Aspergillus oryza* (T4) بطريقة تخمرات الحالة الصلبة

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Absrtract

Forty five fungal isolates belong to different species were tested for their ability to produce lipase, one of these isolates was selected as the best lipase producer. It was identified as a strain of *Aspergillus oryzae* (T4).

The optimum conditions for the production of lipase by solid–state fermentation included culturing of the fungus on cotton seed meal hydrated with 1% ammonium sulfate and1% olive oil, pH 7.0 with 1:2 (w/v) hydration ratio incubated at 30˚C for 7 days .The best extraction solution for the enzyme was 0.2 M phosphate buffer pH 7.0, the productivity reached 2U/g dry weight under these conditions.

المستخلص

اختبرت قابلية خمس واربعون عزله فطريه محليه تعود لأنواع مختلفة على إنتاج إنزيم اللابيز وانتخبت واحده من هذه العزلات كأفضل عزله منتجه للأنزيم ، واظهر التشخيص على أنها إحدى سلالات *Aspergillus oryza* .

وجد إن الظروف المثلى لانتاج أنزيم اللابيز هي تنمية العفن على وسط التخمر الصلب المتكون من بذور القطن المرطبة بـ 1% كبريتات الأمونيوم و1% زيت الزيتون برقم هيدروجيني ابتدائي 7 ونسبة ترطيب 2:1 (وزن/ حجم) وحضنها بدرجة 37 ° م لمدة 7 أيام ، وكان افضل محلول لاستخلاص الأنزيم هو 0.2 مولار من داىء الفوسفات برقم هيدروجيني 7 وبلغت إنتاجية الأنزيم (2وحدة/ غم) تحت هذه الظروف .

Introduction

Lipases (Triacylglycerol acylhydrolase E.C.3.1.1.3) have potential application in food, leather, oil, chemical and pharmaceutical industries [1]. They are widely found in various animals, plants and microorganisms [2], microbial enzymes have many advantages, since the microorganisms can easily grown and usually not difficult to scale up the production conditions [3].

Most studies utilized the liquid media for lipase production from different microorganisms, there are few studies about the production of lipase by solid –state fermentation .The aims of this study were to select a high lipase production fungal isolate, production of lipase in solid-state fermentation (SSF) and studying the effect of some conditions on lipase production, since this technique (SSF) has many economical and technical advantages as high productivity, higher product concentration, simpler equipment, lower contamination and the use of low cost substrates as agroindustry substrates [4]

Materials and Methods

1-Czapek –Dox agar (merk)

2- Lipase detection medium

-Rhan medium [5]

It contains: 5g K_2HPO_4, 5g $(NH_4)2PO_4$,1g $MgSO_4.7H_2O$,1g $CaC_{l2}.6H_2O$, $FeCl.6H_2O$ Trase, 15g agar, 1L D.W. and 1% of sterilized olive oil was added after sterilization.

3-Lipase production media

3.1- Tauson medium [5]

It contains: 1g K_2HPO_4, 2g $NaNO_3$, 0.5g KCl, 0.5g $MgSO_4.7H_2O$, 0.001g $FeSO_4$, 1% tween80, 15g agar, 1L D.W. and 1% of sterilized olive oil was added after sterilization.

3.2- Solid –Slate fermentation media

Wheat bran, rice bran and corn bran were obtained from General Company of Grains. Cotton seed meal, corn seed meal and sunflower seed meal were obtained from General Company of Vegetable oils. These materials were used as substrates for SSF, 10g of each material was hydrated with 30ml of hydration solution, (as described in the methods), in 250 ml conical flasks, the pH was adjusted to 7.0 with 1N NaOH or 1N HCl and autoclaved at 121˚C for 15 min.

4-Source of isolates

The fungal isolates, used in this study, were obtained from Biology Department/College of Science/University of Baghdad, General Company of Grains Ministry of Trade and Biotechnology Department/College of Science/Al-Nahrain University. The fungal isolates were activated and maintained on Czapek-Dox agar slants at 4˚C.

5-Detection of lipase production

Fungal isolates were grown on Rhan medium, the plates were incubated at 32˚C for 72 hrs, the growth and white precipitate was observed.as an induction for lipase production

6- Screening isolates for lipase production on SSF

The fungal isolates were cultured on wheat bran medium, Hydrated with tap water and incubated at 32˚C for 72hrs. The enzyme was extracted with 40ml of tap water, the extract was filtered through a cloth and centerfuged at 6000 rpm at 10˚C for 30 min.

