Isolation and Characterization of Chitinase from Marine Bacteria

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ABSTRACT

A marine bacterium, which can produce an extracellular chitinase, was isolated and identified. The counts of the chitinolytic bacteria in six marine animals ranged from $3.3x10^4$ to $2.3x10^6$ CFU/g, which accounted for 22.03% of the total bacterial count. Ninety-nine isolates were found to produce chitinase. The isolate SH1.13 produced the highest activity of chitinase and was identified as *Bacillus circulans*. The initial pH and temperature for growth and chitinase production were 7.0 and 37°C, respectively, for 48 hrs. The optimum pH and temperature of the enzyme reaction were 4.0 and 37°C, respectively. The chitinase was stable at pH 4.0 and from 20 to 40°C. After purification by 90% ammonium sulfate precipitation followed by Sephacryl S-200 HR chromatography, only one protein peak was obtained. The final specific activity of chitinase was 0.48 unit/µg protein with a purification of 1.28 folds. The subunit molecular weight of the enzyme was estimated by SDS-PAGE to be 107000, 89000, 62000, 42000, 24000 and 17000 daltons, respectively.

Key words: marine bacterium, chitin, chitinase, *Bacillus circulans*

INTRODUCTION

Chitin, a β - (1,4) polymer of N-acetylglucosamine, is one of the most abundant organic compounds in nature. This polysaccharide is an important nutrient and carbon source for marine ecosystems (Yu *et al*., 1991). Degradation of chitin in the environment is an enzymatic process. Two enzymes, chitinase and *N*-acetylglucosaminidase, are important for complete degradation of chitin. Chitinase (EC3.2.1.14) hydrolyses the glycosidic linkages of chitin (Takayanagi *et al*.,1991). Chitinase is commonly found in a wide range of organisms, including bacteria, fungi, higher plants, insects, crustraceans, and some vertebrates (Jeuniaux, 1966; Flach *et al*., 1992). Chitinolytic bacteria, such as *Achromobacter, Aeromonas, Alginomonas, Beneckea, Clostridium, Pseudomonas* and *Vibrio* have been isolated from seawater, marine mud and digestive tracts of fish (Tsujibo *et al*., 1991).

The production of inexpensive

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chitinolytic enzymes is an essential element in the utilization of shrimp processing waste. The alkali-tolerant chitinase producing strain is also useful in freeing the processing wastes of odors generally considered as a pollution problem. Marine waste utilization not only solves environmental problems, but also promotes the economic value of the marine products.

In this study, the isolation and selection of bacteria from marine animals producing chitinase, and chitinase purification and characterization are described .

MATERIALS AND METHODS

Preparation of colloidal chitin

Colloidal chitin was prepared from powdered chitin according to the method developed by Jeniaux (1966). Twenty grams of chitin powder (sieved through a 42-mesh sieve) was added slowly into 800 ml of concentrated hydrochloric acid below 5°C with vigorous stirring. The mixture was filtered through a glass wool and the filtrate was poured into 8 L of deionized water below 5°C while stirring. After 30 min, the stirring was stopped, and then the suspension was kept overnight below 5°C. The supernatant was then decanted out and the remaining mixture was precipitated by centrifugation at 9,000 rpm for 15 min at 4°C. The residue was washed with water until the washing become neutral. The acid -free residue was added into 500 ml of deionized water and resuspended with vigorous stirring to prepare the colloidal chitin solution. This colloidal chitin solution remains stable when stored for a few weeks in a dark place below 5°C.

Isolation of chitinase producing bacteria

Bacteria from six marine animals, namely green mussel (*Perna viridis*; PE), file fish (*Monocanthus chiensis*; FH), octopus (*Octopus* sp.; SQ), swimming blue crab (*Portunus pelagicus*; CR), black tiger shrimp (*Penaeus monodon*; SH), and banana shrimp (*Penaeus merguiensis*; PR) were isolated using the spread plate method. These samples were serially diluted and spread on chitin agar (CHA) plates containing 0.5 % (v/v) colloidal chitin, and 1.5 % agar by sea water. The pH was adjusted to 6.8-7.2 with 0.1 M NaOH and 0.1 N HCl before sterilization at 121°C for 15 min and then incubated at 37°C for 3-5 days. The colonies surrounded by clear zones were isolated and retained for subsequent screening.

