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Production of Chitinase by *Pseudomonas aeruginosa* K-187 Using Shrimp and Crab Shell Powder as a Carbon Source

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ABSTRACT

Shrimp and crab shell powder prepared by treating shrimp and crab processing waste with boiling and crashing was used as a substrate for isolating alkali-tolerant chitinolytic microorganisms. Strain K-187 appeared to be the chitinase-producing strain with the most potential. The organism was identified as a strain of *Pseudomonas aeruginosa*. Maximum chitinase activity was obtained when the strain was grown aerobically in a medium consisting of 3.0% shrimp and crab shell powder, 0.1% CMC, 0.1% (NH4)2SO4, 0.1% K2HPO4, 0.1% MgSO4•D7H2O and 0.1% ZnSO4 (pH 9), at 45°C after 3 days. The optimum pH and temperature of the enzyme reaction were 7 and 40°C, respectively. The chitinase was stable at pH from 5 to 10 and was stable under 60°C.

Key Words: chitinase; chitin; shrimp and crab shell powder; seafood processing waste; *Pseudomonas aeruginosa*.

I. Introduction

Chitin, a homopolysaccharide composed of β-1,4-linked N-acetyl-D-glucosaminopyranosyl residues, is abundant in nature as the integuments of insects and crustaceans and as a component of fungi (Zikakis, 1984). Shrimp and crab shell powder (SCSP) containing chitin, protein, and calcium carbonate etc. is pretreated by size reduction, deproteination and demineralization to yield a chitin material suitable for bioconversion or other uses (Cosio *et al.*, 1982; Carroad and Tom, 1978). Chitin and its derivatives are of interest because they have various biological activities and agrochemicals. Application of chitin, waste of the shellfish industry, has been limited by high processing costs of degrading chitin although chitin itself is substantially lower in cost and appears to be amenable to mechanical and chemical modification. The bulk of the pretreated chitin is mixed with chitinolytic enzyme to hydrolyze it to the monomer or oligomer of N-acetylglucosamine (Murao *et al.*, 1992; Izumi *et al.*, 1992; Mitsutomi *et al.*, 1990; Ohtakara *et al.*, 1990; Yabuki *et al.*, 1986; Ohtakara *et al.*, 1979; Tominaga and Tsujisaka, 1976; Monreal and Reese, 1969).

Chitinase producing strains have been reported by many workers from soils and marine muds by using chitin (Berger and Reynolds, 1958; Monreal and Reese, 1969; Yabuki *et al.*, 1986), colloidal chitin (Ohtakara *et al.*, 1979) or other chitin materials (Ishikawa *et al.*, 1981; Ohtakara, 1961) as a major carbon source for chitinase production. However, isolation of an alkali-tolerant chitinase producing strain by using SCSP as a major carbon source has not been reported.

The production of inexpensive chitinolytic enzymes is an important element in the utilization of shrimp processing waste, and utilization of alkali-tolerant chitinase producing strain is also useful in freeing the processing wastes of odors generally encountered as a pollution problem. Effective utilization of such marine wastes not only solves environmental

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problems, but also promotes the economic value of the marine products.

In this study, the intention was to utilize shrimp and crab processing wastes as a carbon source to produce chitinolytic enzymes. High chitinase producing microorganisms were isolated from the soil of Taiwan. Identification of the alkali-tolerant microorganism, medium composition and the enzymes character were also investigated.

II. Materials and Methods

1. Isolation and Screening of Chitinase Producing Strain First Screening

Microorganisms isolated from soils collected at ten different locations in Taiwan were screened on agar plates containing 0.2% colloidal chitin, 0.1% K2HPO4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% $NaNO_3$ and 2.0% agar (pH 9). Colloidal chitin was used as the sole source of carbon and was incubated at 37°C for 3-7 days. Those colonies which grew well or showed a clear zone around the colonies were isolated and retained for sebsequent screening. Fifty-three bacteria were obtained from the first screening.

2. Second Screening

Those orgaisms obtained from the first screening were cultured in liquid media (containing 3% SCSP, 0.1% K2HPO4 and 0,05% $MgSO_4 \cdot 7H_2O$) in flasks on a reciprocal shaker (37°C). After incubation for 5 days, culture broth was centrifuged, and the supernatants were collected for measurement of enzyme activity. Five out of the 53 isolates from the first screening showed high enzyme activity in the culture broth. The strain K-187, which was isolated from soils collected in central Taiwan, showed the highest capability in producing chitinase. This strain was maintained on nutrient agar and used throughout the study.