7- Determination of lipase activity

Lipase activity was estimated according to the method of Bier [6] with some modification, 10g of olive oil was emulsified by adding to 100ml of 3% aqueous solution of polyvinyl alcohol and homogenized in blender for 5min, the pH was adjusted to 8.5, 1ml of crude enzyme was added to 9ml of emulsified substrate in 100 ml of Erlenmeyer flask and incubated in shaker incubator at 100rpm at 37˚C for 30min, the reaction mixture was stopped with ethanol-aceton 50:50(v/v) the mixture was titrated with 0.05 NaOH. One unit of lipase activity was defined as the amount of enzyme which release 1µmol of fatty acids per min under the determination condition.

8-Optimum conditions for lipase production

8-1 pH and temperature

The method of Grajek [7] was used to determine the optimum pH and temperature for lipase production, Tauson medium was prepared at different pHs (5-10) and incubated at different temperatures (26-40˚C), a disk of each fungal isolate was transferred to the medium surface and incubated for 72hrs. The diameter of fungal colonies and lipolytic region were measured.

8-2 Hydration solution

Wheat bran was hydrated with different hydration solutions 1% ammonium sulphate, 1% ammonium sulphate with 1% sterilized olive oil and tap water and inoculated with 10^5 spores/g wet weight and incubated at 30˚C for 72 hrs the enzyme activity was determined.

8-3 Lipase production media

The solid substrate media described in 3-2 were hydrated with 1% ammonium sulphate with 1% sterilized olive oil, inoculated with 10^5 spores/g wet weight and incubated at 30˚C for 72 hrs, the enzyme was extracted and the activity was determined .

8-4 Hydration ratio

The substrate was hydrated with 1% ammonium sulphate with 1% olive oil at different ratios 1:1-1:4 (w/v) and incubated at 30˚c for 72 hrs, the enzyme was extracted and the activity was assayed.

8-5 Incubation period

The cotton seed meal was hydrated with 1% ammonium sulphate with 1% olive oil and incubated at 30˚C for 1-10 days, two flasks per day were extracted and the enzyme activity was assayed.

8-6 Extraction solution

Different extraction solutions includes tap water, 0.2M sodium phosphate buffer, 0.1M sodium phosphate buffer, 0.1M sodium chlorid and 0.2M sodium chloride were used

Results

1-Screening of lipase producing fungi

Forty five fungal isolates including different genera were screened, all isolates were found to be able to produce lipase but at various levels. Twenty one of them showed heavy growth with dense precipitation in lipase production medium, these isolates were considered as efficient lipase producers. Most of them (85%) was found belong to *Aspergillus* and others belong to *Penicillium,* Table (1).

2-production of lipase in solid state fermentation medium

Six fungal isolates of different genera were selected according to their lipase activity on Rhan medium and grown in solid state fermentation medium (wheat bran), T4 and R2 isolates showed higher enzyme activity. It was found that maximum productivity of these isolates were reached to (1.2U/g) of dry weight fig (1).

Wheat bran medium is a suitable substrate for production of different industrial enzymes such as amylase [8] proteases [9] since it contain the nutritional factor necessary for growth of microorganisms such as carbon and nitrogen. In other study wheat bran used to compare between lipase and protease production from different fungi including the genera *Aspergillus*, *Geotricum*, *Mucor*, *Penicillum* and *Rhizopus*, high lipolytic activity was appeared from

Penicillum candidum, *Mucor miehei* and *Penicillum camembertii* than other isolates [10] .

3-Determination of the optimum conditions for lipase production

3-1 pH

To study effect of pH on growth as well as production ability of the isolate. Three fungal isolates were selected in accordance to their enzyme productivity. It was found that *Aspergillus* R2 isolate grows well at pH (6-9). The optimum pH for growth and lipase production for *Penicillum* P3.4 was (5-7) and *Aspergillus* T4 isolate favored pH (7-8) for growth and lipase production, Table (2).

In general, pH can influence growth and product formation due to its effect on the solubility of nutrients, ionization of the substrate and its availability to the microorganisms [11].

