BIOCHEMICAL STUDIES ON BACTERIAL CHITINASE PRODUCTION

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Abstract

Chitinolytic bacterial candidate was isolated from mature compost and purified. The isolate was identified as *Streptomyces nigrifaciens*. Optimization of growth conditions (peptone N source, 45 °C, pH 9, fermentor agitation speed 300 rpm, air flow rate 1 L/min, 48 hours) to obtain the highest chitinolytic activity, under laboratory scale conditions, led to chitinase production increment, expressed as chitinolytic activity, from 58.816 U/ml up to 2129 U/ml (36 times). Chitinase production in the fermentor was achieved on local chitinic wastes and the resulting crude enzyme was subjected to partial purification steps (ammonium sulphate precipitation and chitin affinity). The resulting partially purified chitinolytic fractions were separated. Three partially purified chitinases were separated from the supernatant (SUP) named S1, S2 and S3 of 1414.7, 616.4 and 641.3 U/mg activities, respectively. From the chitinic debris, two partially purified chitinases having a high affinity to chitin were separated and named P1 and P2 of 748.6 and 5344.3 U/mg activities, respectively. The optimization of temperature revealed the superiority of P2 fraction, as the chitinolytic specific activity increased from 3070.700 U/mg at 37 °C to 9126.421 U/mg at 45 °C raising the purification fold to 2.97 and its yield reached 4 %. The P2 optimum enzymatic conditions for hydrolyzing colloidal chitin were found to be pH 10 and substrate concentration of 22 mg/ml.

INTRODUCTION

Chitin is the second abundant polysaccharide after cellulose in nature. Its structure is composed of straight chains of β-1,4 linked N-acetylglucosamine units and the chains form fibrils and sheets that are arranged in a specific parallel manner β chitin or anti parallel manner α chitin or including both types as γ chitin, (Amy *et al.*, 1997).

Chitinolytic enzymes which act on plant pathogens, that chitin plays an important role in their life, is a major target in biocontrol methods. Soil amendment with chitin or fungal mycelia induced chitinases production in the infected soils that reduced the numbers of chitinic phytopathogens as mentioned by Anna *et al.* (1996).

Shekhar *et al.* (2006) studied the optimum conditions for the production of this biocontrol agent from *Streptomyces violaceusniger* and the effects of various

components in growth medium on growth such as nitrogen and carbon sources. The presence of chitin in the growth media was found to be an essential factor for the appropriate production of the enzyme chitinase. The pH and temperature optima for chitinase were determined as well.

Shigemasa *et al.* (1994) suggested that β-chitin (in fungal cell wall) would elicit more chitinase activity and would be degraded faster than α-chitin (in crab and shrimp shells).

The first aim of the current research was to increase the chitinolytic activity of the isolated and purified strain *Streptomyces nigrifaciens* by optimizing the fermentation conditions. The second aim was to study the strain efficiency in degrading some available chitinic local wastes for maximum chitinase production.

MATERIALS AND METHODS

Microbiological

Streptomyces nigrifaciens which was isolated from mature compost and identified according to Bergey's manual of systematic bacteriology (John *et al.*, 1994) was used in this study for production of chitinase.

The isolate was grown on colloidal chitin medium for chitinase production as described by Kenji *et al.* (1998). One hundred ml of the sterilized medium in 250 ml Erlenmeyer flask was inoculated using 10 ml (had optical density of 1.0) from preculture of same type of medium and incubated on orbital shaker (100 rpm) for 150 hours at 37 °C. Optimization of cultural conditions via N-sources, medium pH and incubation temperature were studied as well as chitinic material sources effect for highest chitinolytic production expressed as chitinolytic activity.

A VirTis omni-culture type 2 liter capacity fermentor was used for 1 L growth fermentation of *St. nigrifaciens* to optimize cultural conditions (agitation speed 100-400 rpm, time course every 25 hours) at air flow rate of 1 L/min. The fermentation was stopped after decline in chitinolytic activity.

The chitin source added to the fermentor medium was a mixture of equal portions from local chitinic wastes collected from shrimp, crab shells and fish scales (*Tilapia nilotica*) from the local markets. Also, *Aspergillus niger* mats from Citro Misr Co. in Egypt for citric acid production was used as a local chitinic waste.

