J. Agric. Sci. Mansoura Univ., 25 (8): 5195 - 5209, 2000

SOME PROPERTIES AND FACTORS AFFECTING CHITINASE PRODUCTION BY *Bacillus licheniformis* AND ITS USE AS ANTIFUNGAL AGENT

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ABSTRACT

Chitinolytic enzymes of *B. licheniformis* , which used as antifungal agent were studied, and the results revealed that the growth of *B. licheniformis* reached its maximum activity in the third day. Maximum enzyme activity was observed in the fifth day. Colloidal chitin + arabinose up to 1.5 + 0.1% were observed as a superior source for enzyme production. Yeast extract enhanced the enzyme biosynthesis. Enzyme production was strongly influenced by aeration. pH 8.0 and 45°C were appeared to be the optimal for chitinase production. Maximum specific activity, recovery and purification fold were found with the use of 60% saturation of ammonium sulphate, being 23.39 units/mg protein, 52.4% and 9.4 folds, respectively. 45°C and pH 7.5 were found as the optimum temperature and pH for enzyme activity. Enzyme was stable up to 60°C and lost 15% only from maximum activity at 70°C, indicating that this enzyme is a thermostable one. Enzyme activity apparently is rather stable with a broad pH ranging from 6.0 to 8.0. Thus, the enzyme has advantageous for use in biotechnology as antifungal agent. $CaCl_2$, $MgSO_4$, $MnSO_4$ and $FeSO_4$ stimulated the enzyme activity, while $HgCl_2$ at 10 mM inhibited the enzyme activity. *B. licheniformis* chitinase succeeded to hydrolyze the three substances tested, which was found as lytic one and may be use as antifungal agent. The addition of *B. licheniformis* chitinase to the nutrient media of some parasitic fungi, inhibited its growth and its sporulation rate. Therefore, this enzyme may be play an important role in the defense against parasitic fungi on higher plants.

Keywords: *Bacillus licheniformis*, chitinase, production, properties, parasitic fungi, lytic enzyme, antifungal agents.

INTRODUCTION

Chitin, the β–1-4 polymer of N-acetylglucoseamine residues, is found in higher plants, in fungi and in the exoskeleton of arthropods (Brine, 1984; Abdel-Fatah, 1995 and El-Sawah, 1999). It is widely utilized by members of genus *Streptomyces.* In recent years, significant research has been directed toward the use of chitin derivatives in fields of drug delivery, in the treatment of dermatitis and fungal infections, as a bacteriostate and fungistate, the development of contact lenses and also effective in wound and burn healing (Hsu & Lockwood, 1975; Brine, 1984; Abdel-Fatah, 1995 and El-Sawah, 1999).

Chitinolytic enzymes are found in a variety of living organisms. But the most convenient sources of these enzymes are microorganisms. Therefore, the production of microbial chitinase has received attention as a step in proposed bioconversion process to treat shellfish waste chitin (Zarain-Herzberg & Arroyo-Begovich, 1983; Abdel-Fatah & Khella, 1995 and Chen & Lee, 1995). Chitinolytic bacteria are common in nature, where they are

important decomposers of chitin polymer (Gupta *et al.*, 1995 and El-Sawah, 1999). Besides the traditional use of selected strains of *Serratia marcescens* and *Streptomyces griseus* for the production of chitinolytic enzymes, fungi have recently been attracting increasing attention (Deshpande, 1986; Vyas & Deshpande, 1991; Tatarinova *et al.*, 1996 and Fenice *et al.*, 1998). However, the only fungal chitinase preparation of any commercial value comes from *Trichoderma harzianum* cultivated by submerged fermentation (Fenice *et al.*, 1998).

Chitinolytic enzymes have been considered important in the biological control of soil borne pathogens because of their ability to degrade fungal cell walls, of which a major component is chitin. In recent years, the use of chitinolytic enzymes in application fields such as biological pest control, the degradation of chitin rich wastes and the production of chitin hydrolysates for pharmaceutical or chemical purposes and for the food/feed industry has become of increasing interest (Di Pietro *et al.*, 1993; Chen & Chang, 1994; Lorito *et al.*, 1994 & 1996; Fenice *et al.*, 1998; El-Sawah, 1999 and Singh *et al.*, 1999). The sensitivity of the fungal cell wall to chitinolytic enzymes has been exploited by releasing chitinolytic bacteria to control plant pathogens in the rhizosphere (Flach *et al.*, 1992 and El-Sawah, 1999).

