J. Microbiol. Biotechnol. (2006), **16**(12), 1868–1873

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

© The Korean Society for Microbiology and Biotechnology

Isolation of a Novel Gellan-Depolymerizing *Bacillus* sp. Strain YJ-1

JUNG, YU-JIN, CHEON SEOK PARK[1], HYEON GYU LEE[2], AND JAEHO CHA*

Department of Microbiology, Pusan National University, Busan 609-735, Korea
[1]Department of Food Science and Technology, Kyunghee University, Yongin 449-701, Korea
[2]Department of Food and Nutrition, Hanyang University, Seoul 133-791, Korea

Received: April 19, 2006
Accepted: July 13, 2006

Abstract A novel microorganism that could degrade high molecular weight gellan was screened and isolated from soil. On gellan plate, the microorganism grew well and completely liquefied the plate. The gellan-degrading microorganism was isolated by pure culture on glucose and nutrient agar medium afterwards. The 16S rDNA sequence analysis and biochemical tests using an API 50CHB/20E kit revealed that the strain belonged to *Bacillus* sp. The isolate, named as *Bacillus* sp. YJ-1, showed optimum gellan-degrading activity in 0.5% gellan medium at pH 7.5 and 37°C. The activity was measured and evaluated by the thiobarbituric acid and thin-layer chromatography method. Mass spectrometry revealed that the major gellan-depolymerized product was an unsaturated tetrasaccharide consisting of Δ4,5-glucuronic acid-(1→4)-β-D-glucose-(1→4)-α-L-rhamnose-(1→3)-β-D-glucose, which is a dehydrated repeating unit of gellan, thus the enzyme was identified as gellan lyase. When the gellan was present in the medium, the gellan-degrading activity was much higher than that in glucose-grown cells. These results indicate that in the presence of gellan, *Bacillus* sp. YJ-1 is able to metabolize the gellan by inducing gellan-degrading enzymes that can degrade gellan into small molecular weight oligosaccharides, and then the gellan-depolymerized products are taken up by the cells and utilized by intracellular enzymes.

Key words: Gellan, gellan-depolymerizing enzyme, screening, thin-layer chromatography, 16S rDNA sequence

Microbial exopolysaccharides have been invaluable ingredients in the food industry, as having well as many attractive pharmaceutical and chemical applications for decades. Gellan is an extracellular heteropolysaccharide produced by *Sphingomonas paucimobilis*, formerly referred to as *Pseudomonas elodea* [14, 15]. It is composed of linear tetrasaccharide repeating units of β-D-glucuronic acid, β-D-glucose, α-L-rhamnose, and β-D-glucose [7, 12]. Occasionally, it carries acetyl and glyceryl groups on glucose units. The degree of ester substitution directly influences the gellan properties in solution and gel [3, 15, 16]. Aqueous solutions of gellan are highly viscous and show high thermal stability. The gellan gum exhibits good stability over a wide pH range of 3.5–8.0. Acid stability of gellan gum is a distinct advantage in the food industry, especially for fruit-based products. According to its property to produce a thermoreversible gel, gellan gum can be substituted for an agar-based cultivating medium [4, 8]. Owing to the diversity of its structure and properties, gellan gum has a wide range of applications in the food, pharmaceutical, and other industries as texturing, stabilizing, thickening, emulsifying, and gelling agents [14, 15]. However, the highly viscous properties have sometimes largely limited its utility, particularly in the food industry. The structural modification by decreasing the molecular sizes may enhance their physical and biological properties [11].

Methods for depolymerization that reduce the molecular sizes are now sought, not only to prepare low-viscosity and low-molecular mass gellans for novel physiological and food technological functions, but also to exploit new areas for the application of gellan in biopolymer-based industries [5]. Two approaches, chemical and enzymatic methods, have been pursued to decrease viscosity. The former has been developed to prepare low-viscous and low-molecular-mass welan [1], whereas the practicality of the latter method at an industrial scale remains as yet unproven. The enzymatic methods for depolymerization could be done by microbial enzymes. So far, two types of enzymes, sphinganase and gellan lyase, from two microorganisms have been reported [9, 15]. Hashimoto *et al.* [6] isolated gellan lyase and proposed the enzymatic depolymerization process by a soil-isolated microbial strain, *Bacillus* sp. GL1.

*Corresponding author
Phone: 82-51-510-2196; Fax: 82-51-510-1773;
E-mail: jhcha@pusan.ac.kr

In this study, we tried to find a novel microorganism that can degrade gellan and examined the enzymes involved in gellan degradation.

MATERIALS AND METHODS

Chemicals and Reagents

Gellan (molecular mass 5×10^5, deacetylated) was purchased from Wako Pure Chemicals Co. (Osaka, Japan). The AccuPower PCR PreMix for PCR amplification was purchased from Bioneer (Daejon, Korea) and the QIAquick gel extraction kit was purchased from Qiagen (Hilden, Germany). For sugar analysis, the silica gel 60 F_{254} thin-layer chromatography (TLC) plate was purchased from Merck (Haar, Germany). *p*-Nitrophenyl-β-D-glucopyranoside (pNPG), *p*-nitrophenyl-α-L-rhamnopyranoside (pNPR), and *p*-nitrophenol (pNP) were obtained from Sigma (St. Louis, MO, U.S.A.). API 50CHB and 20E kit were from bioMerieux (Marci I'Etoile, France).

