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The Discoveery of Agarolytic Bacterium with Agrarse Gene Containing Plasmid, and Sone Enzymology Characteristics

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Abstract: An aerobic, Gram negative extracellular agarase producing bacillus was discovered in Taitung, Taiwan. It has a small plasmid containing an agarase gene with high enzyme activity. This bacterium was identified as *Alcaligene*s sp. strain Yen (AY744384) by 16S RNA sequencing comparison and MicroScan-AS4 automatic microorganism analyzing data. The intact plasmid was successfully transformed into competent *E. coli* HB 101 cells for the first time. The enzyme was purified from culture supernatant. The enzyme had a specific agarase activity of 406 U/mg. The plasmid was estimated 18.4 kb by comparing to electrophoresis markers. This agarase couldn't degrade ι-carrageenan and κ-carrageenan, but could degrade sodium alginate. The enzyme's acting target was β-1,4 bond, so, the enzyme was recognized as β type. This is a discovery of a previously undescribed small agarolytic plasmid and the first time transformation of the intact agarase gene containing plasmid into *E.coli*. Some enzyme characteristics were also described.

Keywords: agarolytic bacterium; agarase plasmid; *Alcaligene*s sp. strain Yen

1. Introduction

Agar, a polysaccharide present in the cell walls of some red algae is composed of agarose (70%) and agaropectin (30%). Agarose is a linear polysaccharide composed of alternating residues of 3-O-linked *β*-D-galactopyranose and 4-O- linked 3,6-anhydro-*α*-L-galactopyranose [1]. Agar-degrading bacteria may utilize agar as sole carbon and energy source [2]. Agar can be degraded by several bacterial strains, including *Agarviorans albus* strain MKT106T [3], *Alteromonas* sp. strain C-1 [4,5] *Pseudoalteromonas citrea* [6], *Pseudoalteromonas antarctica* N-1 [7], *Pseudoalteromonas* sp.strain CKT1[8], *Pseudoalteromonas agarivorans* [9], *Thalassomonas* strain JAMB-A33 [10], *Thalassomonas agarivorans* [11], *Reichenbachia agariperfornas* KMM 3252^T [12], *Zobellia amurskyensis*; *Zobellia laminariae*; *Zobellia russellii* [13,14], *Cellulophaga pucifica* [15], *Echinicola pacifica* [16], *Microbulbifer* strain JAMB-A7 [17], *Bacillus* sp. MK03 [18], and *Paenibacillus* spp. [19] etc.

Agarase can be used to degrade the cell walls of marine algae for extraction of labile substances with biological activities and for

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the preparation of protoplasts [20], as well as isolation of monoclonal hybrids [21]. The polysaccharide fractions can be applied for functional foods [22]. Agarase have applications in food, cosmetics, and medical industries by degrading agar [23]. The polysaccharides produced by hydrolysis of agar can promote immunity in mice by abdominal injection or feeding [22]. Some researches have shown that adding 5% agaropectin to diet suppressed significantly the increasing in cholesterol level in plasma of rats. Anti-hypercholesterolemic effect of rats also was observed [24]. Agarase also could be adapted for molecular biology applications such as extraction of DNA or RNA fragments from agarose gel [25].

In this study, we described the isolation of a plasmid from an agarolytic bacterium *Alcaligenes* sp. strain Yen. The bacterium could produce secretory agarase with high agar-degrading ability. The bacterium was purified from a Taitung city ditch (Taiwan). The plasmid has been transformed into competent *E. coli* HB 101 cell and the transformed cells could form obvious pits on agar surface no more than 18hrs after incubation. We also described the zymogram characterized of the extracellular agarase. We report a previously undescribed small plasmid containing an agarase gene with high agarase activity and for the first transformation of intact agarase gene containing plasmid into *E.coli*.

2. Materials and Methods

Sample collection and screening of agar-degrading microorganisms

For the screening of agarolytic bacteria, city drainer water samples from National Taitung University Taitung City Campus front road side ditch were collected（30 cm wide, 50 cm in depth, with 20 cm deep ordinary house waste water）and spread on C-10 agar plates. The compositions for C-10 medium were (per liter): HEPES (N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid) 1.2 g, $MgSO_4.7H_2O$ 0.25 g, K_2HPO_4 0.05 g, $Ca(NO_3)_2{\cdot}4H_2O$ 0.025 g, KNO_3 1.0 g, Na_2-EDTA (Na_2-ethylenediaminetetraacetic acid) 0.01 g, $Fe_2(SO_4)_3{\cdot}6H_2O$ 0.004 g. For plating, 1.5% agar (Difco) was added. The plates were incubated at 37 ℃ overnight. Colonies formed depressions or pits and clearing zones on agar surface were picked and re-purified by the same cultural method.

Identification of the isolates

Isolates were identified through its morphological and physiological properties according to *Bergey's Manual of Systematic Bacteriology* [26] and MicroScan-AS4 automatic microorganism analyzing system (Baxter Diagnostics, Inc., West Sacramento, Calif. USA. Bacterial identification is based on measurement of the turbidity or color change in the wells of the MicroScan panel. The automated methods directly match to standard biochemical manual methods). The 16S rRNA was amplified by using MicroSeq 500-16S rDNA Bacterial Identification PCR Kit. One and half kb length 16S rDNA fragment enhancements were collected. PCR products were checked by electrophoresis. The DNA sequencing was done by using Applied Biosystems Instruments 310（ABI 310）. The sequence was compared to NCBI GenBank database using BLAST.

Assay for agarase activity of purified enzyme

The isolates were inoculated in 1 liter size Erlenmeyer flasks containing 500 ml of C-10 medium with 0.1% agar and cultured overnight at 37 ℃ with shaking 100 rpm. Cultures were supplemented with 0.1 mM phenylmethyl-sulfonyl fluoride(PMSF) and the cells were centrifuged at 7,000 x*g* for 30 min at 4 ℃ to remove bacterial debris. The

supernatants were brought to 80% saturation with solid ammonium sulfate, the mixture was stirred overnight then centrifuged at 8,000 x*g* for 45 min, 4 ℃. Pellets were resuspended in 1 ml of 20 mM Tris-HCl buffer pH 8.0 and dialyzed against the same buffer at 4 ℃ overnight. The purified enzymes were collected after dialyzation. The dialysate was loaded onto a Sephadex G-100 column (1 x 50 cm) equilibrated with 50 mM Tris-HCl buffer. Elution was carried out with the same buffer. Active fractions were concentrated by 30 kDa MW protein collection centrifuge tubes and then stored at 4 ℃.

