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Production of Chitinase from Shellfish Waste by *Pseudomonas aeruginosa* K-187

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ABSTRACT

The production of chitinolytic enzyme by *Pseudomonas aeruginosa* K-187, using shrimp and crab shell powder (SCSP) as the carbon source, was studied. It was observed that chemically treated SCSP induced a significant increase of enzyme production, as compared with untreated SCSP. Spent HCl and NaOH from the chitin production industry was used to process SCSP. Various strategies of SCSP processing are examined and compared in terms of chitinolytic enzyme production. A three-and-one-half-fold increase of enzyme production (0.68 U/ml to 2.4 U/ml) was attained using HCl/NaOH treated SCSP. The microorganism (K-187) was isolated from soil in Taiwan and has been characterized and reported in a previous paper.

Key Words: Chitinase; chitin; shellfish waste; *Pseudomonas aeruginosa*.

I. Introduction

Shrimp and crab shell (SCS) contain chitin, protein and inorganic compounds, which are composed of calcium carbonate (Carroad and Tom, 1978; Cosio *et al.*, 1982). Traditional methods for the preparation of chitin involve demineralization and deproteinization of the waste material with strong acids and bases (*e.g.*, HCl and NaOH) (Brine and Austin, 1981; Gagne and Simpson, 1993). The production of chitin and its hydrolyzates, such as acetylglucosamine and chitooligosaccharide, from waste of the shellfish industry has been limited due to the high cost of the SCS pretreating process and of chitinase (Wang and Chang, 1997).

Chitinase has been found in microorganisms, plants and animals (Flach *et al.*, 1992; Shaikh and Deshpande, 1993). The major source of chitinase is microorganisms (Roberts and Cabib, 1982; Murao *et al.*, 1992; Okazaki *et al.*, 1995; Ueda *et al.*, 1995). Since chitin represents a vast renewable fermentation feedstock of both carbohydrate and nitrogen, enzymes capable of bioconverting it to low molecular weight fermentable products are potentially of commercial value (Irhuma *et al.*, 1991). Almost all microbial chitinase producers can use chitin, colloidal chitin or other chitin derivatives as major carbon sources for chitinase production. These strains require inducers, such as colloidal chitin or powdered chitin which are produced through demineralization treatment. Colloidal chitin was prepared from demineralized chitin by first soaking it in concentrated hydrochloric acid and then flushing it with an excess amount of water to remove any residual acid. The process of making powdered chitin using a ball mill was greatly limited by the small size of each batch. Furthermore, the process was time consuming. The use of shrimp and crab shell powder (SCSP) directly as a major carbon source to produce chitinase has not been reported. In our previous study, we successfully screened an alkali-tolerant chitinase producing strain, *P. aeruginosa* K-187, from soil of northern Taiwan using SCSP as a major carbon source (Wang *et al.*, 1995).

We have also purified and characterized two bifunctional chitinases/lysozymes from *P. aeruginosa* K-187 cell-free culture broth. This was the first report

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of its kind from prokaryote (Wang and Chang, 1997). In this study, the effects of various carbon sources on production of chitinase by *P. aeruginosa* K-187 utilizing SCSP treated with either hydrochloric acid or sodium hydroxide and the liquid waste produced by the treatment were compared. In addition, the effects of N-acetylglucosamine and some other marine waste products, such as flake chitin, powdered chitosan, SCS extract, and fish powder, were also compared.

II. Materials and Methods

1. Microorganisms

Pseudomonas aeruginosa K-187, an chitinase producing strain, was isolated from the soil of Taiwan (Wang *et al.*, 1995). The organisms were maintained by means of occasional transfer on slants of sagar medium (0.25% glucose, 0.25% beef extract, 0.25% polypepton, 0.25% sodium L-glutamate, and 2% agar, pH 7.0) at 4°C.

2. Shrimp and Crab Processing Waste

The shrimp and crab shell powder (SCSP) used in the experiment was purchased from the Chya-Pau Food Co. (Su-Ao, I-Lan, Taiwan) and stored at –4°C until use.

Seven preparations of the same type of shrimp and crab processing waste were used in this study, namely:

i) Untreated shrimp and crab shell powder (untreated SCSP).

The SCSP used was prepared from shrimp and crab processing waste material, a by-product from the marine food processing industry. This preparation is referred to as untreated SCSP.

ii) SCSP treated with HCl (HCl-SCSP).

