Purification, Characterization and Antifungal Activity of Chitinase from *Serratia marcescens* Isolated from Fresh Vegetables

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Abstract

Seven [35%] and five [25%] *Serratia marcescens* isolates were obtained out of 20 samples of lettuce and 20 samples of spinach, respectively, taken from different locations in a farm in Baghdad city. The isolate that produced chitinase in higher level was chosen to purify chitinase through several stages of purification including: ammonium sulfate precipitation, DEAE-sephadex ion exchange chromatograpgy and sephadex G-200 gel filtration with 89.5- fold purification and 30% recovery.

The purified chitinase was characterized and the molecular weight of enzyme was 59000 daltons by using gel filtration chromatography. The optimum pH and temperature of the purified chitinase were 6.0 and 50° c, respectively, and the purified enzyme was stable on pH 5-7 up to 50° c. The enzyme was activated by Ca^{+2} , Cu^{+2} , Mg^{+2} and inhibited by Hg^{+2}. In addition , Triton x-100 and n-ethylmaleimide increased the chitinase activity while EDTA, methanol, ethanol and acetone inhibited enzyme activity; and this indicates that chitinase is a metaloenzyme. Chitinase showed stronger inhibitory activity to *Fusarium solaini* compared with *Aspergillus flavus* with percent of inhibition 83 and 69%, respectively . Therefore, this research leads to increase interest by using the chitinase as biocontrol agent of phytopathogenic fungi and insects , production of chito –oligosaccharides,preparartion of sphaeroplast and protoplast from yeast and fungi and bioconversion of chitin waste to single cell protein for animal feed.

Introduction

Serratia sp. are gram negative bacteria, classified in the large family of Enterobacteriaceae [1]. The *Serratia* genus includes different species, the best characterized species, and the one most frequently recovered from human , is *Serratia marcescens* [2]. This common microbe is found as a saprophyte in soil, water and plants [3].

*S*erratia is isolated from many plants such as tomatoes, spinach, carrots, coconuts, green onions, lettuce and broccoli [4,5]. In addition, *S*erratia species frequently have been recovered from diseased or dead insects [5]. Serratia sp. have a good set of exoenzymes that may be harmful to insects such as proteases, gelatinase and chitinase[4].

Chitin a homopolymer of N-acetyl-D-glucosamine (GlcNAc) residues linked by β1-4 bonds is widely distributed in nature as a constituent of insects exo skeleton, shells of crustaceans and fungul and algae cell walls [6,7,8]. Chitinase plays an important role in the virulence of *S*erratia for insects and fungi, since this enzyme is active in lysing the cell walls of many insects and fungi that infect the economic plants [8], in addition inhibit spore germination and germ tube elongation of the phytopathogenic fungi [9,10] . This enzyme has also antimicrobial and cell lysis activites against many kinds of bacteria [6,11]. Therefore, chitinase has wide range of biotechnological applications, especially in production of chito-oligosaccharides, preparation of spheroplast and protoplast from yeast and fungal species and bioconversion of chitin waste to single cell protein for animals feed [7-12].

For these reasons, the goal of our research was to purify chitinase, to characterize this enzyme and to study the antifungul activity of chitinase.

Materials and Methods

A total of 40 samples of fresh vegetables, including 20 samples of lettuce (L) and 20 samples of spinach (S), were collected from different locations in a farm in Baghdad city. Each sample was analyzed according to the method that described by [13] . Briefly, 25g of each sample was enriched in peptone water for 24h at 35°c.

Isolation and identification *Serratia marcescens*

One loopfull of plant samples was plated on blood agar and MacConkeys agar, then inucubated at 30°c for 18-24 h. For isolation of *Serratia marcescens*, several biochemical tests were done and these include the following tests: inability to ferment lactose, a negative oxidase, positive results to catalase, DNAase,lysine decarboxylase, ornithine decarboxylase, growth at 40°c and motility tests [5,14]. Besides to API 20E system to differentiate *Serratia marcescens* from the other types.