3. Identification of Strain K-187

From the morphological observation and physiological characteristics, the microorganism was identified according to the description in Bergey's Mannual of Determinative Bacteriology (Krieg and Holt, 1984).

4. Preparation of the Enzymes

Bacterial cells grown on a slant culture was inoculated into a 250-ml Erlenmyer flask containing 100 ml of culture medium (pH 9.0) and incubated with shaking at 37°C for 5 days. The culture broth was centrifuged at 10,000 × g in a refrigerated centrifuge (J2-21M/E, Backmen, U.S.A.) to remove the cells. The supernatants were then taken for measurement of enzyme activity. The experiment was carried out in duplicate, and average values were reported.

5. Preparation of Colloidal Chitin

Colloidal chitin was prepared from powdered chitin by the method of Jeniaux (1966).

6. Assay of Chitinase Activity

Chitinase activity was measured with colloidal chitin as a substrate. Enzyme solution (0.5 ml) was added to 1.0 ml of substrate solution, which contained 1.3% suspension of colloidal chitin in an phosphate buffer (0.125 M, pH 6.0), and the mixture was incubated at 37°C for 10 min. After centrifuging, the amount of reducing sugar produced in a supernatant was determined by the Imoto and Yagishita method (1971) with N-acetylglucosamine as a reference compound. One unit of chitinase activity was defined as the amount of the enzyme which produced 1 μ mole of reducing sugar per min.

7. Effect of SCSP and Chitin

Growth was carried out on minimal synthetic medium (MSM) containing 0.1% K2HPO4 and 0.1% $MgSO_4 \cdot 7H_2O$; the medium was supplemented with various concentrations of SCSP (Chya-Pau Co., Shu-Ao, I-Lan, Taiwan, R.O.C.) or purified chitin powder from crab shells (Sigma Co.). The flasks were removed after 5 days of growth for enzyme assays.

8. Effect of Carbon and Nitrogen Sources

Growth was carried out on SCSP medium (3.0% SCSP + MSM) containing either no additional carbon sources, carboxymethyl cellulose (CMC), xylan, soluble starch, lactose, glucose, glycerol, or sugar cane bagasse (a gift of the Taiwan Sugar Company, Chi-Hu, Taiwan, R.O.C.). The carbon source concentration used was 0.1%. The flasks were removed after 5 days of growth for enzyme assays. The effect of different nitrogen sources on chitinase production was tested in SCSP medium containing 0.1% CMC. The nitrogen sources applied at 0.1% were Polypepton, yeast extract, sodium glutamate, $NaNO_3$, $(NH_4)_2SO_4$, fish powder, and fish extract. The flasks were removed after 5 days of growth for enzyme assays.

9. Effect of Inorganic Salts

Growth was carried out on medium A (3.0% SCSP + MSM + 0.1% CMC + 0.1% ammonium sulfate) and medium B (0.4% chitin + MSM + 0.1% glycerol + 0.1% fish extract). The inorganic salts applied at 0.1% were $FeSO_4$, $ZnSO_4$, $CuSO_4$, and $MnSO_4$. The flasks were removed after 5 days of growth for enzyme assays.

10. Effect of Temperature and Initial pH

Growth was carried out on the medium containing: 3.0% SCSP, 0.1% CMC, 0.1% $(NH_4)_2SO_4$, 0.1% $ZnSO_4$, 0.1% K_2HPO_4, and 0.1% $MgSO_4 \cdot 7H_2O$. The media were adjusted with NaOH and HCl to pH of 5, 7, and 9. The flasks were removed after 5 days of growth at 25, 37, and 45°C, respectively, for enzyme assays.

11. Effect of Medium Volume

Growth was carried out on SCSP-containing medium (pH 9) as described above. 250 ml flasks containing 25, 50, 75, 100, 125, 150, 175, and 200 ml of the culture media were incubated with reciprocal shaking at 45°C. The flasks were removed after 5 days of growth for enzyme assays.