Protein quantification

Protein concentrations were determined at OD_{595} by Bradford's method [27], with a Bio-Rad Protein Assay Kit and using bovine serum albumin as the standard.

Electrophoresis analysis for agarase

Purified agarase sample (30 μl) were mixed with 10 μl of 4-fold sodium dodecyl sulfate (4x SDS) loading buffer, denatured at 95 ℃ for 3 min. The sample was loaded onto gradient polyacrylaminde gel (4~20% Tris-HEPES-SDS), Bio-Rad broad range protein marker was used. Electrophoresis run for 45 min at 20 ℃. Protein bands were stained with Coomassie Brilliant Blue R-250.

Assay of agarase activity

Agarase activity was measured by the method of Miller [28] under standard assay condition, and compared to a standard (Sigma A 6306 agarase from *Pseudomonas atlantica*) using D-galactose reduction equivalents. Enzyme sol (50 μl) was added to 450 μl of sodium phosphate buffer pH 7.0 containing 0.2 % agarose. After incubation at 42 ℃ for 50 min, 1.0 mL DNS (3,5-dinitrosalicylic acid) and K-Na tartrate sol. was added, the reaction was stopped by heating the tube in boiling water for 5 min. at 100 ℃, then cool to room temperature, measured OD_{546} , compared to D-galactose standard curve. One unit of agarase activity was defined as liberating 1 μM of D-galactose per min.

Effect of pH on the activity

One hundred μL(10 U/mL) enzyme sol was added to different pH buffer, 0.9 mL each separately, at 35 ℃, 30 min, then checked the reduced sugar concentration.
Citrate-Na_2HPO_4 buffer (50 mM) (pH Value 3.0 4.0 5.0); Tris-HCl buffer (50 mM) (pH Value 6.0 6.5 7.0 7.5); Sodium phosphate buffer (50mM) (pH Value 7.0 7.5 8.5); Phosphate buffer (50mM) (pH Value 8.0 8.5); Glycine-NaOH (50mM) (pH Value 9.0 10.0) were used.

Effect of temperature on the stability and activity

Made 5U/mL enzyme mixture with 50 mM different pH of citrate-Na_2HPO_4 buffer, pH 3.0-5.0; Tris-HCl buffer, pH 6.0-7.5; Sodium phosphate buffer pH 7.5-8.0; phosphate buffer, pH 8.5; glycine-NaOH buffer, pH 9.0-10.0 , 4℃ , 24 hr. Then 0.1mL of different pH enzyme sol was mixed with 0.2% agarose sol substrate (0.9 mL, separately, 35℃ , 30 min, and then the reduced sugar conc were checked.

The effects of the temperature [18]

Low-melting-point agarose 0.2%, 0.9 mL and 0.2% high-melting-point agarose, in 20、25、30、35、40、45、50、55、60℃ were preheated 5 min, then 0.1 mL(10 U/mL) enzyme sol was added, 30min. and then the reduced sugar conc were checked.

Temperature stability

Enzyme sol 10 U/mL were set in 20、25、

30、35、40、45、50、60 ℃, 30 min, separately, then the enzyme activity was checked as described above.

Effects of Calcium and Sodium ions [29]

Calcium ion effect

Different concentration of $CaCl_2$ (0、0.05、0.1、0.2、0.8、1.0、3.0、5.0、10.0、20.0、100.0 mM) mixed with 0.1 mL 10 U/mL enzyme sol separately, then the enzyme activity was checked as described above.

Sodium ion effect

Different concentration of NaCl (0、20、50、100、200、400、600、800、1,000 mM) mixed with 0.1 mL, 10 U/mL enzyme sol separately, then the enzyme activity was checked as described above.

Transformion of the agarase gene containing plasmid

Plasmid DNA from *Alcaligenes* sp. strain Yen was extracted by alkaline method [30]. The pellet was resuspended in 0.5 ml Tris-EDTA buffer and stored at -20 ℃. The sample was loaded onto the 1% agarose with ethidium bromide and electrophoresised 1 hr at room temperature. Plasmid pRK291 in *E. coli* HB101 (20 kb) and plasmid pC22 in *E. coli* MC1061 (17.5 kb) were used as markers (bought from Food Industry Research Development Institute-FIRDI, Hsin-Chu, Taiwan. Category No. BCRC 41277 and BCRC 41178). All plasmids DNA samples were extracted by the same method. Plasmids DNA were transformed into *E. coli* HB 101 (bought from FIRDI, Category No. BCRC 51534). Competent cells made by the calcium chloride method [30]. Transformants were selected on modified C-10 plate (1% agar with 0.1% yeast extract, 0.5% tryptone and 1% NaCl). After incubation at room temperature overnight, transformants were screened for agarolytic activity by visual inspection of the surface of the agar plate. Colonies that showed shallow depression around the periphery of each colony were confirmed to be agarase positive.

Substrate specificity test

Used high-melting-point agarose、low-melting-point agarose、agar (Agar power, Difco, PCH-D, Agar A)、sodium alginate、ι-carrageenan and κ-carrageenan, (1mg/ml) as substrates, and 0.1 mL(10 U/mL) agarase, 35 ℃, for 30min, then checked the sugar conc as described.

Enzyme Kinetics [31]

Agarase10 U/mL in low-melting-point agarose and high-melting-point agarose as substrates (separately)(conc 0.5、1、1.5、2、2.5、3.0 g/L, 35 ℃, reaction time 30min), then checked the sugar conc as described. Results were used to calculate Michaelis-Menten equation and K_m、V_{max}.

Thin layer chromatography [32]

Silica gel 60 plate; Solvent: n-butanol-acetic acid- water (2: 1: 1); Color developing sol. : naphthoresorcinol (0.2g naphthoresorcinol＋100 mL ethanol＋10 mL H_3PO_4)

3. Results

Isolation and Idetification of agar degrading bacteria

The colonial morphology of *Alcaligenes* sp. strain Yen was white. Pits became obvious formed around the colonies by degrading agar after 16-18 hr incubation at 37 ℃, after 2 days incubation the holes were more clear (Figure1).