Isolation of fungi

Aspergillus Flavus and Fusarium solani were obtained from the laboratory of Agriculture College in Baghdad University and they were identified according to [15].

Detection of chitinolytic activity on plates

Ten µl of *Serratia marcescens* culture was placed into wells (5 mm in diameter) prepared in synthetic medium agar (SM) that contained 2.0g colloidal chitin, 0.5g peptone 0.5g yeast extract, 0.1g KH_2PO_4, 0.01g $MgSO_4.7H_2O$ (pH=6.5) and 1.7%w\w agar in 100ml of water and incubated at 30^0c. After 18-24 h, the chitinolytic activity was indicated by the formation of clear halos around the wells [12,16].

Chitinase assay

Chitinase activity was conducted by a modified method described by [7,10,12]. 0.5 ml of enzyme solution was added to 1 ml of 0.1 M phosphate buffer (pH=6.0) as blank. The reaction initiated by the addition of 0.5ml of enzyme solution to 1ml of 0.2%(wt/vol) colloidal chitin in the same buffer and incubated at 50^0c for 15min, the reaction was stopped by centrifugation (5000xg) for 10min and the addition of 1ml of dinitrosalicylate (DNS) reagent. The reducing sugars released (GLcNAc) were measured by observing the absorbancy at 410nm and returning to standared curve for GlcNAc (N-acetyl-D-glucosamine). One unit of chitinase activity was defined as the amount of enzyme of that released 1µmol of reducing sugars (GLcNAc) in 1 min.

Protein assay

The protein concentration was measured by using the method of [17] by spectrophotometeric assay at 600nm in each stage of chitinase purification and using bovine serum albumin as standard.

Purification of chitinase

Serratia marcescens chitinase was purified by a modification of the method [12]. Cells were grown in M9 medium containing the following: 0.7% Na_2HPO_4, 0.3%KH_2PO_4, 0.1% NH_4Cl, 0.05% NaCl, 0.5% soluble starch and 0.5% colloidal chitin (pH=7.0) in a 500ml flask and incubated at 30°c for 24h [7]. Supernatant was carefully removed after centrifugation at 10000 xg for 30min at 4°C and filtered through 0.22µm millipore filters. Chitinase activity in supernatant was assayed. The purification of chitinase was carried out in three steps. The supernatant was precipitated with ammonium sulfate 40-75% saturation for 1h with gentle stirring. The precipitate was collected by centrifugation at 10000xg for 30min.It was dissolved in 25 mM tris-HCl buffer (pH=7.5) and dialysed against the same buffer.

The dialysed protein was subjected to ion exchanger, DEAE-Sephadex column (2.5 by 25cm). The adsorbed chitinase was eluted with gradient of NaCl from 0.2 to 0.5M in the same buffer. Fractions (5ml)were collected and assayed for chitinase activity. In the final step, the active fractions were pooled, dialyzed against 25mM tris-HCl buffer (pH=7.5) and loaded on sephadex G-200 column (2.0 by 80cm) containing 100ml of sephadex G-200 which had been equilibrated and washed with 10mM tris-HCl buffer (pH=7.5), then the elution was done by the same buffer. The fractions (5ml) were collected and assayed for chitinase activity.

Characterization of purified chitinase

-Evaluation of the molecular weight :

According to the principles that were described by [18] the molecular weight of chitinase was evaluated by gel filtration chromatography by using sephadex G-200 column. This column was equilibrated in 25mM tris-HCl buffer. The void volume (Vo) was determined by using blue dextran. Elution volumes (Ve) of proteins of known molecular mass (Bovine serum albumine [66kDa], ovalbumin [45kDa], trypsin [23kDa] and lysozyme [14kDa] dissolved in 25mM tris-HCl buffer) were measured and used as reference standards in chitinase native molecular mass determination. The relationship between (Ve/Vo) and log molecular weight for standard proteins was plotted to obtain the standard curve.The molecular weight for chitinase was evaluated from incidence (Ve/Vo) value for chitinase on the standard curve.

