Isolation of ι-carrageenase producing marine bacterium and culture conditions for the production of ι-carrageenase

해양으로부터 carrageenase 생산균의 분리와 carrageenase 생산을 위한 최적 배양조건

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Isolation of marine bacterium producing ι-carrageenase and determined optimal conditions for the production of ι-carrageenase

A Dissertation

by

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심 행 선

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요 약

부산 송정 연안에서 채취한 우뭇가사리를 해수와 섞어 분쇄한 다음 십진법으로 희석하여 변형한 ZoBell 2216E 평판 배지에 평판도말법으로 접종하여 25℃에서 5일간 배양 한후 나타난 colony 중에서 고체 평판 배지를 함몰시키는 특성을 갖는 colony 13종을 선별하였다. 이들을 0.5% ι-carrageenan이 든 ZoBell 2216E 배지에 접종한 후 72시간 배양하여 합성기질을 이용하여 흡광도를 측정한 다음 가장 높은 활성을 나타난 한 균주을 최종적으로 선별하였다. 선별된 균주는 생화학 test결과 운동성이 있는 그람 음성의 간균이며 oxidase와 catalase 모두 양성을 나타내었고, 16s rDNA 분석 결과 Pseudomonas

straminea와 94%의 유사성으로 Pseudomonas 속의 신종으로 밝혀졌다. 배양 조건은 탄소원이 ι-carrageenan 뿐만 아니라 λ-carrageenan일 경우에도 미미한 활성이 나타났고, ι-carrageenan 농도가 1%, 배지의 pH가 8.5, 배양시간이 72 시간, 배양 온도가 20℃에서 carrageenase 활성과 균체성장이 가장 높게 나타났다. Carrageemase 생산 최적 배양 조건에서 배양한 배양액을 원심분리하여 얻은 상층액을 조효소로하여 효소특성 실험을 행한 결과, pH 7.0에서 최대 활성을 보였고, pH 6.0 이하, pH 10.0 이상에서는 활성이 급격히 저하되었다. 반응 온도는 35°C에서 최대 활성을 보였고 15°C이하에서 활성이 급격히 낮아졌다. 또한, pH 안정성 실험 결과, pH 7.0에서 최대활성을 보였던 것과는 대조적으로 pH 7.0 이하 pH 10.0 이상의 영역에서 투석결과 상당히 불안정하였으며, preincubation 온도 15°C 이하에서 현저한 활성의 저하를 보였다. 금속 이온이 효소활성에 미치는 영향은 Mg^{2+}에 의해 활성이 증가하였으나 Co^{2+}, Cu^{2+}, Zn^{2+} 등에 의해서 활성이 현저하게 저해되었고 K^{+}와 Ca^{2+}에 의해서는 영향을 받지 않는 것으로 나타났다. p-nitrophenyl α-L-fucopyranoside, p-nitrophenyl β-L-fucopyranoside, p-nitrophenyl β-D-fucopyranoside, p-nitrophenyl α-D-galctopyranosied, p-nitrophenyl β-D-glucuronide, p-nitrophenyl α-D-mannopyranoside, p-nitrophenyl

β-D-mannopyranoside, p-nitrophenol sulfate의 기질에는 효소 활성이 나타나지 않았으나 p-nitrophenyl phosphate에서는 대조구인 p-nitrophenyl β-D-galactopyranoside와 비교하여 77% 정도의 활성을 보였다.

Introduction

The various modified carbohydrate residues that found in marine plants are especially interesting in respect to their functionalities. Three kinds of polysaccharides, agar, alginate and carrageenan are quantitatively major polysaccharides from marine plants and are commercially available in food, pharmaceutical, and cosmetic industries. Carrageenan is a structural polysaccharide in red algae and consists of a linear backbone of galactopyranose residues linked by alternating $\alpha(1\rightarrow3)$ and $\beta(1\rightarrow4)$ linkages (Tristan et al., 2000). The carrageenan has three main branches named kappar-, iota-, and lambda carrageenan, which are well differentiated in linkage of sulfate groups on their galactan backbone (Fig. 1). The polysaccharides exhibit unique rheological properties and are widely used as texturing and moisturizing agents in food and pharmaceutical industries depend on their functionalities (De Ruiter and Rudolph, 1997).

Carrageeanan industry was started in England in 1948, and demand of carrageenan in industry was greatly increased since 1980's. Until

early 1970s, *Chondrus ocellatus Holmes* or *Tenella* was used as a main source of carrageenan. Aquaculture of *Eucheuma cottonii* in Philippines was changed carrageenan source. Fourty six percent of carrageenan produced in the world was covered with cultivated *Eucheuma cottonii* in Philippines. Total production of carrageenan in the world is comprised about 14,000 metric tons, and of these 60% is produced in Europe, 33% in USA and 10% in Asian area, and produce more than 85% in 3 countries such as USA, Denmark, and France. A carrageenan manufacturing industry was overemphasized in the country that do not produce red algae, and the country that have resource is only Denmark. Philippines, Canada and Chile are harvesting carrageenan resource. In Ireland and USA, *Chondrus crispus* is called Irish moss or carrageen and viscous material was extracted from *Chondrus crispus*, Irish moss extract (carrageena) have been used early by purifier of beer and wine and medicinal purposes. These days, according to variety in use of carrageenan in food industry, the countries of carrageenan production is increasing outturn.

Nishino and Nagumo (1992) reported that carrageenan, a sulfated polysaccharide, have very strong anticoagulant and antimicrovial

activity. The anticoagulant activity with respect to APTT (173 units/mg) of an oversulfated fucan (sulfate/sugar ratio, 1.98) was higher than that (167 units/mg) of heparin used as a standard (Nishno and Nagumo, 1992). In general, the oligosaccharides of carrageenan were prepared by degradation with high concentrated inorganic acid. However, degradation of carrageenan with inorganic acid causes side reaction products and deteriorates the quality of carrageenan.

Enzymes degrading carrageenanas, namely κ-, λ- and ι-carrageenase, have been isolated from various marine bacteria (Gurvan et al., 2001). κ-Carrageenase was isolated from the marine bacterium *Pseudomonas carrageenovar* (Yaphe and Baxter, 1955) and produced an extracellular λ-carrageenase in the presence of λ-carrageenan in media (Wegl and Yaphe, 1966). κ-Carrageenase has been purified to electrophoretic homogeneity and hydrolyzed to κ-neocarrabiose sulfate, as a major hydrolytic end product (Weigl and Yaphe, 1966). ι-Carrageenases were purified from a marine Gram-negative bacterium, *Alterodomonas fortis* (Greer and Yaphe, 1984) and *Zovellia galactanovora* (Barbeyron et al., 2000). Enzymes identified as endohydrolases that cleave the internal

β(1→4) linkages of carrageenans yielding products of the neocarrabioses. These two proteins are homologous and have no significant sequence similarities to any other polysaccharidase, including κ-carrageenases. They cleave the internal β(1→4) linkage of ι-carrageenan with overall inversion of the anomeric configuration, whereas κ-carrageenases proceed with retention of configuration (Potin et al., 1995). It was proposed that ι-carrageenases constitute a new structural family of glycoside hydrolases, referred to as family 82 (Barveyron et al., 2000).

In this study, a novel marine bacterium identified as *Pseudomonas* sp. HS5322 was isolated and culture conditions for the production of ι-carrageenase was determined.