Figure 1. Colony morphology of *Alcaligenes* sp. strain Yen, followed by incubation 2 days at RT on C-10 plate.

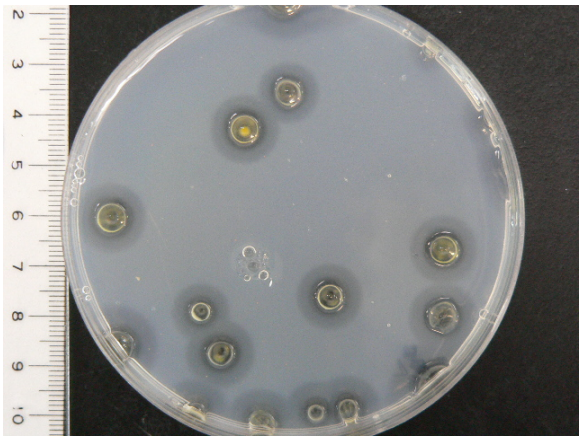


Figure 1. Colony morphology of *Alcaligenes* sp. strain Yen, after incubation for 2 days at RT on C-10 plate.

Bacterium characteristics

The strain was Gram-negative rods, non-fermentative and highly motile. It was positive for oxidase, citrate, malonate, tartrate, acetamide and cetrimide, negative for the production of indole and H2S. The strain was susceptible to penicillin, kanamycin, nitrofurantoin and tobramycin. The result of several biochemical tests were listed in Table 1.

MicroScan AS4 system identified this bacterium and asigned it as Alcaligenes sp. with 97.4% similarity. This finding was comparable to the results of 16S RNA comparison. However our strain showed agarolytic activity. Based on these data and the suggestion of GenBank, we assigned our strain as Alcaligenes sp. strain Yen (NCBI GenBank accession number AY744384). 16S RNA Sequence was obtained

CTGGCTCAGATTGAACGCTAGCGGGAT
GCCTTACACATGCAAGTCGAACGGCAG
CACGGACTTCGGTCTGGTGGCGAGTGG
CGAACGGGTGAGTAATGTATCGGAACG
TGCCCAGTAGCGGGGGATAACTACGCG
AAAGCGTAGCTAATACCGCATACGCCCT
ACGGGGGAAAGCAGGGGATCGCAAGA
CCTTGCACTATTGGAGCGGCCGATATCG
GATTAGCTAGTTGGTGGGGTAACGGCT
CACCAAGGCGACGATCCGTAGCTGGTT
TGAGAGGACGACCAGCCACACTGGGA
CTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTGGGGAATTTTGGACAAT
GGGGGAAACCCTGATCCAGCCATCCCG
CGTGTGCGATGAAGGCCTTCGGGTTGT
AAAGCACTTTTGGCAGGAAAGAAACG
TCGCGGGTTAATACCCCGCGGAACTGA
CGGTACCTGCAGAATAAGCACCGGCTA
ACTACGTGCCAGCAGCCGCGGTAATAC
GTAGGGTGCAAGCGTTAATCGGAATTA
CTGGGCGTAAAGCGTGCGCAGGCGGTT
CGGAAAGAAAGATGTGAAATCCCAGA
GCTTAACTTTGGAACTGCATTTTTAACT
ACCGAGCTAGAGTGTGTCAGAGGGAG
GTGGAATTCCGCGTGTAGCAGTGAAAT
GCGTAGATATGCGGAGGAACACCGATG
GCGAAGGCAGCCTCCTGGGATAACACT
GACGCTCATGCACGAAAGCGTGGGGA
GCAAACAGGATTAGATACCCTGGTAGT
CCACGCCCTAAACGATGTCAACTAGCT
GTTGGGGCCTTCGGGCCTTGGTAGCGC
AGCTAACGCGTGAAGTTGACCGCCTGG
GGAGTACGGTCGCAAGATTAAAACTCA
AAGGAATTGACGGGGACCCGCACAAG
CGGTGGATGATGTGGATTAATTCGATGC
AACGCGAAAAACCTTAYCTACCCTTGA
CATGTCTGGAATTCCGAAGAGATTTGG
AAGTGCTCGCAAGAGAACCGGAACAC
AGGTGCTGCATGGCTGTCGTCAGCTCG
TGTCGTGAGATGTTGGGTTAAGTCCCG
CAACGAGCGCAACCCTTGTCATTAGTT
GCTACGAAAGGGCACTCTAATGAGACT
GCCGGTGACAAACCGGAGGAAGGTGG
GGATGACGTCAAGTCCTCATGGCCCTTA
TGGGTAGGGCTTCACACGTCATACAAT
GGTCGGGACAGAGGGTCGCCAACCCG
CGAGGGGGAGCCAATCCCAGAAACCC
GATCGTAGTCCGGATCGCAGTCTGCAA
CTCGACTGCGTGAAGTCGGAATCGCTA
GTAATCGCGGATCAGCATGTCGCGGTG
AATACGTTCCCGGGTCTTGTACACACC
GCCCGTCACACCATGGGAGTGGGTTTT
ACCAGAAGTAGTTAGCCTAACCGTAAG
GGGGGCGATTACCACGGTAGGATTCAT
GACTGGGGTGAAGTCGTAACAAGGTA
GCCGTATCGGAAGGTGTGGCTGGATCA

The 16S rDNA sequence of the isolate shared 100% sequence similarity with Alcaligenes sp. strain ON5 (GenBank accession number AJ306836).

Table 1. Biochemical characteristics of *Alcaligenes* sp. Strain Yen.