-Effect of temperature on the activity and stability of chitinase

Chitinase activity was assayed at different temperatures ranging from 10-70°c at pH 6.0 in sodium phosphate buffer (50mM). To determine thermostability, chitinase preparation was incubated at the same range of temperatures for 2h then chitinase activity was assayed.

-Effect of pH on the activity and stability of chitinase

Chitinase activity was assayed at different pH values (pH 3 to 10) by using different buffers 50mM such as cirate phosphate buffer (pH 3-6), sodium phosphate buffer (pH 7-8) and glycine of buffer (pH 9-10). To determine pH stability, chitinase preparations in buffer at the same range of pHs were incubater at 50°c for 2h then chitinase activity was assayed.

-Effect of various chemicals on chitinase activity

The effect of various chemicals ($CuSO_4$, $CaSO_4$, $CoCl_2$, $NiSO_4$, Triton x-100, $MgSO_4$, $HgCl_2$, EDTA, n-ethylmaleimide, methanol, ethanol and acetone) on the enzyme ativity were investigated by preincubating the enzyme with chemicals in 50mM sodium phosphate buffer (pH 6.0) for 1 h at 50°c and then measuring the residual activity of chitinase.

-Antifungal activity of chitinase

Agar plates containing potato dextrose agar (PDA) and 10% purified enzyme were prepared for finding the antifungal activity of chitinase , and the same agar plate (without enzyme)was used as a control. Six (6)mm diameter mycelial discs of *Aspergillus flavus* and *Fusarium solani* grown on PDA plates at 28°c for 3 days were placed at the center of the plates. The radial diameter of the colonies was measured after 5 days at 28°c then the percent of inhibition was calculated[10,19].

Results and Discussion

Serratia marcescens was isolated from 35% [7] of lettuce samples (L_1-L_7) and 25% [5] of spinach samples (S_1-S_5). Giri *et.al.*,2004 [1] found that capryllate thallous agar was the best for selecting *Serratia.* DTC (deoxyribonuclease – toludine blue-cephalothin)agar was used for isolating *Serratia,* while DNase medium for isolating *Serratia marcescens*. Vegetables used in salads might bring *Serratia* strains to hospitals and contaminate the patients digestive tract. *Serratia marcescens* , *Serratia liquefaciens* and *Serratia marinorubra* were found in 29%, 28%

and 11% (respectively) in vegetable salad sample[5]. Grimont&Grimont, 1998[4] reported that *Serratia* was isolated from mushrooms, tomatoes, leeks, lettuce, green onions, grass, and spinach and the origin of *Serratia* found on plants is probably the soil.

Chitinolytic activity on plates

Twelve (12) *Serratia marcescens* were tested for chitinase production by measuring the diameter of clear zone of lysis in synthetic medium agar (SM) Figure(1). In this figure, only nine (9) isolates produced chitinase enzyme and *Serratia marcescens* L5 produced chitinase in higher level among the producer isolates, hence this isolate was selected for further study.

Green *et.al.*,2004[20] reported that the optimum conditions (pH 7.0, 32.5°c and 1.0%(w/v) substrate) induced a higher level of enzyme activity. Also [21] showed that the maximum production of *Serratia marcescens* chitinase was between pH 6 and 7 at 30^0c and the optimal shaking speed at 150 rpm.

Chitinase production was reduced by 50% at pH 8.5 of production medium [7]. In a study done by [22] found that among 102 *Serratia marcescens* strains screened, 57strains showed chitinase activity.

Purification of chitinase

The chitinase was purified by using standard techniques such as ammonium sulfate precipitation (40-75%), DEAE-sephadex ion exchange chromatography and sephadex G-200 gel filrtation chromatography. When cell free supernatant was subjected to fractional ammonium sulfate precipitation, Chitinase activity was precipitated in 40-75% salt saturation The yield of chitinase was 71% with a purification fold of 3.1 and specific activity of 6.2 U mg^{-1} protein. The dialyzed protein was loaded on DEAE ion exchanger. After elution with 0.2 to 0.5M NaCl gradient three major peaks of proteins were observed but chitinase activity was observed only in the first peak figure (2).

