Optimization, Production and Antifungal Activity of Chitinase Produced by *Trichoderma harezianum*

تحديد الظروف المثلى وانتاج ودراسة الفعاليه التثبيطيه لأنزيم الكايتينيزالمنتج من العفن *Trichoderma harezianum*

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Abstract

Twelve of *Trichoderma* spp. Isolates were obtained from 30 soils samples collected from different crops fields in Baghdad city. Among them, isolate designated as TLT4 which produced highest chitinase activity in primary and secondary screenings was identified as *Trichoderma harezianum*. The high level of chitinase production was observed in chitinase production media (CPM) amended with 0.4% colloidal chitin with pH6, incubation temperature 30°C, incubation period 6 days, peptone as nitrogen source and inoculum size 1×10^5 spore/ml using submerged cultures. The antifungal activity of crude *T. harezianum*chitinase was investigated against four dermatophytes ncluding: *Trichophytonment agrophytes, T. rubrum, Microsporumcanis, M. gypsum*. The crude chitinase was found to inhibit the growth of all dermatophytes tested with varying degrees. Clotrimazole drug was used to compare.

Key words: Chitinase, optimization, *Trichoderma harezianum*

الملخص

تم عزل اثنى عشرعزلة من الفطر *Trichoderma* spp. من 30 نموذج من نماذج التربه التي جمعت من مناطق زراعيه مختلفه في مدينة بغداد. من بين هذه العزلات تميزت العزله TLT4 بانتاج الكايتينيزبفعاليه عاليه من خلال الغربله الاوليه والثانويه لتحلل الكايتين وشخصت هذه العزله فيما بعد على انها *T.harezianum*. لقد ظهر ان اعلى مستوى لانتاج الانزيم كان عند استخدام الوسط الزرعي CPM المدعم بـ 0.4% من الكايتين الغروي مع درجة حموضه للوسط 6 ودرجة حراره للحضن 30 ومدة حضن 6 ايام واستخدام الببتون كمصدر للنيتروجين ونسبة لقاح $1X\ 10^5$ مع سرعة هز 100دوره/دقيقه. كما درست الفعاليه للانزيم الخام المضاده لبعض الفطريات الجلديه مثل: *T.mentagrophytes, T.rubrum, M.canis, M.gypsum* وقد وجد بان انزيم الكايتينيزالخام قد ثبط نمو جميع انواع الفطريات الجلديه وبدرجات متفاوته واستخدم عقار الكلوتريمازول للمقارنه.

الكلمات المفتاحية: كايتينيز، تحديد الظروف المثلى *Trichoderma harezianum*

Introduction

Chitin, an insoluble linear β-1,4-linked homopolymer of N- acetylglucosamine, is one of the most abundant natural renewable compounds. It is a nitrogen containing polysaccharide, related chemically to the cellulose [1]. It is a major cell wall constituent of higher fungi belonging of chitridiomycets, ascomycetes, basidiomycetes and deuteromycetes, insect exoskeletons and crustacean shells [2]. Chitinase enzyme (EC 3.2.1.14) has responsible for catalyzing the biological hydrolysis of chitin to its monomer N-acetyl-D-glucosamine and has been found to be produced by various types of microorganisms such as bacteria, fungi, yeast etc. Fungi produce chitinase to digest chitin and utilize it as carbon and energy source. As chitin is degraded by chitinase enzyme, this extracellular hydrolytic enzyme is most promptly used in the biological research as a controlling agent for the generation of fungal protoplasts due to its degrading nature of cell wall. Therefore this enzyme was used as nontoxic alternative to chemical fungicides [3].

Studies on medium optimization for chitinase production are a worthwhile technique for multifactor experiments because it is less time consuming and capable of detecting the true optimum level of the factor [4]. Because different medium constituents impact highly on the product of extracellular chitinase from the microorganisms. Therefore in the present work, a chitinolytic fungal strain has isolated and chosen for the production of chitinase and various physical and nutritive parameters were screened to ensure the maximum production of chitinase, and investigating its antifungal activity gainst some dermatophytes.

Materials and Methods

Soil Samples Collection

Thirty random rhizosphere soil samples were collected in sterile polypropelline bags from different crops fields in Baghdad city.

Isolation and identification of *Trichoderma* spp.