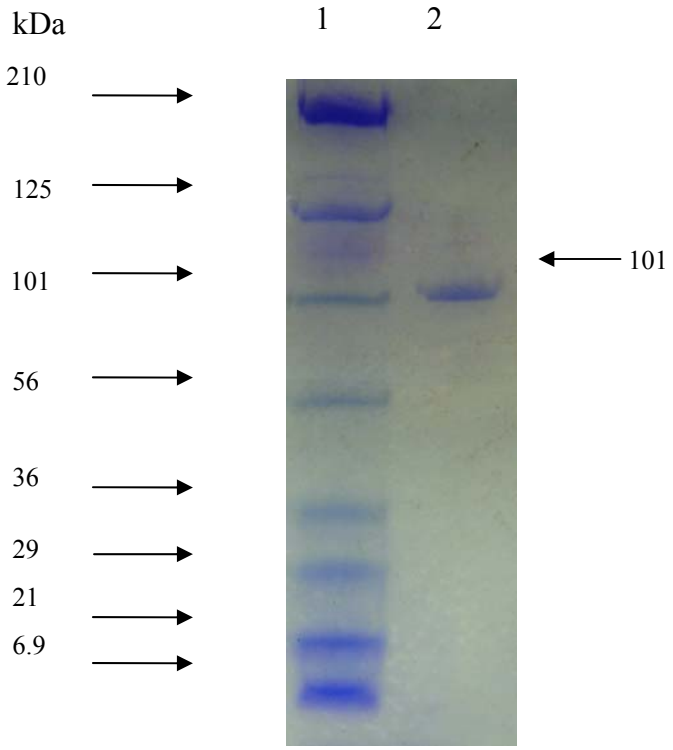
Characteristic tested	Result	Characteristic tested	Result
Motility	m	Ornithine	-
Glucose	-	Tryptophan Deaminase	-
Sucrose	-	Esculin	-
Sorbitol	-	Voges-Proskauer	-
Raffinose	-	Citrate	+
Rhamnose	-	Malonate	+
Arabinose	-	o-Nitrophenyl-beta-D-Galactopyranoside	-
Inositol	-	Tartrate	+
Adonitol	-	Acetamide	+
Melibiose	-	Cetrimide	+
Oxidase	+	OF Glucose	-
Urea	-	Penicillin	S
Hydrogen Sulfide	-	Kanamycin	S
Indole	-	Colistin	R
Lysine	-	Nitrofurantoin	S
Arginine	-	Tobramycin	S
		Nitrate	+

Symbols: +,positive reaction ; -,negative reaction; m,motile; S:susceptible; R:resistible

Properties of purified agarase

Electrophoresis result showed the purified agarase has a molecular mass of 101 kDa as detected by SDS-PAGE (Figure 2).

Figure 2. SDS-PAGE of purified agarase form *Alcaligenes* sp. Strain Yen. Lane 1, molecular mass standard ; Lane 2, purified agarase. Myosine (210 kDa), *b*-galactosidase (125 kDa), Bovine serum albumin (101 kDa), Ovalbumin (56 kDa), Carbonic anhydrase (36 kDa), Soybean trypsin inhibitor (29 kDa), Lysozyme (21 kDa) and Aprotinin (6.9 kDa) were used as markers.



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The measured molecular mass was close to those reported for agarase from *Agarivorans* sp. JAMB-A11 105 kDa [17], but differ from *Pseudomonas* sp. W7 89 kDa [33], *and Alteromnas* sp. SY37-12 39.5 kDa [5]

Effect of pH and temperature optimum on the activity

The optimal pH for the enzyme in different buffers were different (Figure 3).

The enzyme stability for pH profiles was shown in Figure 4.

In pH 3.0 - 8.5, the enzyme was relatively sTable, the enzyme activity decreased sharply as pH raised higher than 8.5. The enzyme activity was sTable between temperature 20 - 35 ℃ (Figure 5).

Then the activity decreased as temperature raised higher than 35 ℃. The optimal temperature for the enzyme was 42 ℃ (Figure 6).

Effects of Calcium and Sodium ions

Calcium ion could increase the activity for Yen-agarase (Figure 7).

Sodium ion could inhibit the activity for Yen-agarase (Figure 8).

Transformion of the agarase containing plasmid

The plasmid DNA was successfully extracted from *Alcaligenes* sp. strain Yen and electrophoresised on the gel. The size of the plasmid was estimated to be 18.4 kb (Figure 9).

Figure 9. Lane 1, 20 kb and 17.5 kb plasmid DNA marker. Lane 2, Plasmids extracted from *Alcaligenes* sp.. Strain Yen showed about 18,400 bp. Lane 3, Lambda DNA was digested by *Eco*R1 restriction enzyme.

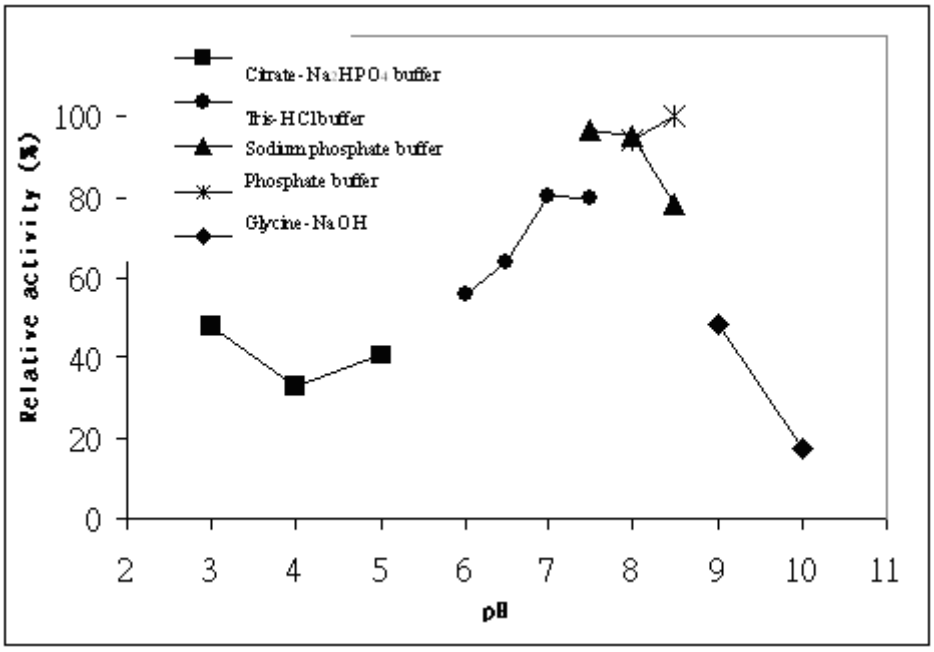

Figure 3. Optimum pH of Yen-agarase in various buffer sol.