First screening

Chitinase-producing microorganisms were screened using chitin agar (0.5 % (v/v) colloidal chitin). The purified isolates were point inoculated on chitin agar plates and incubated at 37°C for 5 days. Chitinase-producing microorganisms formed large clear zones (Figure 1) and were retained for subsequent screening.

Figure 1. Clear zone of chitinolytic bacteria on chitin agar

Second screening

The organisms obtained from the first screening were cultured in sterile chitin broth (CHB) containing 1 % (v/v) colloidal chitin in sea water (pH 6.8-7.2) and shaken at 200 rpm for 5 days at 37°C. After incubation, culture broth was centrifuged at 9,000 rpm for 30 min at 4°C, and supernatants were collected for determination of enzyme activity.

Identification of strain SH1.13

The classification of each bacterial isolate was accomplished according to the diagnostic key described in Bergey's Manual of Systematic Bacteriology (Sneath, 1984). Physiological characteristics were identified using the API test kit (Biomérieux 50 CHB).

Preparation of the enzyme

Bacterial cells, grown on a slant culture, were inoculated into a 500 ml Erlenmeyer flask containing 250 ml of sterile CHB and shaken at 200 rpm for 5 days at 37°C. The culture broth was centrifuged at 9,000 rpm for 30 min at 4°C to remove the cells. The supernatants were then taken to determine enzyme activity.

Assay of chitinase activity

Chitinase activity was determined by measuring the amount of reducing end group, *N*-acetylglucosamine (GlcNAc), degraded from colloidal chitin. A reaction mixture containing 2.0 ml of 0.2 M McIlvaine buffer (pH 4.0) with 0.5 % v/v colloidal chitin, and 1.0 ml of enzyme solution was incubated at 37°C for 60 min (Ueda and Arai, 1992). The amount of reducing sugar produced was measured by Schales method (Imoto and Yagishita, 1971). One unit of activity was defined as the amount of 1 μmol of the GlcNAc liberated per minute under the conditions stated above.

Effect of initial pH

Bacterial cells grown on a slant culture were inoculated into a 500 ml Erlenmeyer flask containing 250 ml of sterile CHB. The media were adjusted with 0.1 M NaOH and 0.1 N HCl to pH 2.0, 4.0, 6.0, 7.0, 8.0, 10.0 and 12.0. The culture media were incubated at 37°C for 48 h, growth was measured by total plate count, and the supernatant was collected to determine chitinase activity.

Effect of temperature

Bacterial cells grown on a slant culture were inoculated in a 500 ml Erlenmeyer flask containing 250 ml of sterile CHB (pH 7.0). The culture medium was incubated for 48 h at 20, 30, 37, 40, 50, 60 and 70°C. Growth was measured by total plate count and the supernatant was collected to determine chitinase activity.

Time course of chitinase production

Bacterial cells grown on a slant culture were inoculated in a 500 ml Erlenmeyer flask containing 250 ml of sterile CHB (pH 7.0). The culture medium was incubated at 37°C, growth was measured by total plate count, and the supernatant was collected to determine chitinase activity.

Optimum pH and temperature of chitinase

Optimum pH of the enzyme activities was determined by mixing the culture broth with substrate at pH 2.0, 4.0, 6.0, 7.0, 8.0 and 10.0 (buffers used were 0.1 M McIlvaine buffer at pH 2.0-7.0, 50 mM Tris-HCl buffer at pH 8.0, and 50 mM glycine-NaOH buffer at pH 9.0-10.0), and incubated at 37°C for 60 min.

To find the optimum temperature, enzyme activity was determined by mixing the culture broth with substrate in 0.1 M McIlvaine buffer pH 4.0 and incubated at 20, 30, 37, 40, 50, 60 and 70 C for 60 min.

pH and temperature stability of chitinase

In the pH stability test, the broth containing the enzymes were pre-incubated at 37°C and various pH levels (buffer used were 0.1 M McIlvaine buffer at pH 2.0-7.0, 50 mM Tris-HCl buffer at pH 8.0, and 50 mM glycine-NaOH buffer at pH 9.0-10.0) for 10 min. Enzyme activity was then determined after the addition of substrate solution buffer at pH 4.0 with 0.2 M McIlvaine buffer.

In the temperature stability test, the culture broth was incubated at various temperatures at pH 4.0 with 0.2 M McIlvaine buffer for 10 min. Enzyme activity was determined similarly with that of the pH stability test.