3-2 Temperature

It was found that optimum temperature for growth and lipase production from *Penicillum* P3.4 was 28˚C, while *Aspergillus* R2 and *Aspergillus* T4 isolates favored (28-30˚C). All isolates could not grow or produce lipase at 40˚c, Table (3).

3-3 Hydration solution

The results showed that 1% ammonium sulphate with 1% olive oil is more suitable for hydration of wheat bran as solid substrate fermentation medium figure (2) this may be attributed to inhibition of protease by ammonium sulphate since most microorganisms secrete proteases to degrade the complex protein to simple nitrogen compound and most proteases undergo catabolic repression i.e., the production of protease is repressed by simple nitrogen source [12] and hence the stability of lipase increases in the presence of ammonium sulphate .

Olive oil may induce lipase production since the production of inducible enzymes increase when this substrate found in the medium which behaves as inducer compound for enzyme production [13].

T4 isolate selected as efficient lipase producer, in addition to its rapid growth, this isolates was identified as *Aspergillus oryzae* according to criteria described by [14]. *Aspergillus oryzae* was recognized as the most widly exploited source of fungal enzyme [15]. Also, *Apergillus oryzae* is classified as Generally Regarded As Safe (GRAS) by FAD (non toxic microorganisms) and allow to be used in many industries such as food industry [16].

3-4 Production of lipase from *Aspergillus oryzae* T4 in different solid fermentation substrate media

All solid fermentation substrate media used in this study was found support fungal growth .However, maximum lipase production was observed in cotton seed meal medium (1.6U/g), figure (3).

In general Solid fermentation substrate media considered as suitable media for fungal growth. Since they contain nutritional factors in sufficient quantity, they permits better air circulation among their particles and mycelial penetration, furthermore they are cheap, available and hence economic for the enzyme production.

3-5 Hydration ratio

Higher enzyme productivity from T4 isolate was obtained when the medium was hydrated with ratio 1:2 (w/v). The productivity reached (2U/g) of dry weight, while decreased to (1.3U/g) and to (0.64U/g) when the hydration was increased to 1:3 and 1:4 (w/v) respectively, figure (4).

The moisture level in solid substrate fermentation may vary depending on the substrate. Generally, the ratio of water to substrate in solid substrate fermentation ranged between 1:1-1:10 (w/v) [3].

3-6 Incubation time

The productivity of lipase increased with increasing the incubation period till the seventh day in which it reached the maximum level, figure (5).

This means that the lipase production begins in early stage of fungal growth and continues during the period of incubation. The production of lipase during the early stage may help the organism in the degradation of fat and liberating fatty acids which are utilized as nutritional factor or enriched supplements to fungal growth Cultured with *Aspergillus oryzae* (T4) after 3 days of incubation at 30˚C.

Table (1): Lipase production ability, of the fungal isolates

	Code of isolates	Lipolytic activity	Genera
1	G10	+ +	*Aspergillus*
2	P3	+	*Aspergillus*
3	T5	+	*Aspergillus*
4	A.p.4	+ + +	*Aspergillus*
5	A.ochra	+ +	*Aspergillus*
6	T2	+ + +	*Aspergillus*
7	L17	+ +	*Aspergillus*
8	H1	+ + +	*Aspergillus*
9	M2	+ +	*Aspergillus*
10	L9	+	*Aspergillus*
11	S1	+ + +	*Aspergillus*
12	T4	+ + +	*Aspergillus*
13	Sb2	+ +	*Aspergillus*
14	T3	+	*Aspergillus*
15	R2	+ + +	*Aspergillus*
16	P25	+	*Aspergillus*
17	T1	+ + +	*Aspergillus*
18	H2	+	*Aspergillus*
19	K2	+ + +	*Aspergillus*
20	D9	+	*Aspergillus*
21	Tz	+ + +	*Aspergillus*
22	L11	+ + +	*Aspergillus*
23	R4	+ +	*Aspergillus*
24	A.p.3	+ + +	*Aspergillus*
25	A.p.5	+	*Aspergillus*
26	A.p.18	+ + +	*Aspergillus*
27	4	+ + +	*Aspergillus*
28	Asp	+ + +	*Aspergillus*
29	I	+ + +	*Aspergillus*
30	25.M.E.a	+ + +	*Aspergillus*
31	Asp.H.	+ + +	*Aspergillus*

