Journal of Microbiology and Biotechnology
Vol. 3, No. 1, 31-38, 1993

Enzymatic Characteristics of an Extracellular Agarase of *Cytophaga* sp. KY-1 and Molecular Cloning of the Agarase gene

KIM, YOUNG HO*, YOUN SOOK KIM, JAE RAN LEE, EUN KYUNG LEE AND JUNG HWN SEU

Department of Microbiology, College of Natural Science
Kyungpook National University, Taegu 702-701, Korea

A bacterial strain KY-1 isolated from sewage was able to produce an extracellular agarase(agarose 4-glycanohydrolase, EC 3.2.1.81). The strain KY-1 was identified as *Cytophaga fermentans* subsp. *agarovorans* based on its morphological and physiological characteristics. The agarase was purified by ammonium sulfate precipitation followed by DEAE-Sephadex A-50, Bio-Gel P-100, and CM-Cellulose column chromatography. The molecular weight of the purified enzyme was 24 kDa by SDS-polyacrylamide gel electrophoresis. The optimum temperature and pH for the enzyme activity were 30°C and 7.5, respectively. The enzyme activity was significantly inhibited in the presence of 0.1 mM $HgCl_2$, whereas it was elevated 3 times by $MnSO_4$ at 1 mM concentration. The Km value and Vmax were 16.67 mg/ml and 3.77 unit/ml·min. The agarase gene was cloned into *Escherichia coli* MC1061 using the plasmid vector pBR322. A 1.4 Kb DNA fragment of *Pst*I-digested chromosomal DNA of *C. fermentans* KY-1 was inserted into the *Pst*I site of pBR322, expressed in the *E. coli*, and up to 60% of the total enzyme was extracellularly secreted. Enzymatic properties of the extracellular agarases produced by both the transformant and the donor were very similar in terms of optimal pH and temperature.

Agar, the complex polysaccharide extracted from marine red algae (*Gelium, Gracilaria, Acanthopeltis, Ceranium* and *Pterocladia*), consists of two major components such as a neutral agarose and a charged agaropectin. The structure of agarose is (1-4)-linked 3,6-anhydro-α-L-galactose alternating with (1-3)-linked β-D-galactose. Agaropectin has the same repeating units, although some of the 3,6-anhydro-L-galactose residues can be replaced with L-galactose sulfate, and there can also be partial replacement of D-galactose residues with the pyruvic acid acetal 4,6-O-(1-carboxyethylidene)-D-galactose (7, 8, 12).

Since agar is relatively stable and forms a gel at low concentrations, it has been widely employed as a gelling agent in microbial culture media as well as in several other biological techniques such as antibiotic assay, viral plaque formation, immunoelectrophoresis, gel electrophoresis and gel chromatography (7, 12).

*Corresponding Author
Key Words: *Cytophaga* sp., agarase gene(dagA), gene expression, *Escherichia coli*

Agar-digesting bacteria have been known to exist since 1902 when Gran isolated a sea water microorganism possessing this property (11). The genera accepted by Bergey's manual relating the agarolytic bacteria are *Pseudomonas, Xanthomonas, Alginomonas, Vibrio, Agarobacterium, Clostridium, Nocardia, Streptomyces, Cytophaga, Chondrococcus* and *Sporocytophaga* (4). However, most of the previous studies are concerned with identification and classification of agar digesting bacteria. Investigations at enzymatic level are restricted to the few genera such as *Pseudomonas* (16, 21), *Vibrio* (9) and *Cytophaga* (20). Molecular cloning and sequence analysis of the agarase gene have been done only in *Streptomyces coelicolor* (1, 5, 13).

Recently we isolated a bacterial strain KY-1 from sewage, which is highly effective in degrading agar. In this study we describe the isolation and identification of the agarolytic bacterium, the purification and some properties of the extracellular agarase, and molecular cloning and expression of the extracellular agarase gene in *E. coli*. The strain KY-1 which exhibited extracellular aga-

rase activity was identified as *Cytophaga fermentans* subsp. *agarovorans* and its agarase gene was able to express the extracellular agarase in *E. coli*.

MATERIALS AND METHODS

Bacterial Strains and Plasmid

Bacterial strains and plasmid used are summarized in Table 1.

Media

The basal medium for the screening of agarolytic bacteria consisted of 0.2% $NaNO_3$, 0.05% Polypeptone, 0.05 % K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$, 0.01% NaCl, 0.002% $FeSO_4 \cdot 7H_2O$, and 0.002% $MnSO_4 \cdot 4\text{-}6H_2O$, pH 7.0. The recipient *E. coli* was cultured in LB containing 1% tryptone, 1% yeast extract and 0.5% NaCl. For the selection of transformants, either 15 μg/ml of tetracycline or 50 μg/ml of ampicillin was added to LB. Agar was added to the media as much as necessary.