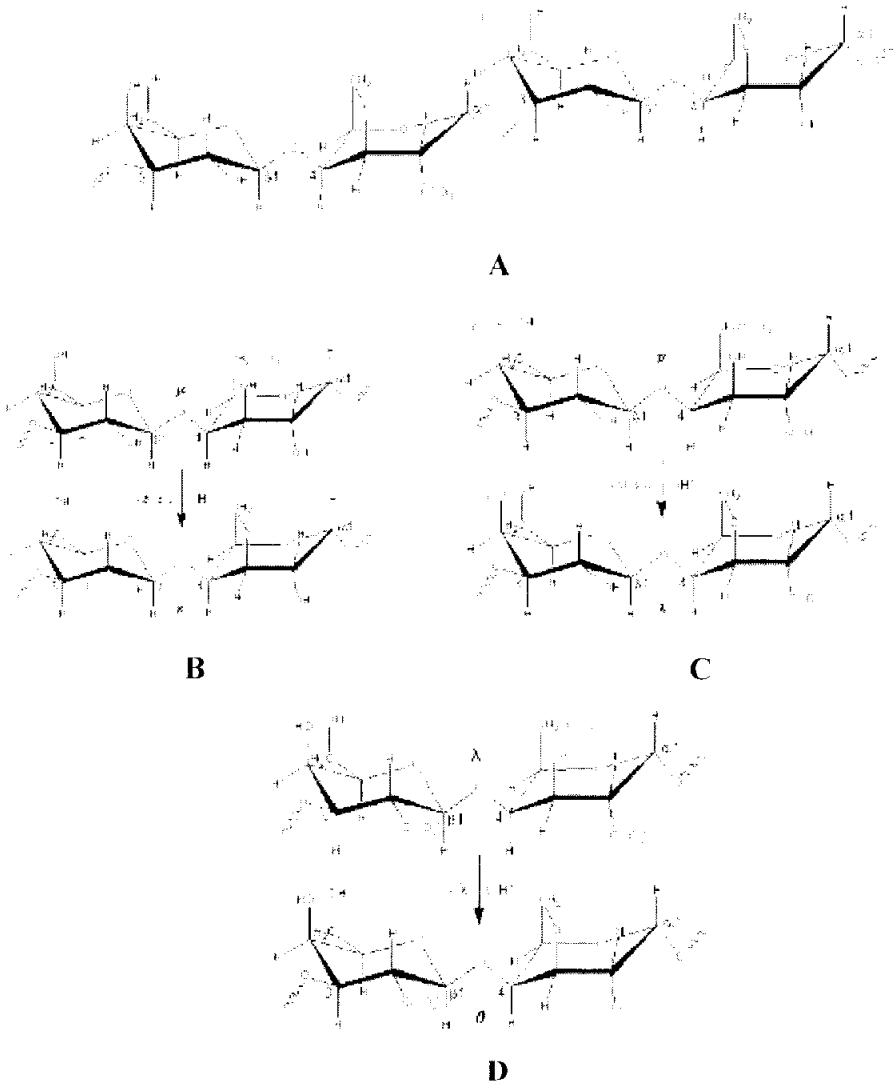


Fig. 1 Structure of carrageenan

A: Crude carrageenan B: κ-carrageenan

C: ι-carrageenan D: λ-carrageenan

Materials and Methods

1. Chemicals

Peptone, yeast extract, and agar were purchased from PC&S (Busan, Korea). ρ-Nitrophenyl β-D-galactopyranoside, ι-carrageenan, and sodium alginate were obtained from SIGMA (St Louis, MO, USA). κ-Carrageenan was purchased from DUCHEFA (Haarlem, Netherlands) and λ-carrageenan was obtained from Wako (Osaka, Japan). ρ-nitrophenyl α-L-fucopyranoside, ρ-nitrophenyl β-L-gucopyranoside, ρ-nitrophenyl β-D-fucopyranoside, ρ-nitrophenyl α-D-galactopyranoside, ρ-nitrophenyl β-D-galactopyranoside, ρ-nitrophenyl β-D-glucuronide, ρ-nitrophenyl α-D-mannopyronoside, and ρ-nitrophenyl β-D-mannopyronoside were obtained from SIGMA.

2. Culture medium.

The medium used for the isolation of bacteria was modified Zobell 2216E plate containing 10 g bacteriological peptone, 10 g yeast extract, 20 g ι-carrageenan, 250 ml distilled water, 750 ml sea water (ME plate). The broth medium used in culture of the bacteria was Zobell 2216E containing 10 g

bacteriological peptone, 10 g yeast extract, 5 g ι-carrageenan, 250 ml distilled water, 750 ml sea water, pH 7.0 (ME).

3. Screening and isolation of bacterial strains producing ι-carrageenase

A seaweed sample (*Geildium amansii*) was collected at coast of Busan in South Korea and blended with 5 volume of autoclaved seawater. The homogenate was diluted to 100, 1,000, and 10,000 times with sterilized seawater and spread onto ME plate. Bacterial colonies showing a hole on the palate after 4 days incubation at 25°C were pick up and isolated by the same plating method. Isolated strain was stored as a glycerol stock at -80°C for further experiments. For liquid culture, the strain was cultured in ME without agar.

4. Phenotypic tests

Cell morphology was observed by optical microscopy of Gram-stained preparation. Motility of the HS5322 was examined using wet mounts.

Biochemical tests were analyzed using VITEK GNI Card (Biomerieux, Marcy-l'Eto, France). Gram staining was done by Gram Stain Kit (DIFCO,

Detroit, USA).

5. 16s rDNA sequencing

Extraction of genomic DNA

Bacterial genomic DNA was prepared using protocols of Genomic DNA extract kit (iNtRon, Kyungki-Do, Korea). Two milliliter of cell culture (OD_{600}: 0.8 - 1.0) was harvested by centrifugation at 13,000 rpm for 1 min. After centrifugation, the supernatant was removed and completely resuspended with resuspension solution by vortexing. 300 $\mu\ell$ of G-buffer solution was added and invert-mixed well. After incubation at 65℃ for 15 min, 250 $\mu\ell$ of binding buffer was added and completely mixed by pipetting. Cell lysate was loaded on a column and centrifuged at 13,000 rpm for 1 min. For washing, 500 $\mu\ell$ of washing buffer A was added to column and centrifuged for 1 min at 13,000 rpm. 500 $\mu\ell$ of washing buffer B was added to column and centrifuged for 1 min at 13,000 rpm. Genomic DNA was eluted with 100 $\mu\ell$ of elution buffer by centrifugation at 13,000 rpm for 1 min.

Amplification of 16s rDNA sequence

Amplification of 16s rDNA sequences was performed with a GeneAmp PCR system 2700 thermocylcer (Perkin-Elmer, MA, USA). 16s rDNA was amplified in 30 $\mu\ell$ of PCR mixture containing 3 $\mu\ell$ of 10x buffer (100 mM Tris-Cl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 0.2 mM dNTP, 0.5 $\mu\ell$ of Taq DNA polymerase (5 U/$\mu\ell$), and 30 pmol of forward primer and reverse primer. PCR primers for 16s rDNA were designed by Moyer (1994) as shown in Table 1. The PCR primers were designed based on *E.coli* 16s rDNA located at 49-68, 1510-1492 and primers were contained *Eco*R I restriction enzyme site in the forward primer, *Bam*H I in the reverse primer (Table 1.). PCR reaction conditions were as follows; predenaturation at 94℃ for 5 min, 35 cycles of 1 min denaturation at 94℃, 1 min annealing at 61℃, 1 min extension at 72℃, and 7 min postextension at 72℃. The PCR products were separated by electrophoresis using 1% agarose gel and visualized by UV illumination.

Table 1. 16s rDNA primers

Forward primer	5' - AGAATTCTNANACATGCAAGTCGANCG - 3'
Reverse primer	5' – GTGGATCCGGYTACCTTGTTACGACTT - 3'

N, degenerate including 4 nucleotides

Y, degenerate including pyrimidine

Restriction enzyme sites were shown by underline.

Cloning with pGEM T-easy vector

PCR products were purified with GeneClean Kit (Q-BIOgene, Canada) from 1% agarose gel and cloned with pGEM T-easy vector (Promega, WI, USA). Purified PCR products were ligated with pGEM T-easy vector. Ligation mixture (75 ng of purified PCR product, 50 ng of pGEM T-easy vector, 5 $\mu\ell$ of 2X ligation buffer, 1 $\mu\ell$ of T4 DNA ligase, upto 10 $\mu\ell$ of dH_2O) was incubated at room temperature for 6 hr. Ligated product was used for transformation in *E. coli* XL1-Blue.

Preparation of competent cell

E. coli strain XL-1 blue [F':: Tn10 · *pro*A+B+ *lacI*q Δ(*lacZ*)M15/*rec*A1 *end*A1 *gry*A96(Nal^r) thi *hsdR*17(rk^-mk^+) *sup*E44 *rel*A1 *lac*] was inoculated into a flask containing LB medium. The cell was incubated at 37℃ with moderate agitation until the cell density was 0.5 at 600nm. The cultured cell was collected into 50 ml centrifuge tube and was chilled on ice for 10 min. The cell was precipitated by centrifugation at 1,000x g for 15 min at 4℃. The pelleted cells were drained thoroughly by inverting the tubes on paper towels and rapping to remove remaining liquid. The cell pellet was resuspended with one third volume of RF1 solution by moderate vortexing (Table 2). The cells were incubated on

ice for 15 min and were precipitated with centrifugation described previously. The cells were resuspended with 1/12.5 volume of RF2 buffer and were incubated on ice for 15 min. The cells were divided into chilled 1.5 ml microcentrifuge tubes. The aliquots were frozen in a liquid nitrogen, and were stored at –70℃.

Table 2. RF1/ RF2 solution

RF1 solution

Compound	Amount/liter	Final concentration
RbCl	12 g	100mM
$MnCl_2 \cdot 4H_2O$	9.9 g	50mM
Potassium acetate	30 ㎖ of a 1 M stock (pH 7.5)	30mM
$CaCl_2 \cdot 2H_2O$	1.5 g	10mM
Glycerol	150 g	15% (w/v)
Adjusted pH 5.8 with 0.2 M acetic acid and filtrated with 0.22 ㎛ membrane filter		

RF2 solution

Compound	Amount/liter	Final concentration
MOPS	20 ml of a 0.5 M stock (pH 6.8)	10 mM
RbCl	1.2 g	10 mM
$CaCl_2 \cdot 2H_2O$	11 g	75 mM
Glycerol	150 g	15% (w/v)
Adjusted pH 6.8 with NaOH and filtrated with 0.22 ㎛ membrane filter		

Transformation

The tubes containing competent cell were thawed on ice. DNA solution was added to the tubes and the tube was swirled to mix the DNA evenly with the cells. After the tubes were incubated on ice for 10 min, the cells were heat shocked in a 42℃ water bath for 90 sec and then were chilled by abruptly returning into ice. Eight hundred microliter of LB medium was added and was incubated at 37℃ for 60 min. The cells were plated on McConkey agar plate (Difco, MI, USA) or LB agar plate containing ampicillin (50 ㎍/㎖) and the plate was incubated at 37℃ for 16 hr.