Purification of chitinase

Ammonium sulfate precipitation

The culture broth was fractionated with ammonium sulfate at 90 % saturation. The protein precipitate was obtained by centrifugation at 9000 g for 30 min. The precipitate was then dissolved in small volumes of 20 mM Tris-HCl buffer (pH 7.0). The solution was dialyzed overnight at 4°C against 4 changes of 4 L each of 20 mM Tris-HCl buffer (pH 7.0). The dialysate was collected and concentrated by lyophilization.

Gel filtration chromatography

Sephacryl S-200 HR was prepared according to the method of Pharmacia Company. Sephacryl S-200 HR was swollen in 20 mM Tris - HCl buffer pH 8.0. The swollen gel was degassed before packing in a column (1.6x75 cm). The column was equilibrated with 20 mM Tris - HCl buffer pH 8.0 at a constant flow rate of 0.5 ml/min. The 1.0 ml of concentrated enzyme that was obtained from lyophilization was applied to the column and eluted with the same buffer. Fractions of 3.0 ml each were collected at a flow rate of 0.5 ml/min. Protein profile was monitored by measuring the absorbance at 280 nm.

Determination of molecular weight

SDS-PAGE was performed according to the method described by Gallagher and Smith (1991) with Bio-Rad Mini-gel Protein II apparatus. Twelve percent acrylamide separating gel and 4.0 % acrylamide stacking gel containing 0.1 % SDS were employed. The gel was stained with Coomassie brilliant blue R-250.

RESULTS

Isolation and selection of chitinolytic bacteria

The aerobic plate counts of six marine animals varied from $1.5x10^5$ to $7.6x10^6$ CFU/g, while the counts of chitinolytic bacteria varied from $3.3x10^4$ to $2.3x10^6$ CFU/g, which accounted for 12.33-27.47 % of the total aerobic bacteria in samples (Table 1). Chen and Chen (1991) reported that the count of chitinolytic bacteria in the soil and whole shrimp were 10^4 to 10^5 CFU/g, which accounted for 2.7% of total bacterial count. Ninety-nine isolations were made. In the primary screening, 27 of 99 isolates produced large clear zones on chitin agar. One isolate screened from black tiger shrimp, strain SH 1.13, showed the highest enzyme activity in culture broth. The strain was kept in tryptic soy agar and used throughout the study.

Table 1. Enumeration of total aerobic bacteria and chitinolytic bacteria from marine animals

Sample	APC[a] (CFU/g)	CCB[b] (CFU/g)	APC/CCB	Average of APC/CCB	Percent ratio
FH[c]	5.0×10^5	0.45×10^5	11.11 : 1		
	1.5×10^5	0.33×10^5	4.55 : 1	7.83 : 1	12.33
PE[c]	9.6×10^5	1.40×10^5	6.86 : 1		
	4.3×10^5	0.90×10^5	4.78 : 1	5.82 : 1	17.18
SH[c]	7.6×10^6	1.8×10^6	4.22 : 1		
	1.6×10^6	3.7×10^5	4.32 : 1	4.27 : 1	23.42
PR[c]	1.0×10^6	3.6×10^5	2.78 : 1		
	8.8×10^5	1.7×10^5	5.18 : 1	3.98 : 1	25.13
CR[c]	8.0×10^5	2.6×10^5	3.08 : 1		
	2.3×10^5	0.52×10^5	4.42 : 1	3.75 : 1	26.67
SQ[c]	5.4×10^6	1.3×10^6	4.15 : 1		
	7.2×10^6	2.3×10^6	3.13 : 1	3.64 : 1	27.47

[a] Aerobic plate count
[b] Count of chitinolytic bacteria
[c] FH - file fish (*Monocanthus chiensis*), PE - green mussel (*Perna viridis*), SH - black tiger shrimp (*Penaeus monodon*), PR - banana shrimp (*Penaeus merguiensis*), CR - swimming blue crab (*Portunus pelagicus*), SQ - octopus (*Octopus* sp.)

Identification of strain SH1.13

The general characteristics of strain SH1.13 are shown in Table 2. The strain SH1.13 was identified as *Bacillus circulans* according to its characteristics listed in Table 2.