Screening of Agarolytic Bacteria

The 50 ml Erlenmeyer flasks containing 10 ml of the basal medium, to which 0.5% agar was added as a sole carbon source, were inoculated with 0.1 ml of sewage sample and incubated at 30℃ on a rotary shaker for 7 days. Subsequently 0.1 ml of the cultures liquefied by agarolytic bacteria were then inoculated to 10 ml of basal media containing 0.5% agar and cultured for another 7 days. One-tenth ml of the cultures were properly diluted and plated on the basal medium containing 1.8% agar. Plates were incubated at 30℃ and examined daily for agarolytic activity, assessed by liquefaction or shallow depressions appearing around the colonies. The purity of culture was checked by further streaking and by microscopic examination.

Enzyme Assay

Quantitative assay for agarase activity was performed by the method of Miller *et al.* (15), which detects reducing sugars released from agarose. One ml of 0.1% agarose in 0.1 M phosphate buffer (pH 7.0) was mixed with 0.5 ml of enzyme solution. The reaction was done at 30℃ for 30 min and the reducing power formed was determined colorimetically by the addition of 1.5 ml of DNS reagent. One unit of the enzyme activity was defined as the amount of the enzyme that produced reducing power equivalent to 1 μM of D-galactose per min.

Table 1. Bacterial strains and plasmid used

Strains and plasmids	Genotypes	Remark
Cytophaga fermentans subsp. *agarovorans* KY-1	wild type	agarase gene donor
E. coli MC1061	hsdR, hsdM$^+$, hsdS$^+$, araD139, Δ (ara-leu) 7697, Δ (lac)X74, galU, galK, rpsL(strr),F$^-$	recipient
Plasmid pBR322	Ap^r, Tc^r	vector

Gel Electrophoresis of Protein

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out as described by Walker (19).

Preparation of Plasmids and Chromosomal DNA

Plasmid DNA was isolated by the alkaline-SDS method of Birnboim and Doly (2), and purified by electroelution or by isopycnic ultracentrifugation (17).

Digestion and Ligation of DNA

The digestion of DNA by the restriction enzymes and the ligation of the DNA fragments were carried out according to the procedures of Dillon and Nestmann (6).

Measurement of Protein

Protein was measured by the procedure of Bradford (3) with bovine serum albumin as a standard.

RESULTS

Isolation and Identification of an Agarolytic Bacterium

A bacterial strain capable of producing extracellular agarase was isolated from sewage samples. The strain could easily be observed to differ from the other isolates since it produced depressions in the screening medium (Fig. 1).

Identification of the Agarolytic Bacterium

Identification was carried out according to the method of Gerhardt *et al.* (10), and Bergey's Manual (4). The characteristics of the agarolytic bacterium KY-1 are shown in Table 2 and Fig. 2. These results indicate that

Fig. 1. An agar plate with agarolytic bacterial colonies.

Table 2. Identification of the isolated agarolytic bacterium KY-1

Characteristics	Characteristics	Characteristics
Shape : flexible rod	Oxidase (+)	Fermentation of
Size : 0.5×3-5 μm	Catalase (+)	Glucose (+)
Spheroplast and	Nitrate reduction (+)	Galactose (+)
distorted cells (+)	Indol production (+)	Fructose (+)
in old culture	Litmus milk reactions (−)	Lactose (+)
Flagella (−)	Hydrogen sulfide (−)	Maltose (+)
Gliding movement (−)	Gelatin hydrolysis (−)	Sucrose (+)
Swarming (+)	Chitin hydrolysis (−)	Trehalose (−)
Colour : pale yellow	Casein hydrolysis (+)	Raffinose (+)
Gram staining (−)		Agar (+)

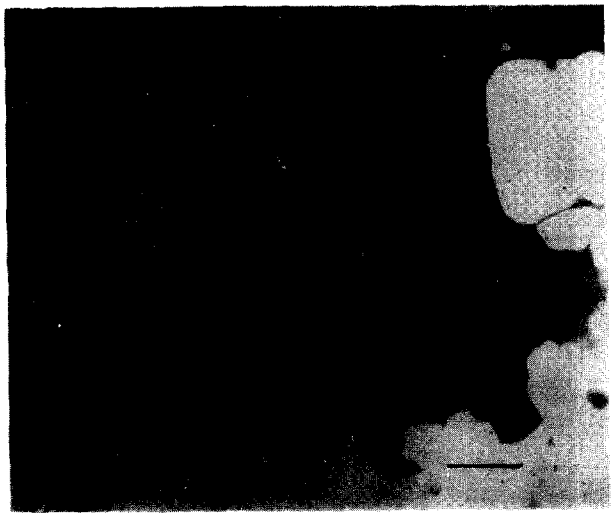

Fig. 2. Electronmicrograph of the agarolytic bacterium KY-1 negatively stained.
The marker represents 1 μm (×15,000).

the strain KY-1 is *Cytophaga fermentans* subsp. *agarovorans*.

