Cloning of alginate lyase gene from *Pseudoalteromonas* sp. and overexpression of recombinant protein in *E. coli*

Pseudoalteromonas sp.의 알긴산 분해효소 유전자 클로닝과 대장균에서의 재조합 단백질 과발현

by

Jae-Jin Choi

A thesis submitted in partial fulfillment of the requirements
for the degree of

Master of Science

In the Department of Microbiology, Graduate School,
Pukyong National University

February 2004

김종오의 이학석사 학위논문을 인준함

2003년 12월 일

주 심 이학박사 이 훈 구 

위 원 이학박사 김 형 락 

위 원 이학박사 최 태 진 

Cloning of alginate lyase gene from *Pseudoalteromonas* sp. and overexpression of recombinant protein in *E. coli*

A Dissertation

by

Jong-Oh Kim

Approved as to style and content by :

Dean of Graduate School

Chariman

Member

Member

December 2003

CONTENTS

Cloning of alginate lyase gene from *Pseudoalteromonas* sp. and overexpression of recombinant protein in *E. coli*

Jong-Oh Kim

Department of Microbiology, Graduate School,

Pukyong National University, Pusan, Korea

ABSTRAT

Alginate lyase producing bacterium was isolated from decayed seaweed at southeast coast in Korea. Purified bacterium was identified as *Pseudoalteromonas* sp. with 16s rDNA sequences analysis. Alginate lyase gene from this bacterium was cloned and sequenced. The sequence comparison of the cloned gene with other genes in the BLAST showed a 99% homology with alginate lyase gene from *Pseudoalteromonas elyakovii*. There are 9 nucleotide substitution in 1197 nucleotide compare with corresponding gene of *Pseudoalteromonas elyakovii* and shows a

new restriction site (Xho I) in the sequences. A recombinant alginate lyase was expressed as insoluble inclusion body in *E.coli* when induced with 1 mM IPTG, and the protein was about 48 kDa. When the cell harboring alginate lyase expressing vector was cultured at 37℃ with or without induction, enzyme activity was not detected. However, alginate lyase activity was shown in the cell harboring alginate lyase-histag construct when the cell was cultured at 25℃ without induction.

INTRODUCTION

As our dietary life is westernized, diseases of adult people such as hypertension, heart attack, and diabetes are increasing. Since Bang and Dyerberg reported that some people who used to eat a marine algae food live longer than other people (1972), nutritious and pharmaceutical interests on marine algae have been increased and many researches have been conducted to find new substance with physiological activity from marine algae (Ito and Tsuchiya, 1972; Suzuki *et al.*, 1993; Kidamikado *et al.*, 1993).

Alginate is linear polysaccharides composed of (1,4)-linked β-D-mannuronic acid and its C-5 epimer, α-L-guluronic acid. Because of its unique physical properties, it has been used in a broad range of applications such as stabilizer in food industries, pharmaceutical, and biotechnological field, and many facets of the properties of this polysaccharide have been studied (Kong *et al.*, 1995).

Most of commercial alginate is obtained from three seaweed genera, *Macrocysis*, *Laminaria*, and *Ascophyllum*. Alginate is also produced by two families of bacteria, the *Pseudomonadaceae* and the *Azotobacteriaceae* (Skjak-Brek *et al.*, 1985). An annual production of crude alginate from all over the

world is about a hundred thousand ton and 41 % of total product is produced in North America and USA.

Although large molecular weight alginate has many biological functions, oligo-alginate has better effect in cholesterol decrease (Suzuki *et al.*, 1993). Especially, about 6-7 dalton alginate has anti-microbial effect and inhibitory effect on angiotension converting enzyme (Kidamikado *et al.*, 1993). In addition, oligo alginate has been reported to have good effect in cystic fibrosis(CF) treatment (Muraka *et al.*, 1993).

Up to now, hydrolytic method has been used to produce oligo alginate. However, this method costs a lot because long time incubation at high temperature is required in this process. Environmental pollution is another concern of this method. Several researches have been performed with bacteria that have an alginate lyase to solve this problem(Thiang *et al.*, 2000).

Alginate lyases are classified poly(M) lyase(EC: 4.2.2.3) and poly(G) lyase(EC: 4.2.2.11), and catalyze the depolymerization of alginate by a β-elimination of the 4-O-linked glycosidic bond and formation of a double bond between C-4 and C-5, resulting an unsaturated sugar residue at nonreducing end of the oligomer (Gacesa, 1987). Alginate-degrading enzymes with various substrate specificities have been isolated from many sources, including marine

algae (Madgwick *et al*, 1973), marine mollusks (Seiderer *et al.* 1982), and wide range of microorganisms. Recent progress in cloning and sequencing of these enzymes has led to structure-function analysis of alginate lyase (Wong *et al.*, 2000). Ju and Lee isolated marine bacteria having alginate lyase activity, and characterized alginate lyase produced by *Vibrio* sp. AL-145 (Ju and Lee, 1993). Algiante lyase gene has been cloned from *Pseudoalteromonas elyakovii* IAM 14594 (Sawabe *et al.*, 1993), *Sphingomonas* sp. A1 (Hashimoto *et al.*, 2000; Miyake *et al.*, 2003) and recombinant enzyme have been produced from the cloned genes (Sawabe *et al.*, 1993; Miyake *et al.*, 2003).

In this study, a marine bacterium *Pseudoalteromonas* sp. that produces alginate lyase was isolated from decayed seaweed. The alginate lyase gene from the bacterium with the highest enzyme activity was cloned and sequenced, and recombinant enzyme was produced in *E coli* from the cloned gene using the pET vector system.

MATERIALS AND METHODS

Isolation of microorganisms

For screening of alginate lyase producing marine bacteria, decayed brown seaweed having pathogenic damage was collected at the southeastern coast (Kijang, Daebyun) in Korea (July-August, 2002) 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 (beef extract, 10 g; peptone, 10 g; sea water, 750 ml; tap water, 250 ml; agar, 20 g; alginate 15g). Bacterial colonies showing a hole on the palate after 4 days incubation at 25°C were picked 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.

Alginate lyase activity test

Alginate lyase activity was confirmed by Somogyi-Nelson method (Nelson, 1944). Nine hundred microliter of substrate (0.5% sodium alginate in 10

mM Tris-Cl buffer) was incubated with 100 $\mu\ell$ of crude enzyme at 37℃ for 30 min. After addition of 1 ml Somogyi solution (Table 1), the mixture was boiled for 10 min and was cooled at room temperature for 5 min and 1 ml of Nelson solution was added (Table 1). The mixture was transferred to microcentrifuge tube and was centrifuged at 12,000 rpm for 1min. Supernatant was measured spectrophotometrically at 510 nm.

Table 1. Somogyi and Nelson solution

- Somogyi solution

10 % $CuSO_4 \cdot 5H_2O$ (10 g/ 100 ㎖); 80 ㎖

Potassium sodium tartrate ($C_4H_4O_6KNa \cdot 4H_2O$); 40 g

$Na_2HPO_4 \cdot 12H_2O$; 71 g

1N NaOH; 100 ㎖

Na_2HO_4; 180 g

And adjust to 1 L with distilled water

- Nelson solution

Ammonium Molybdate $(NH_4)2Mo_7O_{24} \cdot 7H_2O$; 50 g

Sodium Aresentate dibasic ($Na_2HaSO_4 \cdot 4H_2O$); 6 g

H_2SO_4; 42 g

After adjust to 1 L with distilled water, stand overnight at 37℃

and filtrate. Store in the dark condition

Bacterial genomic DNA extraction

Bacterial genomic DNA was extracted with G-spin Genomic DNA Extraction Kit (iNtRON Biotechnology, Korea). Bacterial cell (1-2 ㎖, OD_{600}: 0.8 - 1.0) was harvested by centrifugation at 13,000 rpm for 1min. After centrifugation, the supernatant was removed and the pellet was completely resuspended in resuspension buffer by vortex. G-buffer solution (300 ㎕) was added and invert-mixed well. After incubation at 65℃ for 15 min, 250 ㎕ of binding buffer was added and completely mixed by pipetting. Cell lysates was loaded on column containing silica-membrane and centrifuged at 13,000 rpm for 1 min. For washing, 500 ㎕ of washing buffer A was added to column and centrifuged for 1 min at 13,000 rpm. Washing buffer B(500 ㎕) was added to column and centrifuged for 1 min at 13,000 rpm. Genomic DNA was eluted with 100 ㎕ of elution buffer by centrifugation at 13,000 rpm for 1 min.