Plasmid DNA extraction

Cloned plasmid DNA was purified with Plasmid Extraction Kit (AtmanBio, Korea). 1.5 ml of cultured cell in LB medium containing ampicillin (50 ㎍/㎖) was centrifuged at 14,000 ×g for 1 min. Pellet was resuspended with 250 ㎕ of resuspension buffer by vortex. The cell was denaturated by addition of 250 ㎕ of denaturation buffer through inverting tube. After neutralization with 350 ㎕ of neutralization buffer through inverting tube, centrifugation was performed at 14,000 ×g for 8 min. The supernatant was transfered to the prepared Spin column and centrifuged at 13000 ×g for 1 min at room

temperature. The flow-through from the collection tube was removed and the column was washed with 700 $\mu\ell$ of wash solution by centrifugation for 1 min. the column was washed again with 300 $\mu\ell$ of wash solution by centrifugation for 2 min. Spin column was transferred to a sterilized 1.5 ml microcentrifuge tube. The plasmid DNA was eluted by adding 50 $\mu\ell$ of nuclease-free water to the spin column and centrifuged at maximum speed for 1 min at room temperature in a microcentrifuge. Eluted plasmid DNA was treated with restriction enzyme (100 ng of plasmid DNA, 1 $\mu\ell$ of 10× buffer, 0.5 $\mu\ell$ of *Eco*R I , dH_2O up to 10 $\mu\ell$) and restriction fragment was separated on 1 % agarose gel electrophoresis.

Sequence analysis

Cloned plasmid DNA was purified with Plasmid Extraction Kit (AtmanBio, Korea). PCR products were directly analyzed with ABI 377 XL upgrade DNA sequencer (Perkin-Elmer, MA, USA). The resulting sequences were manually aligned with GenBank databases. Unrooted tree obtained using a neighbour-joining algorithm.

6. Determination of culture conditions

Optimum culture conditions for the growth of isolated strain and ι-carrageenase activity were determined with ME. Erlenmeyer flasks (250 ml) containing 50 ml of the medium adjusted with pH range of 3.0 and 9.5 were incubated for 3 days at 25°C after inoculation of 250 μl of glycerol stock. The effect of carbon source on growth and enzyme activity was performed with ME containing 0.5% different carbon source, ι-carrageenan, κ-carrageenan, λ-carrageenan, agar, and alginate at 25°C for 3 day incubation. The effect of incubation temperature on growth and enzyme activity was determined at 10, 15, 20, 25 and 30°C incubation temperature at pH 7.0 for 3 days. ι-Carrageenan concentration on growth and activity was performed by incubation at 25°C for 3 days with ME (pH 7.0) containing different concentration of ι-carrageenan. The effect of incubation time on the growth and enzyme activity was determined by incubation at 25°C for 12, 24, 48, 72, 96, 120 hours with ME (pH 7.0). Aliquot of culture medium was periodically taken and centrifuged (8000 rpm, 20 min, 4°C) and enzyme activity was determined. Cell growth was determined by measuring

absorbance at 600 nm (Ultrospec 3000, Pharmacia).

7. Enzyme activity assay

The ι-carrageenase activity was determined using ρ-nitrophenyl β-D-galactopyranoside. The reaction mixture containing 250 $\mu\ell$ of enzyme solution, 250 $\mu\ell$ of 1 mM ρ-nitrophenyl β-D-galactopyranoside (NPG) and 500 $\mu\ell$ of 200 mM Tris-HCl (pH 7.0) was incubated 37°C for 20 min. Reaction was stopped by addition of 1 ml 0.5 M NaOH solution and activity was determined by measuring absorbance at 410 nm. The ι-carrageeanse activity (Unit) was expressed as 1 μmol ρ-nitrophenol liberated per min per ml of enzyme solution at 37 °C.

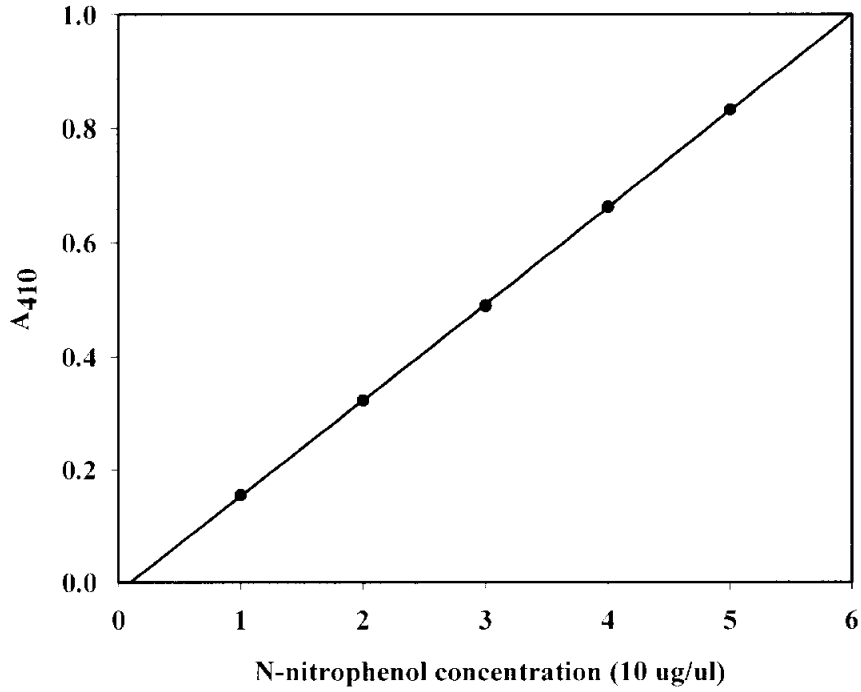


Fig. 2. Standard curve for the determination of ι-carrageenase activity

Equation of reaction velocity: Y = 0.1695X - 0.0163 (R=0.99997163)

8. Preparation of crude enzyme

The *Pseudomonas* sp. HS5322 bacterium was inoculated in ME containing 1% ι-carrageenan and cultured at 25°C for 72 hr at 200 rpm. The medium was centrifugation at 8000 ×g for 10 min at 4°C. The supernatant was used as crude enzyme.

9. Characterization of ι-carrageenase

pH dependence of ι-carrageenase activity

Buffer solutions for the determination of the enzyme activity were prepared to three sequence as follows: 0.1 M citrate - 0.2 M Na_2HPO_4 (pH 4.0 – 6.0), 0.1 M Tris – HCl (pH 7.0 – 9.0) and 0.1 M Na_2CO_3 – 0.1 M $NaHCO_3$ (pH 10.0 – 11.0). Activities were determined in different buffers with overlapping pH points to exclude the possibility of an influence of the buffer species.

Temperature dependence of ι-carrageenase activity

The optimum temperatures of the carrageenase activity were measured at pH 8.5 over a temperature range of 5 to 45°C

Effect of the pH on ι-carrageenase stability

Buffer solutions for the measurement of the enzyme stability were prepared as same buffer used at determination of pH dependence of ι-carrageenase activity. The enzyme was preincubated for 60 min by dialyzing in the various buffer solution of the different pH.

Effect of the temperature on ι-carrageenase stability

Stability of the enzyme at the different temperature was performed at temperature range of 0 to 80°C. The enzyme were preincubated for 30 min at different temperature and residual activities were measured at 37°C.

Optimal enzyme concentration on ι-carrageenase activity

Various enzyme volume of 50, 100, 150, 200, 250, 300, 350 and 400 $\mu\ell$ of enzyme was added at reaction mixture and assayed the enzyme activity at pH 8.0 at 37°C.

Effect of metal ions on ι-carrageenase activity

Enzyme activity in different metal ion solutions was assayed at 37°C, after 20 min reaction time in 200 M Tris-HCl buffer (pH 8.5). 10 $\mu\ell$ of 200 mM

metal ion solution was added at reaction mixture and measured enzyme activity.

Substrate specificity of ι-carrageenase activity

To determine the substrate specificities, the GNP in the assay mixture was replaced with different substrates with a concentration of 8 mM ρ-nitrophenyl α-L-fucopyranoside, ρ-nitrophenyl β-L-fucopyranoside, ρ-nitrophenyl β-D-fucopyranoside, ρ-nitrophenyl α-D-galctopyranosied, ρ-nitrophenyl β-D-glucuronide, ρ-nitrophenyl α-D-mannopyranoside, and ρ-nitrophenyl β-D-mannopyranoside.

Result and Discussion

1. Screening and Isolation of bacterial strains producing ι-carrageenase

Thirteen bacteria capable of carrageenan degradation on the ME plate were isolated from seaweed. Each isolates was separated on the ME plate and confirmed carrageenase activity with hydrolysis of carrageenan after incubation at 25℃ for 3 days. Among them, one isolated strain showing the highest carrageenase activity was selected for this study.

2. Phenotypic and biochemical properties

The isolated strain was found to be Gram-negative and rod-shopped (Fig. 3). The strain was motile and oxidase and catalase-positive (Table. 3).

3. Phylogenetic analysis

Size of PCR product of 16S rDNA based on the genomic DNA extracted from the strain was about 1.4 kb (Fig. 4).