Chitinase activity was measured and then the whole fermentation broth was kept in refrigerator for one day to allow any possible chitinase that had high affinity to chitin substrate to adhere to chitin debris. The broth was then centrifuged and the chitinase bound to the debris was released by shaking in 0.1 M sodium phosphate buffer of pH 6.8 containing 0.1 M sodium chloride according to the method described

by Watanabe *et al.* (1990). The resulting broth supernatant (SUP) and chitin debris (CD) were subjected separately to precipitation for their protein content with successive addition of ammonium sulphate salt gradually at 5 °C and every time the resulting protein precipitate (PPT) was separated by centrifugation under cooling then dissolved and dialyzed against TRIS/HCl buffer (50 mM, pH 7) .

Colloidal chitin from crab shell of SIGMA Co., practical grade chitin, was prepared according to the method described by Rodriguez *et al.* (1983) using concentrated HCl for mild degradation. As mentioned by Tsujibo *et al.* (2003) the chitinase activity in culture filtrate was estimated by the amount of reducing sugars formed during hydrolysis of crab colloidal chitin. The reaction mixture contained 0.25 ml of 0.1 M sodium phosphate buffer (pH 6.8), 0.25 ml of colloidal chitin (10 mg/ml) and 0.25 ml of the sample. Incubation was performed at 37 °C for 10 min. The amount of N-acetyl-glucosamine (NAGA) in 1 ml of reaction mixture was determined using dinitrosalicylic acid method for determination of reducing sugar according to Gail (1959). One unit of chitinase corresponds to the amount of enzyme that liberated reducing sugar corresponding to 1 µmole of (NAGA) from 1 ml of reaction mixture per 1 min under the stated standard conditions of the assay.

Protein determination

Protein content was determined in the media and the successive purification steps spectrophotometrically by the method of Lowry *et al.* (1951) based on the standard curve of bovine serum albumin.

Enzyme partial purification and kinetics

Salting out protein from culture filtrate by Ammonium Sulphate precipitation method and dialysis in TRIS/HCl buffer (50 mM, pH 7) was used to separate the crude enzyme protein from the medium according to Scopes (1988).

The buffers used were: acetate phosphate (McIlvaine) buffer of pH 4 - 6, phosphate buffer of pH 7 - 8, boric sodium borate buffer of pH 9 - 10, glycine sodium hydroxide buffer of pH 8.6 - 10.6 (used for purified fractions instead of Borate buffer) and phosphate buffer of pH 11 - 12 were used for testing effect of pH grade on chitinolytic production and activity according to Daniel and Stuart (1991).

The effect of substrate (crab shell colloidal chitin) concentration on chitinolytic activity of the partial purified enzyme was done using serial concentrations of the substrate in glycine sodium hydroxide buffer of pH 10 (Tsujibo *et al.*, 2003).

The study of the effect of different nitrogen sources was carried out according to Fink *et al.* (1999). The quantities of the nitrogen sources added were calculated on basis of nitrogen content (0.021 % W/V of medium) of ammonium sulphate

concentration (0.1 % of the media W/V) in the colloidal chitin medium described by Kenji *et al.* (1998). .

The significance of the results was determined by the analysis of variance (ANOVA) evaluated by Duncan's multiple range tests (pH 0.05), using Costat software, product of cohort software Inc., Berkley, California, (Duncan, 1955).

RESULTS AND DISCUSSION

Identification of the isolate

The microscopic examination of the isolate gave the preliminary notes needed for the morphological identification. Plate 1 (A and B) indicates that it was Gram+, sporophores monopodially branched, straight or slightly wavy and this was assured by phase contrast Plate 1 (D). The spore surface examined by Electronic microscope at 40,000X was smooth Plate 1 (C). No melanoide pigments were observed when grown on its specified medium for morphology tests. All characteristics observed physiologically and morphologically were insured by the information stated in Bergy's manual of systematic bacteriology (John *et al.*, 1994). The isolate was identified as *Streptomyces nigrifaciens.*