Amplification of 16s rDNA sequence

The PCR primers were degenerate primer from *E.coli* 16s rDNA located at 49-68, 1510-1492 and primers were contained restriction enzyme site *Eco*R I in the forward primer, *Bam*H I in the reverse primer (Table 2). Amplification of 16s rDNA sequences was performed with a GeneAmp PCR system 2700 thermocylcer (Perkin-Elmer, MA, USA). 16s rDNA sequences were 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/ ul), and 30 pmol of forward primer and reverse primer (Table 2). PCR reaction condition was predenaturation at 94℃ for 5 min, 35 cycles of 1 min denaturation at 94℃, 1 min annealing at 61℃, and 1 min extension at 72℃, followed by a 7 min postextension at 72℃. The PCR products were analyzed by electrophoresis in 1% agarose gels and visualized by UV irradiation. 16s rDNA PCR primers were designed by Moyer (1994).

Table 2. Primers for 16s rDNA amplification

Forward primer, 5' - AGAATTCTNANACATGCAAGTCGANCG - 3';
Reverse primer, 5' – GTGGATCCGGYTACCTTGTTACGACTT - 3',
N, degenerate including 4 nucleotides
Y, degenerate including primidines

Cloning with pGEM T-easy vector

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

Preparation of competent cell

E.coli strain XL-1 blue [F'∷ Tn10-*pro*A+B+ *lacI*q $\triangle$(*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 OD_{600} = 0.5. The cultured cell was collected into 50 ml centrifuge tubes such as Falcon 2070 tubes and was chilled on ice for 10 min. The cell was pelleted 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 any liquid. The cell pellet was resuspended by moderate vortexing with 1/3 volume of RF1 buffer (Table. 3). The cells were incubated on ice for 15 min and were pellet again with same method described above. The cells were resuspended with 1/12.5 volume of RF2 buffer and were incubated on ice for 15 min. The cells were aliquoted into chilled 1.5 ml microcentrifuge tubes and flash freezed in liquid nitrogen, then were placed at – 70℃.

Table 3. 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, 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, filtrated with 0.22 ㎛ membrane filter		

Transformation

The tubes containing competent cell were removed from the freezer and were thawed at room temperature until the cell suspension was just liquefied and were placed on ice. The 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 by placing the tubes in a 42℃ water bath for 90 sec and then were chilled by returning tubes immediately to ice. LB medium 800 ㎕ was added and was incubated at 37℃ for 60 min. The cells were plated on McConkey agar plate (Difco, MD, 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). Cultured cell 1.5 ㎖ in LB broth containing ampicillin (50 ㎍/㎖) was centrifuged at 14,000 rpm for 1 min. Pellet was resuspended with 250 ㎕ of resuspension buffer by vortex. The cell was denaturated with 250 ㎕ of

denaturation buffer through inverting. After neutralized with 350 ㎕ of neutralization buffer through inverting, centrifuged at 14,000 rpm for 8 min. Cleared lysate was transfered to the prepared Spin Column. The supernatant was centrifuged at maximum speed in a microcentrigue for 1 min at room temperature. The flowthrough from the collection tube was removed and the column was washed with 700 ㎕ of wash solution by centrifugation for 1 min. the column was washed again with 300 ㎕ of wash solution by centrifugation for 2 min. Spin column was transfered to sterile 1.5 ㎖ microcentrifuge tube. The plasmid DNA was eluted by adding 50 ㎕ of Nuclease-free water to the spin column and centrifuged at maximum speed for 1 min at room temperature in a microcentrifuge. The eluted plasmid DNA was digested with restriction enzyme (100 ng of plasmid DNA, 1 ㎕ of 10X buffer, 0.5 ul of *Eco*R Ⅰ, dH_2O upto 10 ㎕), and analyzed on 1 % agarose gel.

Sequence analysis

The clone was amplified with T7 promoter primer and SP6 promoter primer located at pGEM T-easy vector. The PCR product was gel purified and used as template for the sequencing ABI 377 XL upgrade DNA sequencer

(Perkin-Elmer, MA, USA). The resulting sequences were aligned with GenBank databases through the BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/).

Cloning of alginate lyase gene

For the cloning of alginate lyase gene, the known sequences of alginate lyase gene were collected from GenBank database (Table. 4). Ten primer sets were designed from the known sequences of alginate lyase gene (Table. 5). Amplification of alginate lyase gene was performed with a GeneAmp PCR system 2700 thermocylcer (Perkin-Elmer, MA, USA) using 35 cycles of 94℃ for 30 sec, each annealing temperature of primers for 30 sec and 72℃ for 1 min. The PCR products were cloned into pGEM T-easy vector and the sequences were analyzed.

In order to clone the full ORF of the alginate lyase gene, the primers were re-designed from the GenBank databases (Table. 6). For the ligation into expression vector, primers include a specific restriction enzyme (underlined, *Nde* I in forward primer; *Eco*R I in reverse primer). PCR products purification and cloning procedures were same as described above.

Table 4. Alginate lyase gene enrolled at GenBank

1. Staphylococcus aureus subsp. aureus N315
2. Staphylococcus aureus strain Mu50
3. Paramecium bursaria Chlorella virus 1.
4. Chlorella virus orf1 gene and orf2 gene for alginate lyase
5. Pseudomonas syringae alginate lyase (algL) gene, complete cds.
6. Pseudoalteromonas sp.IAM14594 extracellular alginate lyase(aly)gene.
7. Pseudomonas sp. W7 alginate lyase gene
8. Pseudomonas sp. gene for alginate lyase
9. Pseudomonas sp. genomic DNA for alginate lyase
10. P. alginovora gene for alginate lyase
11. Azotobacter chroococcum algL gene encoding alginate lyase.
12. Azotobacter vinelandii alginate lyase (algL) gene
13. Vibrio halioticoli lyase AlyVGⅢ gene
14. Vibrio halioticoli lyase AlyVG Ⅰ gene
15. Vibrio halioticoli lyase AlyVM Ⅰ gene
16. Vibrio halioticoli lyase AlyVG Ⅱ gene
17. Agarobacterium tumefaciens str, C58 chromosome linear
18. Yersinia pestis strain CO92
19. Bacillus halodurans
20. Salmonella enterica subsp. enterica serovar Typhi
21. Alteromonas sp.
22. Mesorhizobium loti DNA, complete genome, section 19/21
23. Halomonas marina gene for alginate lyase
24. Photobacterium sp. alginate poly(ManA) lyase gene