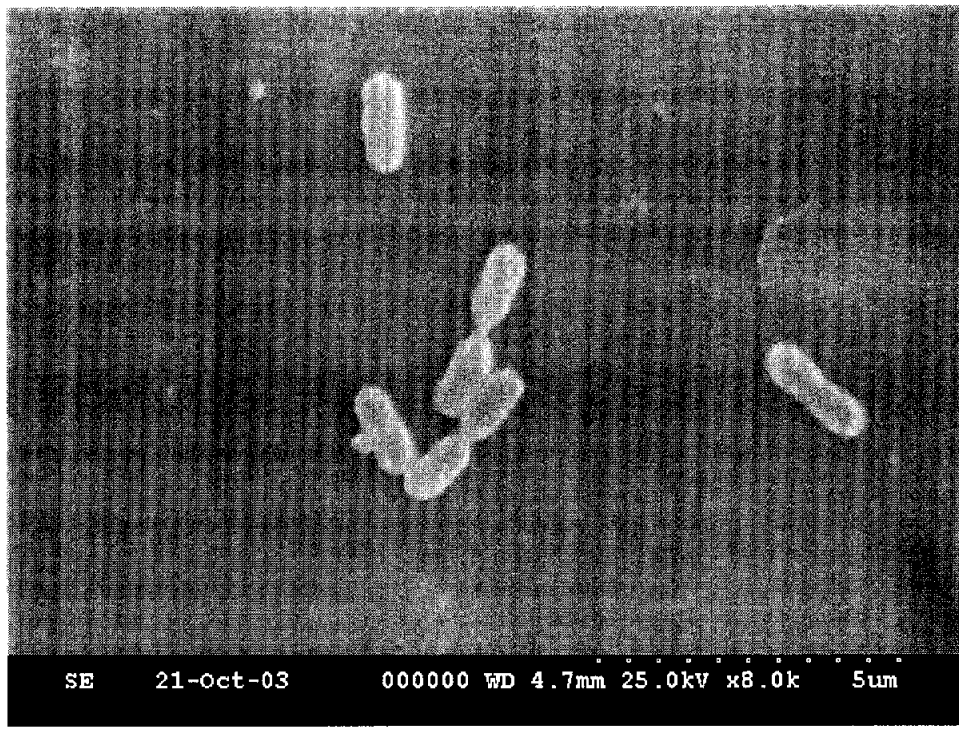


Fig. 3. Transmission electron micrographs showing general morphology of *Pseudomonas* sp. HS5322.

Table 3. Biochemical characteristics of *Psychrobacter* sp. HS5323

Test	Result	Test	Result
*Acid production from		*Specific inhibitor	
Glucose	-	2,4,4'-trichloro-2'-	-
Lactose	-	hydroxydiphenyl ether	
Maltose	-	p-Coumaric	-
Xylose	-	PolymyxinB	-
L-Arabinose	-	*Growth at 35℃ of gram(-)	-
*O-Nitrophenyl	-	strain	
β-D-galactopyranoside		*Utilization as sole carbon	
fermentation		source	
*Decarboxylase Negative	-	Acetamide	-
control		Esculin	-
*Enzyme activity		Citrate	-
Catalase	+	Malonate	-
Urease	+	Glucose	-
Tryptophan deaminase	-	Arginine	-
Arginine digydrolase	-	Lysine	-
Lysine decarboxylase	-	Ornithine	-
Omithine decarboxylase	-	Raffinose	-
Oxidase	+	Sorbitol	-
		Sucrose	-
		Inositol	-
		Adonitol	-

The sequence of 16s rDNA from strain HS5322 was similar to the sequences from *Pseudomonas* species (Table 4). Phylogenetic analysis was done using the sequence date of 11 type *Pseudomonas* species and 4 strains as an outgroup microorganism. The result of the phylogenetic analysis showed that strain HS5322 belongs to the genus *Pseudomonas* (Fig. 5). The homology of HS5322 16s rDNA sequence with *Pseudomoans straminea* strain was 94%, which suggested that strain HS5322 was the closest to *P. straminea*. The sequence of 16s rDNA from the strain was registered in GenBank (Accession No. AY443041.) as *Pseudomonas* sp. HS5322.

4. Optimum culture conditions

Glycerol stock of HS5322 was inoculated in ME adjusted to pH 3.0～9.5 and incubated at 25℃ for 72 hr with 200 rpm in rotary shaker. Cell growth and carrageenase activity of HS5322 were highest at pH 8.5. Carrageenase activity of HS5322 increased with the increase of pH until 8.5 and decreased at the alkaline pH range (Fig 6).

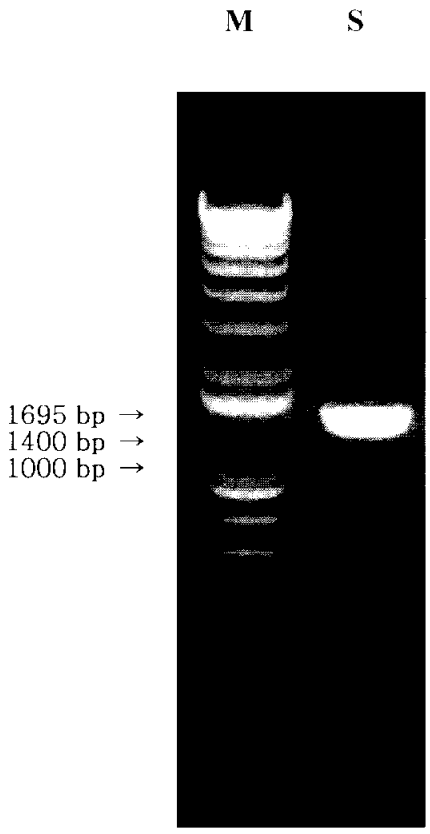
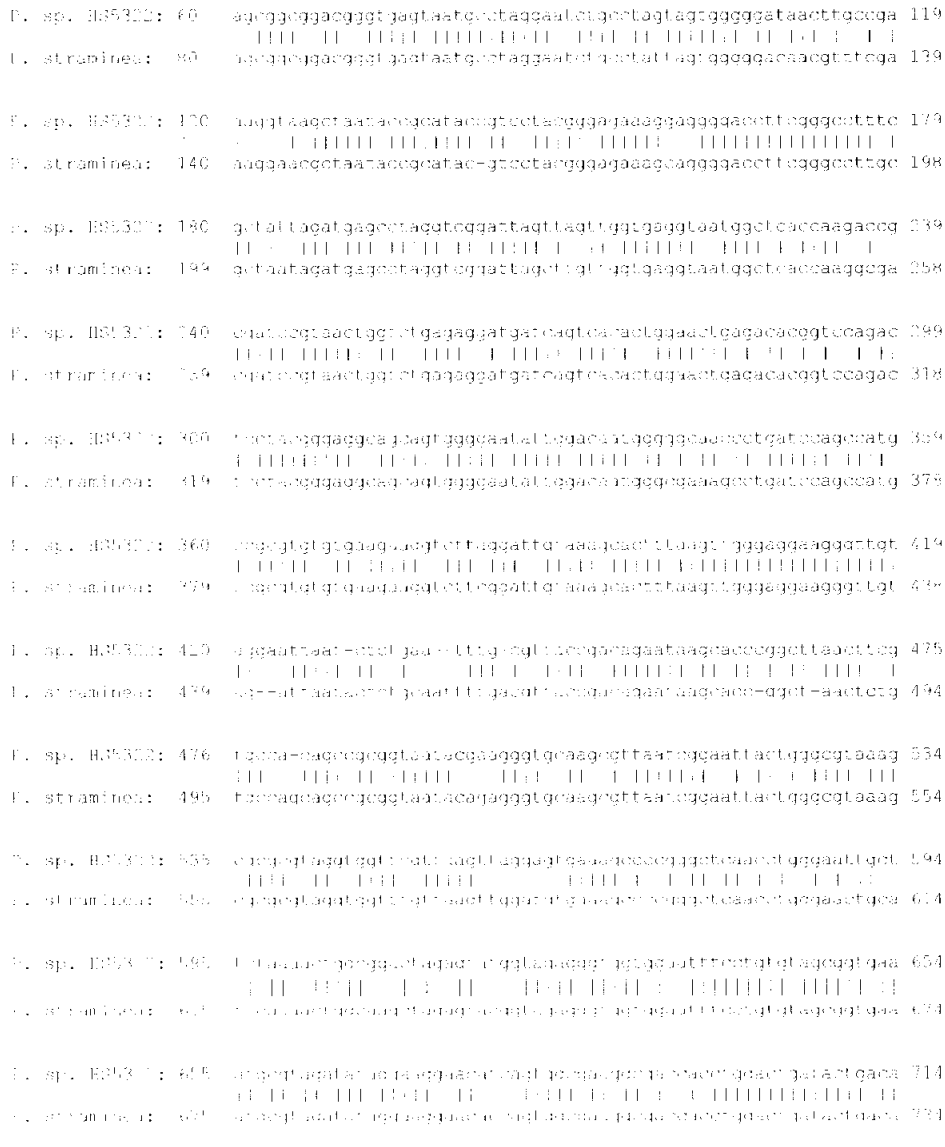