12. Time Course of Chitinase Production

Growth was carried out on SCSP-containing medium (pH 9) to determine chitinase activity. 250 ml flasks containing 175 ml of the culture medium was incubated with shaking at 45°C. The flasks were removed after 3 days of growth for enzyme assays.

13. Enzyme Stability

In the pH stability test, the broth containing the enzymes was preincubated at 37°C and various pHs (buffers used were in a concentration of 25 mM) for 10 min. The enzyme activities were then determined after the addition of substrate solution buffered at pH 6 with 100 mM sodium phosphate buffer. In the thermal stability test, the culture broth was incubated at various temperatures at pH 6.0 for 10 min. The buffered enzyme solutions were then chilled quickly in ice, and the remaining chitinase activity was assayed.

14. pH and Temperature Profiles of the Enzymes

The pHs of the enzyme activities were studied by mixing the culture broth with 50 mM buffer solution at various pHs, and these mixtures were incubated at 37°C for 30 min. For temperature profiles, the enzyme activities were studied at pH 6 and incubated at various temperatures for 10 min.

III. Results

1. Identification of Strain K-187

The bacteriological properties of strain K-187 were as follows: (a) Morphological observation. The vegetative cells were rods (0.6 × 2.0 μm); flagellated; motile; Gram negative. (b) Cultural characteristics. Agar colonies (20 h at 25°C) were circular with a muddy cream colored smooth surface; positive growth on gelatin stab culture and liquefaction of gelatin was observed; moderate growth on medium containing 3% or 6.5% NaCl; aerobic growth on Saboraud dextrose medium and MacConkey agar. (c) Physiological characteristics. Positive tests for catalase, urease, arginine dihydrolase, lecithinase, casein hydrolysis, gelatin liquefaction, Tween 80 hydrolysis, tyrosine utilization, using malonic acid as the sole carbon source, gas production from nitrate, and negative tests for indole production. Production of acids from the following sugars: glucose, arabinose, xylose (Table 1). When strain K-187 was compared with *Pseudomonas aeruginosa* CCRC10944, its physiological and cultural properties were found to be similar only to that of *P. aeruginosa*. Based on the above results and on the primary keys in *Bergey's Manual of Determinative Bacteriology*, strain K-187 was, therefore, identified as a strain of *P. aeruginosa*.

2. Effects of SCSP and Chitin

A series of experiments was carried out to study the effect of different initial SCSP concentrations on enzyme production. As shown in Fig. 1, a tremendous increase in enzyme production was observed by increasing the SCSP concentration from 1.0 to 3.0%. The enzyme production was increased five-fold. However, a further increase of SCSP to 4.0% decreased the enzyme formation remarkably. Fig. 2 shows the effect of chitin on enzyme production. A marked increase in enzyme production was exibited when the chitin concentration increased from 0 to 0.4%. When the concentration increased to 0.6%, the enzyme production clearly decreased. It is obvious that SCSP is more effective than chitin in chitinase production for the strain used.

Table 1. General Characteristics of Strain K-187

Gram stain	G-rod
Shape	
Growth in air	+
Growth anaerobically	−
Motility	+
Catalase	+
Growth at 25°C	+
Growth at 41°C	+
Growth in pH 5 − 10	+
Growth in 3% NaCl	+
Growth in 6.5% NaCl	+
Growth on MacConkey agar	+
Casein hydrolysis	+
Gelatin hydrolysis	+
Tween hydrolysis	+
Fluorescein production	+
Indole formation by using tryptophan	−
Citric acid can be used as the sole source of carbon	+
Urease	+
Gas production from NO_3^-	+
L-arginine dihydrolase	+
Lecithinase	+
Malonic acid can be used as the sole source of carbon	+
Tyrosine utilization	+
Acid is produced from	
L-arabinose, cellobiose, D-glucose, D-fructose, L-inositol, maltose, raffinose, D-xylose	

3. Effect of Carbon and Nitrogen Sources

The production of chitinase by *P. aeruginosa* K-187 was slightly influenced by a carbon source of the medium (Table 1). Chitinase production was slightly enhanced when CMC and glycerol were used as the carbon source of SCSP and chitin medium, respectively. On the other hand, addition of other carbohydrates repressed enzyme production to different degrees.

The secretion of chitinases was also slightly influenced by the nitrogen source used (Table 2). Production of chitinase was slightly higher using 0.1% ammonium sulfate as a nitrogen source.