Table 2. General characteristics of strain SH1.13 (*Bacillus circulans*)

Morphological characteristics	
Form	Rods
Gram staining	Positive
Physiological characteristics	
Catalase	+
Glycerol	+
Erythritol	-
D-arabinose	-
L-arabinose	+
Ribose	+
D-xylose	+
L-xylose	-
Adonitol	-
β-methyl-D-xyloside	+
Galactose	+
D-glucose	+
D-fructose	+
D-mannose	+
Physiological characteristics	
L-sorbose	-
Rhamnose	-
Dulcitol	-
Inositol	-
Mannitol	-
Sorbitol	-
α-methyl-D-mannoside	+
α-methyl-D-glucoside	+
N-acetyl-glucosamine	+
Amygdaline	+
Arbutine	+
Esculine	+
Salicine	+
Cellobiose	+
Maltose	+
Lactose	+
Melibiose	+
Sucrose	+
Trehalose	+
Inuline	-
Melizitose	-
D-raffinose	-
Starch	+
Glycogene	+
Xylitol	-
β-gentiobiose	+
D-turanose	+
D-lyxose	+
D-tagatose	-
D-fucose	-
L-fucose	-
D-arabitol	-
L-arabitol	-
Gluconate	+
2-keto-gluconate	-
5-keto-gluconate	+

Note: + : Positive reaction - : Negative reaction

Effect of optimum pH and temperature on chitinase production

Flask cultivation was carried out in the medium at pH 4.0, 6.0, 7.0, 8.0, 10.0 and 12.0. The results are shown in Figure 2. *B. circulans* SH1.13 produced high enzyme activity when the initial pH ranged from 4.0 to 7.0, with the highest enzyme activity at pH 7.0. Optimum temperature of chitinase production was 37°C (Figure 3).

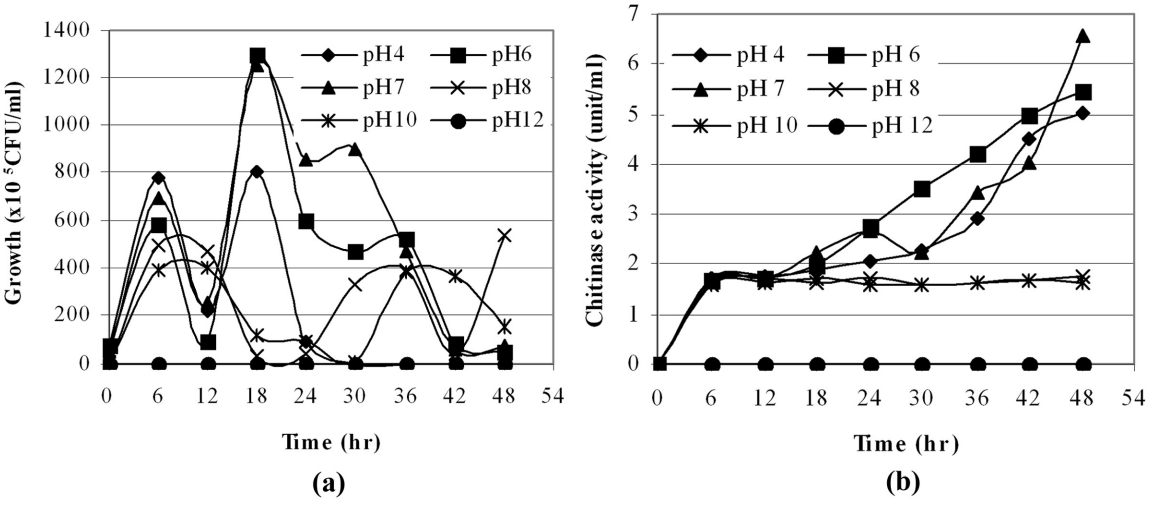

Figure 2. Effects of initial pH on growth and chitinase activity of *B. circulans* SH1.13; (a) growth; (b) chitinase activity