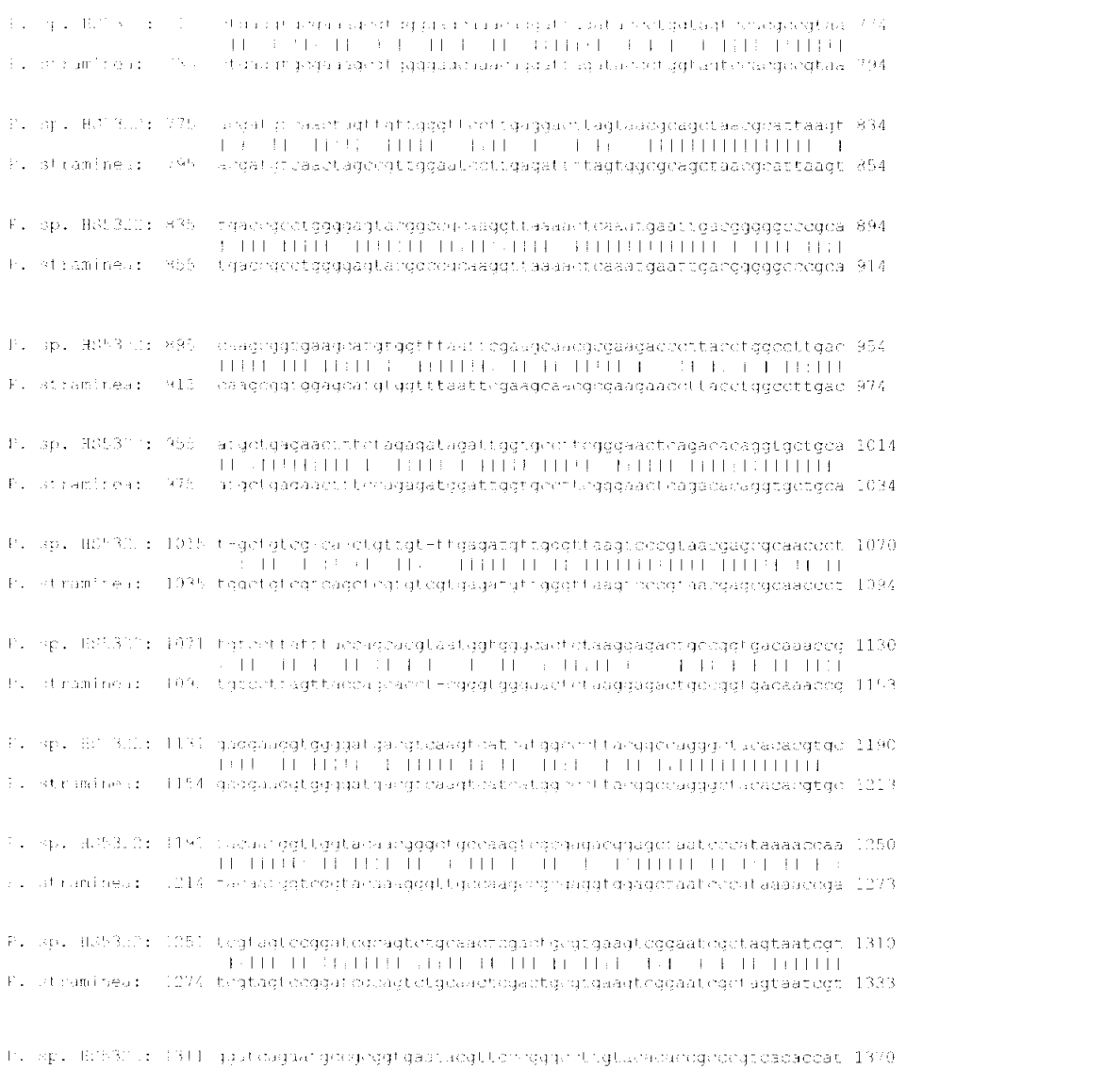
Fig. 4. PCR products of 16s rDNA gene on 1% agarose.

PCR band sizes are about 1.4 kb

(M, 1 kb plus DNA ladder; S, PCR product)

Table 4. Neucleotide sequence of 16S rDNA from *Pseudomonas* sp. HS5322





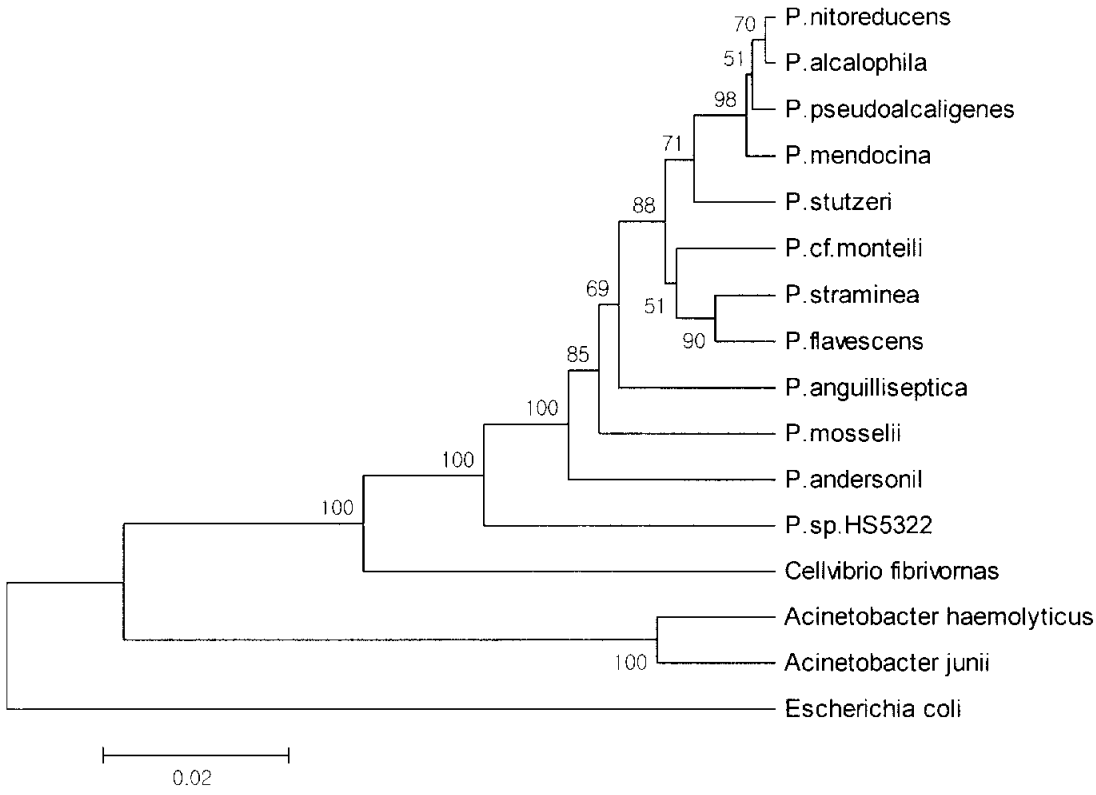


Fig. 5. 16s rDNA dendrogram showing the position of strain HS5322 among some phylogenetically closely related *Pseudomonas* species. Scale bar represents 0.002 substitutions per nucleotide position. Boostrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the branching points.

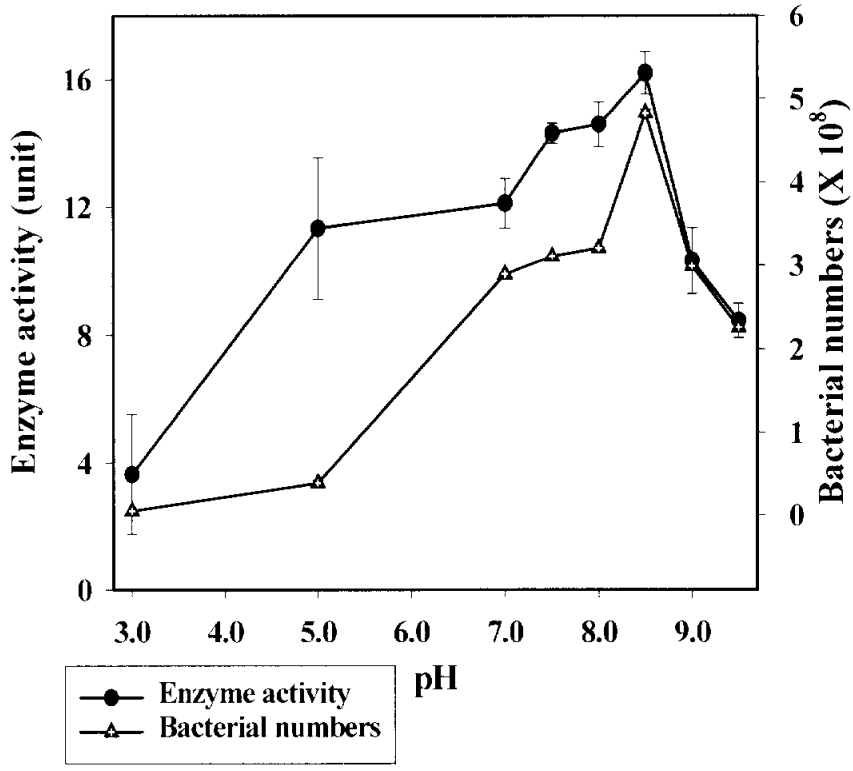


Fig. 6. Effect of pH on the growth and enzyme activity of *Pseudomonas* sp. HS5322

The isolated strain was inoculated in ME (pH 7.0) and incubated at 10, 15, 20, 25 and 30℃ for 72 hr with the same incubation conditions used for optimum pH determination. Cell growth and enzyme activity of HS5322 increased until incubation at 20℃, in which showed the highest cell growth and enzyme activity (Fig 7). Incubation above 25℃ retarded cell growth and decreased enzyme activity.

The isolate had an absolute requirement of NaCl for growth and failed to grow when NaCl was lower than 0.5%. The isolated HS5322 had optimum salt concentration for growth and enzyme activity in the range of 1.5-2.0% NaCl (Fig. 8). Enzyme activity of HS5322 was highest in the presence of 2.0% NaCl in the culture medium.

Incubation time was prolonged until 5 days with optimum pH, temperature, and cell concentration. As shown in Fig 9, enzyme activity of the strain was maximum after 72 hr incubation and decreased with prolonged incubation time.

ι-Carrgeenan concentration for optimum growth and enzyme activity of HS5322 was estimated to be 1.0% ι-carrgeenan in ME (Fig. 10).