Purification of the Agarase

The agarolytic bacterial cells were inoculated into basal medium containing 0.5% agar and cultivation was carried out at 37℃ for 72hrs with shaking. Cells were removed by centrifugation at 15,000 rpm and the supernatant was used as the starting material for purification of the enzyme. During the addition of ammonium sulfate to 160 ml of the culture fluid to 80% saturation with continuous stirring, cohesive suds appeared at the margin of the surface. These suds were spooned out and dissolved in an aliquot of 50 mM phosphate buffer (pH 7.0) and dialyzed against the same buffer. The enzyme solution was subsequently applied on DEAE-Sephadex A-50 column equilibrated with 50 mM phosphate buffer (pH 7.0), and the enzyme fraction was eluted by 0.1 M KCl. The active fractions were combined and applied on Bio-Gel P-100 column and eluted with the same buffer. The active fractions were combined and then applied on CM-Cellulose column equilibrated with 50 mM citrate-phosphate buffer (pH 5.0). The enzyme was eluted in void volume with the same buffer. The purification was 10.6 fold and the yield was 0.8%. A summary

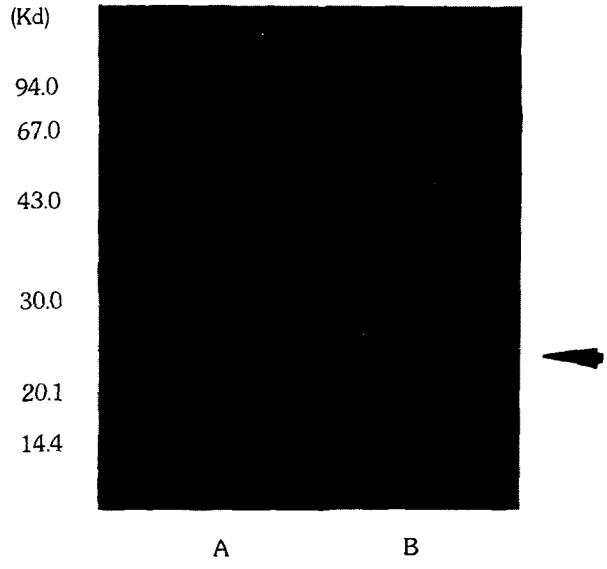

Fig 3. SDS-Polyacrylamide gel electrophresis of the purified agarase.
Symbols: A, size marker; B, agarase

Table 3. Summary of the purification of agarase

Steps	Vol (ml)	Total Enzyme (U)	Total Protein (mg)	Sp. Activity (U/mg)	Purification fold	Yield (%)
Culture fluid	160	1809.0	152.7	11.8	1	100
80% $(NH_4)_2SO_4$	12	638.5	35.1	18.2	1.5	22.9
DEAE-Sephadex	51	311.1	5.9	55.3	4.7	3.9
Bio-Gel P-100	30	197.9	2.8	70.7	6.0	1.8
CM-Cellulose	24	153.0	1.2	125.4	10.6	0.8

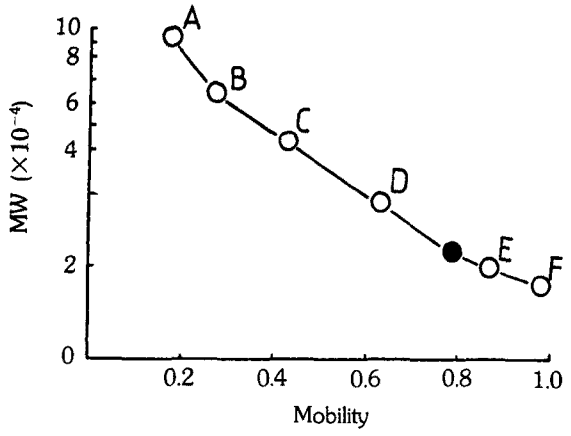

Fig. 4. Determination of the molecular weight of the agarse by SDS-PAGE (15%).
The standard proteins used and their MW were: A, phosphorylase b (94,000); B, albumin (67,000); C, ovalbumin (43,000); D, carbonic anhydrase (30,000); E, Soybean trypsin inhibitor (20,100); F, alphalactalbumin (14,000)