Table 5. PCR primers designed from GenBank databases

Pimer	Sequence
No.1_77f	5'-CAACGCCAGAAAACGATGATAT-3'
No.1_677r	5'-CCCATCTTTTCACACCACTCTG-3'
No.4_31f	5'-CCGATTGAACATAAGGAAAG-3'
No.4_592r	5'-CTGCTCCTAACTTTCGTACC-3'
No.5_118f	5'-GACCGTGCGTATATTTCTCA-3'
No.5_632r	5'-TTGCTATTGACGGAAACATT-3'
No.6_1f	5'-ATGAATGGAAACGACAACTG-3'
No.6_952r	5'-GTTGCCACATGATCCTATTC-3'
No.10_249f	5'-CAAAGTCGTGATGCAGTACA-3'
No.10_734r	5'-GGCAGTGCATAGTTGTGATA-3'
No.14_206f	5'-CGGCAGATAATGAATTTGTC-3'
No.14_650r	5'-CTAGGTCCGTTGCTGTATTG-3'
No.19_450f	5'-CTACCTGCGCCTGAAGTTCT-3'
No.19_944r	5'-TCGGTCTCCAGATCCTCCAT-3'
No.21_28f	5'-TTGGCACCGACACTACTGAG-3'
No.21_481r	5'-TCAGGCGAATGTACGAAGAC-3'
No.24_71f	5'-GCACTGCTGAAAAACCAATT-3'
No.24_649r	5'-CGTGCCTAAAAGTAACCGTC-3'
No.26_58f	5'-GCAGGTGTCGAATTTTCTAA-3'
No.26_690r	5'-GCCTACGGTAATGGTGAAAT-3'

Table 6. Re-designed PCR primers for the cloning of full ORF of alginate lyase gene

Pimer	Sequence	
Alg_pseudo_446f	5'-CGAGTT**CATATG**TACAGGTTTGGAG -3'	Nde I
Alg_pseudo_1658r	5'-T**GAATTC**TTAATTCGTCCTACGCG -3'	EcoR I
Alg_pseudo_1658r _BamHI	5'-T**GGATCC**TAAATTCGTCCTACGC -3'	BamH I

* Alg_pseudo_446f/ Alg_pseudo_1658r primer combination was used for cloning of pALPET44a clone.

* Alg_pseudo_446f/ Alg_pseudo_1658r_BamHI primer combination was use for cloning of pALPET25b-His clone.

Subcloning of alginate lyase gene with pET expression vector system

Alginate lyase gene was subcloned into pET expression vector system for overexpression. For the cloning of alginate lyase gene expressed with native form, pALT4 (pGEM T-easy vector including alginate lyase gene) and pET44a clones were double digested with Nde I /EcoR I and DNA band on the agraose gel was purified with GeneClean Kit (Q-BIOgene, Canada). The purified insert and vector was ligated at room temperature for 6 hrs and transformed into BL21 (DE3) *E.coli* strains. The resulting pALPET44a clone (Fig. 1) was used for overexpression of the alginate lyase. For purification of recombinant enzyme, pALT4' was subcloned into pET25b expression vector with double digestion of Nde I /BamH I . Cloning procedures were same as described above. The resulting pALPET25b-His clone was used for purification of alginate lyase (Fig. 1).

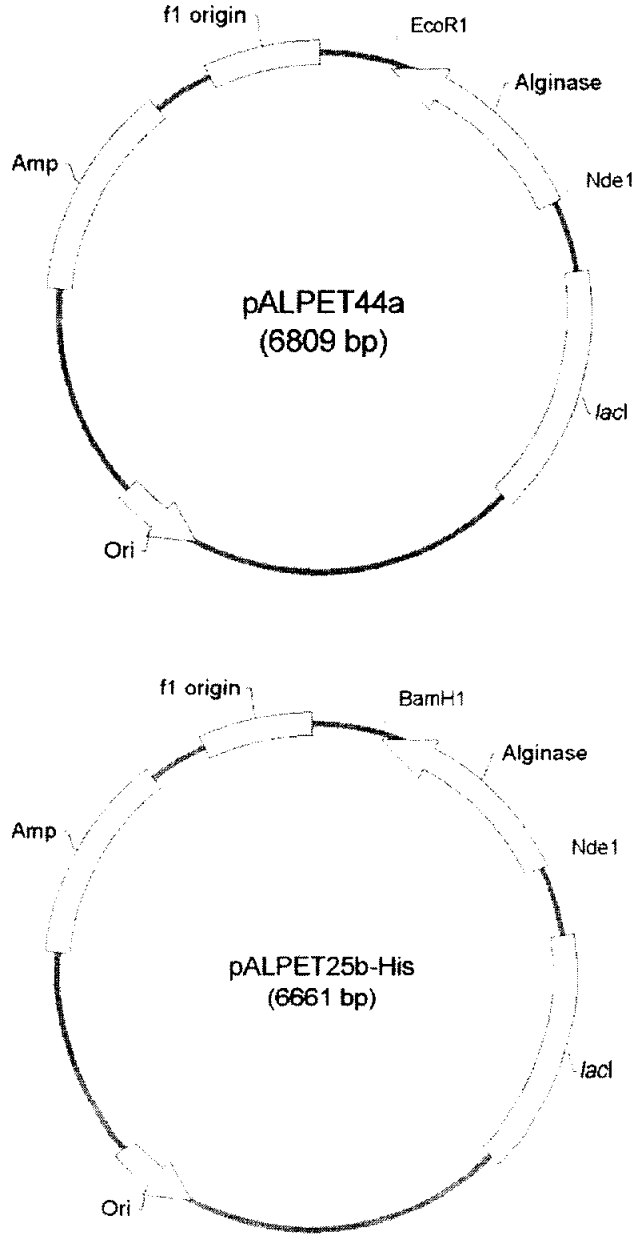


Fig. 1. plasmid map of pALPET44a and pALPET25b-His

Overexpression and purification of alginate lyase

E. coli transformed with the pALPET44a clone and the pALPET25b-His clone were cultured in LB broth containing ampicillin (50 ㎍/㎖). When OD_{600} reach to 0.5, the cell was induced with IPTG at the final concentration of 1 mM. After induction, 3 ml of cell was harvested at 2, 4, 6 hours. The cell was centrifuged and the pellet was heated at 100℃ for 5 min with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer. SDS-PAGE analysis of the expressed protein was performed using 12.5 % polyacrylamide gel according to the method of Laemmli (1970). Overexpressed alginate lyase from pALPET25b-His was purified with His-Bind Purification Kit (Invitrogen, CA, USA) according to protocols.

RESULTS

Isolation of Microorganisms

Eighteen bacteria strains showing alginate lyase activity were isolated from decayed brown seaweed, sea water, soil and sea tangle (Table. 7). Four strains showing high activity were selected and alginate lyase activity was tested by Somogyi-Nelson method. Alg-250 strain that showed the highest activity of alginate lyase was used to clone the alginate lyase gene.

Table 7. Samples used for the isolation of alginate degrading bacteria and the number of bacterial strains isolated from the samples.

Source	Strain number
Soil	4 strains
Sea water	1 strains
Brown seaweed	6 strains
Sea tangle	7 strains

Table 8. The alginate lyase activity of isolated strains.

Strain	Cell growth (OD_{600})	Reducing sugar (mg/ml)*
Alg-250	1.201	0.632
Alg-12	0.963	0.421
Alg-11	1.102	0.365
Alg-04	0.812	0.142

Table. 9. Biochemical characterization of *Pseudoalteromonas* sp.

Biochemical tests were conducted using VITEK GNI Card (Biomerieux, Marcy-l'Eto, Frnace)

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 ℃	-
* O-Nitrophenyl		* Utilization as sole carbon source	
β-D-galactopyranoside	-	Acetamide	-
Fermentation		Esculin	+
* Decarboxylase Negative control	-	Citrate	-
* Enzyme acitivity		Malonate	-
Catalase	+	Glucose	-
Urease	-	Arginine	+
Tryptophan deaminase	-	Lysine	-
β-galactosidase	-	Ornithine	-
Arginine digydrolase	+	Raffinose	-
Lysine decarboxylase	-	Sorbitol	-
Ornithine decarboxylase	-	Sucrose	+
Oxidase	+	Inositol	+
		Adonitol	-

16s rDNA sequencing analysis

The result of 16s rDNA sequence analysis of marine bacterium showing alginate lyase activity was aligned with 16s rDNA sequences in GenBank databases through the BLAST search. The BLAST search result was showed that the sequences had a 99% homology with *Pseudoalteromonas elykovii*.