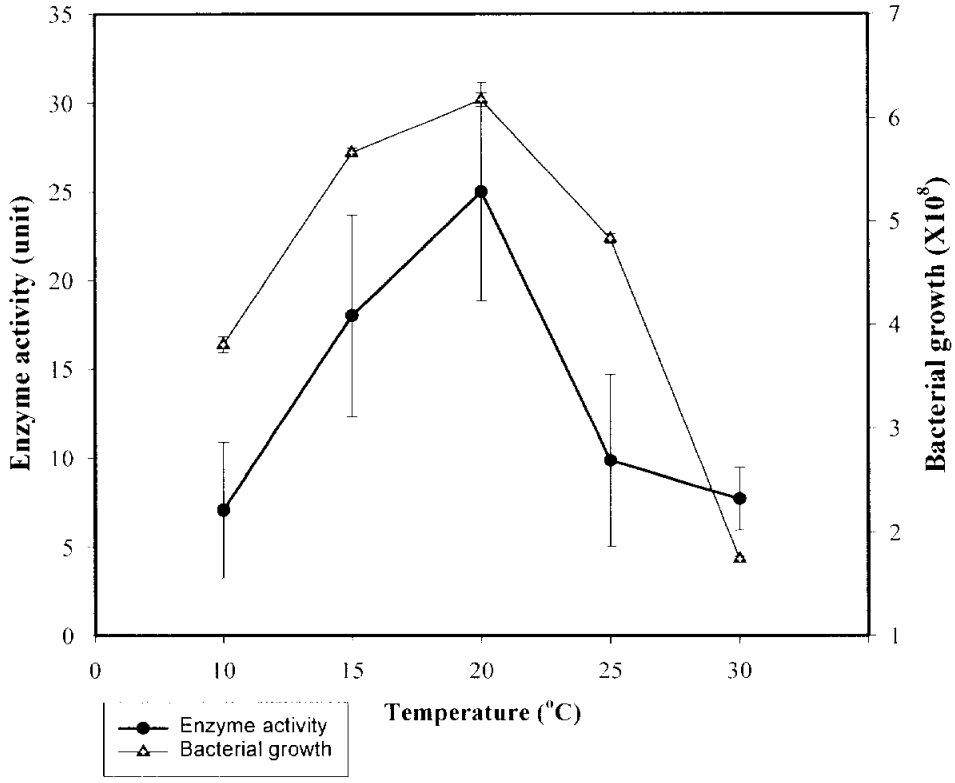


Fig. 7. Effect of temperature on the growth and enzyme activity of *Pseudomonas* sp. HS5322

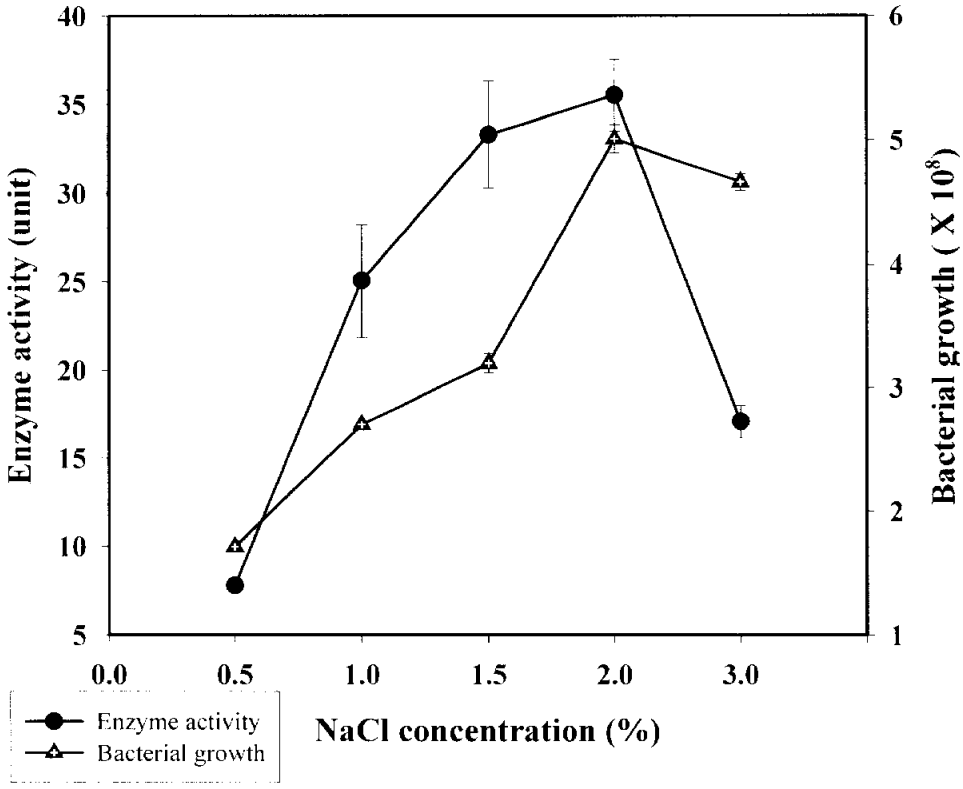


Fig. 8. Effect of NaCl concentration on the growth and enzyme activity of *Pseudomonas* sp. HS5322

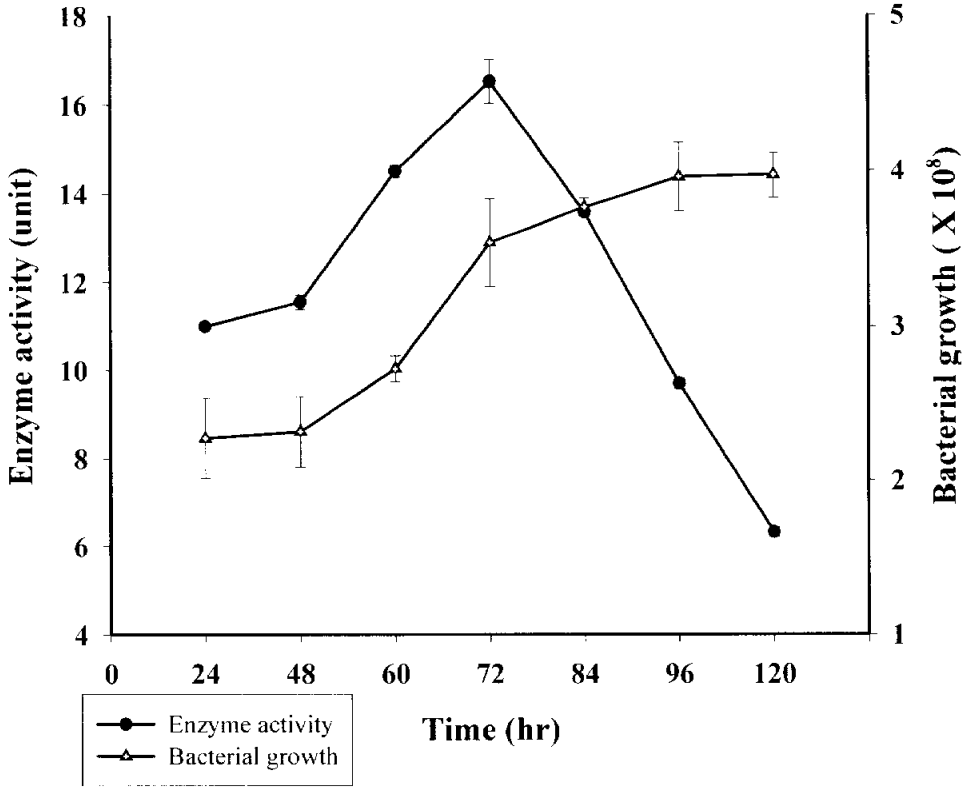


Fig. 9. Effect of incubation time on the growth and enzyme activity of *Pseudomonas* sp. HS5322

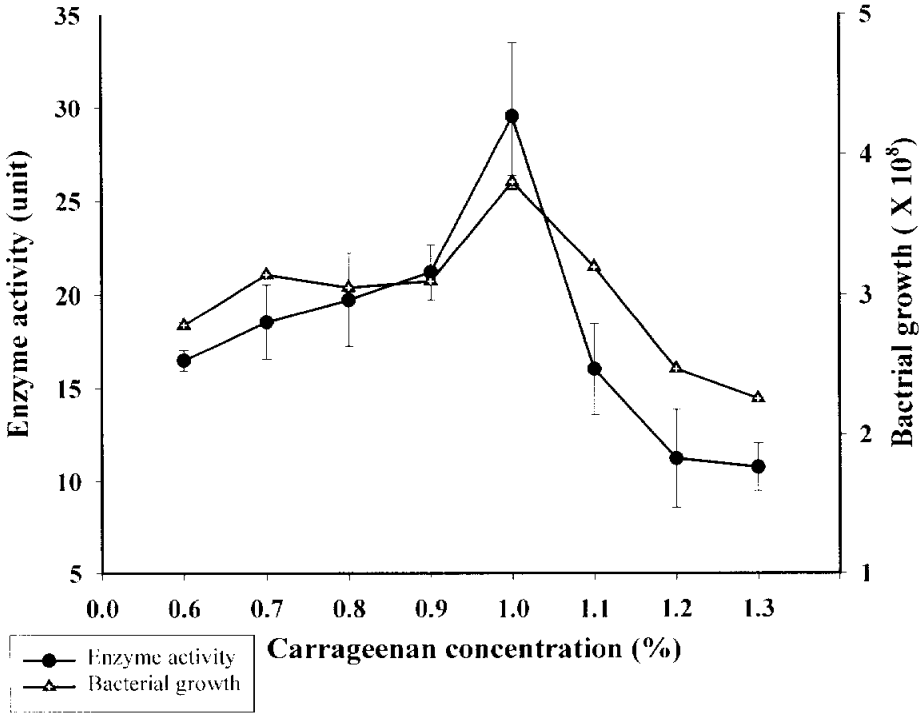


Fig. 10. Effect of ι-carrageenan concentration on the growth and enzyme activity of *Pseudomonas* sp. HS5322

Over 1% of ι-carrageenan concentration, the enzyme activity and cell growth was rapidly decreased. The ι-carrageenase activity of HS5322 was not shown in ME containing κ-carrageenan, agar or alginate, however, the activity showed in the presence of ι-carrageenan as well as λ-carrageenan (Table. 5).