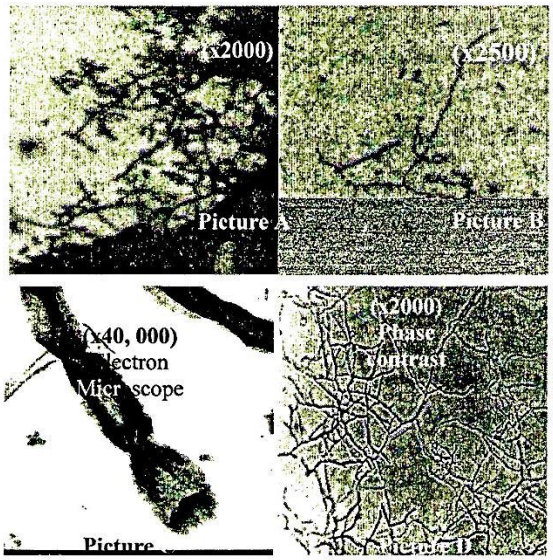

Plate 1. Microscopic examination of the isolate (A, B, C and D)

Increasing the chitinolytic activity by optimizing the fermentation conditions

N-source

Figure 1 illustrated the beneficial effect of peptone as an organic nitrogen source on chitinase produced by *St. nigrifaciens*. The chitinase excretion increased from 53 U/ml after 100 hours (control) to 99U/ml after 50 hours only. Little is known on the effect of N-source on chitinase production by *Streptomyces* sp. because mostly chitin was used as the only carbon and nitrogen sources as mentioned by Manucharova *et al.* (2004). On the other hand, N-source did affect many metabolic pathways in *Streptomyces* sp., as mentioned by Fink *et al.* (1999) who stated that glutamine synthetase activity in *Streptomyces coelicolor* decreased using ammonium salt as N- source. That is why the role of nitrogen source in chitinase production was important to be revealed in this test.

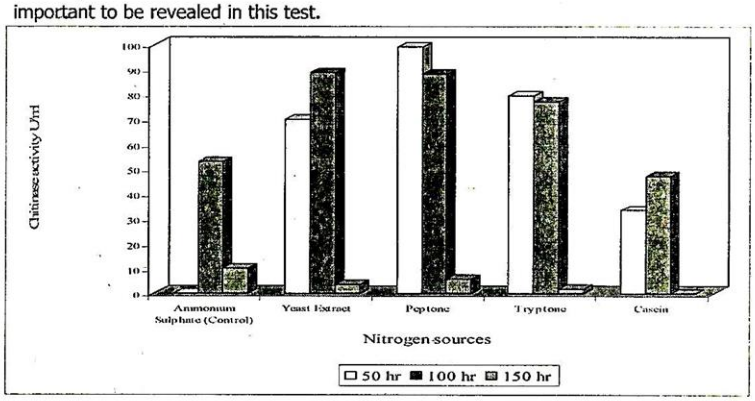

Figure 1. The effect of N source on chitinolytic activity in relation to time.

Initial pH

Changing the initial pH of growth medium affected the chitinase production as shown in Figure 2. The reduction in chitinolytic activity of the control result from 99 U/ml down to 43.5 U/ml was noted when acetate phosphate buffer was used to control the pH of 6.0, in order to resemble the original medium pH which was not controlled by any buffering means. This was due to the chemical nature of the buffer mixture added, that may have affected the nutritional needs for the microbe.

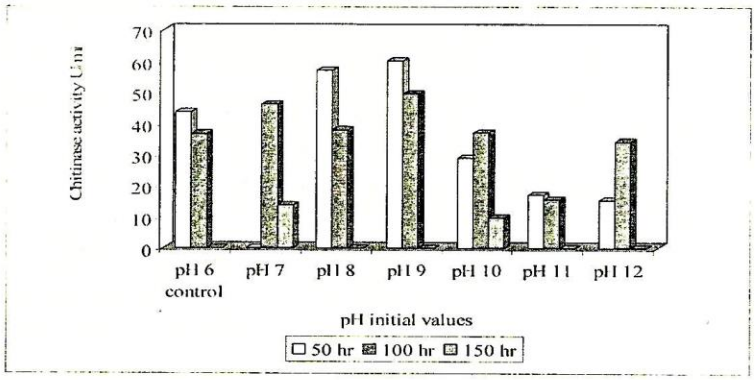

Figure 2. Effect of medium initial pH on chitinolytic activity in relation to time.