Here the yield of chitinase was 42% with a purification fold 13.2 and specific activity 26.6 Umg^{-1} . Gel filtration chromatography with sephadex G-200 was the last step of chitinase purification. The eluted fractions of this step contained the chitinase two protein peaks,only the second peak contained the chitinase activity figure (3). The yield of chitinase was 30% with a purification fold 89.5 and specific activity 179.1 Umg^{-1} . The results of chitinase purification were summarized in table(1). The increase in the specific activity for chitinase may refer to the efficiency of the purification process and the decrease of the contaminating materials.

Chitinase was purified from the culture filtrate of *Enterobacter , Fusarium* and *Sreptomyces* and purified by using ion- exchange chromatography and gel filtration as mentioned at [9, 12, 23].

On the other hand, *Serratia* sp. chitinase was purified by using DEAE Bio-Gel, chromatofocusing with PBE 96 and gel filtration with sephacryl S-200 [24]. In addition, *Serratia marcescens* chitinase was purified by affinity adsorption, hydroxylapatite and sephadex G-200 [25]. *Serratia* chitinase showed high sequence homology with chitinase from *Bacillus* and *Streptomyces* [26].

Characterization of purified chitinase

-Evaluation of the molecular weight of chitinase

By using gel filtration with sephadex G-200, the molecular weight of purified chitinase was evaluated. The result revealed that purified chitinase had approximately 59000 daltons figure (4). The molecular weight of *Enterobacter* sp. chitinase was estimated to be 60kDa by SDS-PAGE and sephadex G-200 gel filtration, suggesting that the purified chitinase is a monomer type (12). Wang& Change, 1997. Frankowski *et.al.,* 2001[6,27] found the molecular mass for *Pseudomonas*

aeruginosa and *Serratia plymuthica* were 60 and 60.5kDa, respectively. In a study done by [28] reported that *Serratia marcescens* strains grown in the presence of Mitomycin C revealed the presence of four extracellular proteins with chitinase activity and molecular masses of 21, 38, 52 and 58kDa.

-Effect temperature on activity and stability of chitinase

The effect of temperature on the activity of chitinase with colloid chitin as substrate of various temperatures ranging from 10 to 70^0c figure (5) . The enzyme showed a good activity between 40-60^0c with maximum activity at 50^0c. Considerable decrease in the chitinolytic activity was observed for lowest and highest temperatures, reaching 25, 38 and 20% activity at 10, 20 and 70^0c. Thermostability of the enzyme was observed in the temperatures up to 50^0c after its thermal exposure for 2h figure(5).

Incubations above 50^0c promoted inactivation of this enzyme. The chitinase produced by *Enterobacter* sp., *Bacillus* sp., *Serratia marcescens* showed optimum activity at 45, 45-55 and 40^0c, respectively[12,29,30] .Moreover, the chitinase produced by *Serratia* sp. and *Bacillus* sp. were more thermostable than *Fusarium* sp. chitinase[9, 25, 29].

-Effect of pH on activity and stability of chitinase

The chitinase activity was evaluated at different pH values at 50^0c by using colloid chitin as substrate figure (6). The optimum activity for colloid chitin hydrolyisis reached at pH 6.0.
Chitinase mantained 88 and 80% of its initial activity when incubated for 2h at pH 5 and 7, with a decrease in chitinase activity at pH lower and higher than the above pH values. The enzyme was stable at PH 5 to 7 figure(6). The chitinase produced by *Serratia marcescens* and *Stretomyces* exhibited maximum activity at pH 6.0 and 5.0, respectively [30]. In other study done by [31] reported that the chitinase produced by *Serratia* sp. was an exochitinase and exhibited a greater pH range [5.0-10.0].