Petri dishes containing Sabouraud dextrose agar (SDA) were inoculated with (1ml) of the 10^{-3} diluted soil suspension. The plates were incubated at 28°C and the growths of the colonies were accompanied up to 48h. Fragments of the individual colonies were transferred separately to the same medium and the growth was accompanied for 5 days to identification. All isolates were identified on the basis of macro morphological (colony appearance, colony colour, colony growth rate) and micro morphological characteristics (conidial shape and size, phialide shape and size) could separate all species of *Trichoderma* using SDA, Czapekdox agar, Malt extract agar and slide culture technique.

Preparation of spore suspension

All isolates of *Trichoderma* spp. were subcultured on PDA slants and incubated at 30°C for 5 days. Spores from the slants were suspended in sterile saline(0.085% NaCl) containing 0.01% Tween 80 to obtain 1×10^6spore /ml determined by haemo cytometer.

Preparation of choloidal chitin

Five grams of chitin powder was taken from crab shells (sigma) and added slowly to (60ml) of concentration HCl (Merck) and left at room temperature overnight with vigorous stirring. The mixture was added to (200ml) ice cold (95%) ethanol and incubated overnight at room temperature with vigorous stirring. The precipitate was collected by centrifugation at 8000rpm for (20 min.) at 4°C and transferred to a glass funnel with filter paper .The colloidal chitin was washed with sterile distilled water until colloidal chitin became neutral (pH 7.0), it was lyophilized and stored in a dark place at (4°C) for farther studies [5].

Primary screening of chitin hydrolysis

The isolates of *Trichoderma* spp. were screened for overproducing chitinolytic activity, by using the method of Kotasthane and Agrawal [6]. Here to study the chitinase activity, chitinase detection medium was used. The final chitinase detection medium consist of (g/L): 4.5g of colloidal chitin, 0.3g of $MgSO_4.7H_2O$, 3.0g of $(NH_4)SO_4$, 2.0g of KH_2PO_4, 1.0g of citric acid monohydrate, 15.0g of agar, 0.15 g of bromocresol purple and 200 µl of tween-80, pH was adjusted to 4.7 and was autoclaved at 121°C for 15 min. The medium was poured into the 90mm Petri's plates. The fresh culture plugs of the *Trichoderma* spp. to be tested for chitinase activity was inoculated and incubated at 25°C for 3-4 days. Formation of the purple colored zone was observed and recorded.

Secondary screening of chitin hydrolysis

chitinase production medium (CPM) was used for detection of chitinase production in broth culture. The final CPM consist of (g/L):0.5g of NaCl, 0.5g of $CaCl_2$, 3g of KH_2PO_4 , 1g of K_2HPO_4 , 0.7g of $MgSO_4.7H_2O$, 1.4g of $(NH_4)_2SO_4$ and 5g of colloidal chitin , pH was adjusted to 4.7 and the CPM was distributed into 250ml Erlenmeyer flask containing 50ml of the medium and autoclaved at 121°C for 15 min. Isolates of *Trichoderma* spp. were inoculated into CPM with 0.5ml of 1×10^6 spore/ml and incubated in rotary shaker incubator with 140rpm at 30°C for 5 days. Culture filtrate was harvested after 5 days and the enzyme assay as well as the protein content was measured.

Measurement of enzyme activity

Extracellulary Chitinase activity was determined colorimetrically by detecting the amount N-acetyl glucosamine (GlcNAc) released from colloidal chitin substrate. The reaction mixture consisted of 0.3ml of crude enzyme and 0.2ml of colloidal chitin (2%) in 50 mmol/l acetate buffer, pH 4.6. The mixture was boiled for 10 min., chilled and centrifuged at 5000g for 10min. to remove insoluble chitin. The resalting adduct of reducing sugars were museared by dinitro salicylic acid(DNS) method [7]. Absorbance was measured at 530 nm using UV `activity was defined as the amount of enzyme that released 1µmol GlcNAc from colloidal chitin per minute under the specified conditions.

Determination of protein concentration

Protein concentration was determined by the method of [8], with bovine serum albumin as the standard.

Optimization of chitinase production

The optimization study of the following parameters was done for better growth and production of the enzyme. Each experience was conducted in three replicates.

1. **Effect of colloidal chitin concentration on chitinase production**

Six flasks of 250ml Erlenemyer flask prepared, every flask contain 50 ml of CPM with different substrate (colloidal chitin) concentration of (0.1 %, 0.2%, 0.3%, 0.4%,0.5% and 0.6%), pH was adjusted to 4.7 and autoclaved at 121°C for 15 min., then all flasks inoculated with 0.5ml of 1×10^6spore/ml, and incubated in rotary shaker incubator with 140rpm at 30°C for 5 days. Culture filtrate was harvested after 5 days and the enzyme assay as well as the protein content was measured.