In this work, optimal culture conditions leading to high yield of chitinase by chitin-degrading *B. licheniformis* were studied. The obtained enzymatic preparations were tested for antifungal activity toward fungi parasiting on higher plants.

MATERIALS AND METHODS

Microorganisms

The extracellular chitinase producing bacterial strain used in this study was *B. licheniformis* NRRL NRS-1264. The cultures were maintained and monthly transferred on medium of Frändberg and Schnürer (1994). *Rhizoctonia solani*, *Aspergillus niger* and *Fusarium oxysporum* were obtained from Plant Pathol. Dept., Fac. of Agric., Mansoura Univ., Mansoura, Egypt. The tested parasitic fungi were maintained and monthly transferred on potato-dextrose agar (PDA).

Growth technique

The culture medium described by Frändberg and Schnürer (1994) was used for enzyme production. This medium had the following composition (g/l): K_2HPO_4, 1.5; KH_2PO_4, 1.5; $MgSO_4.7H_2O$, 0.7; NaCl, 0.5; KCl, 0.5; $CaCl.2H_2O$, 0.13 and yeast extract, 0.5. The pH was adjusted to 6.6. Fifty-ml portions of the fermentation medium were dispensed in 250 ml Erlenmeyer flasks, sterilized at 121°C for 15 min. It was supplemented with 0.5% colloidal chitin from crab shell (Sigma) as a carbon source. Chitin was sterilized separately to avoid browning. The media were inoculated with cell suspension. For preparing the inoculum, the cultures were grown on agar slants of the above medium for 24 h, scraped using 5 ml sterile tap water. The inoculum was thus used to inoculate the liquid medium (in the sense of

one tube for each fermentation flask). After incubation on a rotary shaker (200 rev/min) for 8 days at 30°C, the flasks were filtered and centrifuged at 8 000 rpm for 15 min at 4°C. The clear supernatants were collected and used as a crude chitinolytic enzyme source.

Assay of chitinase activity

Chitinase activity was determined spectrophotometrically by estimating the amount of free reducing groups formed after colloidal chitin hydrolysis. The reaction mixture was composed of 1.0 ml of 1% (w/v) colloidal chitin suspended in 0.05M phosphate buffer (pH 7.0) and 0.5 ml of the culture supernatant (El-Sawah, 1999). After 10 min of incubation at 40°C with reciprocal shaking (60 strokes/min), N-acetylglucose amine (NAGA) was estimated by the method of Reissig *et al*. (1955). A standard curve was constructed using N-acetylglucoseamine (Sigma, St. Louis, Mo.) as a standard. A unit of chitinase activity was defined as an amount of enzyme required to produce 1 μmol of NAGA / min at 40°C.

Ammonium sulphate precipitation:

The precipitation of *B. licheniformis* chitinase was carried out by adding various amounts of ammonium sulphate to the supernatant maintained at 4°C to give percentage saturation from 20-90%. The mixture was left overnight at 4°C and then centrifuged (10 000 rpm) under cooling. Each fraction precipitate was dissolved immediately in 20 ml (0.05 M) phosphate buffer (pH 7.5). The enzyme solution was centrifuged at 10 000 rpm for 10 min to remove the undissolved particles. The dissolved fractional precipitate were tested for both chitinase activity and protein content (Abdel-Fatah and Khella, 1995).

Protein assay:

It was determined by the method of Lowry *et al*. (1951), with bovine serum albumin as a standard.

Effect of temperature on chitinase activity and stability

Chitinase activity was determined by incubation enzyme solution in 1.0 ml of 1% colloidal chitin suspended in 0.05M phosphate buffer (pH 7.5) at various temperatures.