In this process, the SCSP was treated with 2N HCl at room temperature for 2 days. The ratio of SCSP to solvent was 1:8 (w:v). This is identical to the demineralization method for the preparation of crustacean chitin (Shimahara and Takiguchi, 1988). The demineralized material was recovered by filtration, thoroughly rinsed with deionized water, and dried at 65°C in a forced air oven. This preparation is referred to as HCl-SCSP.

iii) The filtrate from SCSP treated with HCl (HCl-extract).

This extract was the filtrate obtained from the HCl-SCSP preparing process as described above. The filtrate was adjusted to pH 7 by means of NaOH solution. This preparation is referred to as HCl-extract.

iv) SCSP treated with NaOH (NaOH-SCSP).

In this process, SCSP was treated with 2N NaOH at 100°C for 30 min. The ratio of SCSP to solvent, 3:40 (w:v), was referred from the deproteination method for the preparation of crustacean chitin (Gagne and Simpson, 1993). The deproteinized material was recovered by means of filtration, thoroughly rinsed with deionized water, and dried at 65°C in a forced air oven. This preparation is referred to as NaOH-SCSP.

v) The filtrate from SCSP treated with NaOH (NaOH-extract).

This extract was the filtrate obtained from the NaOH-SCSP preparation process described above. The filtrate was adjusted to pH7 using HCl solution. This preparation is referred to as NaOH-extract.

vi) SCSP treated with HCl/NaOH (HCl/NaOH-SCSP).

In this process, SCSP was first treated with HCl, washed and dried, and then treated with NaOH. The methods used were the demineralization and deproteination methods described above. This preparation is referred to as HCl/NaOH-SCSP.

vii) The filtrate from HCl-SCSP treated with NaOH (HCl/NaOH-extract).

This extract is the NaOH-treated filtrate obtained from the HCl/NaOH-SCSP preparation process described above. The filtrate was adjusted to pH 7 using HCl. This preparation is referred to as HCl/NaOH-extract.

The concentrations of the six preparations other than the raw SCSP are expressed as the weight percentage of raw SCSP used before treatment. For example, X g of raw SCSP, treated with acid or alkali, produced X weight percent of SCSP in the acidic/alkaline solution (w/v).

3. Chemicals

Powdered chitin was purchased from the Wako Co. (Japan). Flake chitin and powdered chitosan from crab shells were purchased from the Biotech Co. (Kau-Shoun, Taiwan). N-acetylglucosamine was purchased from the Sigma Co. (USA). Colloidal chitin was prepared from powdered chitin using the method of Jeniaux (1966). Fish extract, SCS extract, and fish powder were purchased from the Chya-Pau Co., Su-Ao, I-Lan, Taiwan. All other chemicals used were of analytical grade.

4. Preparation of Enzymes

Bacterial cells were inoculated into a 250-ml Erlenmeyer flask containing 175 ml of culture medium (pH 7.0) (which will be described later) and incubated at 37°C. The culture broth was centrifuged at

10,000 × g to remove the cells. The supernatants were then used to measure the enzyme activity.

5. Assay of Enzyme Activity

The reaction was carried out by mixing 0.5 ml of enzyme solution and 1 ml of 1.3% colloidal chitin in 0.125 M phosphate buffer, pH 6. This mixture was then incubated at 37°C for 10 min. After centrifuging, the amount of reducing sugar produced in a supernatant was determined using the modified Schales method (Imoto and Yagishita, 1971) with N-acetylglucosamine as a reference compound. One unit of chitinase activity was defined as the amount of enzyme which produced 1 μmole of reducing sugar per min.

6. Comparison of the Inducing Effect of SCSP with the Other Chitin-related Commercial Products

Cultivation was carried out on medium A (0.1% CMC + 0.1 % $(NH_4)_2SO_4$ + 0.1% K_2HPO_4 + 0.1% $MgSO_4$ + 0.1% $ZnSO_4$) and medium B (0.1% glycerol + 0.1% fish extract + 0.1% K_2HPO_4 + 0.1% $MgSO_4$) as described in a previous study (Wang *et al.*, 1995). Medium A was supplemented with 0 – 4% of SCSP or pellet chitin whereas medium B was supplemented with 0 – 1% of powdered chitin or powdered chitosan. The flasks were removed after 3 days of growth for enzyme assays.