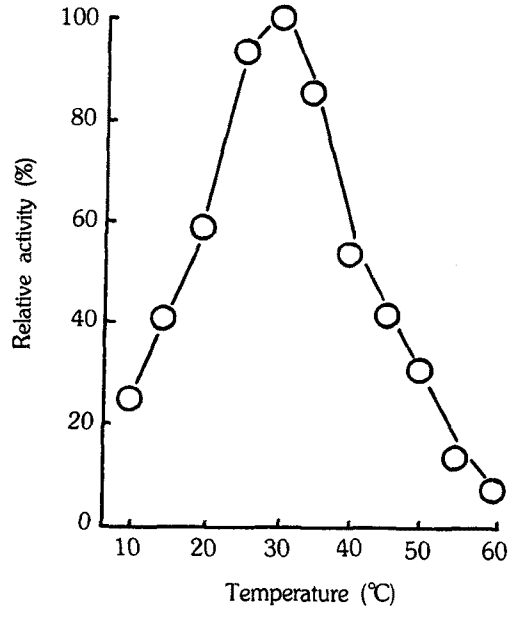

Fig. 5. Effect of temperature on the agarase activity.
The agarase activity in reaction mixture at various temperatures was estimsted.

of the purification procedure is presented in Table 3. The purified enzyme showed a single band upon 15% SDS-polyacrylamide gel electrophoresis (Fig. 3) and the molecular weight was estimated to be 24 kDa (Fig. 4).

Biochemical and Enzymatic Characteristics of the Agarase

The agarase showed maximum activity at 30℃ and

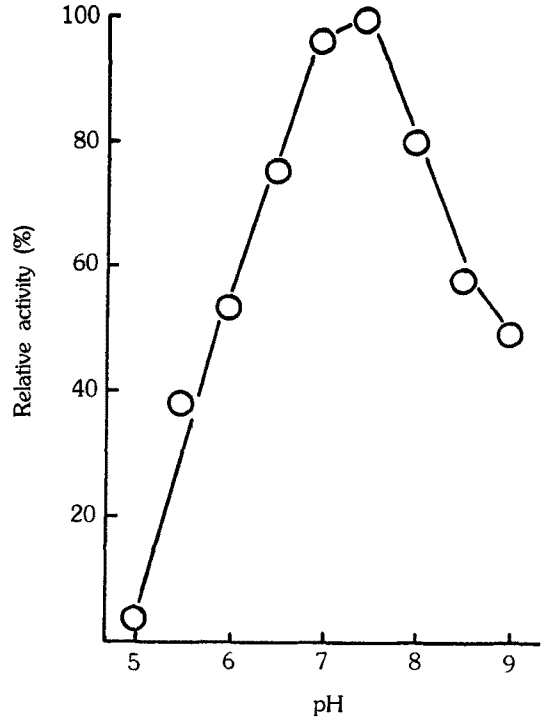

Fig. 6. Effect of pH on the agarase activity.
The buffers used were: 0.1 M Citrate-Na_2HPO_4 (pH 5-7), 0.1 M K_2HPO_4-Na_2HPO_4 (pH 7-8), 0.1 M H_3BO_3-KCl-NaOH (pH 8-9).

approximately 40% of the maximum activity at both 15° C and 45℃ (Fig. 5). The optimal pH of the enzyme activity was 7.5 and it showed approximately 60% of the maximum activity at pH 6.0 and 8.5 (Fig. 6). Beyond pH 7.0, the optimum pH for enzyme stability, the enzyme was considerably unstable and lost 55% of the activity by incubation at pH 3.0 for 1 hr at 30℃ and 80% at pH 10 (Fig. 7). While the enzyme was stable at 30℃, about 70% and 30% of the original activity were retained after incubation for 90 min at 40℃ and 50℃, respectively (Fig. 8). Table 4 indicates the effect of various metal ions on the enzyme activity. Hg^{2+} significantly inhibited the activity, whereas Mn^{2+} strongly activated the activity at concentration of 0.1 mM. The agarase activity was increased 3 times more by the addition of 1 mM of $MnSO_4$ (Fig. 9). The K_m value and V_{max} of the enzyme were 16.67 mg/ml and 3.77 unit/ml·min, respectively (Fig. 10).

Construction of Recombinant Plasmid

Chromosomal DNA of the strain KY-1 was partially digested with *PstI* and then ligated into pBR322 that was also cleaved with *PstI*. The ligation mixture was transformed into *E. coli* MC1061. A transformant which formed a shallow depression around the periphery of

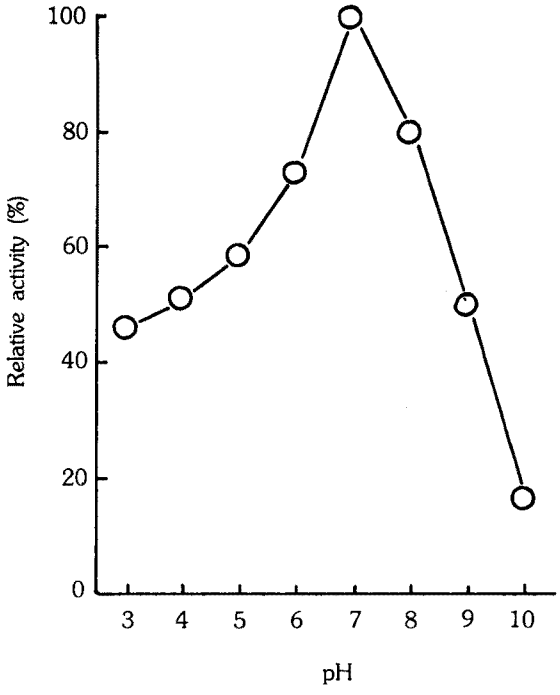

Fig. 7. pH stability of the agarase.
The enzyme solution at various pH was placed for 1 hr at 30℃. After adjustment of pH with acid or alkali solution, the residual enzyme activity was measured.