Chitinase stability was determined by incubating the enzyme solution in 0.05M phosphate buffer (pH 7.5) for 60 min at various temperatures (30-90°C). The enzyme was cooled to the optimal temperature, then the residual activity was measured.

Effect of pH on chitinase activity and stability

Chitinase activity was assessed as above, using the following buffers (0.05M) sodium citrate (pH 3.0-5.5), potassium phosphate (pH 6.0-7.5), Tris-HCl (pH 8.0-8.5) and glycine-NaOH (pH 9.0-10.5).

Chitinase stability was assessed by determination the residual activity after incubating the enzyme solution in an appropriate buffer (pH 4-10) at 30°C overnight.

Preparation of target fungi cell wall

The fungi *R. solani, A. niger* and *F. oxysporium* were grown in potato dextrose agar, incubated at 30°C and then harvested after 72 h growth. Cell

walls were obtained from the mycelium of the three tested fungi as described by Skujins *et al.* (1965). Mycelium of the fungus was autoclaved and homogenized at 4000 rev/min for 3 min in a tissue homogenizer. The homogenate was further subjected to ultrasonic disintegration in ice bath for 6 min in 12 cycles each of 30 s in MSE ultrasonicator. The pellet obtained by centrifugation at 5000 rev/min for 25 min at 4°C in a refrigerated centrifuge was washed four times with distilled water and dried.

Preparation of colloidal chitin

Colloidal chitin was prepared by the method described by Lingappa and Lockwood (1961).

Biological activity of chitinase against fungal growth and sporulation rate:

To determine the activity of the enzymatic preparations in vitro of target fungi used, fungi were plated on petri dishes with a potato-dextrose agar containing enzymatic preparations (0.1%) and incubated at 20-22°C for 8 days. The diameters of the fungal colonies were then determined. After 10 days of incubation, 20 ml of water was added to every dish, and a spore suspension was obtained to determine the intensity of sporeulation. The suspension was homogenized for 1 min at 1500 rpm and filtered through a sterile cotton cloth. The suspension was filtered through a sterile cotton cloth. The spores were counted in a Goryaev Chamber (Tatarinova *et al.* (1996).

RESULTS AND DISCUSSION

Optimizing culture conditions for chitinase-producing *B. lichenifofmis:*
Time course:

The growth of *B. licheniformis* reached its maximum in the third day, then decreased slightly. At this point, the cell count was log 9.2 cells /ml, thereafter, the cell density decreased slightly. The enzyme activity appeared related to the cell growth through the cultivation up to 5 days. Chitinase activity in the culture fluid was detectable after one day and increased rapidly, which reached its maximum activity (0.26 U/ml) in the fifth day, but decreased rapidly thereafter (Fig. 1). The results indicated that this enzyme is not constitutive one, which induced with its substrate and its activity appeared in the surrounding media after 24 hours. Therefore, 5 days as the suitable cultivation period was chosen for the subsequent experiments. El-Sawah (1999) found that *Paenibacillus pabuli* chitinase reached its maximum activity after 72 hours.

Fig. 1: Time-course of chitinase production by *B. licheniformis.*

Carbon sources:

Some chitin types and chitin derivatives were investigated for the induction of extracellular chitinase. Results in Table (1) showed that, colloidal chitin + arabinose served as a superior source for chitinase production, (0.31 U/ml). Chitin and chitin + xylose were found as a good promoter for enzyme biosynthesis. While, glucose and N-AGA were completely repressed the enzyme synthesis. These results were easily indicated that this enzyme was inducible one with chitin or substrate-containing chitin (Gupta *et al*., 1995). Neugebauer *et al.* (1991) found that chitinase production by *Streptomyces lividance* was induced by chitin, but not by NAGA. Similar results have been reported with chitinase from *B. licheniformis* (Takiguchi and Shimahara, 1989), and *B. circulans* WL-12 (Watanabe *et al*., 1990). However, most of the chitinolytic systems reported in the literature are induicable (Ulhoa & Peberdy, 1991 and Gupta *et al*., 1995). El-Sawah (1999) found that colloidal chitin served as a superior source for chitinase production.