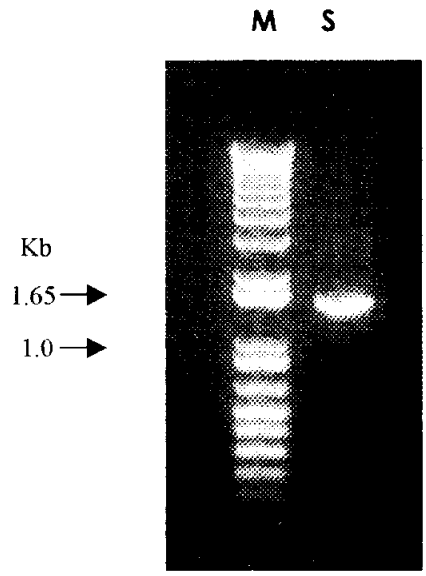


Fig 2. PCR products of 16s rDNA gene on 1% agarose. PCR band sizes are about 1.4 kb (M, 1 kb plus DNA ladder; S, PCR product)

GTGGATCCGGCTACCTTGTTACGACTTCACCCCAGTCATGAATCACTCCGTGGTAAACGTCCTCC
CGAGGGTTAGACTATCTACTTCTGGAGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAG
GCCCGGGAACGTATTCACCGCGTCATTCTGATACGCGATTACTAGCGATTCCGACTTCATGGAGT
CGAGTTGCAGACTCCAATCCGGACTACGACGCACTTTAAGTGATTCGCTTACTCTCGCGAGTTCG
CAGCACTCTGTATGCGCCATTGTAGCACGTGTGTAGCCCTACACGTAAGGGCCATGATGACTTGA
CGTCGTCCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCTTAGAGTTCTCAGCATTACCTGCT
AGCAACTAAGGATAGGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACAACACGAGCTGA
CGACAGCCATGCAGCACCTGTATCAGAGCTCCCGAAGGCACCAAACCATCTCTGGTAAGTTCTCT
GTATGTCAAGTGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGT
GCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCTACTTAA
TGCGTTAGCTTTGAAAAACAGAACCGAGGCTCCGAGCTTCTAGTAGACATCGTTTACGGCGTGGA
CTACCGGGGTATCTAATCCCGTTTGCTCCCCACGCTTTCGTACATGAGCGTCAGTGTTGACCCAG
GTGGCTGCCTTCGCCATCGGTATTCCTTCAGATCTCTACGCATTTCACCGCTACACCTGAAATTC
TACCACCCTCTATCACACTCTAGTTTGCCAGTTCGAAATGCAGTTCCCAGGTTGAGCCCGGGGCT
TTCACATCTCGCTTAACAAACCGCCTGCGTACGCTTTACGCCCAGTAATTCCGATTAACGCTCGC
ACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGTCAGAGCGTCACA
GCTAGCAGGTATTAACTACTAACCTTTCCTCCTGACTGAAAGTGCTTTACAACCCGAAGGCCTTC
TTCACACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTC
CCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCATCCTCTCAAACCAGCTAGGGA
TCGTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCCCACTTGGGCCAATCTAAAGGCGA
GAGCCGAAGCCCCCTTTGGTCCGTAGACATTATGCGGTATTAGCAGTCGTTTCCAACTGTTGTCC
CCCACCTCAAGGCATGTTCCCAAGCATTACTCACCCGTCCGCCGCTCGTCAGCAAAGTAGCAAGC
TCCTTTCTGTTACCGCTCGACTTGCATGTCTAAGAATTCT

Fig. 3. Nucleotide sequences of 16s rDNA

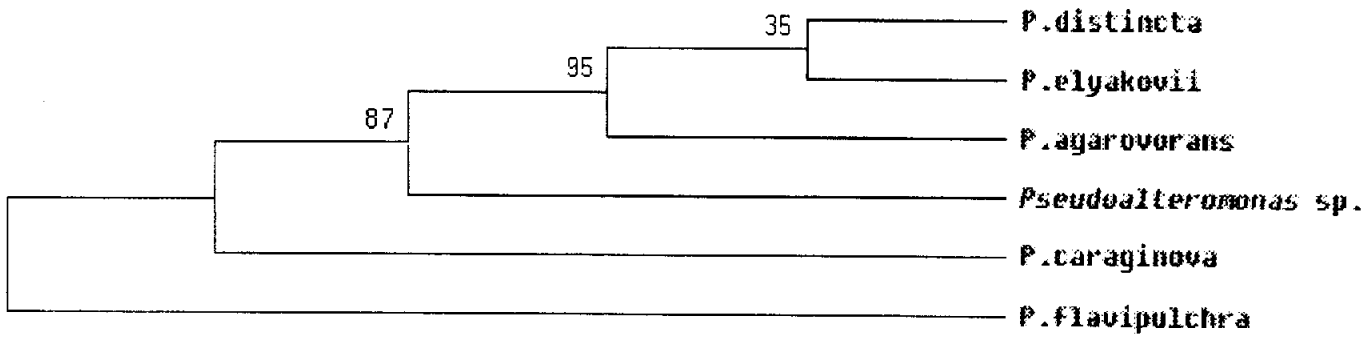


Fig 4. Phylogenic tree of *Pseudoalteromonas* sp. Unrooted tree obtained using a neighbour-joining algorithm

Cloning of alginate lyase gene

A PCR product of 1197 bp was obtained from the PCR with Alg_pseudo_446f and Alg_pseudo_1658r primer(Fig. 2). Purified PCR product was cloned into pGEM T-easy vector and analyzed with ABI 377 XL upgrade DNA sequencer. The analyzed sequences show in Fig. 3 and these sequences were aligned with other gene sequences in GenBank database through the BLAST search. It had a 99% homology with alginate lyase gene from *Pseudoalteromonas elyakovii*. There are 9 nucleotide nucleotide substitution in 1197 compare with *Pseudoalteromonas elyakovii* from GenBank databases and shows a new restriction site (Xho I , underlined, bold in Fig. 5) in the sequences.