The optimum pH 9.0 was achieved by using phosphate buffer mixture which raised the activity up to 60.224 U/ml. The inductive effect of the optimum pH may trigger the Figure 2. Effect of medium initial pH on chitinolytic activity in relation to time synthesis and secretion of the chitinase as had been explained by Raymond *et al.* (1998). They stated that the ambient pH played a major role in gene expression of secreted chitinase in *Metarhizium anisopliae*. They also found that the optimum pH for chitinase production is the same optimum pH for the chitinase activity in vitro.

Incubation temperature

Results in Figure 3 show the effect of temperature on chitinase activity. Optimizing growth temperature to 45 °C raised the chitinase activity up to 178.199 U/ml (3 times that of control).

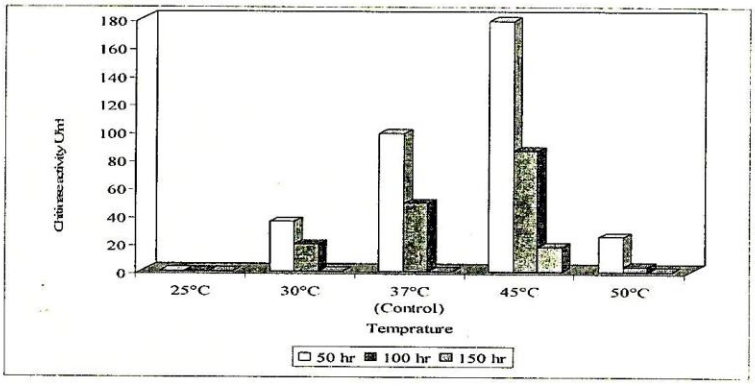

Figure 3. Effect of growth temperature on chitinolytic activity in relation to time.

Peptone concentration:

Optimizing peptone concentration added to growth medium affected the chitinase activity as shown in Figure 4. Decreasing peptone concentration from 0.18 % (control) to 0.15 % increased chitinolytic activity from 178.045 up to 187.467 U/ml.

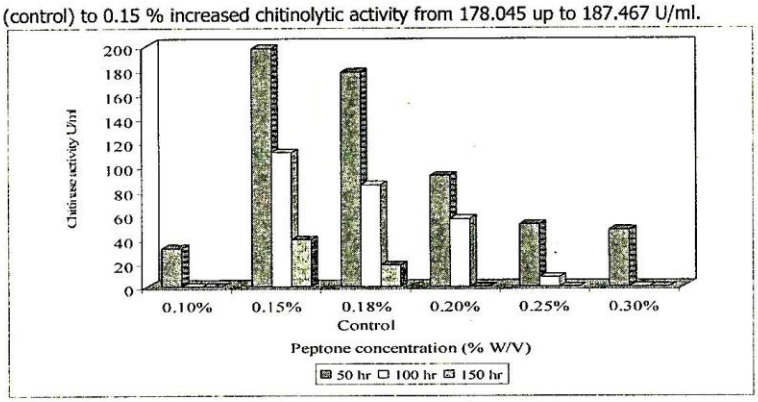

Figure 4. Effect of peptone concentration on chitinolytic activity in relation to time.

The apparent optimum growth conditions for the highest chitinolytic activity were using peptone at a concentration of 0.15 % as N-source, initial pH 9 and 45 °C.