4. Effect of Inorganic Salts

The production of chitinases was significantly higher with zinc sulfate in medium than with ferric sulfate, copper sulfate, or manganess sulfate and without inorganic salt (Table 3). Enzyme production was observed upon increasing the zinc sulfate concentration from 0 to 0.1%. However, a further increase of zinc sulfate to 0.25% decreased enzyme production remarkably.

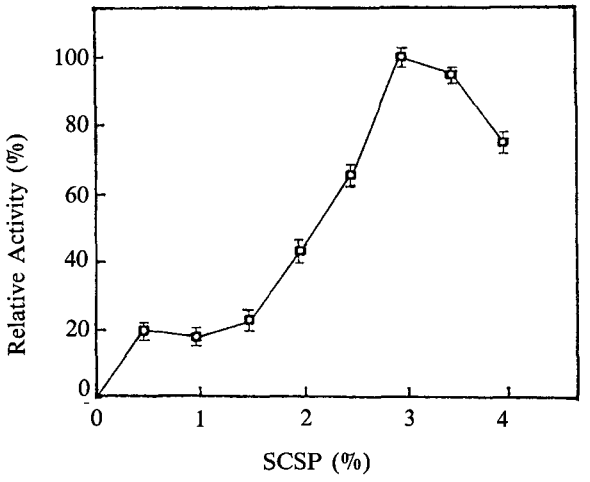

Fig. 1. Chitinase production by *P. aeruginosa* K-187 in the presence of various concentration of SCSP.
The basal medium consisted of 0.10% K2HPO4 and 0.05% $MgSO_4 \cdot 7H_2O$ (pH 9) in distilled water. The solution was autoclaved at 121°C for 48 hrs on a reciprocal shaker. 100% relative activity equal 0.30 U/ml. Datum points represent the means of three measurements. Standard deviations are indicated by error bars.

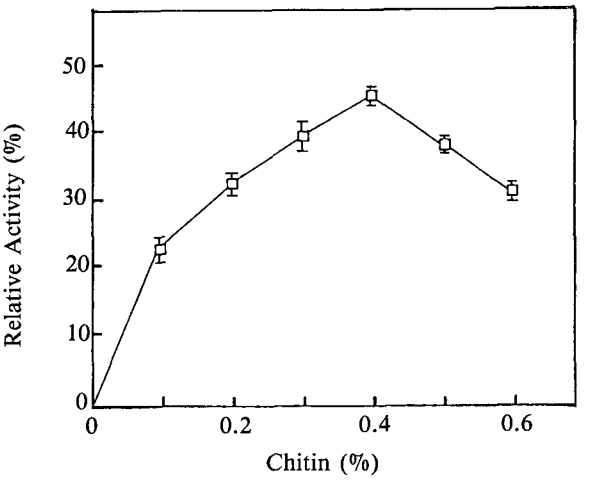

Fig. 2. Chitinase production by *P. aeruginosa* K-187 in the presence of various concentration of chitin.
Experimental details are described in Fig. 1. Data points represent the means of three measurements. Standard deviations are indicated by error bars.

5. Effect of Temperature, Initial pH, and Culture Volume

Flask cultivation was carried out in the optimum medium at three initial pHs and temperatures for 5 days. The results are shown in Table 4. The strain K-187 produced higher enzyme activity when the

Table 2. Effect of Various Carbon Sources on Enzyme Production

Carbon source	Concentration (%)	Relative activity (%)	
		SCSP	Chitin
None	0	100	34
CMC	0.1	107	38
Xylan	0.1	94	24
Soluble starch	0.1	86	20
Lactose	0.1	68	7
Glucose	0.1	73	38
Glycerol	0.1	83	52
Bagasse	0.1	88	33

Table 3. Effect of Various Nitrogen Sources on Enzyme Production

Nitrogen source	Concentration (%)	Relative activity (%)	
		SCSP	Chitin
None	0	100	68
Polypepton	0.1	122	64
Yeast extract	0.1	82	50
Sodium glutamate	0.1	108	72
Sodium nitrate	0.1	78	66
Ammonium sulfate	0.1	132	48
Fish powder	0.1	112	45
Fish extract	0.1	117	81

initial pH and temperature were in the range of 7 to 9 and 37 to 45°C, respectively. Compared with pH 5 (45°C), chitinase activity was significantly higher under the conditions of pH 9 and 45°C. The production of chitinase has not significantly difference between pH 7 and 9.