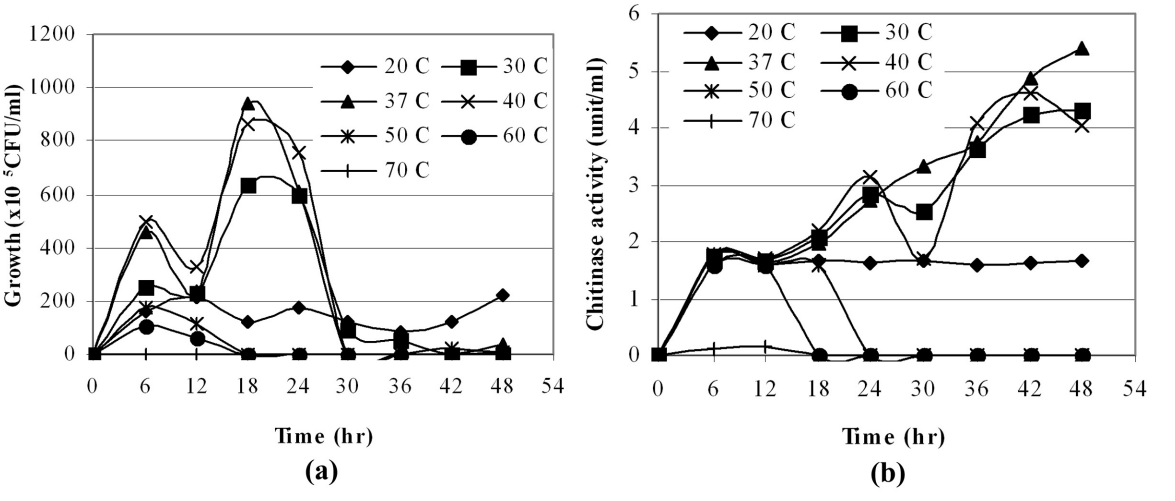

Figure 3. Effects of temperature on growth and chitinase activity of *B. circulans* SH1.13; (a) growth; (b) chitinase activity

Time-course of cultivation

B. circulans SH1.13 was grown at an initial pH of 7.0 and temperature of 37°C. During the process of incubation, chitinase, cell growth and pH in the broth were measured every six hours. A typical time course of chitinase production is shown in Figure 4. Chitinase activity increased in the death phase period, and the highest activity was 13 units/ml.

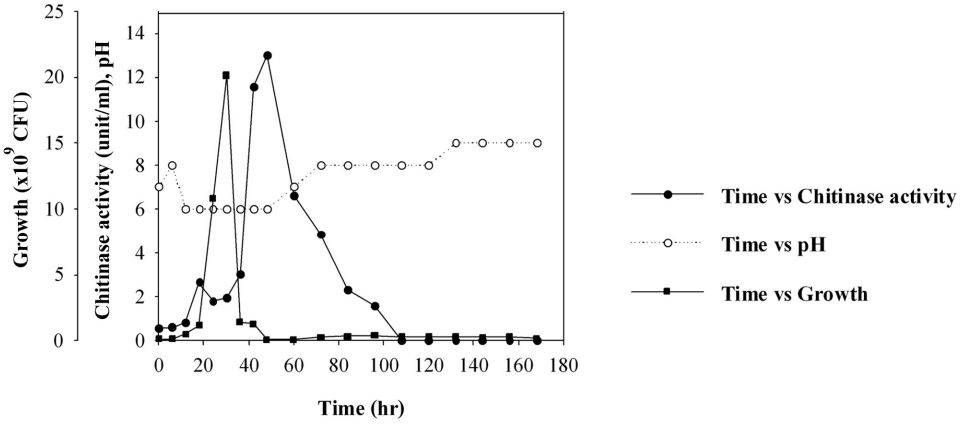

Figure 4. Time-course on growth and chitinase activity of *B. circulans* SH1.13

Stability of and optimum pH and temperature of chitinase

The optimum pH and temperature levels for chitinase reaction were 4.0 and 37°C, respectively (Figure 5). The pH and temperature stability of the crude enzyme were 4.0 and between 20 and 40°C, respectively (Figure 6).