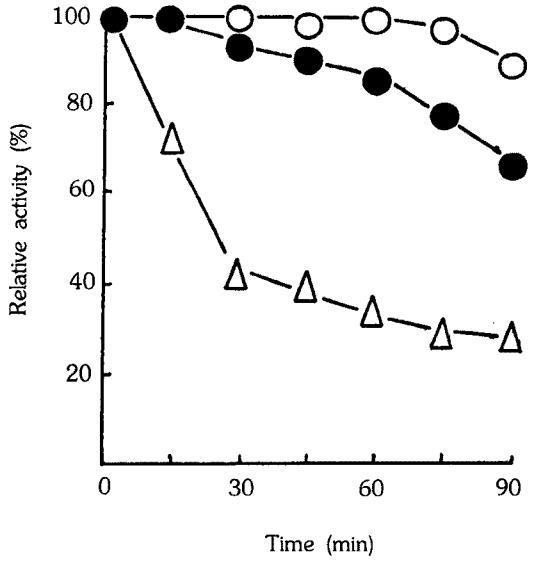

Fig. 8. Heat stability of the agarase.
The enzyme solution was preincubated at 30℃ (○), 40℃ (●), or 50℃ (△) for various intervals. After preincubation, the residual enzyme activity was measured.

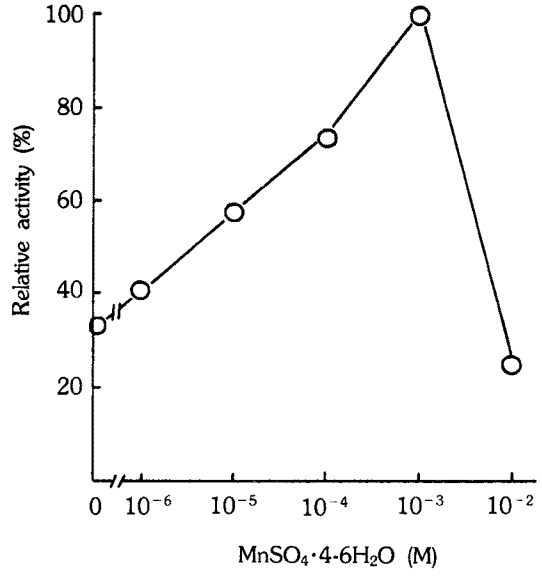

Fig. 9. Effect of manganase sulfate concentration on the agarase activity.
Agarase activity in reaction mixtures containing various concentrations of manganese sulfate was estimated.

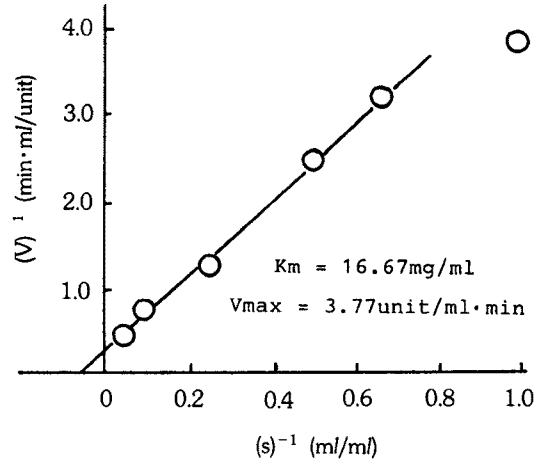

Fig. 10. Lineweaver-Burk Plots of initial rate of hydrolysis of agarose by agarase at different substrate levels.

the colony was selected among 3,420 transformants resistant to tetracycline but sensitive to ampicillin (Fig. 11). The recombinant plasmid designated as pBA322 was subjected to further study.