32	2.M.E.A	+ + +	*Aspergillus*
33	Fus.1	+	*Fuzarium*
34	Fus.2	+	*Fuzarium*
35	P.18	+ +	*Penicillum*
36	P.12	+ +	*Penicillum*
37	Pen.3.	+ +	*Penicillum*
38	Pen3.4	+ + +	*Penicillum*
39	Pen.H.B.1	+	*Penicillum*
40	Pen.C.	+ +	*Penicillum*
41	Pen.A.	+ +	*Penicillum*
42	Pen.1.	+ + +	*Penicillum*
43	3.1	+ + +	*Penicillum*
44	R1	+	*Rhizopus*
45	R3	+	*Rhizopus*

Lipolytic activity : + Little precipitation ++ Modrate ppt +++ Dense ppt.

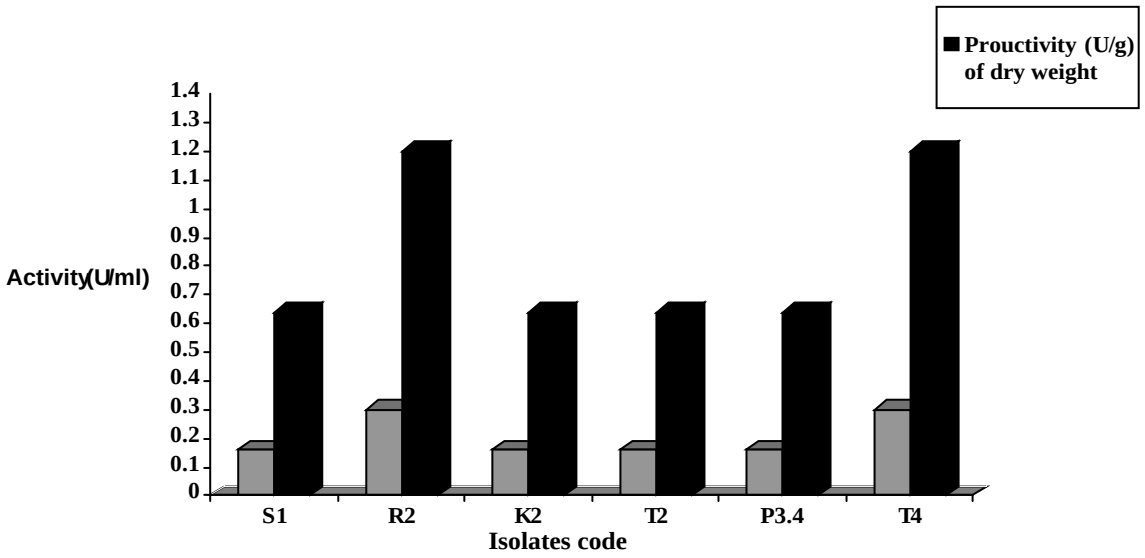


Figure (1): The productivity of lipase from different fungal isolates in wheat bran medium hydrated with tap water 1:3 (w/v) at pH 7.0 .

Table (2): The growth and ability of isolates for lipase production at different pH

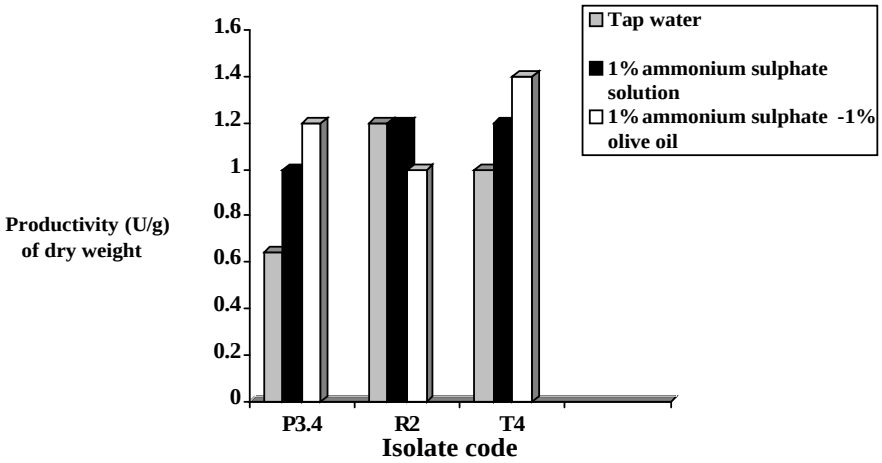
	pH					
The ratio Of lipolytic area / Diameter of colony	**5**	**6**	**7**	**8**	**9**	**10**
P3.4	1.5 / +++	1.6 / +++	1.6 / ++	1.1 / +	1.2 / +	1.1 / +
R2	1 / +	1.6 / ++	1.6 / ++	1.5 / +++	1.5 / ++	1.4 / +
T4	1.3 / ++	1.2 / ++	1.5 / +++	1.6 / ++	1.4 / ++	1.1 / +