In previous studies (Greer et al., 1984), the production of ι-carrageenase in the cell-free medium of marine bacterium was closely paralleled early exponential growth, but enzyme activity disappeared as cells going to stationary stage. Bellion et. al. (1984) demonstrated that no protease activity was detected in the cell-free medium. The induction of carrageenase, level of enzyme activity, and the production time of the enzyme were related to the carrageenan concentration on the culture medium. Increasing the carrageenan concentration in the culture medium increased enzyme production, and maximum enzyme production achived in later stage of the growth cycle (Bellion. 1979). The exhaustion of carrageenan in the growth medium appeared to play a role in the reduction of enzyme activity, but when

Table 5. Effect of carbohydrate source on ι-carrageeanse activity

Carbohydrate source	Activity (A_{410nm})
Alginate	0.060
Agar	0.003
κ-Carrageenan	0.021
λ-Carrageenan	0.199
ι-Carrageenan	0.279

Tween 80, Triton X-100, or BSA were added in the culture medium, enzyme activity showed plateaus and did not decrease until late in the stationary phase. The role of detergents in preventing the reduction of enzyme activity in the stationary phase of growth suggested that ι-carrageenase in the cell-free medium is stabilized by the agents that act as surfactants, or interact with detergents in both the cell-free medium and in association with cell membranes or other cell wall components (Greer and Yaphe, 1984). Similar observations have been reported for alginate lyase (Doubet and Quatrano, 1984) and carboxymethylcellulase (Groleau and Fosberg, 1983).

5. Characterization of ι-carrageenase

pH dependence of ι-carrageenase activity

The pH dependence of ι-carrageenase from *Pseudomonas* sp. HS5322 is shown in Fig. 11. The ι-carrageenase activity was highest at pH 7.0 and decreased rapidly in higher than pH 7.0 when enzyme activity was assayed at 37℃ and higher than

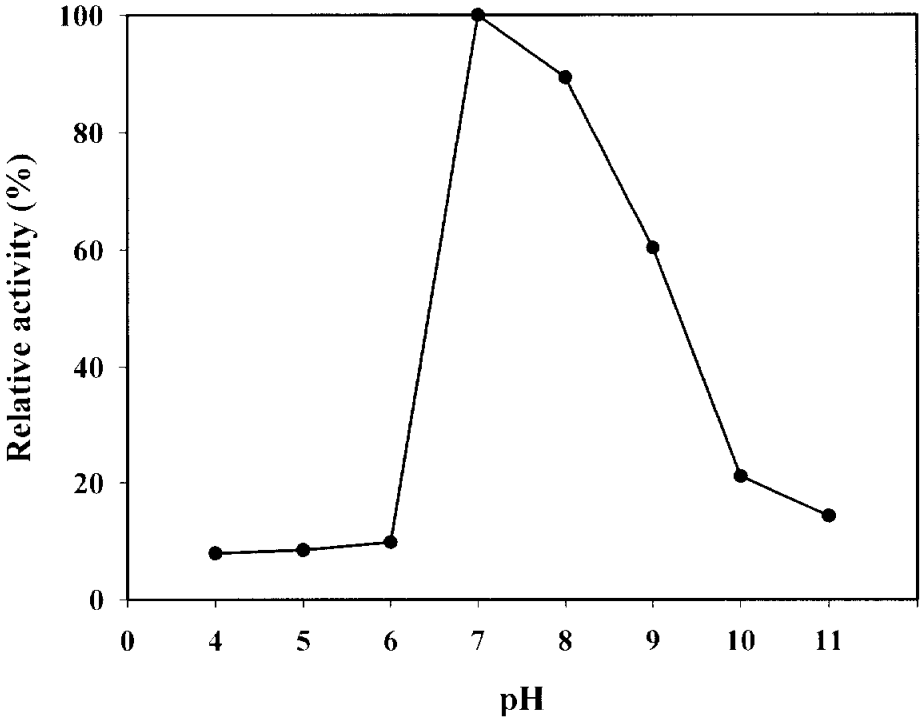


Fig. 11. pH dependence of the Enzyme activity

The used buffer in the reaction mixture were
0.1 M citate - 0.2 M $NaHPO_4$ (pH4.0 - 6.0),
0.1 M Tris - HCl (pH 7.0 - 9.0), 0.1 M Na_2CO_3
- 0.1 M $NaHCO_3$ (pH 10.0 - 11.0).
Substrate concentration : 8 mM NPG
Reaction condition : substrate 250 ul,
buffer soln. 500 ul, enzyme soln. 250 ul
temperature 37°C, reaction time 20 min.

pH. 9.0. Especially, the enzyme activity was not displayed at lower than pH 6.0. ι-Carrageenase of marine bacterium isolated from decomposing algae exhibited a pH optimum of 8.0 at 40℃. However, pH optima was shifted to pH 7.0 when enzyme activity was assayed at 25℃ (Greer and Yaphe, 1984).

Temperature dependence of ι-carrageenase activity

The enzyme activity was measured in the temperature range of 5 to 45℃. The highest activity of the ι-carrageenase was observed around 35℃. The catalytic activity of the enzyme was rapidly reduced at 10℃ (Fig. 12). Purified ι-carrageenase of marine bacterium showed a temperature optimum of 40℃ and the enzyme was completely deactivated at 60℃, and 30% of maximal activity was expressed at 20℃ (Park, 1996). However, the enzyme from *Pseudomonas* sp. HS5322 had 94% of maximal activity at 20℃ and retained 90% of maximal activity at 45℃.

pH stability of ι-carrageenase

The stability of the ι-carrageenase activity was highest

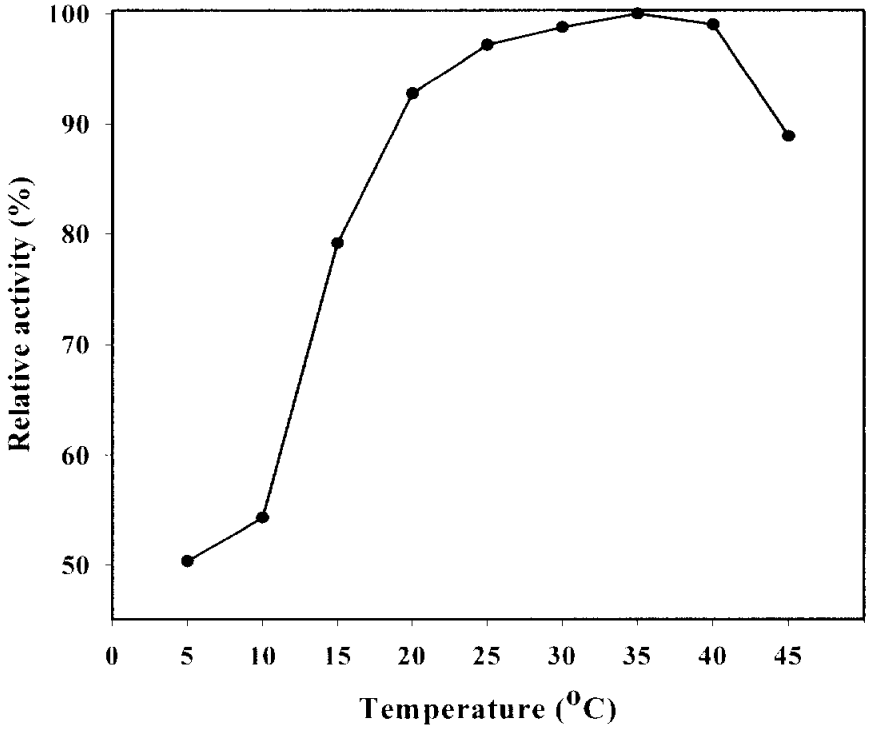


Fig. 12. Temperature dependence of the enzyme activity

The used buffer in the reaction mixture were 20 mM Tris - HCl buffer, pH 8.5.

Substrate concentration : 8 mM NPG

Reaction condition : substrate 250 ul, enzyme soln. 250 ul, buffer soln. 500 ul, reaction time 20 min.

at pH 8.0 in contrast with optimal pH of ι-carrageenase activity (Fig. 13). The enzyme activity was rapidly decreased at pH lower than 7.0 and higher than 9.0. Activity at pH 7.0 showed 80% of maximal activity at pH 7.0, while purified κ-carrageenase of *Cytophaga* sp. was stabled in the range of pH 5.0-8.0 (Potin et al., 1991).