-Effect of various chemicals on chitinase activity

Chitinase of *Serratia marcescens* was treated with many chemicals some of these chemicals showed activation effect, others had inhibition effect. Co^{+2} , Ni^{+2} , Hg^{+2} inhibited chitinase activity to 82, 70 and 23% respectively, also EDTA, methanol,ethanol and acetone were inhibited the activity to 30, 45, 68 and 44%, respectively table(2). On the other hand, the other metal ions Ca^{+2} , Cu^{+2} , and Mg^{+2} increased chitinase activitor to 120,150 and 170% respectively, hence these metals can be evaluated as activator compounds for chitinase activity. In addition, n-ethylmaleimide and Triton x-100 increased the activity to 110 and 140%, respectively. The results may prove that chitinase was metaloenzyme.

Serratia plymuthica chitinase activity was stimulated by 120, 150 and 240% in presence of mMCa^{+2} , Co^{+2} or Mn^{+2} and inhibited by 80% in presence of 10 mM Cu^{+2} [27]. Chitinase from *Enterobacter* sp. was stimulated by Ca^{+2} , K^{+2} and Mg^{+2} strongly inhibited by Hg^{+2}, Ag^{+1} , Cu^{+2} and Co^{+2} [12]. In a study done by [29] found that Ca^{+2} , Ni^{+2} and Triton x-100 stimulated the activity up to 20% whereas Ag^{+1} and Hg^{+2} inhibited the activity up to 50%.

- Antifungal activity of chitinase

The purified chitinase showed inhibitory activity against *Aspergillus flavus* and *Fusarium solani*. Chitinase showed much stronger inhibitory activity to *Fusarium solani* compared with *Aspergillus flavus* figure (7 and 8). The percent of inhibition for *Fusarium solani* was 83% while 64% for *Aspergillus flavus* table (3). This experiment increased the benefit by using the chitinase as biocontrol agent against phytopathogenic fungi. El-Katatny, *et.al.,*2005 [10] found that the purified endochitinase of *Trichoderma* has shown antifungal activity against *Sclerotium rolfsii* and the inhibition of *Sclerotium rolfsii* depended on the carbon source used and correlated with the level of chitinase activity. In addition, the chitinase was able to induce morphological altration in both *Aspergillus flavus* and *Fusarium moniliforme*. In a study done by [32] reported that chitinase of *Serratia marcescens* showed antifungal activity against *Sclerotium rolfsii*.

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Table(1): Purification of chitinase from *Serratia marcescens* (L_3 isolate)

Purification step	Size ml	Protein conc. mg/ml	Activity U/ml	Specific activity U/mg	Total activity	Purification fold	Yeild (%)
Crude enzyme	120	41.1	82.4	2.0	98885	1	100
$(NH_4)_2SO_4$	50	23	142.2	6.2	7110	3.1	71
DEAE-Sephadex	21	7.4	196.8	26.6	4128	13.3	42
Sephadex G-200	14	1.2	215	179.1	3010	89.5	30

Table(2): Effect of various chemicals on chitinase activity

Substrate	Conc.	Relative activity (%)	Substrate	Conc.	Relative activity (%)
$CuSO_4$	1.0mM	150*	EDTA	5.0mM	50
$CaSO_4$	"	120	n-ethylmaleimide	5.0mM	110
$CaCl_2$	"	82	Triton x-100	1%	140
$NiSO_4$	"	70	Methanol	50%	45
$MgSO_4$	"	170	Ethanol	50%	68
$HgCl_2$	"	23	acetone	50%	44

* The chitinase activity before treatment equals 215 U/ml

Table(3): Antifungal activity of *Serratia marcescens* chitinase

Fungus	Diameter of colony(mm)	Percent of inhibition(%)
Aspergillus flavus	13	69
Fusarium solani	5	85

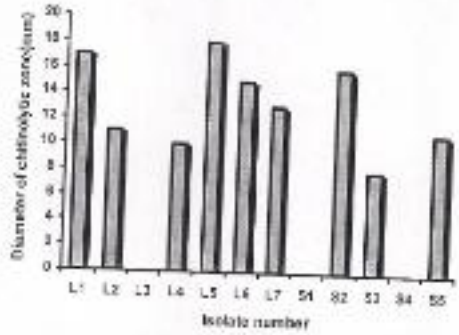

Fig.(1): Diameter of chitinolytic zones for all *Serratia marcescens* isolates by using Synthetic Medium agar (SM).
(L)= lettuce samples , (S)= spinach samples.