Table 1: Effect of different carbon sources on chitinase production by *B. licheniformis.*

Carbon sources	Chitinase activity (U/ml)
Chitin	0.26
Chitosan	0.04
Chitobiose	0.06
N-Acetyl glucose amine (N-AGA)	0.00
Cell wall	0.17
Glucose	0.00
Chitin + glucose	0.21
Laminarin	0.05
Chitin + laminarin	0.11
Chitin + N-AGA	0.08
Chitin + arabinose	0.31
Chitin + xylose	0.25
Chitin + ribose	0.20
Chitin + lactose	0.15

Effect of chitin concentration in the presence of arabinose:

Increasing the amount of chitin in the medium (Table 2) up to 1.5% + 0.1% arabinose stimulating the biosynthesis of *B. licheniformis* chitinase. The enzyme activity increased gradually and reached its maximum yield (0.43 U/ml) at 1.5% chitin in the production medium. For routine experiments, 1.5-2.0% chitin + 0.1% arabinose in the culture medium is suitable for maximum yield of this enzyme. Young *et al.* (1985); Abdel-Fatah (1995) and El-Sawah (1999) found similar results.

Effect of chitin/arabinose ratio:

Results in Table (3) show the appropriate proportion of arabinose in the culture medium. Accordingly, the production of enzyme was investigated using culture media containing different chitin/arabinose ratios. In any case,

the sum of chitin + arabinose was 15 g/l. Arabinose (1%) plus colloidal chitin (14 g/l) increased chitinase production (Table 3). Also, the results show that with the increasing of chitin and decreasing concentration of arabinose, the enzyme biosynthesis enhanced greatly and reached its maximum activity at 14 g chitin + 1 g/l arabinose. This means that, the lowest amount of arabinose is necessary for the stimulation enzyme formation, but the highest concentration of arabinose repressed the synthesis of chitinase. A synergistic effect of arabinose with colloidal chitin was observed by Gupta *et al.* (1995). They suggested a possible relationship with the area operon or its product in inducing of the chitinolytic system.

Table 2:Effect of chitin concentration in the presence of 0.1% arabinose on the production of chitinase by *B. licheniformis*.

Colloidal chitin conc. %	Chitinase activity (U/ml)
0.25	0.15
0.50	0.21
0.75	0.24
1.00	0.31
1.50	0.40
2.00	0.39
2.50	0.38
3.00	0.35

Table 3: Effect of chitin/arabinose ratio on the production of chitinase by *B. licheniformis.*

Chitin/arabinose ratio (g:g)	Chitinase activity (U/ml)
7.5:7.5	0.30
12.5:2.5	0.35
13.5:1.5	0.44
14:1	0.48

Effect of nitrogen source:

On an equivalent N basis, yeast extract enhanced the enzyme production and found as the best nitrogen source, which being 0.48 U/ml (Table 4). Also, peptone and meat extract were found as an excellent promoters for enzyme biosynthesis. In general organic nitrogen sources promoted enzyme synthesis than inorganic one. Finally, it appears from the results (Table 4) that nitrogen sources were greatly affecting on chitinase biosynthesis. In this respect, Abdel-Fatah (1995) and Osawa & Koga (1995) indicated the nutritional value of chitin as carbon and nitrogen source. El-Sawah (1999) found similar results.

Effect of aeration on enzyme biosynthesis:

Fig. (2) shows the effect of aeration rate (flask vol/culture vol) on the chitinolytic activity of *B. licheniformis*. The enzyme production was strongly influenced by aeration. Chitinase activity was highest (0.48 U/ml) at 18:02 aeration rate of the culture through the cultivation. Lower levels of enzyme

activity was present at aeration rates, this is probably due to insufficient oxygenation that could be partially compensated by increasing the aeration rate. Similar results were reported by Fenice *et al*. (1998).