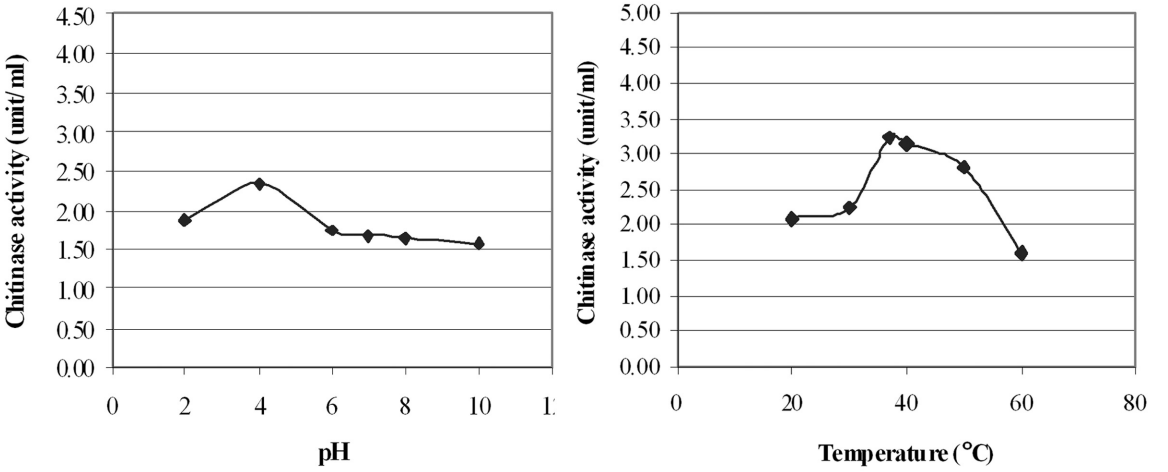

Figure 5. The optimum pH and temperature of chitinase from *B. circulans* SH1.13

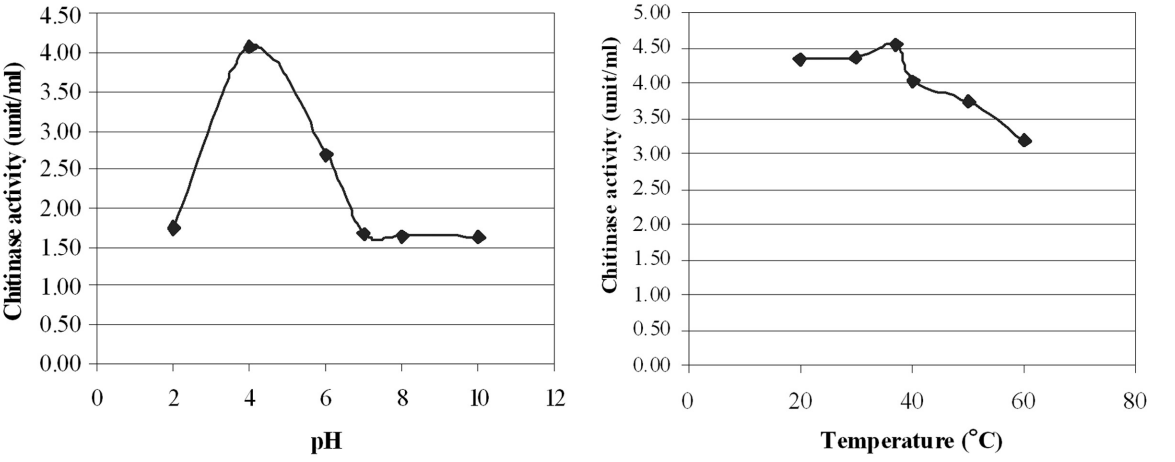

Figure 6. The stability of pH and temperature of chitinase from *B. circulans* SH1.13

Purification of chitinase

B. circulans SH1.13 released chitinase into the culture broth. The released crude enzyme was purified by precipitation in 90% ammonium sulfate and then dialyzed. The dialyzed enzyme had a specific activity of 0.41 unit/µg of protein and 1.1 folds of purity. The dialyzed solution was subjected to Sephacryl S-200HR column. Fractions 15-19 showed high specific enzyme activities (Figure 7). The highest specific enzyme activity was 0.48 unit/µg of protein with purity of 1.28 folds (Table 3).