Strain efficiency in degrading some local wastes for best chitinase production

An application test for revealing the chitinolytic production behavior of *St. nigrifaciens* was done using the available local wastes rich in chitin content instead of expensive purified crab shell colloidal chitin. Results illustrated in Figure 5 revealed the superiority of *Aspergillus niger* mycelial waste as source for chitin for higher chitinase activity after 100 hours of fermentation in comparison to other wastes. Because of its tightly packed, antiparallel strands of α-chitin, it was more difficult for chitinases to gain access and hydrolyze crab shell chitin as had been mentioned by Shigemasa *et al.* (1994). This result was confirmed by the study of Anna *et al.* (1996) who used dead mycelia of *Aspergillus* sp. in fermentation media and Melent'ev *et al.* (1999) who used fruiting bodies of *Polyporus squamosus* as source of chitin. They succeeded to increase the chitinolytic activity of *Bacillus* sp. up to 108 U/ml when it was grown on medium containing fruiting bodies of *P. squamosus* as source of chitin, while *St. nigrifaciens* chitinolytic scored 663.824 U/ml when grown on medium containing mycelia waste of *Aspergillus niger* as source of chitin.

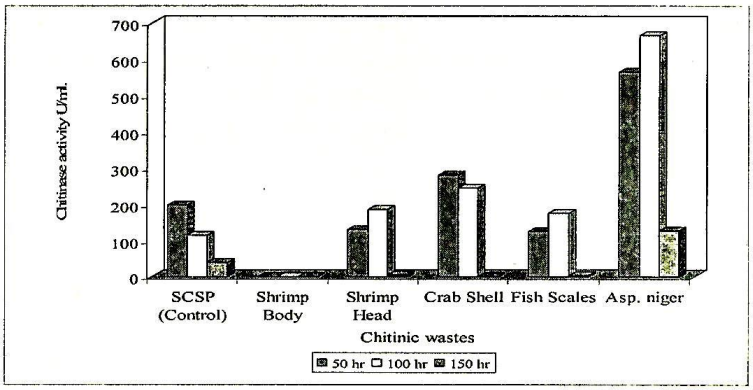

Figure 5. Effect of chitinic wastes on chitinolytic activity

The fermentor agitation speed test

St. nigrifaciens was grown on the optimized fermentation medium conditions previously mentioned (peptone 0.15 %, 45 °C and initial pH 9). Figure 6 indicates that 300 rpm was the best agitation speed that gave the highest activity after 50 hours. It was noticed that with all the agitation speeds the chitinolytic activity declined after 50 hours of fermentation. It was also observed that the high agitation speeds (300 rpm) achieved a higher chitinolytic production than lower ones, meaning that aeration is much important for this process. This was restricted by the fact that streptomycetes have an oxidative type of metabolism that uses a wide range of organic compounds as sole carbon source for energy and growth (John *et al.*, 1994). In spite of that, there was a decrease in chitinolytic activity with 400 rpm after 25 hours, which may be due to the destructive effect of the agitation on *St. nigrifaciens* mycelia form. The highest result was 2129.626 U/ml at 300 rpm after 50 hours of fermentation.

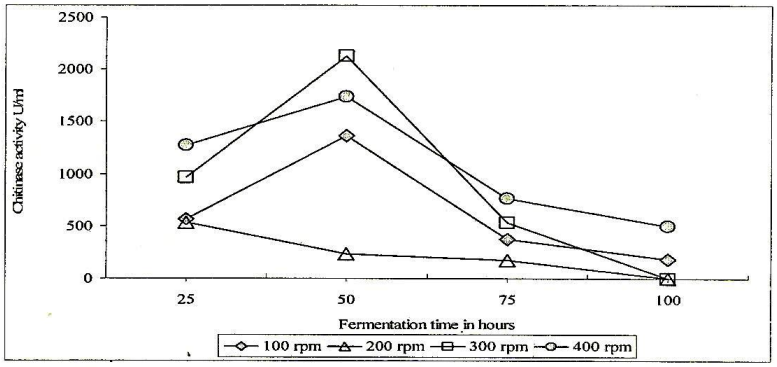

Figure 6. Effect of agitation speed (in rpm) on chitinase production expressed as chitinolytic activity (U/ml) in relation to time (hour).

Production of chitinase using local chitinic wastes and purification

Studying the chitinolytic activity of different protein portions separated by precipitation from the fermentation medium by the effect of different ammonium sulphate concentrations. From Figure 7, the fermentation broth supernatant (SUP) did give three chitinolytic fractions at 25 % (S1), 65 % (S2) and 85 % (S3) ammonium sulphate salt concentrations, while Figure 8 demonstrates that the fermentation chitinic debris (CD) did give two chitinolytic fractions at 65 % (P1) and 85 % (P2).