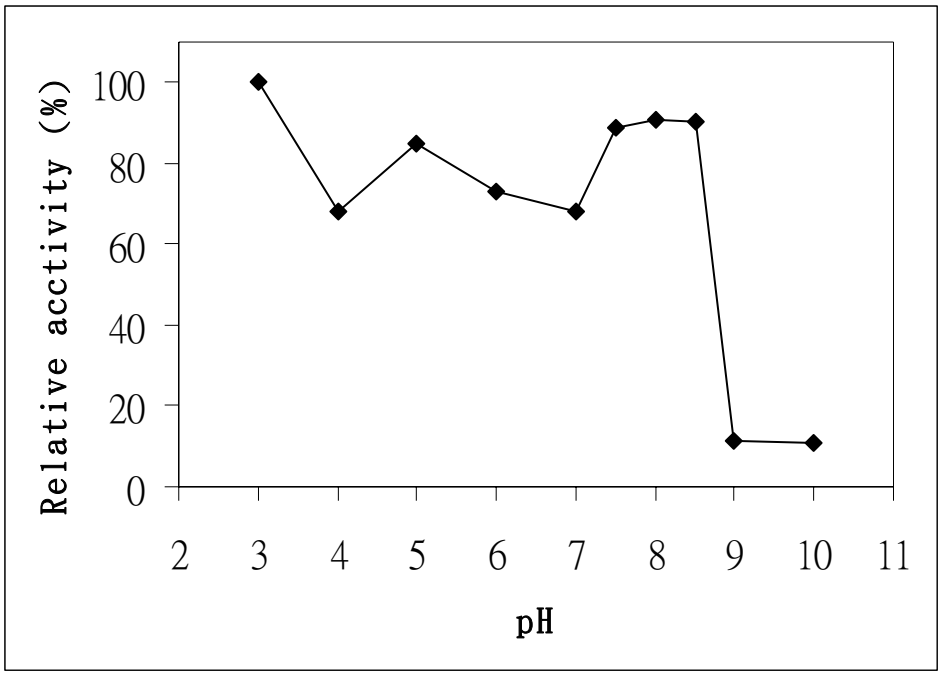

Figure 4. Effect of pH on the stability of Yen-agarase.

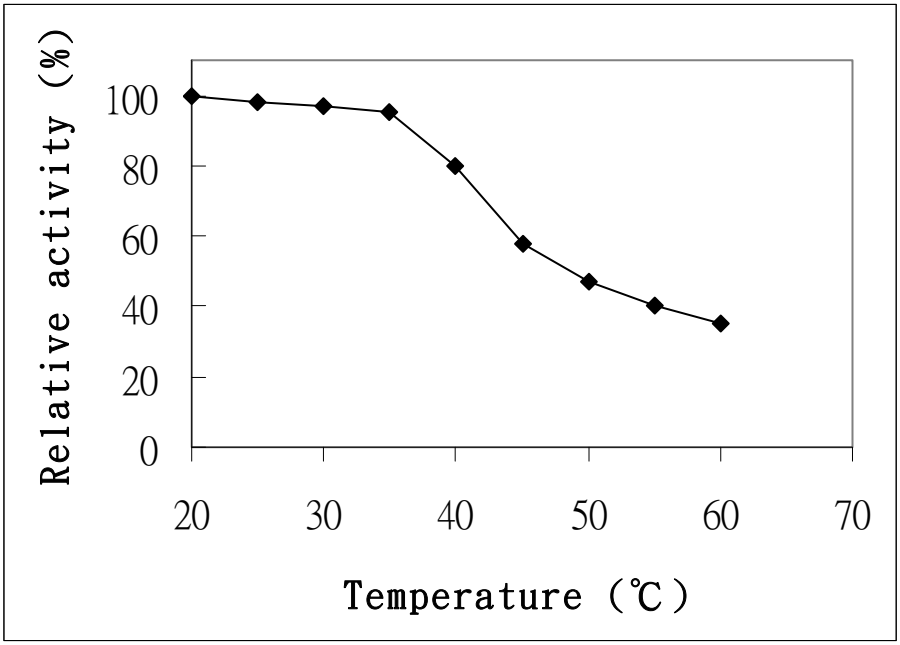

Figure 5. Thermostability of Yen-agarase.

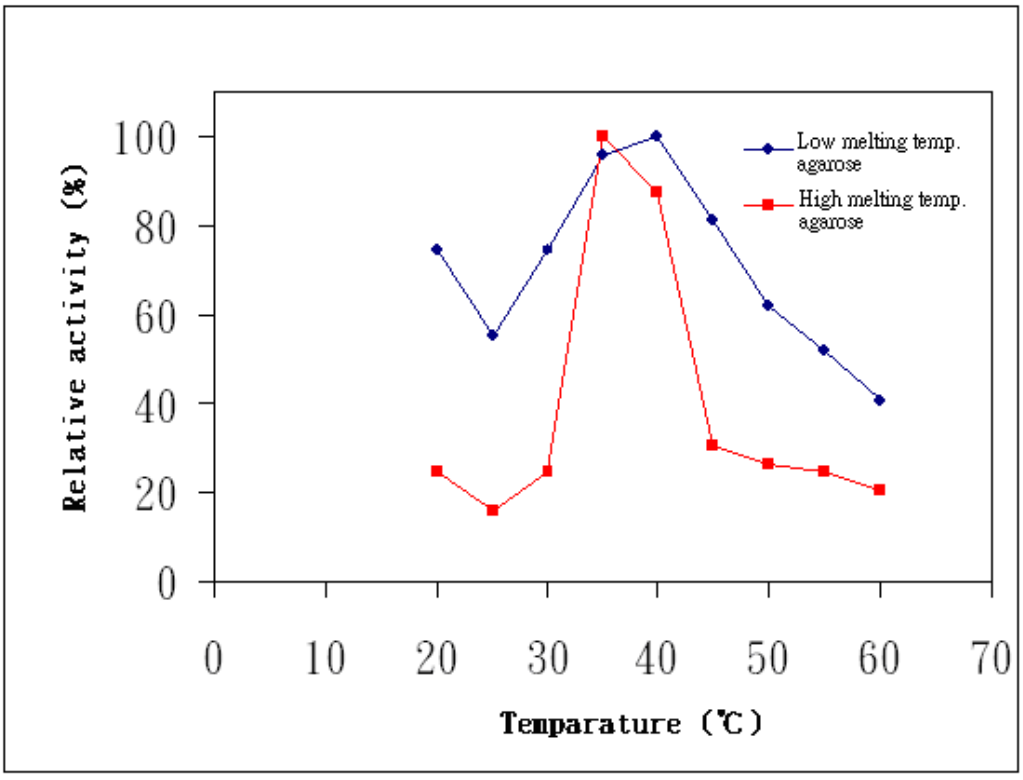

Figure 6. Optimum temperature of Yen-agarase.