7. Effect of Nitrogen Sources

The effects of nitrogen sources on chitinase production were tested in SCSP medium (3% SCSP + medium A), flake chitin medium (2% flake chitin + medium B), and powdered chitin medium (0.5 % powdered chitin + medium B), containing additional nitrogen sources of either polypepton, yeast extract, fish extract, SCS extract, fish powder, sodium L-glutamate, sodium nitrate, ammonium sulfate, or N-acetylglucosamine. The concentration of the nitrogen source used in the media was 0.1%. The flasks were removed after 3 days of growth for enzyme assays.

8. Comparison of the Inducing Effects of Seven Preparations of SCSP on Chitinase Production

Cultivation was carried out on medium A containing seven additional preparations as described above. The media were adjusted using NaOH and HCl to pH 7.

III. Results

1. Comparison of the Inducing Effects of SCSP with the Other Chitin-related Commercial Products

In our previous study (Wang *et al.*, 1995), we found that SCSP was a more suitable carbon source than powdered chitin for chitinase production by strain K-187. To further compare the inducing effect of SCSP with the other chitin related materials, the cheaper commercial products of flake chitin and powdered chitosan were used as the carbon sources. As shown in Fig. 1, SCSP was the best carbon source, followed by flake chitin, powdered chitin, and powdered chitosan. The best concentrations were 3.0, 2.0, 0.5, and 0.25%, respectively. Therefore, 3.0% SCSP, 2.0% flake chitin, and 0.5% powdered chitosan were chosen and added into the cultures in the following experiments.

2. Effect of Nitrogen Source

The effects of nitrogen sources induced by other inexpensive marine processing waste products, such as fish extract, SCS extract, and fish powder were compared with those of other chemicals. As shown in Table 1, the chitinase activity for the medium containing 2.0% flake chitin increased about 1.3 fold following the addition of 0.1% N-acetylglucosamine. There was no pronounced change in the chitinase production when the medium contained 3% SCSP or 0.5% powdered chitin. Addition of other nitrogen sources, on the other hand, depressed enzyme production to different levels. A further comparison of the effect of N-acetylglucosamine on chitinase production in 2% flake chitin containing medium is shown in Fig. 2. No remarkable increase in chitinase production was observed even after increasing the concentration of N-acetylglucosamine from 0.1 to 0.3%. A further increase of the concentration of N-acetylglucosamine to above 0.4% resulted in a marked decrease of enzyme production.

3. Comparison of the Effects of SCSP with Other Related Preparations

A series of experiments was carried out to study the effects of the seven preparations on enzyme production. The enzyme production with strain K-187 is shown in Fig. 3. It is seen that, except for NaOH-extract, the preparations were all more effective than SCSP in chitinase production as carbon sources for the strain used. Maximum chitinase activity was ob-