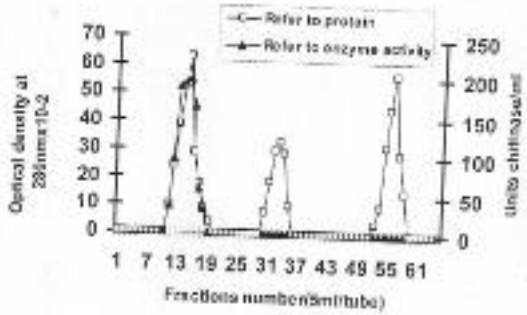

Fig.(2): DEAE-Sephadex chromatography of *Serratia marcescens*.
The fraction was collected and eluted with gradient of NaCl from 0.2 to 0.5M in the tris-HCl buffer (pH=7.5), Fractions (5ml) were collected at speed (ml^{-1}).

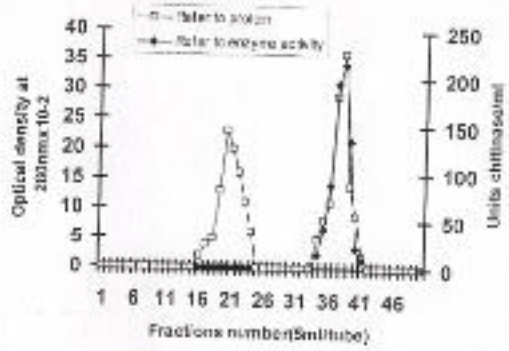

Fig.(3): Sephadex G-200 chromatography of *S. marcescens* chitinase

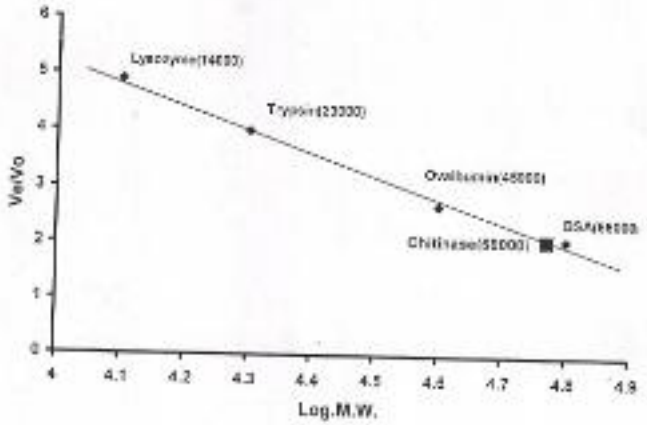

Fig.(4): The standard curve of determination of molecular weight for chitinase by gel filtration on Sephadex G-200