ATGTACAGGTTTGGAGAAATAAAGATAATGATTAACCAAAAATCCCTATTTATGCTTGCA

M Y R F G E I K I M I N Q K S L F M L A

GCCATGACTGCTAGCTCAAGTTTTGTACAAGCGGCGACGATCAATAATGCTGGGTTTGAA

A M T A S S S F V Q A A T I N N A G F E

GATGGTTGGAGTAACTGGAATGAAACAGAACCTGCAGCTATCTCAGGTAGTGCTTATAAG

D G W S N W N E T E P A A I S G S A Y K

GGCTCTAAATCATTAAAAATTCAAGGTAGCCCAGGGCGTGTTTACCAGAATGTAGATGTA

G S K S L K I Q G S P G R V Y Q N V D V

GATCGAAATACTCAGTACACGTTAAGTGCGTATGTACTTGGTAAGGGTCAAATCGGCATT

D R N T Q Y T L S A Y V L G K G Q I G I

AACGATCTTAATGGCTTGTTTAAAAATGAGAAATTTAACGTTTCTTCATGGACAAAAGTA

N D L N G L F K N E K F N V S S W T K V

TCTAGAACTTTCACCACAGCAAATACAGGTACGCTACAAGTATTTGCCAAGCACGATAAG

S R T F T T A N T G T L Q V F A K H D K

AG**CTCGAG**CGATGTTCGTTTTGATAACTTTTCATTAACTAAGGGCGCTTCAGGTGGCGGT

S S S D V R F D N F S L T K G A S G G G

GATACTGGCGGCGGCGACACTGGTAGTGGCTCTGGTATAGCAAGCAACATCACCAACGGT

D T G G G D T G S G S G I A S N I T N G

AGTATTTTTGACCTTGAAGGTAACAATCCTCATCCATTAGTAAATAGCAATACTTTAGAG

S I F D L E G N N P H P L V N S N T L E

TTTGTTCCTCTANAAGCGCGTCATATCACTCCAAACGGTAATGGCTGGCGTCATGAATAT

F V P L X A R H I T P N G N G W R H E Y

AAAGTAAAAGAAAGTGCTCGTGCTGCAATGACCGAAACCTATGAGGTTTTTGAGGCAACC

K V K E S A R A A M T E T Y E V F E A T

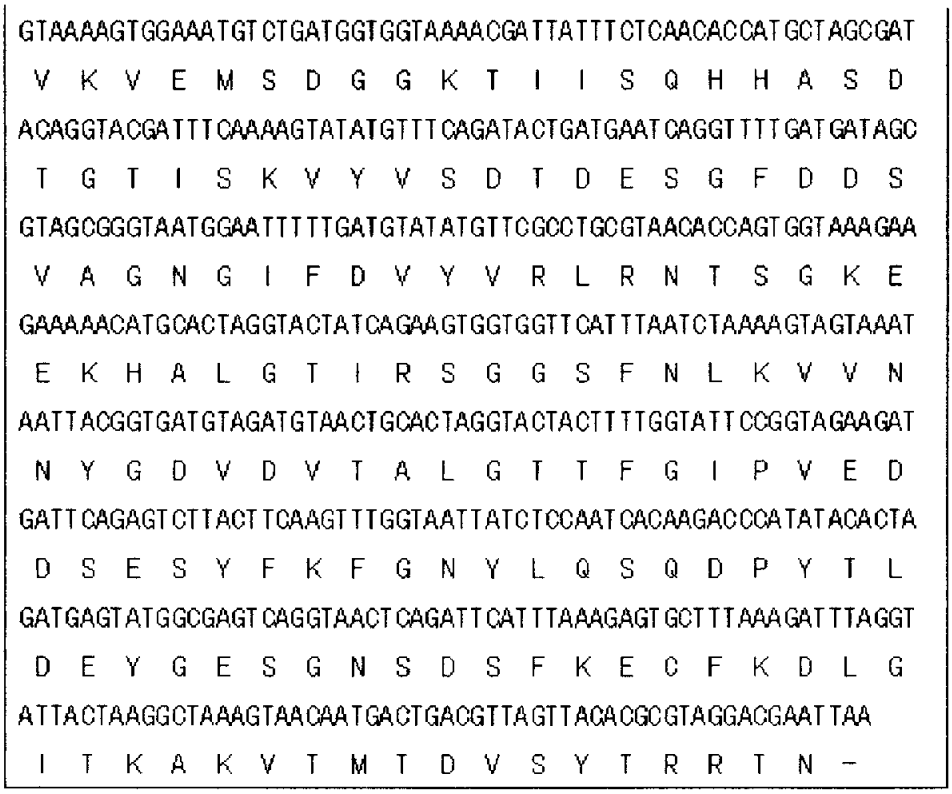


Fig. 5. Alginate lyase sequence of *Pseudoalteromonas* sp.

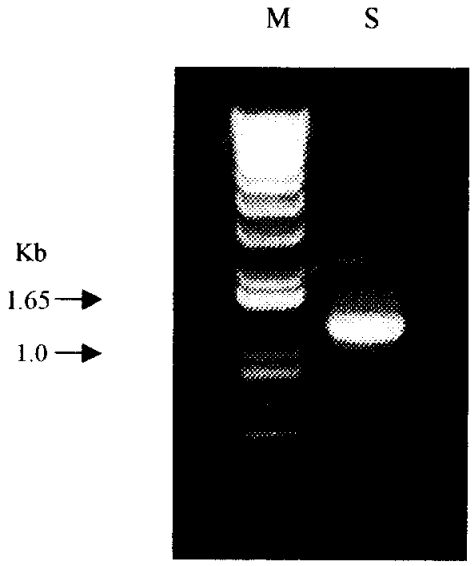
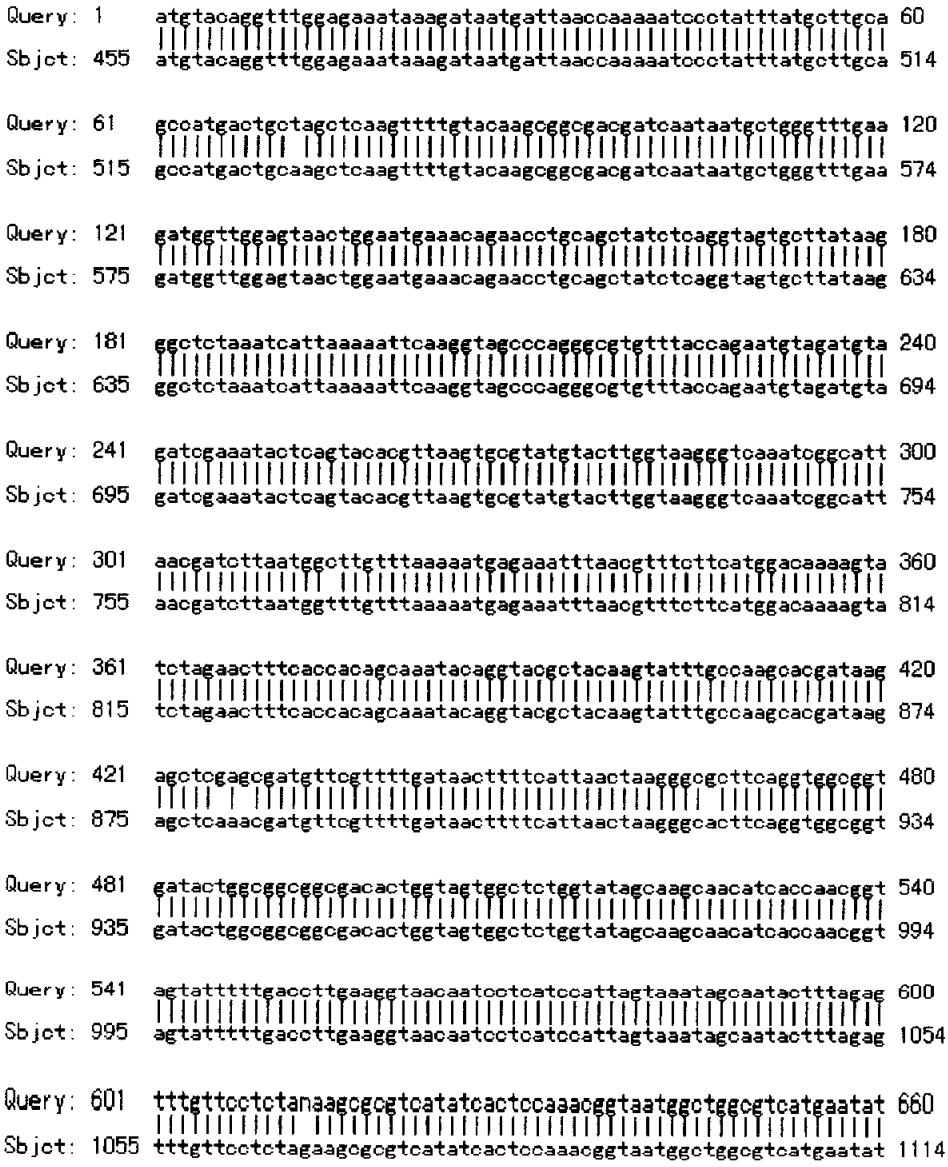


Fig. 6. PCR product of alginate lyase gene on 1% agarose gels. PCR band sizes are about 1.2 kb (M, 1kb plus DNA ladder; S, PCR product)