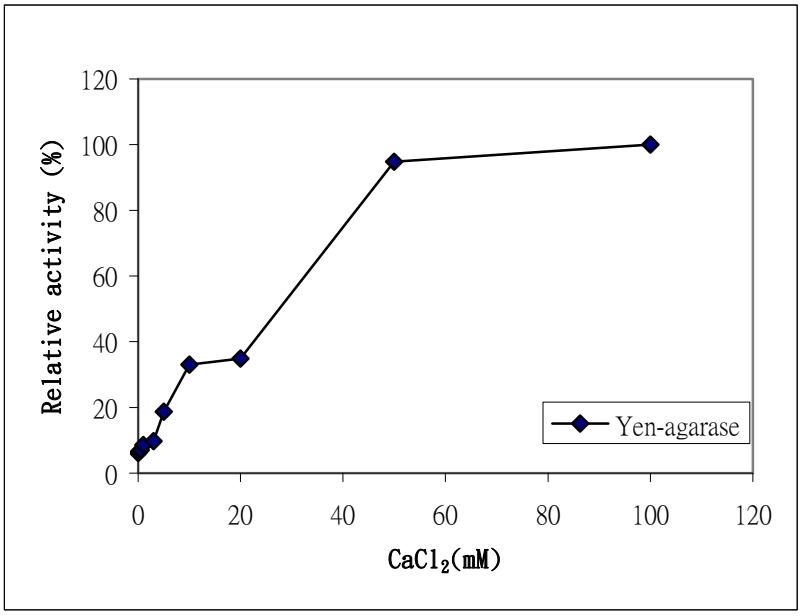

Figure 7. Effect of $CaCl_2$ concentration to Yen-agarase on their activities.

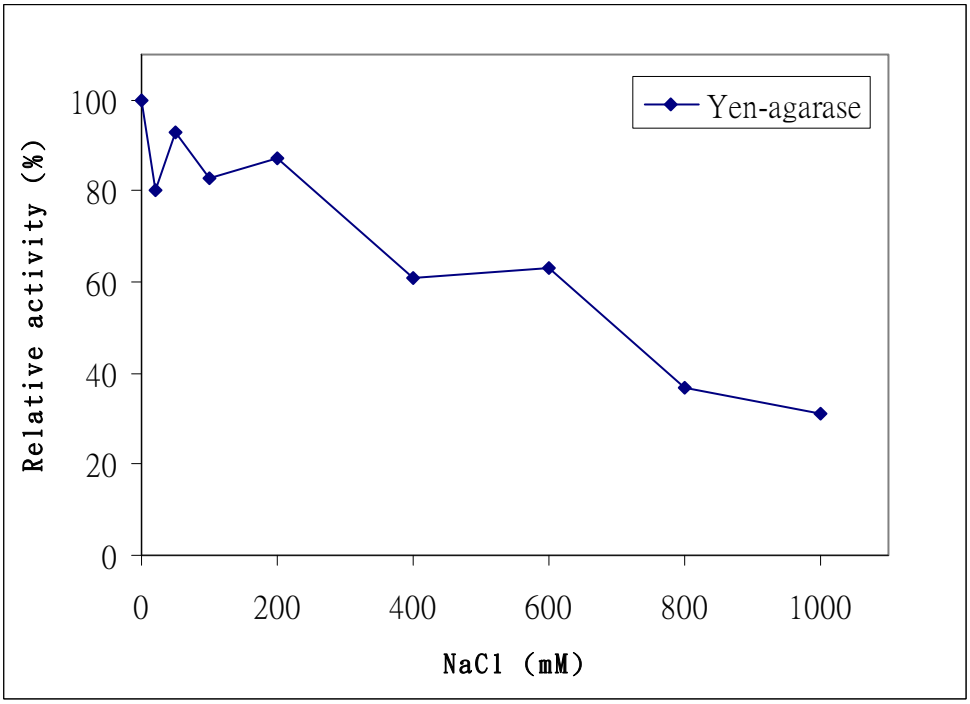

Figure 8. Effect of NaCl concentration on Yen-agarase on its activities.

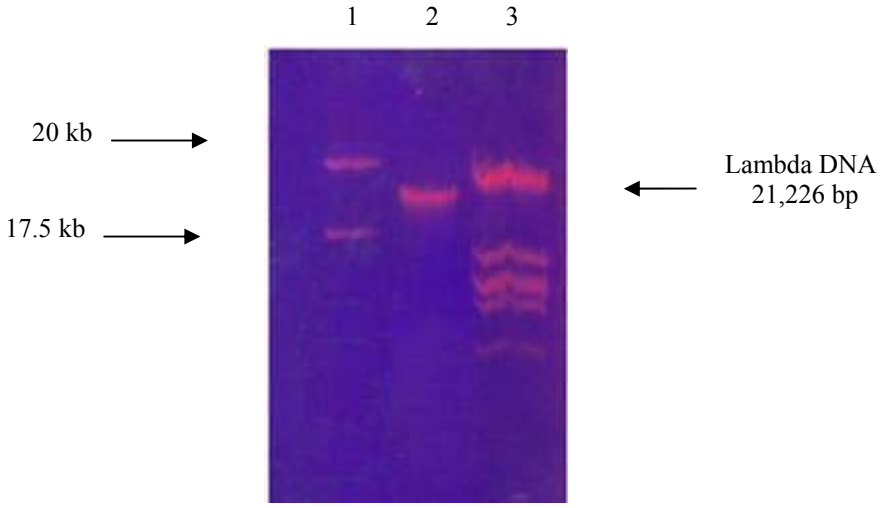

Figure 9. Lane 1, 20 kb and 17.5 kb plasmid DNA marker. Lane 2, Plasmids extracted from *Alcaligenes* sp. Strain Yen showed about 18,400 bp. Lane 3, Lambda DNA was digested by *Eco*R1 restriction enzyme.