2. Effect of pH on chitinase production

Seven flasks of 250ml Erlenemyer flask was prepared, every flask contain 50 ml of CPM and pH of the medium was adjusted to(3.0,4.0,4.5,5.0,5.5,6.0,6.5,7.0 and 8.0) .

3. Effect of temperature on chitinase production

Five flasks of 250ml Erlenemyer flask was prepared, every flask contain 50 ml of CPM, and pH was adjusted to 6, and incubated at different temperatures (20,25,30,35,40°C).

4. Effect of different nitrogen sources on chitinase production

Four flasks of 250ml Erlenemyer flask was prepared, every flask contain 50 ml of CPM with different nitrogen sources, organic such as: $(NH_4)_2SO_4$, NH_4Cl and inorganic such as: pepton, tryptone, separately. These were added with concentration (1%).

5.Effect of incubation period on chitinase production

Eight flasks of 250ml Erlenemyer flask were prepared, every one contains 50 ml of CPM, and cultures were incubated in rotary shaker incubator with 140rpm for 1-8 days.

6.Effect of inoculums size on chitinase production

Seven flasks of 250ml Erlenemyer flask was prepared, every flask contain 50 ml of CPM, flasks were inoculated with1×10^2 -1×10^8 spore/ml.

Utilization of Fungal biomass as a source of chitin

Fusariumsolani, Aspergillusniger, A.flavus, Rhizopusoryzae, Penicilliumdigitatum were grown on czapek-Dox broth. After 15 day of incubation, the fungal mats were collected and sterilized by autoclave at 121°C for 20min. The sterilized fungal mats were washed twice with sterile distilled water and dried in an oven at 80°C till constant weight [9].The dried fungal mats was powdered and used as chitin source with ratio 5g/lc c for chitinase production.

Collection of dermatophytes

Medically pathogenic dermatophytic fungi including: *Trichophyton mentagrophytes*, *T. rubrum*, *Microsporumcanis* and *M. gypsum* were obtained from the dermatologicalde department of Baghdad teaching hospital, and they were inoculated into Sabouraud dextrose agar (SDA) and incubated at 28 - 30 °C for 10 days. All isolates were identified on the basis of macro morphological and micro morphological characteristic using SDA, and slide culture technique, according to [10].

Antifungal Activity of crude Chitinase

The crude chitinase was assyed for antifungal activity against some dermatophytes including : *T. mentagrophytes, T. rubrum, M.canis*and *M. gypsum* by agar dillution method on PDA plates. A fungal plug (6 mm diameter) was removed from the 10 day old culture. The plug was transferred onto the center of the PDA plates with different concentration of crude chitinase (1%, 5%, 10%, 15%, 20%).The plates were incubated for 10 days at 30ºC and was monitored for a fungal growth inhibition. The percentage of inhibition of growth was calculated as:

Fungal growth inhibition (%) = (A-B)/A x 100

where A = microorganism growth in control,

and B = microorganism growth in media with different concentrations of chitinase. Clotrimazole drug was used to compare.

Statistical analysis

Duncan test method was used for statistical analysis.

Results and discussion

Isolation and identification

T. harezianum is a filamentous fungus that is widely distributed in the soil, plant material, decaying vegetation, and wood. Morphological features of the conidia and phialides help in differentiation of this species from each other.

Macromorphological features

At 25°C and on potato dextrose agar, the colonies are wooly and become compact in time. Colony rate9.8cm in 4 days, whitish to bright green, floccose, reverse is drab colour, show much pigmented yellowish mycelial growth than other species.

Micromorphological features:

Septate hyaline hyphae, Conedial shape is smooth and globose to subglobose with size 2.7×2.5µm and yellow to pale green, phialide is flask shaped with size 5.2×2.4µm.

Primary screening of chitin hydrolysis

All fungal isolates were chitinase producer, among them *Trichoderma* sp.TLT4 was the most efficient one, specific activity of chitinase was (8.3 U/mg). According to this results, isolate TLT4 was selected for improving chitinase production, since it gave the highest productivity, and identified as*Trichoderma harezianum*.