* Colony diameter : + ≤ 1cm + + ≤ 2 cm +++ ≤ 3 cm

Table (3):Growth and ability of isolates for lipase production at different Temperatures, pH 7.0 for 72 hrs.

The ratio Of lipolyt area / Diameter of colony	Temperature 26 ˚C	28 ˚C	30 ˚C	33 ˚C	37 ˚C	40 ˚C
P3.4	1.4 / ++	1.4 / +++	1.3 / ++	1.3 / ++	1.3 / +	0 / 0
R2	1 / +	1.4 / ++	1.2 / +++	1.1 / +	1.1 / +	0 / 0
T4	1.3 / ++	1.5 / ++	1.6 / +++	1.4 / +++	1.2 / ++	0 / 0

* Colony diameter: + ≤ 1cm + + ≤ 2 cm +++ ≤ 3 cm



Figure(2): Lipase productivity in wheat bran medium hydrated with diffrenet solution in a ratio of 1:3 (w/v), pH 7.0 inoculated with 10^5 spores/g and incubated at 30c˚ for 3 days.

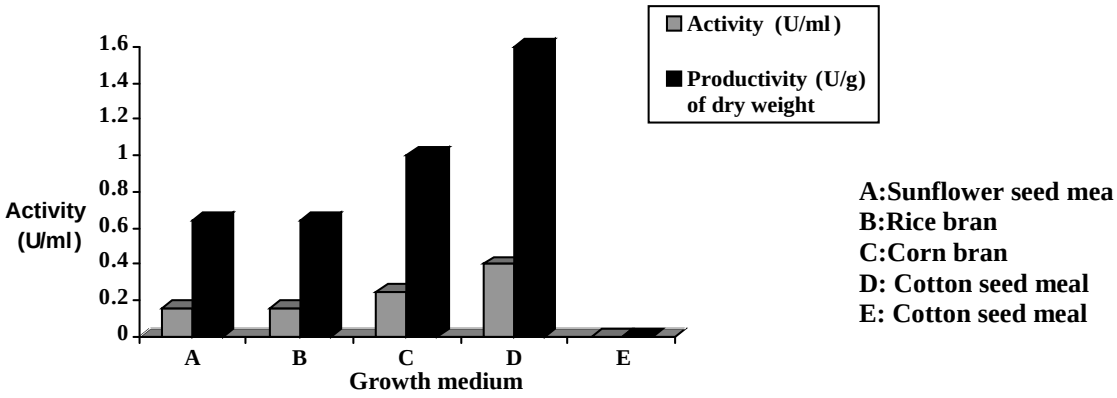


Figure (3):Lipase productivity from *Aspergillus oryzae* (T4) in different growth media hydrated with 1% ammonium sulphate 1% olive oil at ratio of 1:3 (w/g) ,pH 7.0 inoculated with 10^5 spores and incubated at 30˚c for 3 days.

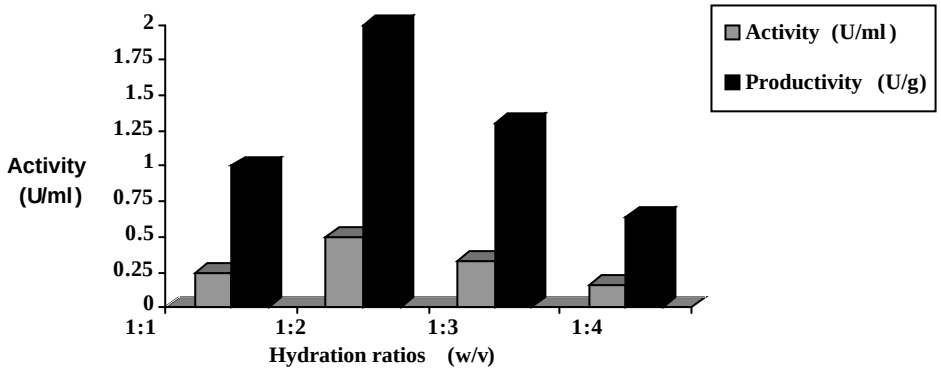


Figure (4): lipase production in cotton seed meal hydrated with different hydration ratios