Characterization of pBA322

The restriction enzyme analysis revealed that the recombinant plasmid contained 4.3 kb of *Pst*I-cleaved pBR 322 and 1.4 kb of *Pst*I fragment of donor chromosomal DNA (Fig. 12). Additional restriction enzyme analysis al-

Table 4. Effect of metal salts on the agarase activity

aa' M	Agarase activity (U)
$CaCl_2$	54.1
$CuSO_4$	42.2
$FeSO_4$	61.1
KCl	62.5
$BaCl_2$	36.2
$MgSO_4$	63.3
$MgCl_2$	56.6
$MnSO_4$	96.2
NaCl	54.4
$NiSO_4$	54.0
Na_2HAsO_4	60.0
Na_2WO_4	59.2
TiO_2	51.1
$K_4Fe(CN)_6$	62.9
$HgCl_2$	7.6
EDTA·2Na	41.4
$ZnCl_2$	46.3
$CoCl_2$	67.3
None	44.4

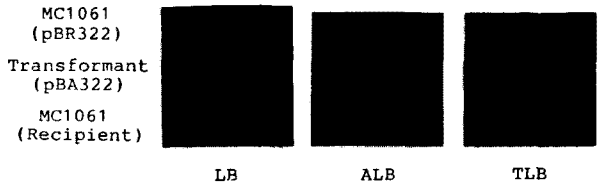

Fig. 11. Identification of transformant on LB agar medium containig ampicillin (50 μg/ml) and Tetracycline (15 μg/ml).

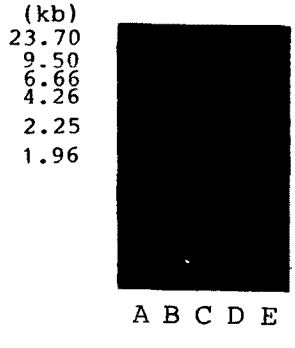

Fig. 12. Restriction map of plasmid pBA322.
Symbols: A,lambda DNA-*Hin*dIII; B, pBR322; C, pBR322-*Pst*I; D, pBA322-*Pst*I; E, pBA322.

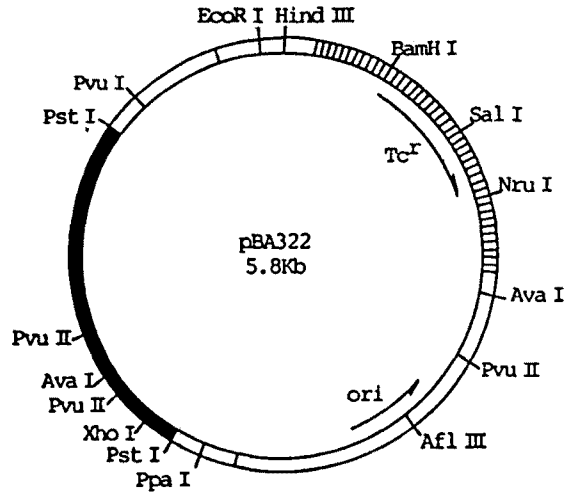

Fig. 13. Restriction map of plasmid pBA322.

Table 5. Amounts and localization of agarase produced by *Cytophaga fermentans* subsp. *agarovorans* KY-1 and *E. coli* MC1061 harbouring the plasmid pBA322

Strains	Extra cellular	Peri-plasmic	Cellular	Total
C. Fermentans subsp. *agarovorans* KY-1	98.0	7.8	22.9	138.4
E. coli MC1061 harbouring pBA322	22.8	4.3	5.4	37.4
E. coli MC1061	–	–	–	–

lowed the construction of a restriction map of recombinant plsmid pBA322 (Fig. 13). The 1.4 kb of *Pst*I fragment of donor chromosomal DNA containing the agarase gene has unique *Ava*I, unique *Kpn*I, unique *Xho*I, and two *Pvu*II sites, whereas it has no sites of *Eco*RI, *Hin*dIII, *Bam*HI, *Sal*I, *Nru*I and *Bgl*I.

Agarase Localization

The enzyme localization of both the donor *Cytophaga fermentans* subsp. *agarovorans* KY-1 and the *E. coli* transformant harboring the recombinant plasmid pBA 322 was followed as described previously (14). As shown in Table 5, approximately 80% of the total agarase produced by the donor was extracellularly secreted, while 60% of the total agarase was extracellularly secreted by the transformant.

Enzymatic Characteristics of the Agarase Produced by Transformant

The agarases produced by both transformant and donor were compared with respect to optimal pH as well as optimal temperature. As the results shown in Fig. 14 and Fig. 15, the optimum pH and temperature for the enzyme activity of both agarases were pH 7.5 and 30℃,