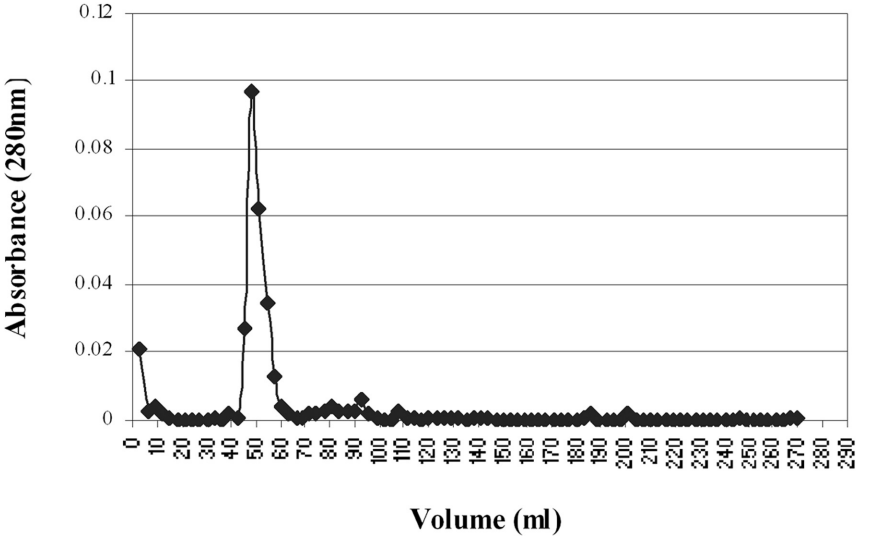

Figure 7. Elution profile of chitinase from Sephacryl S-200HR

Table 3. Purification of chitinase from *B. circulans* SH1.13

Step	Volume (ml)	Total protein (μg)	Total activity (unit)	Specific activity (unit/μg)	Yield (%)	Purification (fold)
Culture supernatant	500	3,310.14	1,238.43	0.37	100.00	1.00
Dialysis	25	188.20	77.27	0.41	6.24	1.10
Sephacryl S-200HR	15	63.45	30.40	0.48	2.45	1.28

Molecular weight

The molecular weight of each enzyme was calculated on the basis of semi-logarithmic plots of the mobilities of the bands on SDS-PAGE, using a standard curve established with proteins of known molecular weights. The molecular weights of protein bands a, b, c, d, e and f were estimated to be 107000, 89000, 62000, 42000, 24000 and 17000 daltons, respectively (Figure 8).

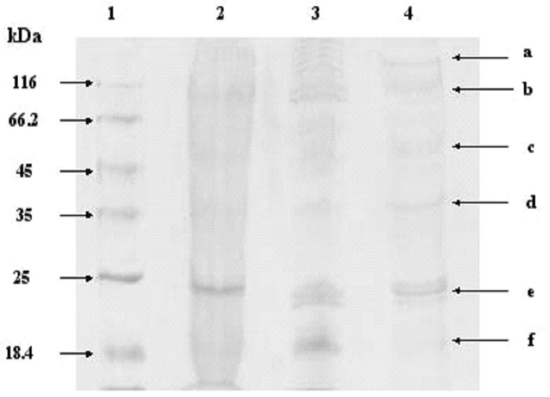

Figure 8. SDS-PAGE of chitinase from *B.circulans* SH1.13; (1) protein marker; (2) crude enzyme; (3) enzyme solution from ammonium sulfate precipitation and dialysis; (4) enzyme fraction from Sephacryl S-200HR chromatography

DISCUSSION

Marine bacteria isolated from octopus were mostly chitinase-producing bacteria, in contrast with bacteria isolated from other animals (shrimp, swimming blue crab, black tiger shrimp, green mussel and file fish). It has been shown that octopus, shrimp and crab have chitin in their structures, so chitinase-producing bacteria isolated from these groups were similar to chitinolytic bacteria, *Aeromonas caviae* D1, isolated from shrimp (*Penaeus monodon*) shell (Chen and Chen, 1991). Moreover, chitinase -producing bacteria of the genus *Bacillus* have also been reported e.g. *Bacillus circulans* No.4.1 (Wiwat *et al*., 1999), *B. alvei* E1, *B. sphaericus* J1, *B. cereus* J1-1 (Wang and Hwang, 2001), and *Bacillus* spp. K9, K14, and K20 (Frändberg and Schnürer, 1994).

Chitinolytic activity was found in the isolated bacteria from black tiger shrimp (*P. monodon*), and later identified as *B. circulans* SH1.13. The optimum initial pH for growth of this bacterium ranged from pH 6.0 to 7.0, however, the highest enzyme activity was at pH 7.0. Thus, the initial optimum pH for growth and chitinase production could be at pH 7.0 which was the same result of *B. circulans* No.4.1 (Wiwat *et al*., 1999),

B. cereus J1-1 (Wang and Hwang, 2001), *B. licheniformis* X-7u (Takiguchi and Shimahara, 1989), *Bacilllus* sp. 13.26 (Yuli *et al*., 2004) and *Aeromonas* sp. No.10S-24 (Ueda and Arai, 1992). The optimal temperature for growth and chitinase production of *B. circulans* SH1.13 was 37°C similar to *B. circulans* No.4.1 (Wiwat *et al*., 1999), *B. sphaericus* J1 and *B. cereus* J1-1 (Wang and Hwang, 2001), and *P. aeruginosa* K-187 (Wang and Chang, 1997). Moreover, the optimal time for culture of *B. circulans* SH1.13 was 48 hrs or after its death phase which was not different from the study in *B. cereus* J1-1 at pH 7.0 and 37°C, *B. alvei* E1, and *B. sphaericus* J1 at pH 9.0 and 45°C (Wang and Hwang, 2001), and *P. aeruginosa* K-187 at pH 9.0 and 45°C (Wang and Chang, 1997).