The plasmid DNA was successfully transformed into competent *E.coli* HB 101 cells and positive clones expressing agarolytic activity were detected as colonies forming pits on the agar surface after overnight incubation (Figure 10).

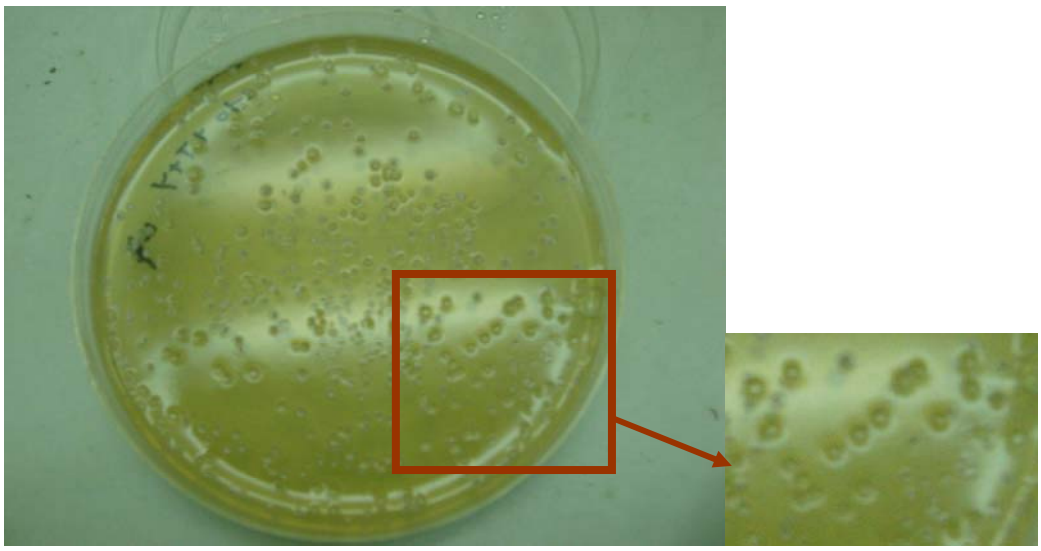

Figure 10. The identification of transfected *E.coli* HB101 colonies.

Substrate specificity

No degradation could be detected for ι-carrageenan and κ-carrageenan, but the enzyme could degrade sodium alginate, as well as low or high temp melting agarose (Table 2).

Table 2. Substrate specificity of agarase-Yen against nine polysaccharides
[1]: μmole D-galactose equivalent/min/mL.
[2]: Could not be determined.

Substrate	Yen-agarases
High-melting-point agarose	51.45[1]
Low-melting-point agarose	42.60
Difco agar	30.72
Agar power	24.84
Agar A	32.68
PCH-D agar	24.78
Sodium alginate	9.28
ι-Carrageenan	---[2]
κ-Carrageenan	---

K_m 、V_{max}

High and low-melting-point agarose were used as substrates separately, the catalytic rate was shown by Lineweaver-Burk plots and Michalis-Menten equations. When the high-melting-point agarose was used as substrate, K_m was 2.76 g/L, V_{max} was 68.03 μmole min^{-1}(Figure 11)

when low-melting-point agarose was used as substrate, K_m was 1.54 g/L, V_{max} was 36.9 μmole min^{-1}(Figure 12).

The Yen-agarase had higher affinity for low-melting-point agarose than high-melting-point agarose.Thin layer chromatography

Neoagarobiose, neoagarohexaose and galactose were detected (Figure 13). **it was the results of the beta type agarase.**

4. Discussion

Previous studies have showed that the agar-degrading bacterium *Paenibacillus* spp.[19] took 10 days（28 ℃） to form depressions on agar surface, and *Pseudoalteromonas antarctica* N-1 [7] took 48 hr（25 ℃）. Our strain shown pits on agar surface surrounding the colony in less then 18 hr. The purified enzyme shown high agarase activity as 406 U/mg. *Pseudoalteromonas antarctica* strain N-1 [7] had reported agarase activity 290 U/mg , *Thalassomonas* sp. strain JAMB A33 40.7 U/mg [10], *Agarivorans* sp. JAMB-A11 371 U/mg (Ohta et al. 2005a) and *Microbulbifer* strain JAMB-A7 398 U/mg [34] respectively. A marine bacterium JAMB-A94 had reported agarase activity 517 U/mg [35]. *Alcaligenes* sp. strain Yen had different pH maximal enzyme activity in different buffers. The optimal enzyme activity shown at 42 ℃. Compare to other agar-degrading bacteria. Agarase from *Bacillus* sp. MK03 had optimal pH at 7.6 [18], *Thalassomonas* sp. strain JAMB-A33 8.5 [10], *Pseudomonas* sp. SK38 9.0 [36]. JAMB-A94 had optima temperature at 55 ℃ [35], *Microbulbifer* strain JAMB-A7 50 ℃ [34]. The transformed *E. coli* HB101 cells had optima growth at 25-30 ℃. This plasmid had the smallest size among all plasmids containing an agarase

gene that have been found in nature. Some other findings were much larger than this [37]. This plasmid could be used to transform competent bacterial cells directly or be modified for cloning vectors. For the successfully transformation in *E. coli* HB101 and got agarase expression. The plasmid's agarase gene promoter region should be recognized by *E.coli* HB101 transcription facilities. The agarase activity raised sharply at 30 - 42 ℃, afterward it decreased. The further study is to prepare a new cloning vector with agarase gene and another marker gene such as β-galactosidase or green fluorescent protein without antibiotic resistant gene as an innovative molecular biology tool. The new tool can select positive colonies without using environmental contaminating antibiotics. In this study, we used C-10 plates to culture the bacterium without adding organic material. This was the first time of all agarase research that used inorganic material plate to select agarolytic bacteria. Agarases have low activity and low productivity are not economically useful for industrial application. Our strain secreted extacelluar agarase with high activity. It has a highly biotechnological significant agarolytic plasmid for industry application. The results showed *Alcaligene*s sp. strain Yen could use agar as its carbon and energy source. Transformed *E. coli* HB101 cells needed yeast extract as supplement for growth. When yeast extract was not added in the medium, the transformed *E. coli* grew poorly and formed tiny pits on the agar surface.