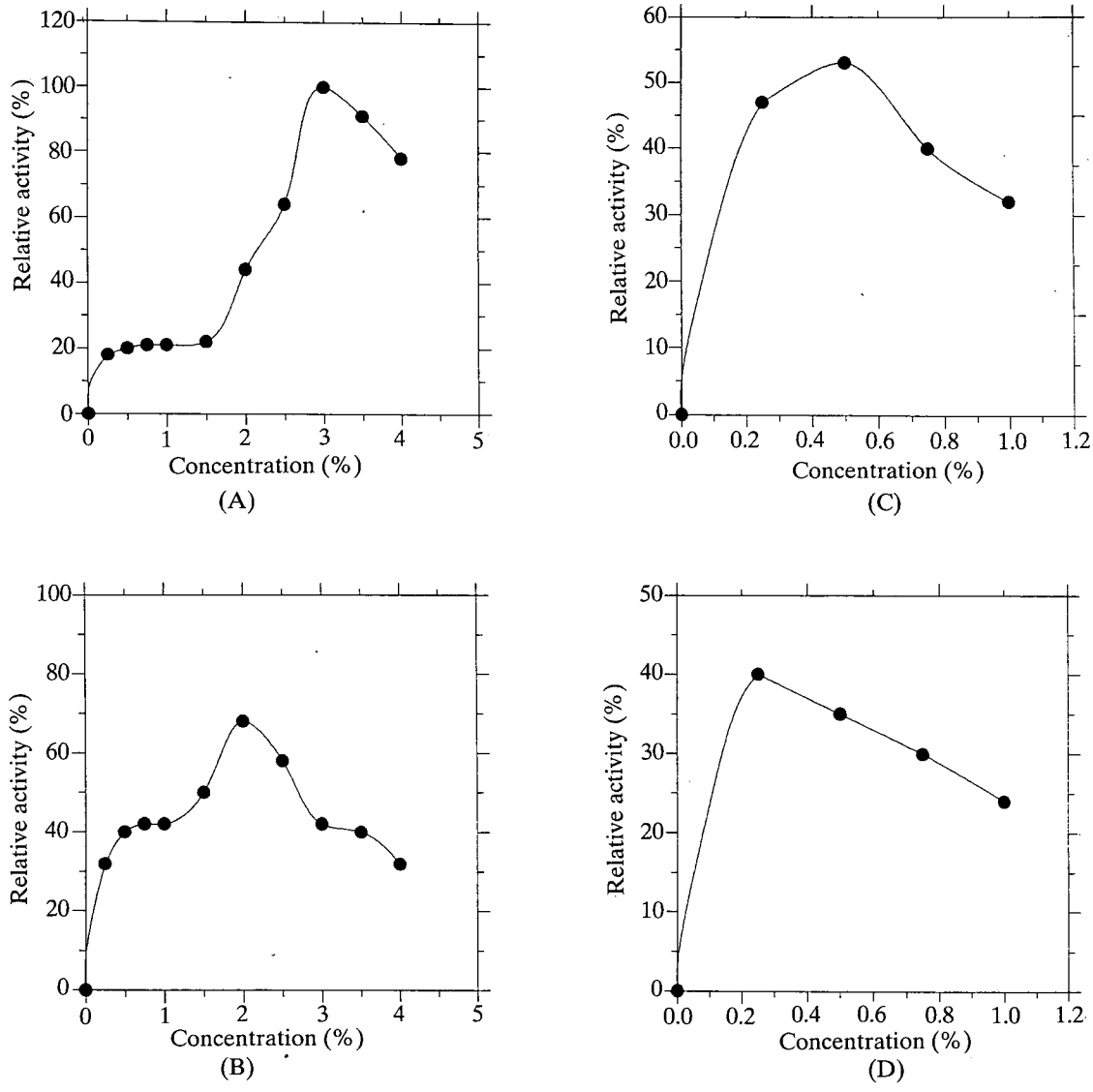

Fig. 1. Comparison of the inducing effect of SCSP with other chitin related products. A, SCSP; B, flake chitin; C, powdered chitin; D, powdered chitosan.

Table 1. Effect of Various Nitrogen Sources on Chitinase Production

Nitrogen source	Concentration (%)	Relative activity (%)		
		3% SCSP	2% Flake chitin	0.5% Powdered chitin
None	0	100	67	53
Polypepton	0.1	79	59	45
Yeast extract	0.1	75	54	25
Fish extract	0.1	88	48	30
SCS extract	0.1	85	48	32
Fish powder	0.1	84	45	30
SCSP	0.1	100	68	55
Sodium L-glutamate	0.1	82	40	49
Sodium nitrate	0.1	64	45	40
Ammonium sulfate	0.1	72	39	24
N-acetylglucosamine	0.1	60	90	50

100% = 0.68 U/ml

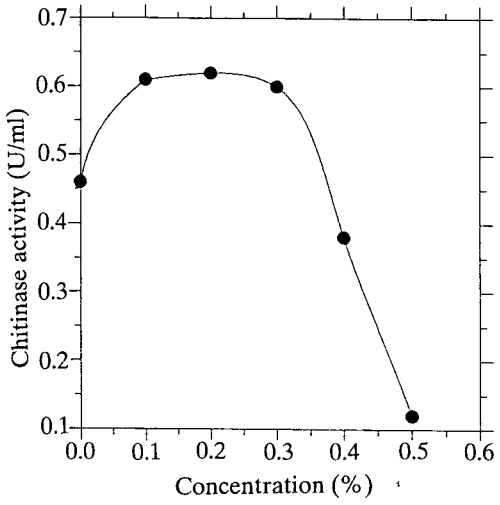

Fig. 2. Chitinase production in the presence of various concentrations of N-acetylglucosamine in 2% flake chitin containing medium.

tained when the strain was grown in the medium containing HCl/NaOH-SCSP, followed by HCl-SCSP, HCl/NaOH-extract, NaOH-SCSP, HCl-extract, untreated SCSP, and NaOH-extract.