Query: 1 atgtacaggtttggagaaataaagataatgattaaccaaaaatccctatttatgcttgca 60
Sbjct: 455 atgtacaggtttggagaaataaagataatgattaaccaaaaatccctatttatgcttgca 514
Query: 61 gccatgactgctagctcaagttttgtacaagcggcgacgatcaataatgctgggtttgaa 120
Sbjct: 515 gccatgactgcaagctcaagttttgtacaagcggcgacgatcaataatgctgggtttgaa 574
Query: 121 gatggttggagtaactggaatgaaacagaacctgcagctatctcaggtagtgcttataag 180
Sbjct: 575 gatggttggagtaactggaatgaaacagaacctgcagctatctcaggtagtgcttataag 634
Query: 181 ggctctaaatcattaaaaattcaaggtagcccagggcgtgtttaccagaatgtagatgta 240
Sbjct: 635 ggctctaaatcattaaaaattcaaggtagcccagggcgtgtttaccagaatgtagatgta 694
Query: 241 gatcgaaatactcagtacacgttaagtgcgtatgtacttggtaagggtcaaatcggcatt 300
Sbjct: 695 gatcgaaatactcagtacacgttaagtgcgtatgtacttggtaagggtcaaatcggcatt 754
Query: 301 aacgatcttaatggcttgtttaaaaatgagaaatttaacgtttcttcatggacaaaagta 360
Sbjct: 755 aacgatcttaatggtttgtttaaaaatgagaaatttaacgtttcttcatggacaaaagta 814
Query: 361 tctagaactttcaccacagcaaatacaggtacgctacaagtatttgccaagcacgataag 420
Sbjct: 815 tctagaactttcaccacagcaaatacaggtacgctacaagtatttgccaagcacgataag 874
Query: 421 agctcgagcgatgttcgttttgataacttttcattaactaagggcgcttcaggtggcggt 480
Sbjct: 875 agctcaaacgatgttcgttttgataacttttcattaactaagggcacttcaggtggcggt 934
Query: 481 gatactggcggcggcgacactggtagtggctctggtatagcaagcaacatcaccaacggt 540
Sbjct: 935 gatactggcggcggcgacactggtagtggctctggtatagcaagcaacatcaccaacggt 994
Query: 541 agtatttttgaccttgaaggtaacaatcctcatccattagtaaatagcaatactttagag 600
Sbjct: 995 agtatttttgaccttgaaggtaacaatcctcatccattagtaaatagcaatactttagag 1054
Query: 601 tttgttcctctanaagcgcgtcatatcactccaaacggtaatggctggcgtcatgaatat 660
Sbjct: 1055 tttgttcctctagaagcgcgtcatatcactccaaacggtaatggctggcgtcatgaatat 1114

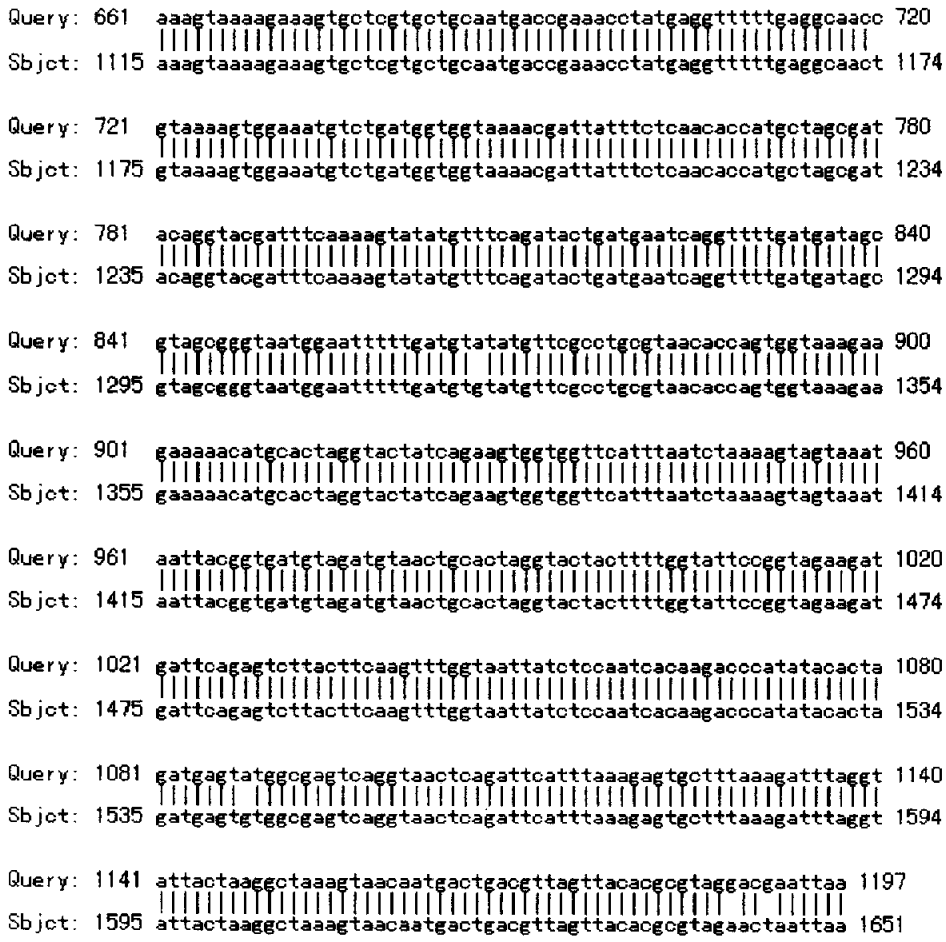


Fig. 7. Alignment of alginate lyase gene with *Pseudoaltero-monas elyakovii* algiante lyase gene Query, sequences of pALT4 clone; Sbjct, alginate lyase gene of *Pseudoalteromonas elyakovii*

Expression, purification and activity test of recombinant enzyme

The overexpressed alginate lyase having the pALPET44a construct was found to form an insoluble inclusion body because the protein band was detected in the pellet of *E.coli* lysate(Fig. 8). The protein was purified with His-Bind Purification Kit (Invitrogen, CA, USA). The molecular weight of purified protein was estimated to be 52 kDa (Fig. 8). When the cell harboring pALPET44a or pALPET25b-His was cultured at 37℃ with or without induction, enzyme activity was not detected. However, alginate lyase activity was detected in the cell harboring pALPET25b-His cultured at 25℃ without induction (Fig. 9).

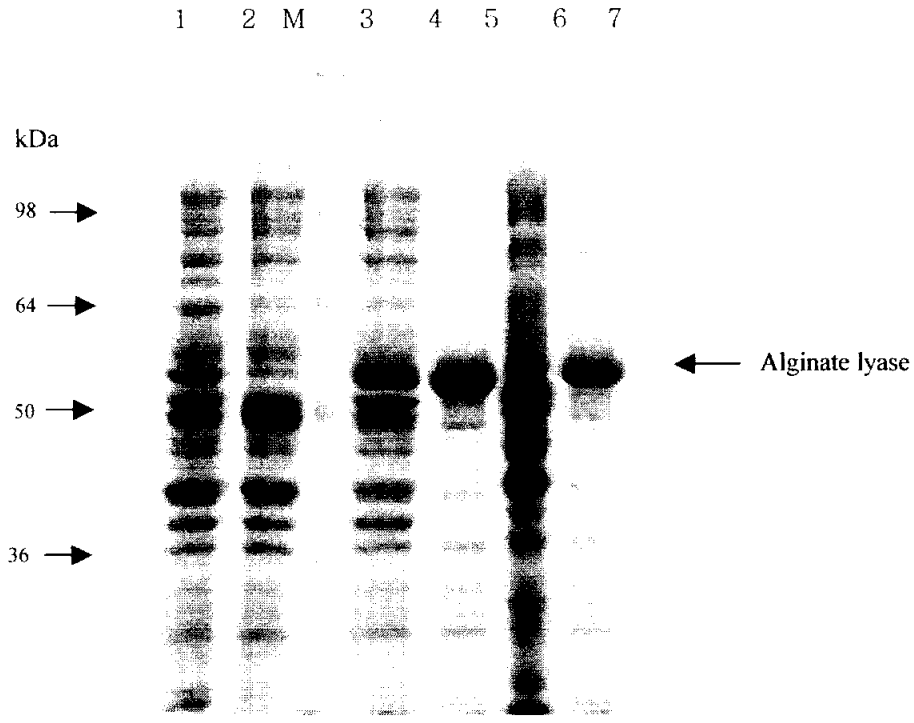


Fig. 8. SDS-PAGE of overexpressed and purified alginate lyase. Lane M, protein marker; lane 1, BL21(DE3); lane 2, induced native alginate lyase (pALET44a); lane 3, pALPET25b-His without inductoin; lane 4, total cell of pALPET25b-His with induction; lane 5, soluble fraction of induced pALPET25b-His; lane 6, insoluble fraction of pALPET25b-His with induction; lane 7, purified alginate lyase with His-Bind Purification Kit.