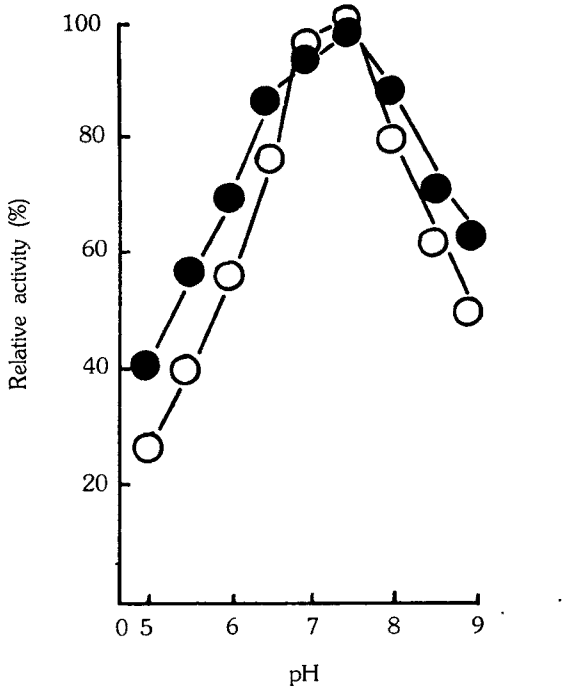

Fig. 14. Effect of pH on the agarase activity.
Symbols: ●, *E. coli*, MC1061 harbouring pBA322
○, *Cytophaga* sp. KY-1

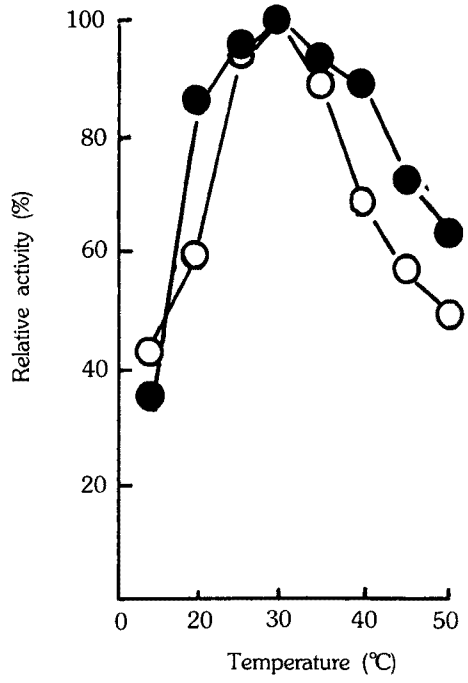

Fig. 15. Effect of temperature on the agarase activity.
Symbols: ●, *E. coli* MC1061 harbouring pBA322
○, *Cytophaga* sp. KY-1

respectively. These results indicate that the agarase expressed in the transformant is the same with that of the donor.

DISSCUSSION

Although most of the previously known agarolytic bacteria were isolated from marine environment, enrichment culture of agarolytic ba teria from fresh water sewage sample by using subculture twice in a screening medium containing 0.5% agar as a sole carbon source allowed us to isolate a bacterial strain KY-1 that shows the extracellular agarase activity. Based on its both morphological and physiological characteristics, the strain KY-1 turned out to be *Cytophaga fermentans* subsp. *agarovorans*. The extracellular agarase was induced by polysaccharides such as agar and agarose, but not by monosaccharides including glucose and galactose (data not shown), indicating that the agarase is inducible by some degradation products of either agar or agarose and may be regulated by carbon catabolite repression.

The agarase was purified by the routine protein purification steps involving ammonium sulfate precipitation, DEAE-Sephadex A-50, Bio-Gel P-100 and CM-Cellulose column chromatography. The molecular weight of the purified enzyme was 24 kDa on SDS-PAGE and smaller than those of β-agarase I (35 kDa) of Pseudomonas atlantica (16), extracellular agarase (29 kDa) of *Streptomyces coelicolor* A3 (5), extracellular agarase (50 kDa) of *Vibrio harveyi* (9) and extracellular agarase (26 kDa) of *Cytophaga flevenis* (20).

The agarase showed maximum activity at 30℃ and pH 7.5 which was similar to the cases of *Pseudomonas atlantica* (pH 7.0), *Vibrio purpureus* (pH 7.4) and *Agrobacterium pastinator* (pH 7.0), but slightly different from the cases of *Vibrio harveyi* (pH 6.0) and *Pseudomonas* sp. PT-5 (pH 8.5) (9, 16, 18, 21).The enzyme was stable in the neutral pH region suggesting that it can be useful for isolating DNA fragment from agarose gel following electrophoresis, since the running buffer is pH 8.0. The enzyme activity was not much affected by NaCl but significantly enhanced by $MnSO_4$, while the agarase activity of agar-digesting bacteria isolated from marine environment was elevated in the presence of NaCl (9, 18, 21).

In an attempt to elucidate the mechanism of gene expression regulation as well as the genomic sequence of the agarase, the gene was cloned from *C. fermentans* subsp. *agarovorans* KY-1 into *E. coli* and localized to 1.4 kb fragment DNA. Since the agarase appeared as

a 24 kDa that would require a coding sequence of about 650 bp for the structural gene, and since the agarase gene expressed an active enzyme in *E. coli* and upto 60% of total agarase was secreted extracellularly, these results indicate that the inserted 1.4 kb fragment DNA may contain the full sequence of structural gene as well as the signal peptide sequence. This prediction was further supported by comparing the enzymatic properties of agarase produced by both donor and recipient, which showed the same patterns in terms of optimal pH and temperature. Further studies characterizing the genomic sequence, and the mechanism of both the gene expression regulation and the agarase secretion are currently being conducted.