In order to investigate the optimum concentrations of the seven preparations for chitinase production, strain K-187 was grown aerobically in 175 ml of the medium containing various concentrations of the seven preparations in a 250 ml Erlenmeyer flask at 37°C for 2 days. The results are shown in Fig. 4. The optimum concentrations for chitinase production of the seven preparations were in the order of 3% HCl/NaOH-SCSP, 4% NaOH-SCSP, 2% HCl-extract, 3% HCl-SCSP, 2% HCl/NaOH-extract, 3% untreated SCSP, and 1% NaOH-extract.

In order to obtain more complete data for enzyme production, it was necessary to measure the time-course of cultivation in medium containing the

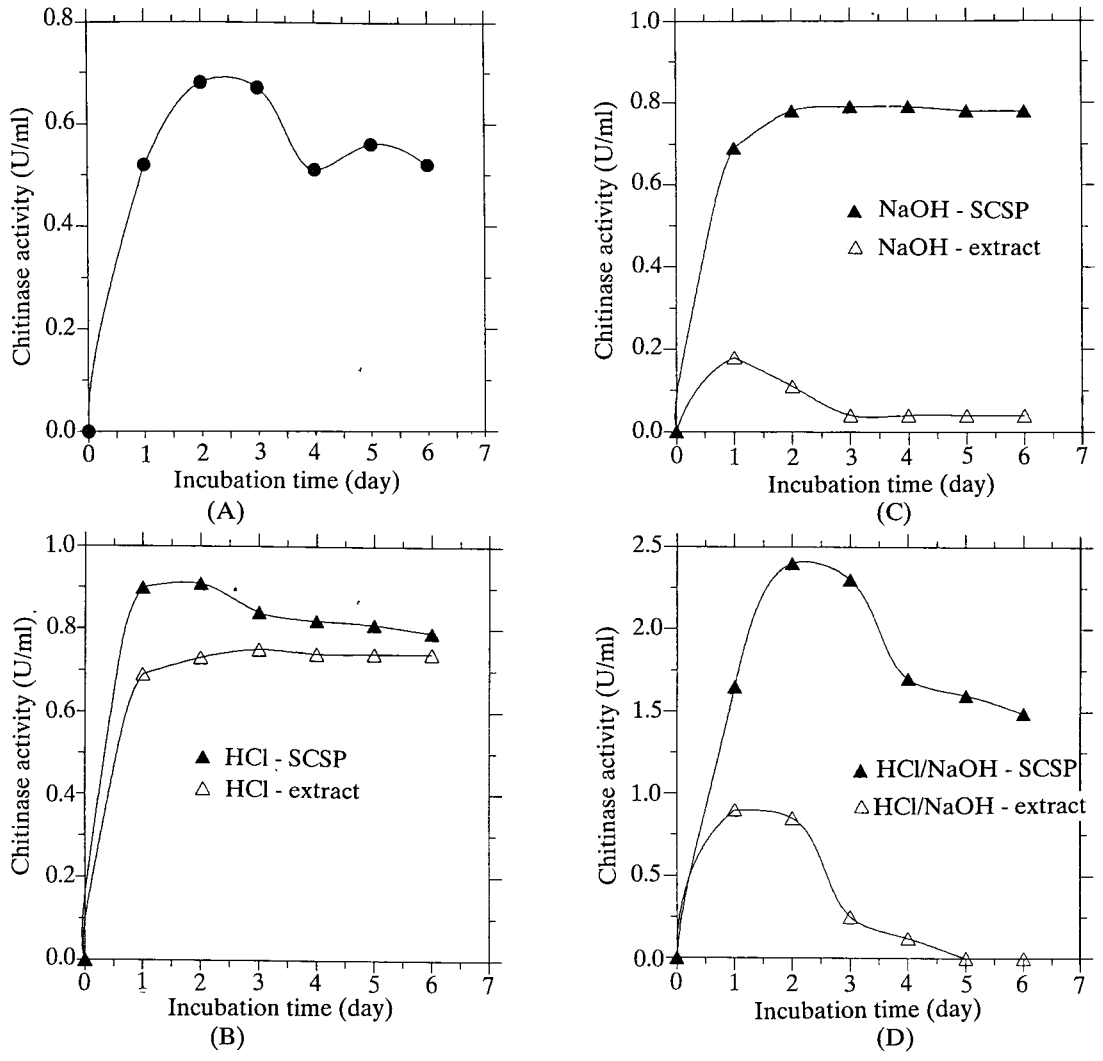

Fig. 3. Production of chitinase by *P. aeruginosa* K-187 grown on 3% of the seven preparations. A, untreated SCSP; B, HCl-treated (▲, HCl-SCSP; △, HCl-extract); C, NaOH-treated (▲, NaOH-SCSP; △, NaOH-extract); D, HCl/NaOH-treated (▲, HCl/NaOH-SCSP; △, HCl/NaOH-extract).

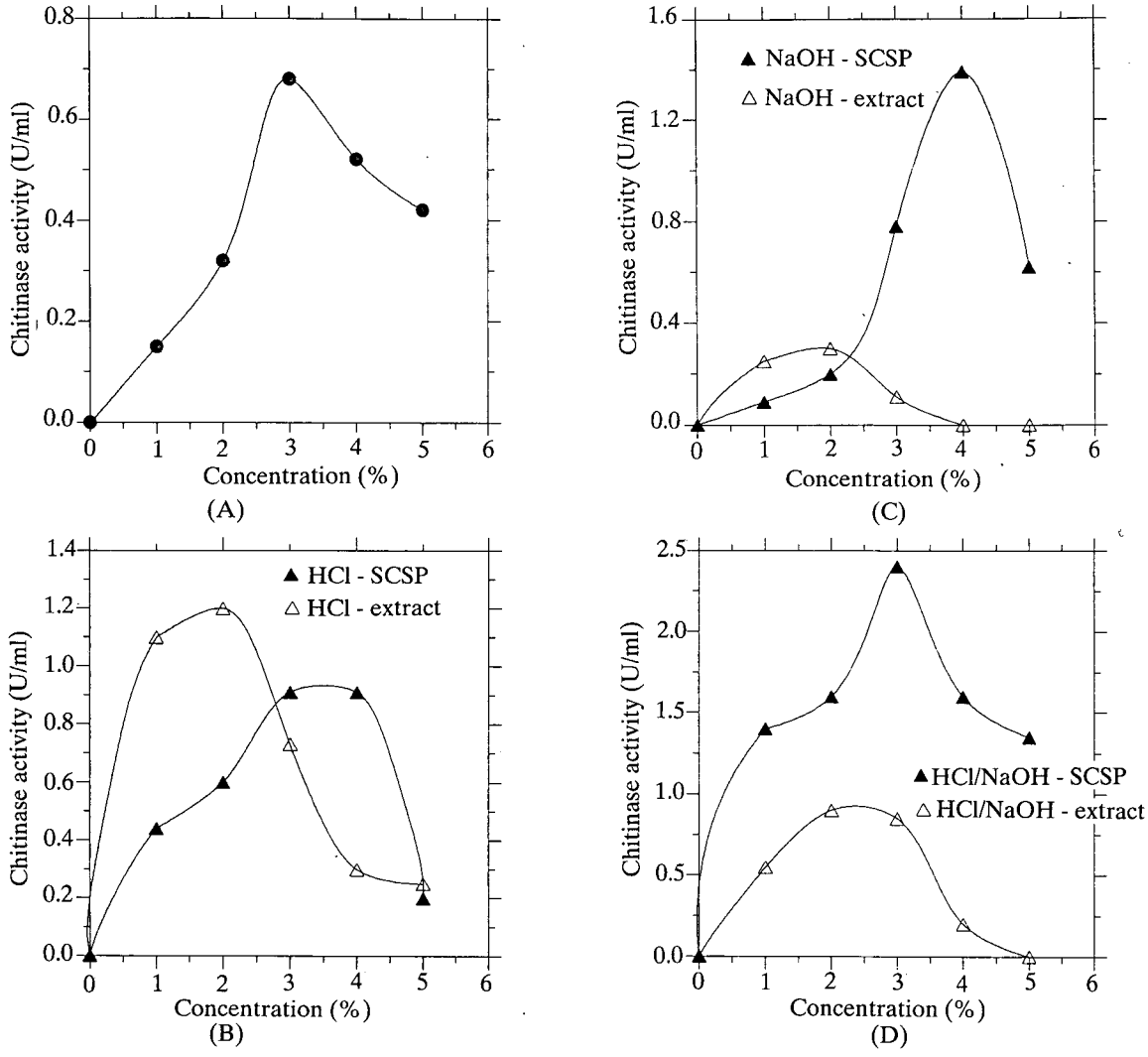

Fig. 4. Chitinase production in the presence of various concentrations of the seven preparations after 2 days of cultivation. The symbols are the same as those in Fig. 3.