The optimum pH for chitinolytic reaction of the enzyme was at pH 4.0 or in acidic conditions. This is similar to previous studies which showed that the optimum pH for chitinolytic reaction was in the range of pH 3.5-8.0, such as the strain of bacteria and other related factors for the reaction (Cabib, 1988; Ohtakara, 1988; Watanabe *et al*., 1990; Sherief *et al*., 1991; Ueda and Arai, 1992; Sakai *et al*., 1998 and Koga *et al*., 1999). The chitinase was stable at pH 4.0 similar to *Aeromonas* sp. No.10S-24 at pH 4.0-9.0 (Ueda and Arai, 1992), *Serratia marcescens* at pH 4.8-7.2 (Monreal and Reese, 1969) and *Saccharomyces cerevisiae* at pH 3.0 (Correa *et al*., 1982). The optimum temperature for chitinolytic reaction of the enzyme was 37°C. The general optimal temperature for chitinase reaction ranges from 40-50°C such as with *B. circulans* No. 4.1 (Wiwat *et al*., 1999), *Vibrio* sp. (Ohtakara *et al*, 1979), *P. aeruginosa* K-187 (Wang *et al*., 1995), *A. hydrophila* H-2330 (Hiraga *et al*., 1997), *Aeromonas* sp. No.10S-24 (Ueda and Arai, 1992), *B. alvei* E1 and *B. cereus* J1-1 (Wang and Hwang, 2001). Moreover, some chitinase producing bacteria have their reactions at high temperatures (thermophilic bacteria) such as *B. licheniformis* X-7u which has its optimal reaction at 70-80°C (Takayanagi *et al*., 1991).

The chitinase enzyme produced from *B. circulans* SH1.13 was stable at 20-37°C which was in the same range of chitinase FI and FII from *P. aeruginosa* K-187 at 20-50°C (Wang and Chang, 1997) and *Vibrio* sp. at lower than 40°C (Zhou *et al*., 1999).

The chitinase produced from *B. circulans* SH1.13 consisted of 6 subunits with their molecular weights of 107000, 89000, 62000, 42000, 24000 and 17000 daltons. The result from this study was similar to the previous report of Wang and Chang (1997) which reported that molecular weights of chitinase from microorganisms ranged between 20000 and 120000 daltons, whereas bacterial chitinase ranged between 60000 and 110000 daltons.

CONCLUSION

The results from a selection of chitinase producing marine bacteria from 6 marine animals showed that chitinolytic bacterial counts ranged from 2.6×10^4 to 2.3×10^6 CFU/g with chitinolytic activity found to be highest in bacteria isolated from *Octopus* sp. Moreover, marine animals that contain chitin in their structures had more chitinolytic activity than those without chitin.

Ninety-nine isolates were found to produce chitinase. The isolate SH1.13 from *Penaeus monodon* showed the highest activity and was identified as *B. circulans*.

This bacterium is Gram positive, rod shaped, and can produce extracellular enzyme.

The optimum conditions for culture and chitinase production of *B. circulans* SH1.13 were found to be pH 7.0 at 37°C for 48 hours. Chitinase produced from *B. circulans* SH1.13 had the highest activity at pH 4.0 and 37°C, and was stable at pH 4.0 and 20-37°C.

Purification of crude enzyme through 90% ammonium sulfate precipitation and dialysis yielded enzyme specific activity of 0.41 unit/μg of protein and 1.1 fold of purity. Further purification with Sephacryl S-200HR column resulted in enzyme specific activity of 0.48 unit/μg of protein and 1.28 folds of purity.

The molecular weights of the enzyme subunits were estimated by SDS-PAGE to be 107000, 89000, 62000, 42000, 24000 and 17000 daltons, respectively.

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