Table 4: Effect of different nitrogen sources on the production of chitinase by *B. licheniformis.*

Nitrogen source	Chitinase activity (U/ml)
Yeast extract	0.48
Peptone	0.45
Meat extract	0.43
Corn steep liquor	0.40
Soybean	0.20
Urea	0.10
$(NH4)_2SO_4$	0.22
$0NaNO_3$	0.20
Without N	0.15

Fig 2: Effect of aeration on chitinase production by *B. licheniformis*.

Initial pH:

The effect of the initial medium pH was studied in shaken cultures in the pH range 5.5-9.0 (steps of 0.5 pH). The results (Fig. (3) show that enzyme was not produced at pH 5.5. This means that the acidity greatly affected the biosynthesis of this enzyme, which prevented its production at pH lowest 6.0. At pH 6.0 and above enzyme activity increased gradually, which reached its maximum at pH 8.0, then decreased. An initial pH of 8.0 appeared to be optimal for chitinase production. At this pH level, the enzyme activity reached 0.71 U/ml, with 11% approximately higher than that 0.64 U/ml obtained at pH 7.0 as the initial culture pH. Fenice *et al*. (1998) reported that pH 4.0 appeared to be optimal for *Penicillium janthinellum* chitinase production.

Effect of incubation temperature:

The effect of incubation temperature (25-60°C) on enzyme formation was tested in shaken cultures (Fig. 4). Enzyme production was highest in the temperature range 40-50°C with an optimum at 45°C. Enzyme activity reached 1.30 U/ml at 45°C. Above or below, enzyme activity decreased sharply. Fenice *et al*. (1998) found that the highest level of enzyme activity was obtained at 24°C as the optimum growth temperature.

Fig. 3: Effect of initial pH on chitinase production by *B. licheniformis.*

Fig. 4: Effect of temperature on chitinase production by *B. licheniformis.*

Enzyme properties:

Ammonium sulphate precipitation:

The results presented in Table (5) reveal that the specific activity, recovery and purification (fold) in the precipitate increased as the ammonium sulphate solution increased reaching their maximal values at 60% saturation, being 23.39 Units/mg protein, 52.4% and 9.4 folds, respectively. These parameters decreased by increasing ammonium sulphate concentration, also most of the enzyme activity could be precipitated using ammonium sulphate concentration of 60-80 saturation, which contained highest of the enzymatic activity. Therefore, enzyme at this saturation (60%) was used for further studied (enzyme properties and its application). Similar observations were obtained by Abdel-Fatah and Khella (1995).

Optimum temperature:

As shown in Fig. (5), the results reveal that the optimal temperature for maximal activity of *B. licheniformis* chitinase was 45°C. Decreasing or increasing the temperature than 45°C led to appreciable decrease in the enzyme activity. Abdel-Fatah and Khella (1995) found that 35°C is the optimum temperature for *Streptomyces cellulosae* chitinase activity. While, El-Sawah (1999) observed that 50°C is the temperature optima for *Paenibacillus pabuli* enzyme activity.

Table 5: Fractional precipitation of *B. licheniformis* chitinase with ammonium sulphate.

Ammonium sulphate conc. %	Volume (ml)	Enzyme activity (Units)	Total activity (Units)	Total protein (mg)	Specific activity (U/mg protein)	Recovery %	Purification (fold)
Culture supernatant	100	1.50	1500	604	2.48	100	---
20	20	9.6	192	20.5	9.37	12.8	3.8
40	20	17.7	354	27.9	12.69	23.6	5.1
60	20	39.3	786	33.6	23.39	52.4	9.4
80	20	25.7	514	38.2	13.46	34.3	5.4
90	20	19.1	382	41.6	9.18	25.47	3.7

Thermal stability:

Results illustrated in Fig. (6) clearly reveal that the enzyme was stable up to 60°C, at which it lost only 3% from its maximum activity. While exposure to 70°C led to loss 15% of its maximum activity at 90°C. This means that this enzyme was stable one, which showed higher stability against the higher temperature. *B. licheniformis* chitinase was stable up to 70°C. The optimum temperature of activity was 70°C for chitinase (Trachuk *et al.*, 1995)

Optimum pH:

The highest chitinase activity was observed when the pH was 7.5, being 1.8 units/ml (Fig. 7). Other pHs levels affecting greatly and reduced the enzyme activity. Abdel-Fatah and Khella (1995) found that pH 7.0 is the optimum pH for chitinase activity. While, El-Sawah (1999) observed that pH 6.5 was the optimum pH for enzyme activity.