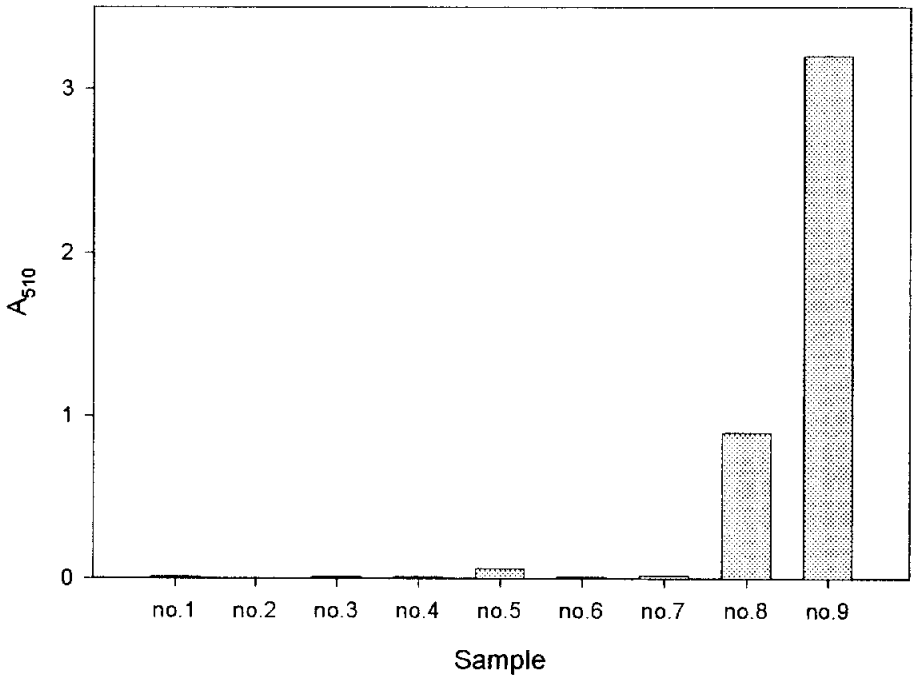


Fig. 9. Activity of alginate lyase. No.1- 4; cell grown at 37℃ (1; pALPET44a with induction, 2; pALPET44a without induction, 3; pALPET25b-His with induction, 4; pALPET25b-His without induction). No.5- 8; cell grown at 25℃ (5; pALPET44a with induction, 6; pALPET44a without induction, 7; pALPET25b-His with induction, 8; pALPET25b-His without induction). No. 9; alginate lyase activity of wild type strain

DISCUSSION

As new source of substances with physiological activity on diseases of adult people such as hypertension, heart attack, and diabetes, nutritious and pharmaceutical interests to marine algae have been increased. The effect of oligosaccharide from marine polysaccharide such as carrageenan, alginate, agar on these diseases have been reported (Bang and Dyerberg, 1972; Ito and Tsuchiya, 1972; Suzuki *et al.*, 1993; Ju and Lee, 1993). Up to now, hydrolytic method has been used to produce oligo alginate. But this method costs a lot because of long incubation at high temperature. This method also causes problem of environmental pollution. Several researches have been performed with bacteria that have alginate lyase to solve this problem (Suzuki *et al.*, 1993; Ju and Lee, 1993).

In order to screen marine bacteria producing alginate lyase, decayed brown showing pathogenic damage was collected at the southeastern coast of Korea. An alginate lyase producing bacterium was identified as *Pseudoalteromonas* sp. by sequence anlysis of its 16s DNA (Fig. 3). The new genus *Pseudoalteromonas* was proposed on the basis of the phylogenetic study by Gauthier *et al.*(1995). Small subunit (16S) ribosomal RNA sequence analysis

was performed on *Alteromonas, Shewanella, Vibrio,* and *Pseudomonas* species using three different phylogenetic algorithms. *Pseudoalteromonas* strains are arerobic, Gram-negative, nonsporeforming, straight or slightly curved, rod-shaped bacteria of 0.2-1.5 ㎛ x 1.8-4.0 ㎛. Strains of most species are motile by means of single unsheathed polar flagellum. The majority of *Pseudoalteromonas* species is characterized by high hydrolytic activities, and also produce gelatinase, lipase, caseinase, lecithinase and DNAses. They utilize D-glucose as a sole carbon source, but are negative for utilization of D-ribose, L-rhamnose, turanose, salicin, glucuronate, DL-glycerate, erythritol, sorbitol, meso-inositol, adonitol, L-valine, L-ornithine and m-hydroxybenzoate. Some species display amylase, alginase and chitinase activities(Novic and Tyler, 1985; Enger et al., 1987).

For the cloning of alginate lyase gene from the isolated bacterium, the known sequences of alginate lyase gene from 24 species were collected from GenBank database (Table. 4). In order to design a degenerative PCR primer from these sequences, multiple alignment with whole sequences was conducted. However, there was wide range of variation such as its sources, sequence lengths, and homologies. Therefore, the sequences were grouped by its sources and homologies, and PCR primers were designed from each group using multiple

alignment (Table. 5). The PCR reactions were conducted with conditions proper each primer and a PCR product of about 600 bp was obtained with the No. 6 primer in Table. 5. The PCR product was cloned and showed 99% homology with alginate lyase gene from *Pseudoalteromonas elyakovii*. In order to clone the full ORF of the alginate lyase gene, primers were re-designed from a previous research (Sawabe *et al.*, 2001). The full length PCR product was cloned into the pGEM T-easy vector, resulting the pALT4 (pGEM T-easy vector + alginate lyase gene, including a Nde I and EcoR I restriction enzyme site for subcloning). There are 9 nucleotide substitution in 1197 compare with *Pseudoalteromonas elyakovii* from GenBank databases and shows a new restriction site (Xho I , underlined, bold in Fig, 5) in the sequences. Another T-vector clone named pALT4' has Nde I and BamH I restriction enzyme sites for subcloning into pET vector system. The inserts from pALT4 and pALT4' were subcloned to construct pALPET44a and pALPET25b-His, respectively.

The overexpressed alginate lyase from the pALPET44a clone formed an insoluble inclusion body and the molecular weight was about 47 kDa (Fig. 8). It is bigger than the alginate lyase from *Alteromonas* sp. strain H-4 (32 kDa, Sawabe *et al.*, 1992), *Halomonas marina*(39kDa, Kraiwattanapong *et al.*, 1999), *Pseudoalteromonas alginovara* (28kDa, Byen *et al.*, 1990), *Vibrio* sp.(40 kDa,

Takeshita *et al.*, 1999). The overexpressed alginate lyase from pALPET25b-His also fromed an insoluble inclusion body and its molecular weight was about 52 kDa (Fig. 8). However, the protein was easily purified with His-Bind Purification Kit (Invitrogen, CA, USA) after denaturation using the histidine tag at the end. When the cell harboring the pALPET44a or pALPET25b-His was cultured at 37℃ with or without induction, enzyme activity was not detected. Alginate lyase activity was detected in the cell harboring the pALPET25b-His construct when the cell was cultured at 25℃ without induction (Fig. 9). However, the activity was only one third of alginate lyase activity from wild type crude enzyme. Because the enzyme activity test was not conducted with same amount of protein, it looks like the expression level of the enzyme in un-induced E.coli is very low. So the recombinant enzyme doesn't have an activity than wild type. Therefore, more researches such as protein refolding, chaperone co-expression will be acquired to improve alginate lyase activity.