Optimization of chitinase production

1. Effect of different concentrations of colloidal chitin

The effect of different concentrations of colloidal chitin on chitinase production was evaluated. Among six different concentrations tested, highest specific activity for extracellular chitinase was (8.2U/mg) in the presence of (0.4%) colloidal chitin and considerably enhanced the chitinase activity, followed by(0.5%).Beyond (0.5%) and less(0.4%), Figure (1).

The same results was observed by [11] which reported that maximum chitinase production of *Trichoderma harezianum* was in presence of (0.5%) chitin, while chitinase production by *Streptomyces lividance* is induced by(1%) colloidal chitin [12].

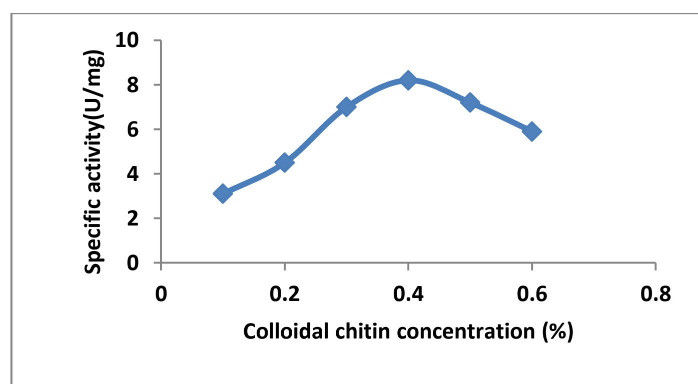

Fig. (1): Effect of different concentrations of chollidal chitin on chitinase production by *T. harezianum*. Inoculum size 1X10^6, incubationperiod 5 days,temperature 30°C and pH7.

2. Effectof pH on chitinase production

Chitinase productivity was variable at different pH values. It was observed that maximum chitinase productivity was reached pH6 with specific activity (8.8U/mg).Results in figure (2) showed that pH values between 5-7was the best for chitinase production, the minimum chitinase productivity was occurred at pH3.

pH my change during the incubation time as a result of fungus metabolism and my effect on enzyme activity, it is effect on medium characterization such as solubility and translocation of nutritional materials and uptake by microorganisms [13].

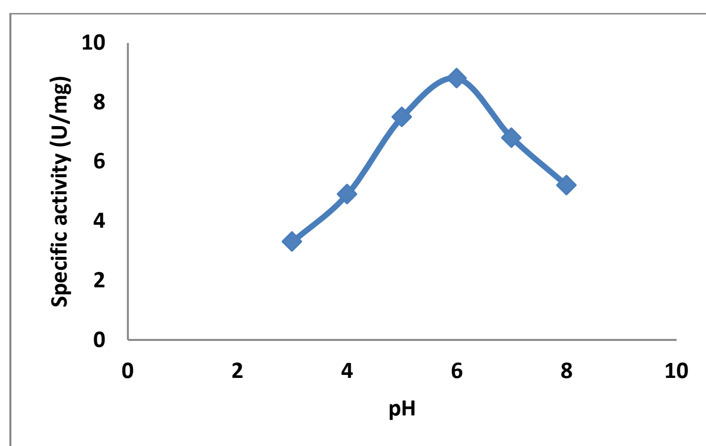

Fig. (2): Effect of different pHs on chitinase production by *T. harezianum* .Inoculum size 1X10^{6}, incubation period 5 days, temperature 30°C.

3. Effect of temperature on chitinase production

The optimum temperature for chitinase production by *Trichoderma harezianum* was found to be 30°C with specific activity (9.5 U/mg), However, the decrease or increase in the incubation temperature lead to decrease the enzyme production as it was illustrated in figure (3).

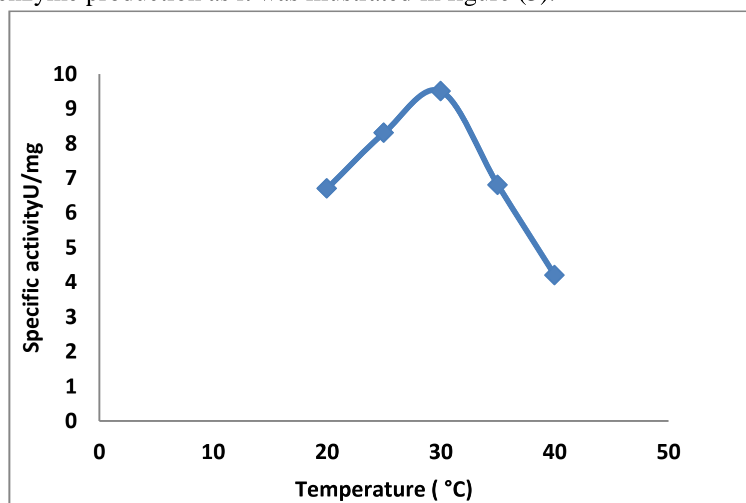

Fig. (3): Effect of temperatures on chitinase production by *T. harezianum* .Inoculum size 1X10^{6}, incubation period 5 days, pH6 and 0.4% colloidal chitin