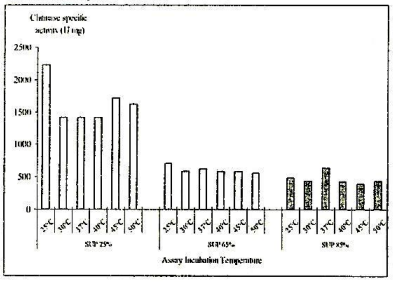

Figure 7. Effect of ammonium sulphate purification step on the chitinolytic activity of the resulting fractions from fermentation broth SUP (free chitinase in broth) in relation to temperature effect.

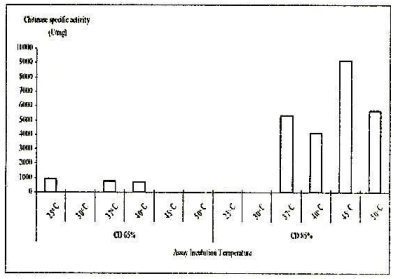

Figure 8. Effect of ammonium sulphate purification step on the chitinolytic activity of the resulting fractions from fermentation chitinic debris CD (bound or adherent chitinase to chitin debris) in relation to temperature effect.

The specific chitinolytic activity for chitinase bound to fermented chitin debris (CD) which was named (P2) was much higher than its corresponding non bound chitinase from fermentation broth (SUP) as shown from the least significant difference calculated between results and summarized in Table 1 in relation to the temperature assay effect.

Table 1. The chitinolytic activity of ammonium sulphate fractions resulting from SUP and CD of fermentation broth in relation to best temperature effect.

STEP	Best Temperature (°C)	Total Activity (U)	Total Protein (mg)	Mean Specific Activity (U/mg) ± SE			
SUP 25 % (S1)	25	15961.200	7.178	2223.783	±	122.922	B
SUP 65 % (S2)	25	19755.920	28.014	705.216	±	3.375	CD
SUP 85 % (S3)	37	18217.520	28.406	641.338	±	2.606	D
CD 65 % (P1)	25	10833.200	11.963	905.597	±	1.934	C
CD 85 % (P2)	45	17864.970	1.958	9126.421	±	42.872	A
LSD	225.746						

Watanabe *et al.* (1990) studied the chitinase system produced during fermentation of *Bacillus circulans*. They stated that the purified chitinase showing a high affinity to insoluble substrate chitin (bound chitinase) was more efficient in degrading chitin substrate (higher activity) than other chitinase from the same fermentation broth that had no ability to bind to chitin (free chitinase). The reason was that the free chitinase was generated proteolytically from the bound chitinase in the same fermentation growth of *Bacillus circulans* by removal of its C-terminal region. This region plays the main role in binding to the chitin substrate for more efficient hydrolyzes. This explanation may emphasize the presented results in this work, except that there were no evidence study about structural relationship between the P2 fraction and others resulting from supernatant.

The propagation of chitinolytic specific activity through purification steps and the most active resulting fraction are shown in Table 2. The SUP activity was still near to that of fermentation broth, while the decrease in yield indicates the loss of a great part of the chitinolytic enzymes that appeared adherent and active in the resulted chitin debris. The adherent chitinases had high affinity towards chitin waste debris and this increased its purification fold by 0.6.

Table 2. Propagation of specific chitinolytic activity in relation to separation and purification steps held out.

Purification step	Temperature (°C)	Volume (ml)	Total Activity (U_T)	Total Protein Content (mg)	Mean Specific Activity ± SE (U/mg)			Yield %	Purification Fold
Total Broth	37	1000	446142.000	145.290	3070.700	±	55.170	100	1
SUP portion	37	600	305299.080	93.177	3276.550	±	12.231	68.43	1.067
CD portion	37	400	202712.240	39.846	5087.392	±	9.291	45.44	1.657
P2 fraction from CD	45	50	17864.970	1.958	9126.421	±	42.872	4.004	2.972

From the ammonium sulphate purification step, the highest active portion was separated from chitin debris (CD) at 85 %. This protein had a specific chitinolytic activity of 9126.421 U/mg higher than that of original broth (3070.700 U/mg) by 2.972 purification fold and lower in yield by 96 % due to its lower protein content and its higher affinity to chitin substrate which lead to its approach to the substrate that did improve its hydrolyzing action much more than free chitinase as mentioned before by Watanabe *et al.* (1990).