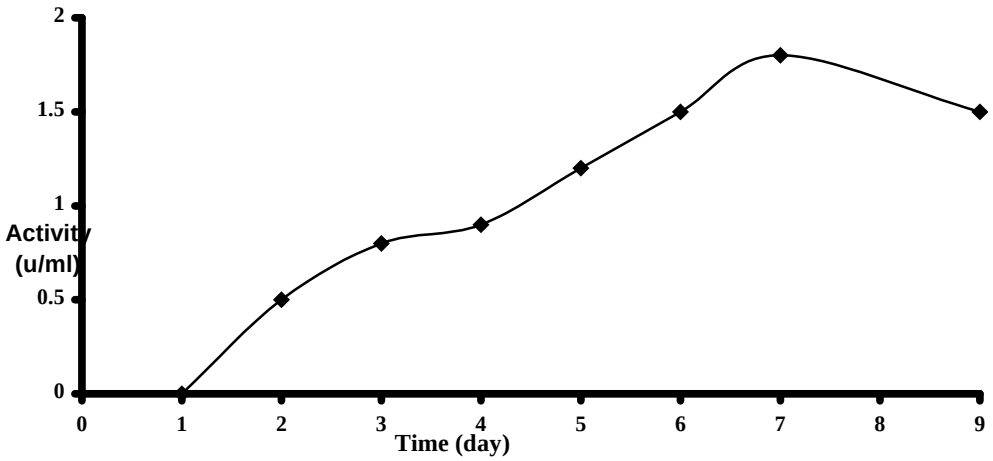


Figure (5): Production of lipase from *A. oryzae* (T4) during different incubation periods at 30 ˚C.

3-7 Extraction solution

The best extraction solution for lipase from cotton seed meal medium was phosphate buffer at pH 7.0 and phosphate buffer-sodium chlorid solution, the activity in these solution reached to (0.7U/ml), followed by sodium chlorid solution (0.6U/ml) while the activity reached (0.5U/ml) when the enzyme was extracted with tap water figure (6) .

The efficiency of phosphate buffer in enzyme extracton may belong to its ability to dissolve the enzyme and maintains a suitable pH for the stability of enzyme.

The decreasing in lipolytic activity with tap water may be due to that the tap water has no buffering capacity and ionic strength which facilitate the extraction of enzyme from the medium.

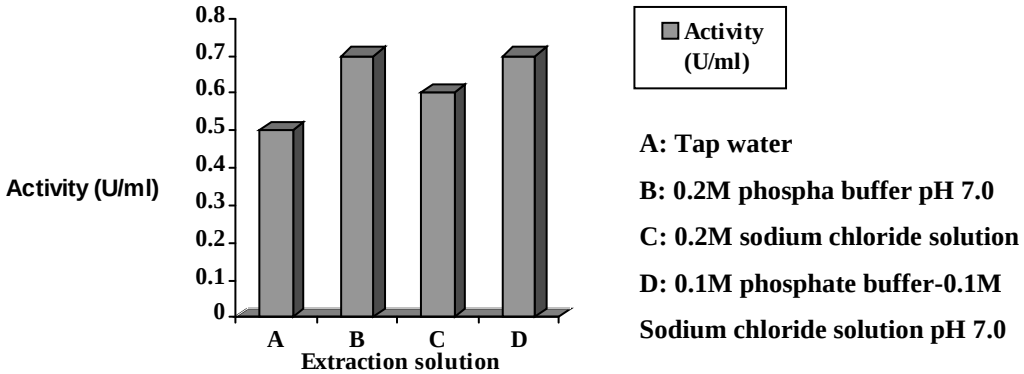


Figure (6): Activity of lipase produced by *Aspergillus oryzae* (T4) growing in cotton seed meal medium and extracted with different extraction solutions

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