This is a report of finding an agarase secretion bacterium with smallest agarolytic plasmid ever been found, and the first time transformation of the intact agarolytic plasmid into *E. coli* and got agarase gene expression. The enzymology analysis was also reported.

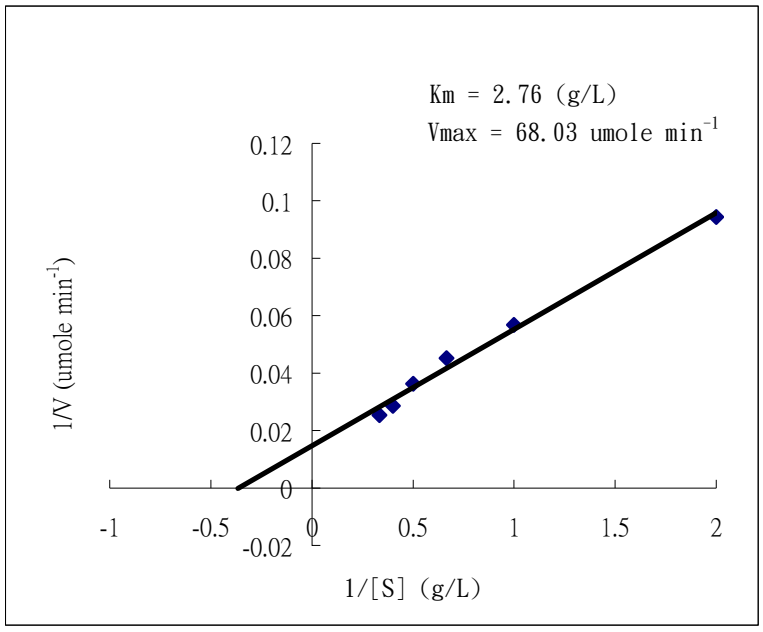

Figure 11. Lineweaver-Burk plot of Yen-agarases using high-melting-point agarose as substrate. (Yen-garase conc.10 U/mL . Substrate conc. 0.5、1 、1.5、 2、 2.5 g/L ,30min, Used the reduced sugar conc. Plot substrate conc vers reversed reaction rate. Calculated K_m *and* V_{max} (unit g/L ; velocity(V) μmole min^{-1})

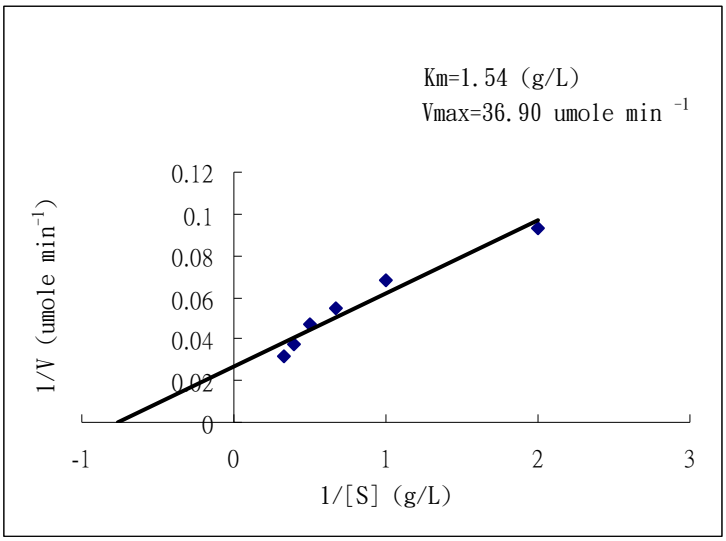

Figure 12. Lineweaver-Burk plot of Yen-agarases using low-melting-point agarose as substrate. (Agarase conc. 10 U/mL, substrate conc. 0.5、1 、1.5、 2、 2.5 g/L, 30min, Used the reduced sugar conc. Plot substrate conc vers reversed reaction rate. Calculated K_m *and* V_{max} .(Unit g/L ; velocity (V) unit μmole min^{-1})

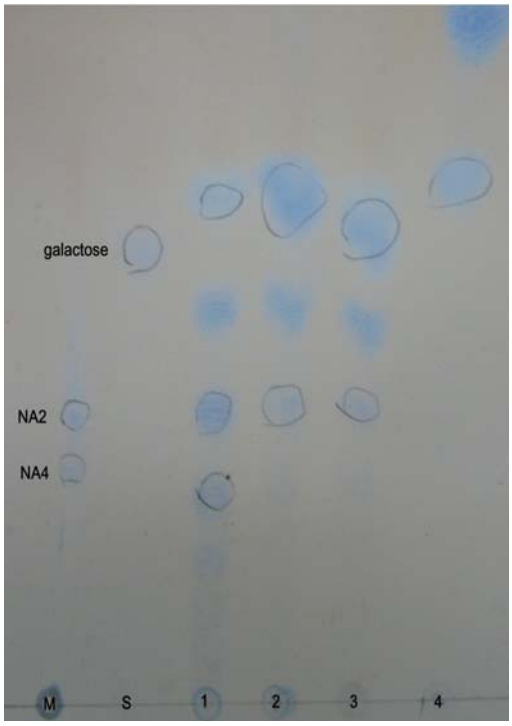

Figure 13. Thin layer chromatography of the products of agarose hydrolysed by Yen-agarase (NA2: neoagarobiose; NA6: neoagarohexaose; G: galactose. lane 1~ 4 : 5U/mL Yen-agarase reacted with 0.2 % agarose 35 ℃, after 0.5、1、5、24 hr. M：mark；S：galactose；Lane 1：0.5 hr； Lane 2：1 hr；Lane 3：5 hr；Lane 4：24 hr)

Authors' contributions

The bacterium was isolated by Yen Lee. Its agarolytic plasmid and the plasmid was transformable into *E. coli* HB101 were also discovered by Yen Lee. The enzymological analysis were done cooperatively by Yu-Fong Sie and Hui-Chun Yang**.**

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