Acknowledgement

This study was supported by a research grant from the Ministry of Education in Korea.

REFERENCES

1. **Bibb, M.J., G.H. Jones, R. Joseph, M. Buttner and J. Ward.** 1987. The Agarase Gene (dagA) of Streptomyces coelicolor A3(2): Affinity Purification and Characterization of the Cloned Gene Product. *Eur. J. Microbial.* **133**: 2089-2096.
2. **Birnboim, H.C. and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**: 1513.
3. **Bradford, M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry.* **72**: 248-254.
4. **Breed, R.S., E.G.D. Murray and N.R. Smith.** 1957. 7th Bergey's Manual of Determinative Bacteriology, Edited R.E. Buchanna and N.E. Gibbons, Waverly press.
5. **Buttner, M.J., I.M. Fearnley and M. Bibb.** 1987. The agarase gene(dagA) of *Streptomyces coelicolor* A3(2): nucleotide sequence and transcriptional analysis. *Mol. Gen. Genet.* **209**: 101-109.
6. **Dillon, A.R., A. Nasim and E.R. Nestmann.** 1985. Recombinant DNA Methodology, Willey press. Pp. 35-40.
7. **Duckworth, M. and W. Yaphe.** 1971. The structure of agar. I. Fractionation of a complex mixture of polysaccharides. *Carbohydrate Research* **16**: 189-197.
8. **Duckworth, M. and W. Yaphe.** 1971. The structure of agar. II. The use of bacterial agarase to elucidate structural features of the charged polysaccharide in agar. *Carbohydrate Research* **16**: 435-445.
9. **Fukasawa, S. and H. Kobayashi.** 1987. Properties of the Agarase from a Luminous Bacterium, *Vibrio narveyi. Agric. Biol. Chem.* **51**: 269-270.
10. **Gerhardt, P., R.G.M. Murrury, R.N. Costilow., E.W. Nester., W. A. Wood., N.R. Krieg and G.B. Phillips.** 1981. Manual of Methods for General Bacteriology, ASM press.
11. **Gran, H.H.** 1902. Studien uber Meeresbakterian. II. Veber die Hydrolysis des Agar durch ein neues Enzyme die Gelase. Bergen Museums Aarbog, No. 2 [Cited in Stanier, R.Y. (1941). Studies on marine agar-digesting bacteria. *Journal of Bacteriology* **42**: 527-559].
12. **V. Hofsten, B and M. Malmqvist.** 1975. Degration of agar by a Gram-negative bacterium. *Journal of General Microbiology* **87**: 150-158.
13. **Kendall, K. and J. Cullum.** 1984. Cloning and expression of an extracellular-agarase gene from *Streptomyces coelicolor* A3(2) in *Streptomyces lividans* 66. *Gene.* **29**: 315-321.
14. **Kim, Y.H, J.H. Seu., D.Y. Jun and S.D. Hong.** 1986. Cloning of *Bacillus amyloliquefaciens* amylase gene using YRp7 as a vector-I. Expression of cloned amylase gene in *Escherichia coli. Kor. J. Appl. Micobiol. Bioeng.* **14**: 161-168.
15. **Miller, J.M., R. Blum, W.E. Glennon and A.L. Barton.** 1960. Measurement of Carboxymethyl cellubase activity. *Anal. Biochem.* **1**: 127-
16. **Morrice, L.M., M.W. Mclean, F.B. Williamson and W.F. Long.** 1983. β-Agarases I and II from *Pseudomonas atlantica*: purification and some propteries. *Eur. J. Biochem.* **135**: 553-558.
17. **J. Sambrook, E.F. Fritsch and Maniatis, T.** 1989. Molecular cloning, A Laboratory Manual. Pp. 1.42-1.46. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor press, N.Y.
18. **Sampietro, A.R. and M.A. Vattuone De Sampietro.** 1971. Characterization of the Agarolytic System of *Agarbacterium pastinator. Biochem. Biophs. Acta.* **244**: 65-76.
19. **Smith, B.J.** 1984. SDS polyacrylamide gel electrophoresis, Pp. 41-45 In Walker, J.M. (ed.), Methods in Molecular Biology. I. The humana press Inc. Crescent Manor, Clifton, N.J.
20. **Van der Meulen, H.J. and W. Harder.** 1976. Charterization of the neoagarotetraase and neoagarobiase of Cytophaga flevensis. *Antonie van Leeuwenhook.* **42**: 81-94.
21. **Yamura, I., T. Matsumoto, M. Funatsu, H. Shigeri and T. Shibata.** 1991. Purification and some properties of Agarase from *Pseudomonas* sp. PT-5 Agric. *Biol. Chem.* **55**: 2531-2536.

(Received 22 February, 1993)