Enzymatic properties of partially purified chitinase (P2)

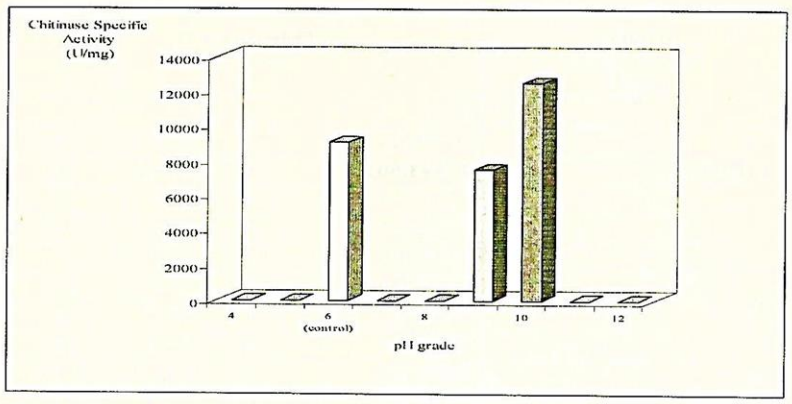

Fig. 9. Effect of pH grade on chitinolytic activity of semi purified fraction (P2)

The pH influence on the chitinolytic activity of P2 is demonstrated in Figure 9. The occurrence of more than one maximum chitinolytic specific activity value proved the presence of more than one chitinase type acting individually at its own suitable pH grade. Maximum specific chitinolytic activity was at pH 10 raising the chitinolytic activity of P2 fraction from 9108.102 U/mg obtained at pH 6 (control) to 12515.867 U/mg. The presence of more than one type of chitinases that differ in their affinity towards chitin substrate, optimum temperature and pH made a possibility for *St. nigrifaciens* to hydrolyze the chitinic wastes and in turn most chitinic phytopathogens with high efficiently. Given the great diversity of possible chitin structures is not surprising that actinomycetes typically produce more than one type of chitinase. This was so obvious in the study done by Tsujibo *et al.* (2003) on *Nocardiopsis prasina* an alkaliphilic actinomycete isolated from soil which secreted three types of chitinases in the presence of chitin.

The effect of substrate concentration on chitinolytic activity was tested on P2 fraction using successive concentrations (W/V) from prepared colloidal chitin in glycine sodium hydroxide buffer 0.1 M at pH 10. The concentrations prepared were according to the standardized assay steps. The substrate concentration that gave maximum chitinolytic activity at 6.5 % was actually 2.167 gm in 100 ml. The statistical calculations in Table 3 show that there are no statistical differences between the chitinolytic activities for the last four concentrations.

Table 3. Statistical result for arranging best substrate concentration corresponding to highest chitinolytic activity achived.

Substrate concentration % (W/V)	Chitinase activity U/mg ± SE			
0	0			
0.5	10490.01	±	2.592	M
1 (Control)	**12493.501**	**±**	**2.642**	**L**
1.5	21918.758	±	2.666	K
2	26048.461	±	4.198	J
2.5	29324.826	±	3.179	I
3	36131.559	±	5.592	H
3.5	36568.217	±	3.695	G
4	37591.368	±	3.769	F
4.5	42871.081	±	3.323	E
5	47935.462	±	3.711	D
5.5	48509.254	±	5.954	C
6	49724.277	±	4.181	B
6.5	**49749.813**	**±**	**4.271**	**A**
7	49753.587	±	4.658	A
7.5	49752.437	±	2.926	A
8	49752.437	±	2.926	A
LSD 0.05	11.201			

The chitinolytic least significant difference 11.201 calculated obviously showed the great difference between the control concentration (1 %) and the best concentration (6.5 %) influencing chitinolytic activity. A presentation of the substrate effect on chitinolytic activity is shown in Figure 10.