Fig. 5: Temperature optima of *B. licheniformis* chitinase.

Fig. 6: Thermal stability of enzyme.

Fig. 7: pH optima of *B. licheniformis* chitinase.

pH stability:

Enzyme apparently is rather stable within a broad pH ranging from 6.0 to 8.0 (Fig. 8). The enzyme showed a higher stability toward pH 7.0. However, this enzyme was characterized by a relatively wide optimum pH (6.0 to 8.0), and stability within the same range. These characteristics can be advantageous for this bacterium for use in biotechnology, and as antifungal agent. The *B. licheniformis* chitinase revealed activity optima against colloidal chitin at pH 9.0-9.5 and it was rather stable at pH 4.0-9.5 (Trachuk *et al.*, 1996).

Effect of some metal salts and some inhibitors on enzyme activity:

The effect of some metal and some inhibitors on enzyme activity was tested by incorporating different concentrations of each (0.1, 1.0 and 10 mM) into a reaction mixture during normal enzyme assay. Data in Table (6) reveal that the enzyme, which can hydrolyze colloidal chitin was stimulated by addition of $CaCl_2$, $MgSO_4$, $MnSO_4$ and $FeSO_4$ at 10 mM. Addition of NaCl, KCl did not exert any effect on the enzyme activity. While, $HgCl_2$ at 10 mM inhibited the enzyme activity completely. Other substances affecting greatly, which reduced sharply the enzyme activity. Such inhibition may be due to the participation of a metal cation or sulfhydryl groups of this enzyme (Abdel-Fatah & Khella, 1995). Hg^{2+} ions effectively inhibited activity of *B. licheniformis* chitinase activity (Trachuk *et al.*, 1996).

Fig. 8: pH stability of the enzyme.

Table (6):Effect of some metal salts and some inhibitors on *B. licheniformis* chitinase activity.

Metal salts and inhibitors	Enzyme relative activity % Concentration (mM)		
	0.1	**1.0**	**10**
Control (without salts)	100 %		
NaCL	100	100	100
KCl	100	100	100
$CaCl_2$	100	105	111
$MgSO_4$	100	107	110
$MnSO_4.H_2O$	100	105	109
$CuSO_4$	91	75	53
$ZnCl_2.7H_2O$	100	101	104
$FeSO_4.7H_2O$	102	109	115
$HgCl_2$	35	15	0.00
$AgNO_3$	67	55	40
EDTA	83	60	40
Na-Azide	89	79	68
Iodine	95	78	67

Enzyme applications:

1- Substrate hydrolysis:

The results in Table (7) reveal that culture supernatant of *B. licheniformis* chitinase as a lytic enzyme has antifungal activity against all the three cell wall substrates of the target fungi. Also, the results showed that, enzyme activity increased with time of hydrolysis with all tested substrates. Enzymatic preparation obtained from the culture fluid of *B. licheniformis* displayed a high antifungal activities toward all substrates tested and the maximum antifungal activity was observed against the cell wall preparation. Preliminary studies also indicate that other metabolites are also produced (Singh *et al.*, 1999). Similar results were obtained by Abdel-Fatah & Khella (1995) and Singh *et al.* (1999)

2- Fungal growth and sporulation rates:

The addition of *B. licheniformis* chitinase preparation to the nutrient medium (potato-dextrose-agar) decreased the growth rate and the sporulation rate of the three fungal colonies by 47-57% and 69-71%, respectively, (Table 8). The maximum antifungal activity was observed against *F. oxysporium* growth (Table 8). These results also show that this enzyme displayed the maximum efficiency and chitinase activity as lytic enzyme. From these results, this enzyme may be play an important role in the defense against parasitic fungi on higher plants. Similarly to the inhibition of *Trichoderma viride* growth by plant chitinases and the inhibition of wheat fusariosis by *Streptomyces kurssanovii* chitinase (Tatarinova *et al.*, 1996).