Temperature is affected in all vital events in the cell directly through influence in the genetic material and enzymes and lipids in the cell membrane and lead to influence in the quantity and speed of growth. When temperature increases to more than the optimum degree, this will lead to a rapid decline in the velocity of growth due to denaturation of the enzyme, this is because the rupture of weak bonds in the secondary and tertiary of enzyme construction, and this change is very significant.

In the case of low temperature to less than optimal temperature, this will lead to slow down the crossing of the solutes through the cytoplasmic membrane in the cell and this lead to the slow of enzyme action [14].

4. Effect of different nitrogen sources on chitinase production

The effect of supplementation of different nitrogen sources (organic and inorganic) on chitinase production was evaluated. Data obtained revealed that Pepton and $(NH_4)_2SO_4$were potent inducers for chitinase production by *T.harezianum*, in which the specific activity were (10.7U/mg) and (9.4U/mg), respectively. On the other hand NH_4Cl (7.2U/mg) was the less inducer for enzyme production Figure (4).

It can be concluded that the organic nitrogen compound enhancing chitinase production more than inorganic compounds, this may be because organic sources support the growth and biosynthesis of protein, nucleic acid and many other cell constituents by providing cell with nitrogen, carbon and

energy, while inorganic compounds need to assimilate into organic molecules to involve in biosynthesis [15].

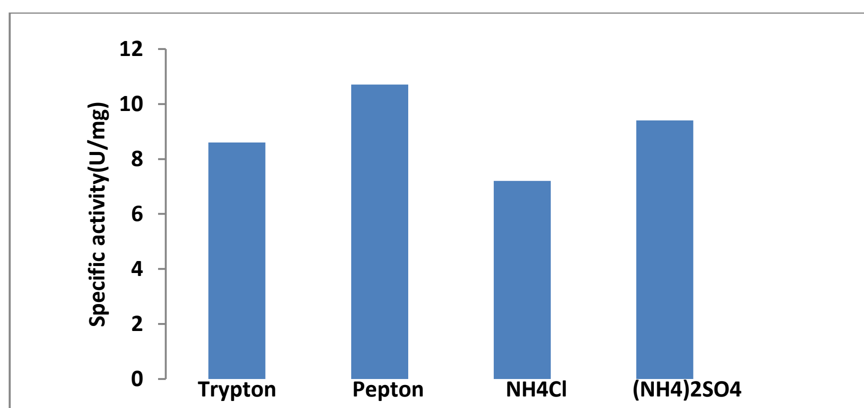

Fig. (4): Effect of different nitrogen sources on chitinase production by *T. harezianum*. Inoculum size 1X10^6, incubation period 5 days, pH6 and 0.4% colloidal chitin

5. Effect of incubation period on chitinase production

Chitinase production by *T. harezianum* was observed during 1 to 8 days. Results revealed that maximum specific activity of chitinase (11.3 U/mg) was achieved after 6 days; however it decreased down to (10.2 U/mg) at the 7th day figure (5).

Results in figure (5) clearly show pronounced chitinase production with the increasing of fermentation period up to 6 days and then decreased.

The decline in enzyme activity after 6th day of fermentation may be due to the secretion of proteolytic enzymes which are known to cause the denaturation of chitinase. This may also be attributed to decrease in nutrient availability in the medium at the end of the cultivation process or catabolic repression of enzyme [16].