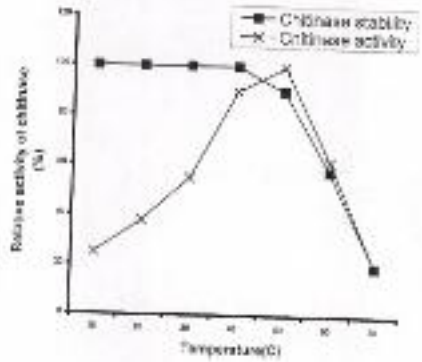

Fig.(5): Effect of temperature on the activity and stability of chitinase

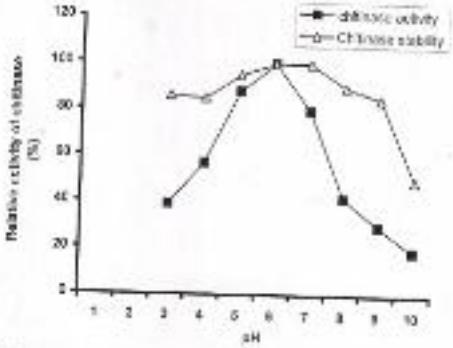

Fig.(6): Effect of pH on the activity and stability of chitinase

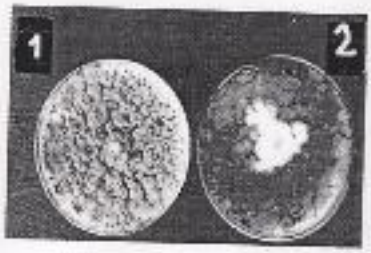

Fig. (7): Inhibitory activity against *Aspergillus flavus*
1- Control(without chitinase) 2- With chitinase

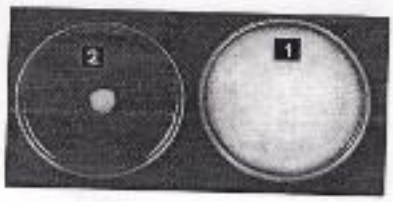

Fig. (8): Inhibitory activity against *Fusarium solani*
1- Control(without chitinase) 2- With chitinase

عزل وتوصيف ودراسة الفعالية المضادة للفطريات لانزيم الكايتينيز المنتج من بكتريا*Serratia marcescens* المعزولة من الخضراوات الطازجة

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الخلاصة

تم الحصول على 7 عزلات (بنسبة 35%)، و5 عزلات (بنسبة 35%) تعود لبكتريا *Serratia marcescens* من مجموع 20 عينة لنبات الخس، و 20 عينة لنبات السبانج، على التوالي، مأخوذة من مواقع مختلفة في مزرعة في مدينة بغداد.

أختيرت العزلة التي انتجت الكايتينيز بأعلى مستوى لاستخلاص وتنقية انزيم الكايتينيز بعدة مراحل تتضمن الترسيب بكبريتات الامونيوم، والتبادل الايوني بأستخدام DEAE-Sephadex ، والترشيح الهلامي باستخدام Sephadex G-200 بعدد مرت تنقية 89.5 وبحصيلة نهائية 30% .

تم توصيف انزيم الكايتينيز المنقى ووجد أن وزنه الجزيئي بحدود 59000 دالتون باستخدام كروماتوغرافيا الترشيح الهلامي. وقد وجد أن الرقم الهيدروجيني الامثل ودرجة الحرارة المثلى لفعالية الانزيم المنقى هي 6.0 و 50^0 م ، على التوالي،ووجد أن الانزيم المنقى مستقر في الرقم الهيدروجيني المتراوح بين 5-7 ودرجة الحرارة تصاعديا الى 50^0 م. تم تنشيط أنزيم الكايتينيز بوجود Mg^{+2} Cu^{+2} , Ca^{+2} ، وثبطت فعاليته بوجود Hg^{+2} . فضلا" عن ذلك وجد أن Triron x-100 و n-ethylmaleimide ادت الى زيادة فعالية انزيم الكايتينيز ، أما EDTA ، الميثانول، الايثانول والاسيتون فأدت الى تثبيط الفعالية وهذه النتيجة تؤكد أن انزيم الكايتينيز هو انزيم معدني. كذلك اظهر انزيم الكايتينيز اعلى فعالية تثبيطة لفطر *Fusarium solani* بالمقارنة مع فطر *Aspergillus Flavus* وبنسبة تثبيط 83 و 69 % على التوالي. لذا أدى هذا البحث الى زيادة الفائدة باستخدام الكايتينيز عامل سيطرة بايولوجية للفطريات والحشرات الممرضة للنبات، انتاج سكريات بسيطة كايتينية، تحضير البروتوبلاست والسفيروبلاست من الخمائر واعفان وتحويل المخلفات الكايتينية الى بروتين احادي الخلية لتغذية الحيوانات.