Table (7): Activity of *B. licheniformis* chitinase produced on specific fungal cell wall and colloidal chitin (3:1) on dissolution of cell wall of different target fungi.

Cell wall substrate for dissolution	Enzyme activity (U/ml)						
	1h	2h	3h	4h	5h	6h	12h
Rhizoctonia solani	0.03	0.05	0.06	0.06	0.07	0.09	0.20
Aspergillus niger	0.02	0.04	0.05	0.06	0.08	0.08	0.12
Fusarium oxysporium	0.01	0.03	0.04	0.04	0.06	0.07	0.10

Table 8: Fungal growth and sporulation rates on PDA containing 0.1% *B. licheniformis* chitinase.

Target fungi	Growth rate %	Control	Sporulation %	Control
Rhizoctonia solani	46	100	29	100
Aspergillus niger	53	100	32	100
Fusarium oxysporium	43	100	31	100

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خصائص إنزيمات الكيتنيز المنتجة من *Bacillus licheniformis* وإستخدامها فى تثبيط بعض الفطريات الممرضة

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اتجهت الأبحاث فى الآونة الأخيرة نحو استخدام إنزيمات الكيتنيز فى كثير من مجالات التقنيات الحيوية الحديثة مثل إنتاج أدوية لعلاج الإصابات الفطرية وكذلك إستخدامها فى مجال الغذاء وتغذية الحيوان وفى معالجة المخلفات الغنية فى محتواها من الكيتين وفى مقاومة الآفات الضارة مثل الفطريات والحشرات وغيرها ونظراً لأهمية هذه الإنزيمات فقد إستهدف هذا البحث دراسة العوامل المؤثرة على إنتاج ونشاط هذه الإنزيمات المنتجة من ميكروب *B. licheniformis* وإستخدامها فى تثبيط والقضاء على بعض الفطريات الضارة وقد أظهرت النتائج الآتى :

- وصل أعلى معدل لنمو البكتيريا فى اليوم الثالث فى حين لوحظ أعلى إنتاج للإنزيم فى اليوم الخامس من التحضين .
- حث إستخدام الكيتين + الأرابينوز بمعدل 1.5% + 0.1% على إنتاج الإنزيم
- كان مستخلص الخميرة هو أفضل مصادر النيتروجين المستخدمة حثاً على إنتاج الإنزيم .
- كانت درجة pH 8 و ˚45م هما المثاليتين لإنتاج الإنزيم .
- أدى إستخدام 60% تشبع من كبريتات الأمونيوم إلى الحصول على أعلى نشاط نوعى للإنزيم و recovery fold و purification rate حيث وصلت إلى 23.39 وحدة (مجم بروتين) ، 52.4% ، 94 مرة على الترتيب .
- كانت ˚45م ودرجة pH 7.5 هما المثاليتين لإنتاج الإنزيم .
- أظهر الإنزيم ثباتاً حرارياً حتى ˚60م وفقد 15% فقط من نشاطه عند ˚70م مما يعنى أنه إنزيم متحمل للحرارة .
- أظهر الإنزيم ثباتاً تجاه درجات الـ pH فى المدى من 6 – 8 وهذا يضفى عليه مزايا عديدة فى إستخدامه كمضاد للفطريات الضارة .
- كان لكلوريد الكالسيوم وكبريتات الماغنسيوم والمنجنيز والحديد تأثير حثى وتنشيطى للإنزيم فى حين ثُبط النشاط كلياً بإستخدام 10 ملليمول من كلوريد الزئبقيك .
- نجح هذا الإنزيم فى تحليل جدر الثلاث فطريات المستخدمة مما يعنى إمكانية إستخدامه كإنزيم مثبط لنمو الفطريات الضارة .
- أدى إضافة هذا الإنزيم لبيئة نمو وتجرثم بعض الفطريات المتطفلة إلى تقليل معدل نموها وتجرثمها مما يعنى أهمية إستخدامه فى المقاومة الحيوية لهذه الفطريات الضارة .