No significant differences were observed in chitinase production when the culture volume was increased from 25 to 175 ml.

6. Time-Course of Cultivation

Strain K-187 was grown aerobically in 175 ml of the optimum medium in a 250-ml Erlenmeyer flask at 45°C. During the process of incubation, chitinase, cell growth and pH in broth were measured every 24 h. A typical time course of chitinase production is shown in Fig. 3. The pH value decreased steadily to about 9.0. The chitinase activity increased to 0.68 U/ml within 3 days and remained constant thereafter.

7. Some Properties of the Enzyme

The pH stability of the crude enzyme retained its 90% activity within a pH range of between 6 and 10. For thermal stability, the enzyme was stable under 60°C. The optimum pH for chitinase reaction

Table 4. Effect of Various Inorganic Salts on Enzyme Production

Inorganic salts	Concentration (%)	Relative activity (%)	
		SCSP	Chitin
None	0	100	74
$FeSO_4$	0.1	115	70
$ZnSO_4$	0.1	146	64
$CuSO_4$	0.1	54	71
$MnSO_4$	0.1	93	70

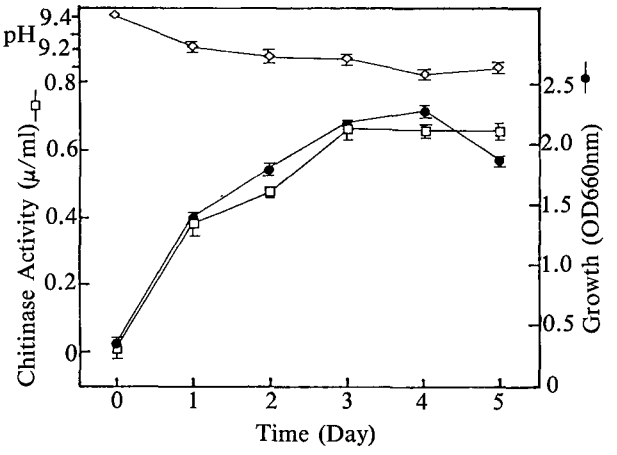

Fig. 3. Time course of cultivation of *P. aeruginosa* K-187. Data points represent the means of three measurements. Standard deviations are indicated by error bars. -□-, chitinase activity; -●-, growth; -◇-, pH.

was within a range of between 5 to 9. The optimal temperature for chitinase reaction was within a range of between 37 to 90°C.

8. Comparison of Culture Condition for Various Microorganisms

The major carbon source and cultural conditions of other chitinase producing microorganisms reported in the literature are listed in Table 5. Except strain K-187, almost all the other chitinase producing strains were found to use chitin or colloidal chitin as a carbon source for chitinase production. Also, no strains have been reported that can be cultivated in a high temperature (45°C) and alkaline (pH 9) environment.

IV. Discussion

Monreal and Reese (1969) reported that the optimal carbon source for chitinase production of *Serratia marcescens* was 1.5% chitin. Ohtakara *et al.*

Table 5. Effect of Temperature and pH on Enzyme Production

Temperature	Relative activity (%)		
	pH 5	pH 7	pH 9
25°C	84	82	80
37°C	98	116	100
45°C	77	91	110

(1979), using 0.2% colloidal chitin as a major carbon source to enhance the chitinase production of *Vibrio* sp. (Yabuki *et al.*, 1986), indicated that 1.0% chitin was the optimal carbon source for chitinase production of *Aeromonas hydrophila*, and that production decreased with an increase in chitin concentration. In this study, SCSP was a more useful carbon source and inducer of chitinase production than chitin by *P. aeruginosa* K-187. Why this strain showed higher enzyme production with SCSP than with chitin as the carbon source is not clear. The shell of shrimp and crab is composed mainly of chitin impregnated with protein, that has been variously modified with lipid and mineral salts. The impregnated mineral salts are primarily calcium carbonate with minor amounts of magnesium, phosphate, silica, and sulfur (Chen and Yang, 1994). The reason why SCSP is more effective than chitin in chitinase production by K-187 may be the difference in composition of SCSP and chitin.