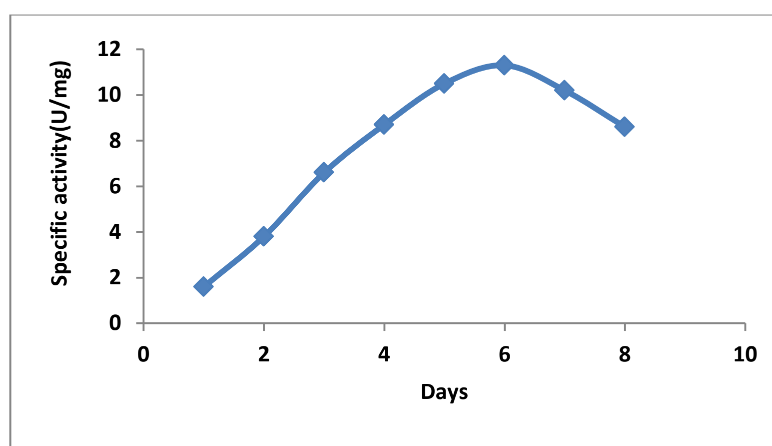

Fig. (5): Effect of incubation period on chitinase production by *T. harezianum* ..Inoculum size 1X10^6, temp. 30°C, pH6 and 0.4% colloidal chitin

6. Effect of inoculum size on chitinase production

It was noticed that chitinase production increase gradually with increasing of inoculum rate. The higher specific activity for chitinase was (12.2U/mg), when the medium inoculated with 1 x 10^5 spore / ml Figure (6).

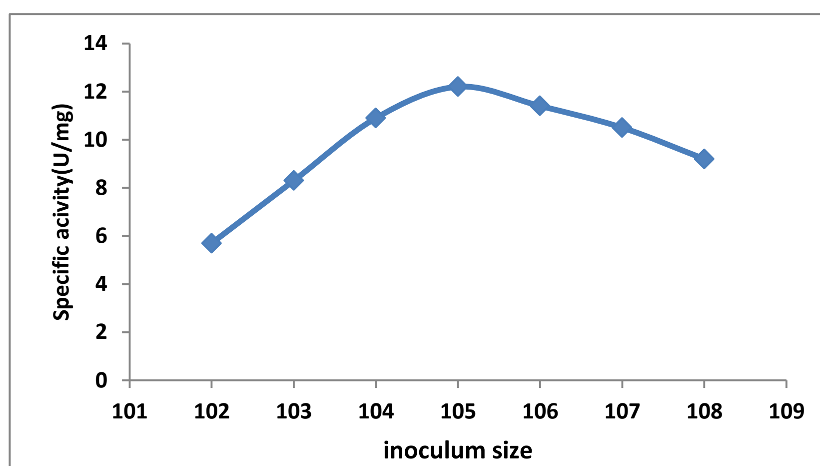

Fig. (6): Effect of inoculums size on chitinase production by *T. harezianum.*temp. 30°C, pH6 and 0.4% colloidal chitin

Low enzyme activity was recorded above and below of optimum inoculum size, at high inoculum rate the viscosity of fermentation medium might increase due to the tremendous growth of fungi, resulting in nutritional imbalance in the medium or may be using up the nutrients before they are physiologically ready to start enzyme production.

Low chitinase production below the optimum inoculum rate may be due to insufficient fungal biomass. inoculum size plays an important role in fermentation process; in a suitable inoculum rate, sufficient amount of nutrient and oxygen will be accessible for growth [17].

Utilization of Fungal biomass as a source of chitin

Results showed that medium containing colloidal chitin as chitin source was more suitable than media containing other crude chitin sources to produce extracellular chitinase, since the specific activity was (12.3U/mg), followed by the medium containing *Rhizopusoryzae* powder (9.4U/mg) Figure (7).

Using of crude chitin for chitinase production instead of colloidal chitin reducing the cost and efforts of it's extraction beside it represents a waste and can be remove by this method [18]. Productivity of chitinase was high in medium containing colloidal chitin comparing with other natural chitin sources. This is may be attributed to that the colloidal chitin provides high concentration of chitin as a substrate which enhancing chitinase production, while fungal mycelium containing lower concentration of chitin [19].