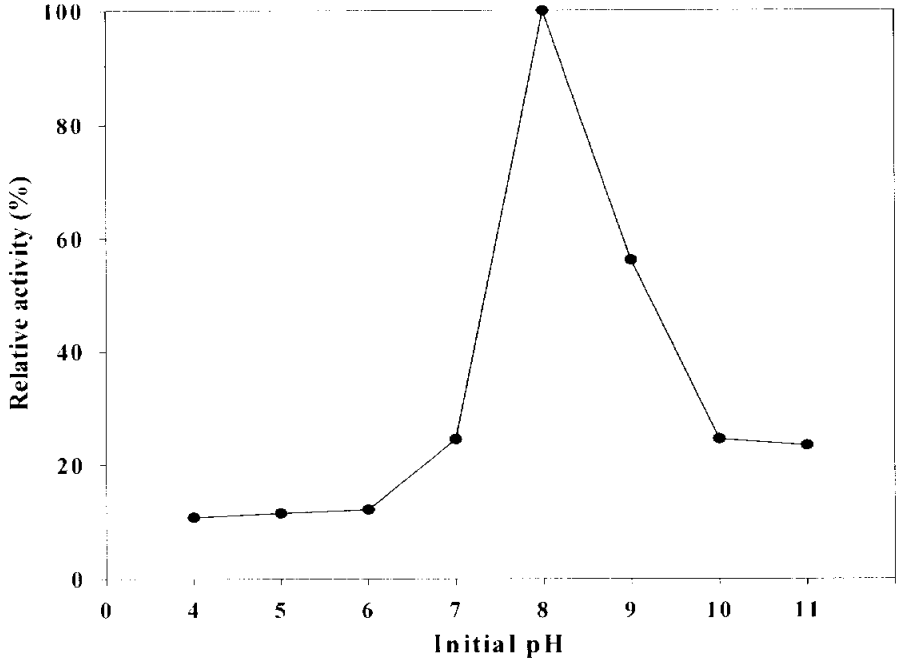


Fig. 13. Stability of the enzyme at the different pH under preincubation condition

The enzymes were preincubated for 60 min by dialyzing in the various buffer solution of the different pH.

Substrate concentration : 8 mM NPG

Reaction condition : substrate 250 ㎕, buffer soln. 500 ㎕, enzyme soln. 250 ㎕, temp. 37°C, reaction time 20 min.

Effect of the temperature on ι-carrageenase stability

To confirm thermostabilites of the ι-carrageenase, the enzyme was preincubated for 30 min at different temperature and residual activity was determined at 37℃. As displayed in Fig. 14, the enzyme showed much higher thermostability at 20 -40℃. The highest enzyme activity was at 40℃ and 45% of maximal activity was expressed at 50℃. Incubation at 0℃ and 10℃ for 30 min inactivated enzyme activity which is not reported until now.

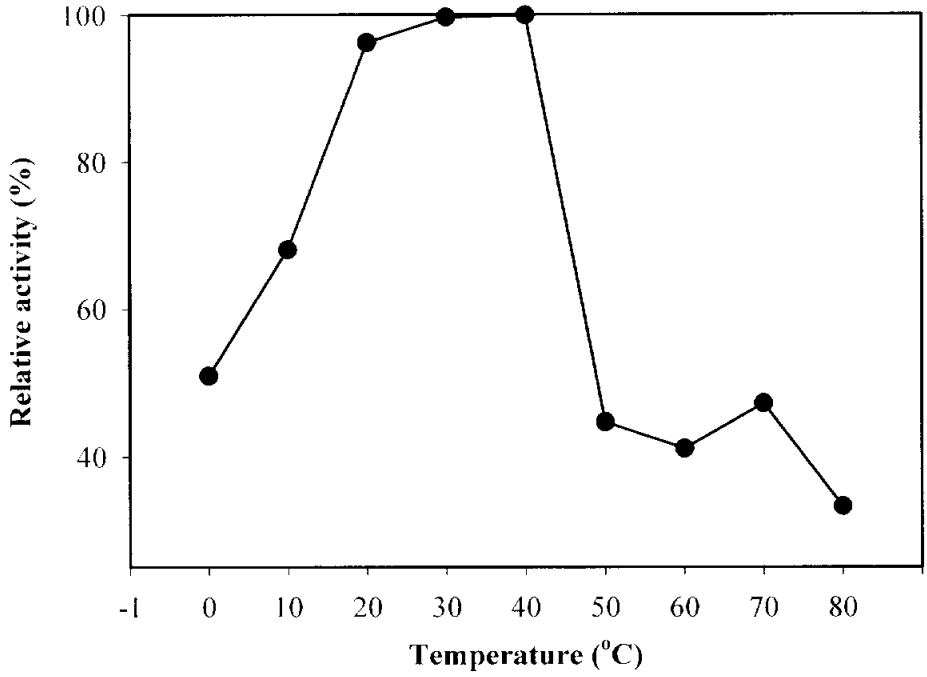


Fig. 14. Theraml stability of the i-carrageenase from *Pseudomonas* sp. HS5322.

The enzymes were preincubated for 30 min at different temperature.

Substate concentration : 8 mM NPG

Reaction condition : substrate 250 ul, buffer soln. 500 ul, enzyme soln. 250 ul, temp. 37°C, reaction time 20 min.

Optimal enzyme concentration on ι-carrageenase activity

Fig. 15 showed effect of enzyme concentration of the reaction mixture on the ι-carrageenase activity. The enzyme activity was linearly increased with enzyme volume upto 200 $\mu\ell$ in 1 ml of reaction solution.

Effect of metal ions on ι-carrageenase activity

The effect of metal ions on the enzyme activity was shown in Table 6. The ι-carrageenase activity was decreased by Co^{2+}, Cu^{2+} and Zn^{2+}, however, increased about 10% by Mg^{2+}, and K^{+}, and Ca^{2+} were not influence to the enzyme activity.

Substrate specificity of ι-carrageenase activity

As displayed in Table 7, the ι-carrageenase has high activity for NPG and less activities on other substrate. These result suggest that the enzyme is endohydrolases that cleave the internal β(1→4) linkages of carrageenans. Enzymes that degrade carrageenans

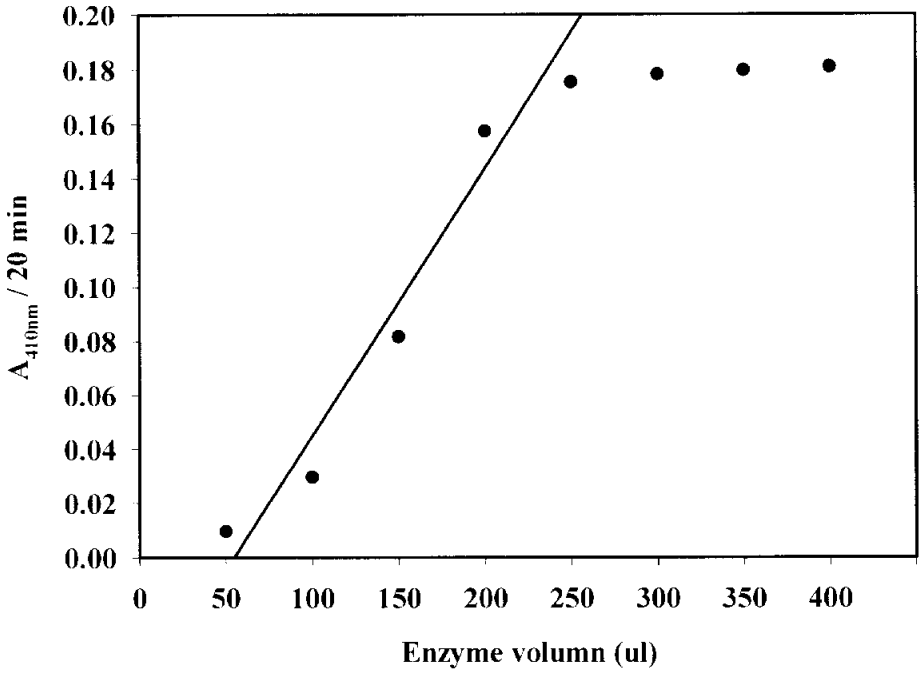


Fig. 15. Effect of enzyme concentration of the reaction mixture on the enzyme activity

The used buffer in the reaction mixture were 200mM Tris - HCl buffer, pH 8.5.
Substrate concentration : 8 mM NPG
Reaction condition : substrate 250 ul, enzyme soln., buffer soln. 500 ul, temperature 37°C, reaction time 20 min.
Equation of reaction velocity : Y = 0.0496X-0.0544 (R=0.9697)

Table 6. Effect of metal ions on the enzyme activity

Metal ions*	Relative activity (%)
Control	100
K^{+}	102.75
Ca^{2+}	98.79
Co^{2+}	42.28
Cu^{2+}	20.03
Mg^{2+}	110.15
Zn^{2+}	23.81

* chloride form

Table 7. Substrate specificity of the carrageenase

Substrate	Relative activity (%)
ρ-nitrophenyl α-L-fucopyranoside	15.81
ρ-nitrophenyl β-L-fucopyranoside	19.22
ρ-nitrophenyl β-D-fucopyranoside	19.22
ρ-nitrophenyl α-D-galctopyranosied	25.30
ρ-nitrophenyl β-D-galactopyranoside	100
ρ-nitrophenyl β-D-glucuronide	14.03
ρ-nitrophenyl α-D-mannopyranoside	23.25
ρ-nitrophenyl β-D-mannopyranoside	23.22

isolated from various marine bacteria, display a strict substrate specificity, obviously recognize the sulfation pattern on the digalactose repeating unit and thus provide an opportunity for investigating the structure-function relationships of hydrolases that degrade sulfated polysaccharide (Michel et al. 2001). Purified ι-carrageenase hydrolyzed the β(1→4) linkage in ι-carrageenan producing ι-neocarrahexaose sulfate as the major end products (Greer and Yaphe, 1984).

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