The optimal pH for chitinase production of *Bacillus licheniformis* (Takayanagi *et al.*, 1991), *Serratia marcescens* (Monreal and Reese, 1969), *Vibrio* sp. (Ohtakara *et al.*, 1979), *Aeromonas hydrophila* (Yabuki *et al.*, 1986), *Streptomyces griseus* (Berger and Reynolds, 1958), *Streptomyces orinentalis* (Tominaga and Tsujisaka, 1976) and *Streptomyces thermoviolaceus* (Tsujibo *et al.*, 1993) all were found to be pH 7, and production decreased with an increase in pH. In this study, the initial pH of the medium for maximal chitinase production by *P. aeruginosa* K-187 has not significantly different between pH 7 and 9. In addition, chitinase production by this strain was better at higher cultivation temperature of 45°C. This result also differs from other results described above except for *S. thermoviolaceus* (50°C). In addition to the alkali-tolerant properties described above, the enzymes of this strain showed a specific characteristic of wide pH stability (pH 5 to 10) compared with the enzymes reported for other microbial chitinases (Zarain-Herzberg and Arroyo-Begovich, 1983; Tsujibo *et al.*, 1993; Ohtakara *et al.*, 1979; Ueno *et al.*, 1990; Tominaga and Tsujisaka, 1976) except for pH 4 to 10 of *S. erythraeus* (Hara *et al.*, 1989). On the other hand, some properties of the enzymes of this strain are similar to those of other enzymes: the optimum

Table 6. Comparison of Culture Conditions and Enzyme Optimum pH for the Enzyme Reaction from Various Microorganisms

Organism*	Major carbon source	Culture condition			Optimum pH
		pH	Temp. (°C)	Days	
Pseudomonas aeruginosa K-187	3.0% SCSP	9.0	45	3	7
Serratia marcescens	1.5% chitin	7.7	30	6-7	6
Aeromonas hydrophila	1.0% chitin	7.0	30	3	7
Streptomyces griseus	0.25% chitin	7.0	28	6-7	8
Vibrio sp.	0.2% colloidal chitin	7.0	30	2	6-8
Streptomyces sp.	0.2% colloidal chitin	7.0	30	5	5
Pycnoporus cinnabarinus	4.0% sucrose	7.0	30	6	4.5

*from Ohtakara (1988).

pH in the neutral range of the enzymes from K-187 were similar to that of the enzymes from *Aeromonas hydrophila* subsp. *anaerogenes* (Yabuki *et al.*, 1986), *Bacillus stearothermophilus* (Sakai *et al.*, 1994), *Vibrio* sp. (Ohtakara *et al.*, 1979) and the tobacco hornworm (Turner *et al.*, 1981) while many other enzymes have shown pH optima in the acidic range.

Environmental and energy restrictions have forced the nation's seafood processors to consider alternatives to inland dumping or hauling out to sea of waste materials (Carroad and Tom, 1978). Chitin bioconversion to yeast single-cell protein has been proposed as a waste treatment alternative to the disposal of shellfish waste (Carroad and Tom, 1978; Cosio *et al.*, 1982). Experimental results indicate that these conditions of K-187 are suitable for carrying out chitinolytic activities of biomass utilization. Such a high pH condition is desirable in enzyme production because under a high pH value, the culture is more resistant to contaminants and also can be used as a measure to prevent the chitin from containing wastes from odors generally encountered as problem. The results presented in this article represent those for a shake-flask scale test, which needs to be adapted for real applications.

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利用蝦蟹殼粉爲碳源以 *Pseudomonas aeruginosa* K-187 生產幾丁質酶

王 三 郎 • 張 文 智 • 呂 明 洲

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摘 要

利用蒸煮粉碎處理過之蝦蟹殼粉作爲篩選耐鹼性幾丁質酶生產菌之基質。菌株K-187具較佳之酵素生產性。此菌株經鑑定係爲 *Pseudomonas aeruginosa*。於含3%蝦蟹殼粉，0,1% CMC，0.1% $(NH_4)_2SO_4$，0.1% K_2HPO_4，0.1% $MgSO_4 \cdot 7H_2O$，以及0.1% $ZnSO_4$ (pH9)之培養基，經三天培養（45°C）可得較佳之酵素活性。酵素反應之最適pH與溫度分別爲 7 及40°C，而pH及熱穩定性則係分別爲5-10及60°C。