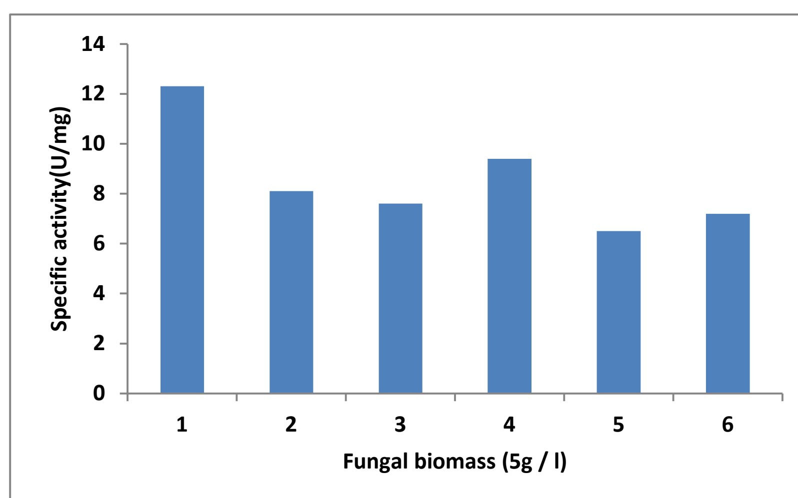

Fig. (7): Utilization of Fungal biomass as a source of chitin) 1) colloidal chitin (2)*Aspergillusniger* (3)*Aspergillusflavus* (4)*Rhizopusoryzae* (5)*Penicilliumdigitatum* (6)*Fusariumsolani*.

Antifungal activity of crude chitinase against some dermatophytes

Data obtained revealed that crude chitinase production from *T.harezianum* has different antifungal activity on different dermatophytes *in vitro*. The inhibitory effect on the growth of dermatophytes were investigated under different concentrations of crude chitinase (1%, 5%, 10%, 15%, 20%) by agar dilution method on PDA plates. The concentration of (20%) yielded the maximum inhibition against all dermatophytes were investigated, while high crude chitinase activity effect it was against *T. rubrum* Table (1).

Several studies have demonstrated that chitinases can cause eformation of viable hyphae and result in inhibition of hyphae of the test fungi, this inhibition is due to degradation of cell wall chitin of fungi [20].

Treatment of dermatophytes infection with antibiotic have many disadvantages including toxicity for human and a detoxification of drugs by the infectious fungi, i.e., antibiotic resistance, therefore the chitinase was overcome this disadvantages. The current usage of synthetic drugs leads to either side effects in human or resistant fungal varieties due to prolonged use. Since these pathogenic dermatophytes are eukaryotae, their chemical treatment with antifungal drugs may also affect host tissue cells [15], Table (2).

Table (1): Antifungal activity of crude chitinase against some dermatophytes

Fungus	*T. mentagrophytes*		*T. rubrum*		*M. canis*		*M. gypsum*	
Conc. (%)	Colony diameter average (mm)	Fungal growth inhibition (%)	Colony diameter average (mm)	Fungal growth inhibition (%)	Colony diameter average (mm)	Fungal growth inhibition (%)	Colony diameter average (mm)	Fungal growth inhibition (%)
Control	63.4 a	0	27.5 a	0	80.4 a	0	55.2 a	0
1	61.6 a	2.84	26.2 a	4.73	79.5 a	1.12	53.4 a	3.26
5	54.8 b	13.57	20.0 b	27.28	76.2 a	5.22	49.3 a	10.69
10	46.1 c	27.29	14.3 c	48.0	70.1 b	12.81	42.5 b	23.0
15	35.8 d	43.53	7.4 c	73.10	66.4 b	17.41	38.0 c	31.16
20	22.5e	64.51	0 d	100	59.7 c	25.75	34.6 c	37.31

Different vertically letters mean no significant differences at the level of probability ($p \leq 0.05$) according to Duncan test .

Table (2): Antifungal activity of clotrimazole against some dermatophytes

Fungus	*T.mentagrophytes*		*T.rubrum*		*M.canis*		*M.gypsum*	
Conc. %	Colony diameter average (mm)	Fungal growth inhibition (%)	Colony diameter average (mm)	Fungal growth inhibition (%)	Colony diameter average (mm)	Fungal growth inhibition (%)	Colony diameter average (mm)	Fungal growth inhibition (%)
Control	63.4 a	0	27.5 a	0	80.4 a	0	55.2 a	0
1	60.1 a	5.2	23.4 a	14.9	69.3 b	13.8	49.4 b	10.5
5	48.4 b	23.6	19.6 b	28.7	40.0 c	50.2	33.5 c	39.3
10	26.1 c	58.8	9.2 c	66.5	21.5 d	73.2	18.2 d	67.0
15	10.5 d	63.4	0 d	100	8.0 e	90.0	9.3 e	83.1
20	0 e	100			0 f	100	0 f	100

Different vertically letters mean no significant differences at the level of probability ($p \leq 0.05$) according to Duncan test .

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