국문초록

최근 식생활이 서구화됨에 따라 비만, 동맥경화, 협심증, 당뇨병 등의 여러 가지 성인성 질병에 대한 예방과 치료에 식이섬유의 약리효과가 입증됨으로써 해조 다당류의 이용에 대한 관심과 기술개발이 집중되고 있다. 해조 다당류의 식이섬유 효과와 약리효과는 그 중합도를 낮추었을 때 원래의 다당류에 비해 그 효과가 증대되며 갈조류 중에 다량 함유되어 있는 알긴산은 혈장 콜레스테롤 저하효과, 항혈액 응고효과, 항암효과 등의 약리효과와 식이섬유로서의 영양상의 효과가 높은 것으로 밝혀졌다(주동식 et al.1993a). 본 연구에서는 해양 미생물(*Pseudoalteromonas* sp.) 유래 알긴산 분해 효소 유전자를 분리, 클로닝하여 재조합 효소생산을 통한 저분자화 알긴산 생산의 기초를 마련하고자 하였다.

다시마 병반 부위에서 분리한 alginate lyase 생성 균주는 16S rDNA sequence 분석결과 *Pseudoalteromonas* sp.로 동정되었고, NCBI의 여러 문헌과 DB를 이용하여 얻은 본 연구의 alginate lyase clone은 sequence 분석결과 *Pseudoalteromonas elyakovii* 균주의 alginate lyase gene과 99% 이상의 homology를 보였다. 본 연구에서 알려진 두 염기서열간의 차이점은 Pseudoalteromonas elyakovii 균주의 algiante lyase gene과 본 연구의 algiante lyase gene이 전체 1197개의 염기서열중에서 11개의 염기치환으로 서열차이를 보였으며, 이 염기치환으로 인해 서열내에 Xho I 이라는 새로운 제한효소 사이트가 확인되었다. Full ORF를 포함하여 발현시킨 pALPET44a 균주는 overexpression 결과 47 kDa 정도의 크기를 나타내었으며, His bind purification Kit를 이용해 정제한 pALPET25b-His 균주는 52 kDa 정도의 단백질 크기를 보였다. *E. coli*에서 발현되는 대부분의 단백질이 inclusion body를 형성하는 바와 같이 본 연구의 alginate lyase 역시 inclusion body를 형성하여 효소의 활성이 나타나지 않았지만, 여러 조건에서의 발현을 확인해 본 결과 25℃에서 induction 없이 배양한 균주에서 알긴산 분해효소능이 가장 높게 나타난 것으로 확인되었다.

REFERENCES

Bang, H.O. and Dyerberg, J., 1972. Plasma lipids and liporteins in Greenladic West Coast Eskimo. *Acta. Med. Scand.*, 192: 85-94

Hashimoto, W., Miyake, O., Momma, K., Kawai, S., and Murata, K. 2000. Molecular identification of oligoalginate lyase of Sphingomonas sp. strain A1 as one of the enzymes required for complete depolymerization of alginate. *J. Bacteriol.* 182: 4572-4577.

Ito, K. and Tsuchiya, Y. 1972. The effect of agal polysaccharides on the depressing of plasma cholesterols in rat. In "Proc. of 7'th Int. Seawed Symp." Nishizawa, K. ed., Univ. Tokyo Press. Tokyo. Japan. Pp 558-561

J. Kraiwattanapong, H. Tsuruga, T. Ooi and S. Kinoshita, 1999. Cloning and sequencing of a *Deleya* marina gene coding for alginate lyase. *Biotechnology Letters*, 21: 169-174

Joo, Dong-Sik, Jung-Suk Lee, Jung-Je Park, Soon-Yong Cho, Chang-Bum Ahn and Eung-Ho Lee, 1995. Purification and characterization of the intracellular

alginase from *Vibrio* sp. AL-145. *Kor. J. Appl. Microbiol. Biotechnol.* 23: 432-438

Joo, Dong-Sik, Jung-Suk Lee, Soon-Yeong Cho, Sung-Jae Shin and Eung-Ho Lee, 1995. Changes in functional properties of alginic acid by enzymatic degradation. *Korean J. Food Sci. Technol*, 27: 86-91.

Kennedy, L., McDowell, K., and Sutherland, I.W., 1992. Alginases from *Azotobcter* species. *J. Gen. Microbiol.* 138: 2465-2471.

Kidamikado, M., Hishimura, M., Yamaguchi, K., and Tseng, C-H., 1993. Bacteriostatic action of oligosaccharides prepared from alginate by enzymatic degradation. *Nippon Susian Gakkaishi*. 59: 315-320.

Kong, In-Soo, Young-Ok Kim, Jin-Man Kim, Sung-Koo Kim, Doo-Hwan Oh, Ju-Hyun Yu and Jai-Yul Kong, 1995. Alginate lyase production of Halophilic *Pseudomonas* sp. by recombinant *E.coli*. *J. Microbiology and Biotechnology*. 5: 92-95

Michael J. Franklin and Dennis E. Ohman, 2002. Mutant analysis and cellular localization of the AlgI, AlgJ, and AlgF proteins required for O acetylation of alginate in *Pseudomonas aeruginosa*. *Journal of Biotechnology*. 184: 3000-3007.

Murata K, Inose T, Hisano T, Abe S, and Yonemoto Y, 1993. Bacterial alginate lyase: enzymology, genetics and application. *J. Fermen. Bioeng*. 76: 427-437.

Nelson N, 1944. A photometric adaptation of the somogyi method for the determination of glucose. *J. Biol. Chem*. 153: 375-380

Peter Gacesa and Frederick S. Wusteman, 1990. Plate assay for simultaneous detection of alginate lyases and determination of substrate specificity. *Applied and Environmental Microbiology*, 56: 2265-2267.

R. Scott Doubet and Ralph S. Quatrano, 1984. Properties of alginate lyases from marine bacteria. *Applied and Environmental Microbiology*, 47: 699-703.

Sawabe T, Ohtsuka M and Ezura Y, 1997. Novel alginate lyase from marine bacterium *Alteromonas* sp. strain H-4. *Carbo. Res*. 304: 69-76.

Suzuki, T., Nakai, Y. Yoshie, T., Shiral, T., and Hirano, T. 1993. Effect of sodium alginates rich in guluronic and mannuronic acids on cholesterols and digestive organs of high-cholesterol fat rats. *Nippon Suisan Gakkaishi*. 59: 545-551.

Thiang Yian Wong, Lori A. Preston and Neal L. Schiller, 2000. Alginate Lyase: review of major sources and enzyme characteristics, structure-function analysis, biological roles. *Annu. Rev. Microbiol.* 54: 289-340

Tomoo Sawabe, Hiromasa Takahashi, Yoshio Ezura and Peter Gacesa, 2001. Cloning, sequence analysis and expression of *Pseudoalteromonas elyakovii* IAM 14594 gene (alyPEEC) encoding the extracellualr alginate lyase. *Carbo. Res.* 335: 11-21.

Yasuhito Matsubara, Ryoko Kawada, Ken-ichi Iwasaki, Tatsuya Oda, and Tsuyoshi Muramatsu, 1998. Extracellular poly(α-L-guluronate) lyase from Corynebacterium sp.: Purification, characteristics, and conformational properties. *Journal of Protein Chemistry*, 17: 29-36

Yoshiko Iwamoto, Kenichi Iriyama, Kiyoshi Osatomi, Tatsuya Oda, and Tsuyoshi Muramatsu, 2002. Primary structure and chemical modification of some amino acid residues of bifunctional alginate lyase form a marine bacterium *Pseudoalteromonas* sp. strain NO. 272. Journal of Protein Chemistry, 21: 455-463