Table 2. Summary of the Optimal Culture Conditions for Chitinase Production by *P. aeruginosa* K-187 Grown on the Seven Preparations

Type of SCSP	Concentration (%)	Maximum chitinase yield (U/ml)	Day of maximum yield
Untreated SCSP	3	0.68	2-3
HCl-SCSP	4	0.91	1-2
HCl-extract	2	1.20	2-3
NaOH-SCSP	4	1.39	2-3
NaOH-extract	1	0.25	1
HCl/NaOH-SCSP	3	2.40	2
HCl/NaOH-extract	2	0.90	1

optimum concentrations of the seven preparations. K-187 was grown aerobically in 175 ml of optimum medium in a 250-ml Erlenmeyer flask at 37°C. During the period of incubation, the chitinase activity was monitored in 24 hr intervals for up to 6 days. As shown in Table 2, HCl/NaOH-SCSP was the most suitable carbon source for chitinase production by strain K-187. Maximal chitinase activity increased

from 0.68 to 2.40 U/ml when HCl/NaOH-SCSP was substituted for untreated SCSP as a carbon source for chitinase production.

IV. Discussion

The bacteria species used in this study was a *P. aeruginosa*, strain K-187, which was isolated from soil of northern Taiwan in our previous study (Wang *et al.*, 1995). Takiguchi *et al.* (1985) reported chitin production using a similar *P. aeruginosa*, strain E-139. Takiguchi's culture medium included 1.54% decalcified prawn cuticles and a 1.0% flake chitin. After 8 to 10 days and 4 days of shake-flask culture using the above two media, respectively, chitinase activity was detected in the broth of both culture schemes. The maximum chitinase productivity for both was 0.01 U/ml without lysozyme activity. On the other hand, strain K-187, in this study, using a culture medium containing only SCSP (without colloidal or powdered chitin), showed a maximum chitinase activity of 0.68 U/ml in the culture supernatant.

Strain K-187 also has potential for producing chitinase using the acid and/or alkali liquid waste from SCSP treatment (Table 2). The results showed that strain K-187 can be applied to acid and/or alkali liquid waste in the chitin production process. It was found that, when a 2% flake chitin was supplemented with 0.2% N-acetylglucosamine, the chitinase productivity was raised from 0.46 U/ml to 0.63 U/ml (Fig. 2). This result is similar to that published by Takiguchi and Shimahara (1985), but it differs from that published by Ohtakara (1979), who indicated that glucosamine inhibited the production of chitinase. When SCSP was used as the main carbon source, the addition of N-acetylglucosamine inhibited chitinase production. This finding is similar to Ohtakara's. SCS extract, fish extract, and fish powder were not effective in chitinase production. As far as we are aware, no chitinase productivity as high as 2.4 U/ml for strain K-187 (Table 2) has been published.

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以 *Pseudomonas aeruginosa* K-187 發酵蝦蟹加工廢棄物生產幾丁質酶

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摘 要

利用蝦蟹殼粉經由酸及/或鹼前處理所得之各種不同製品作爲幾丁質酶生產菌 *Pseudomonas aeruginosa* K-187 生產幾丁質酶之碳源。蝦蟹殼粉經由適當前處理則比未處理更適於用來作爲生產幾丁質酶之碳源。當以酸/鹼處理過之蝦蟹殼粉代替未處理過之蝦蟹殼粉作爲碳源結果發現，幾丁質酶之最大生產量由 0.68 U/ml 提升爲 2.4 U/ml。此結果顯示 *P. aeruginosa* K-187 於發酵蝦蟹殼粉及其酸鹼處理廢液生產幾丁質酶之同時，亦能用來回收再利用幾丁質生產業所衍生之此類酸鹼廢液。