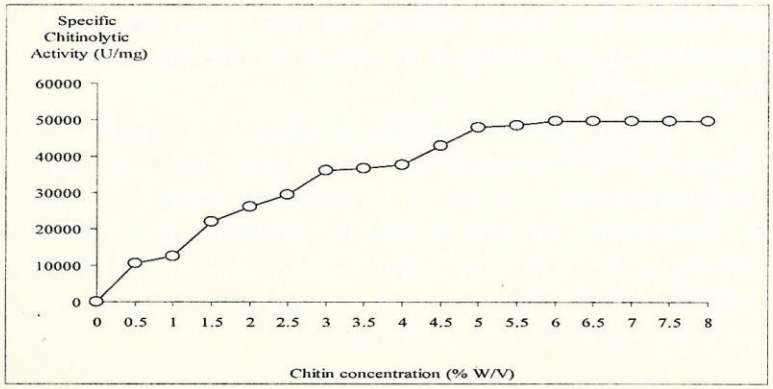

Figure 10. Effect of colloidal chitin concentration on chitinolytic activity under optimized assay conditions.

The results of Figure 10 indicate the high capacity of P2 chitinase to hydrolyze bigger quantity of chitin substrate under the new optimized conditions. The high capacity and high affinity for chitin degradation of the produced chitinases under the high alkaline conditions of pH 10 (Figure 9) beside the *St. nigrifaciens* ability to produce highly active chitinases at high temperature 45 °C (Figure 3) made it suitable for biological control of phytopathogens in the alkaline soils of Egypt. Beside that, the strain appeared to secrete a complex system of free and substrate-binding enzymes to degrade the chitinic waste mixture efficiently.

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دراسات كيميائية حيوية على إنتاج الكايتينيز البكتيرى

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تم فى هذا البحث دراسة عزلة بكتيرية منتجة لانزيم الكايتينيز من الكومبوست الناضج وتنقيتها و تعريفها على انها *Streptomyces nigrifaciens* .

تمت دراسة ظروف النمو المختلفة لزيادة الإنتاج الإنزيمى (متمثلا فى قياس النشاط الانزيمى) حيث أمكن رفعه من ٥٨,٨١٦ وحدة / ملليلتر الى ٢١٢٩ وحدة / ملليلتر(أى تضاعف النشاط الإنتاجى ٣٦ مرة) بإستخدام بيئة للنمو تحتوى على البيبتون كمصدر للنتروجين والنمو ٤٥°م ، الأس الأيدروجينى ٩ ، سرعة قلاب المخمرأثناء النمو ٣٠٠ لفة/الدقيقة ، حجم الهواء الداخل هو ١ لتر/ الدقيقة و مدة النمو ٤٨ ساعة.

أستخدمت مخلفات السوق المحلى الغنية بالكايتين لتنمية السلالة لإنتاج الكايتينيز فى المخمر معمليا. اجزاء من ألإنزيم الخام الناتج تم فصلها من راشح و راسب البيئة المتخمرة وتنقيتها جزئيا. أعطى الراشح ثلاثة أجزاء إنزيمية تمت تسميتها S3, S2, S1 ذات نشط انزيمى مقداره ١٤١٤,٧ ، ٦١٦,٤ و ٦٤١,٣ وحدة / ملليجرام بالترتيب. أما الراسب فقد أعطى جزئين إنزيميين تمت تسميتهما P2 , P1 ذات نشط انزيمى مقداره ٧٤٨,٦ و ٥٣٤٤,٣ وحدة / ملليجرام.

بدراسة تأثير درجات الحرارة على نشاط هذه الأجزاء ، أعطى P2 أعلى نشاط عند ٤٥°م و قدره ٩١٢٦,٤٢١ وحدة / ملى جرام رافعا نقاوة الإنزيم ٢,٩٧ عن مقدار الإنزيم الخام و لتصبح الحصيلة ٤%. بدراسة أفضل الظروف لأعلى نشاط إنزيمى للكايتينيزP2 فقد كانت عند درجة الأس الأيدروجينى ١٠ و بإستخدام تركيز كايتين ٢٢ ملليجرام / ملى لتر.