Screening and Isolation of Gellan-Degrading Microorganism

Soil samples were cultured in 0.2% gellan medium consisting of 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.2% $Na_2HPO_4 \cdot 12H_2O$, 0.01% $MgSO_4 \cdot 7H_2O$, 0.01% yeast extract, and 0.2% gellan, pH 7.2, at 30°C for seven days. Culture fluid was inoculated to 0.5% gellan medium for further cell culture, and then smeared on the 0.75% agar medium including 1% gellan. On 1% gellan solid medium, gellan-degrading microorganisms liquefied the plates after prolonged incubation. The gellan-degrading microorganism on 1% gellan medium was subcultured on 1% gellan medium and then transferred on 1% glucose agar medium. Each colony on the glucose agar medium was picked and inoculated into the 0.5% liquid gellan medium for the examination of gellan-degrading activity. The grown cells in gellan medium were then smeared on LB agar medium. Finally, the microorganism on LB agar medium was subcultured on nutrient agar medium and isolated as a gellan-degrading microorganism.

Identification of the Gellan-Degrading Isolate and Phylogenetic Relationship with Other Microorganisms

The identification of the isolate was carried out by 16S rDNA sequence analysis and API method. Total genomic DNA was extracted by the modified method of Rodrigues and Tait [13]. The concentration of DNA was determined by measuring its absorbance at 260 nm using a spectrophotometer (Ultrospec 2100Pro, Amersham, Uppsala, Sweden) and the purity was evaluated by the A_{260}/A_{280} ratio. Only samples with ratios $A_{260}/A_{280}>1.8$ were used for PCR amplification. Amplification of 16S rDNA was performed with universal primers, 27F (5'-AGAGTTTGA-TCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTT-GTTACGACTT-3'). PCR amplification was carried out on a thermal cycler (GenAmp PCR System 2400, Perkin Elmer, Wellesley, MA, U.S.A.) with a program of a predenaturation at 94°C for 3 min, followed by 30 cycles (denaturation at 94°C, 1 min; annealing at 52°C, 1 min; extention at 72°C, 1 min), and a final incubation at 72°C for 5 min. The PCR product was isolated by a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced with a DNA Analyzer (ABI PRISM 3700). Sequence similarity searches were performed using the BLAST of the NCBI database (http://www.ncbi.nlm.nih.gov/blast). Based upon the result of 16S rDNA sequencing of the isolate, API 50CHB and API 20E kits for *Bacillus* sp. identification were also used. The biochemical profile obtained by API kits can be examined using the identification software, APILAB. The phylogenetic tree of the gellan-degrading isolate was visualized according to the results of 16S rDNA sequencing and API method. The 16S rDNA sequences of the isolate and closest microorganisms were collected and then aligned by the Clustal X program and Bioedit program. After editing, the file was converted to construct a phylogenetic tree with the Mega2 program. Bootstrap values were expressed as percentages of 1,000 replications.

Optimization of Culture Conditions for Gellan Degradation

Temperature, pH, or nitrogen concentration was examined to determine the optimum culture condition for gellan degradation. For the pH and temperature, cells were aerobically incubated at the range of pH 6–9 and 20–45 in 0.5% gellan liquid medium and the activity was measured every 12 h by the thiobarbituric acid (TBA) method and TLC. The growth was also examined by optical density at 660 nm. The concentration of yeast extract was used in the range of 0.01–0.5%.

Enzyme Assays

Gellan lyase activity was assayed as described by Weissbach and Huritz [17]. Enzyme reaction was performed in a mixture of 0.05% gellan, 50 mM HEPES buffer (pH 7.5), and enzyme, at 30°C. An aliquot (0.1 ml) of the reaction mixture was added to 0.125 ml of 0.025 N H_5IO_6 in 0.125 N H_2SO_4. After 20 min at room temperature, 0.25 ml of 2% sodium metaarsenite in 0.5 N HCl was added with shaking, and the solution was permitted to stand for 2 min. One ml of 0.3% TBA was added and the mixture was then heated in boiling water for 10 min. After cooling the mixture, the absorbance at 548 nm was measured. One unit of enzyme activity was defined as the amount of enzyme required to increase 1 optical density at 548 nm per 1 h at 30°C under the above assay conditions.

β-D-Glucosidase and α-L-rhamnosidase activities were assayed using 0.4 mM pNPG and pNPR as substrates in

50 mM potassium phosphate buffer (pH 7.0). The reaction mixture was incubated at 30°C and terminated by boiling. After cooling the reaction mixture, the color development was read at 405 nm by a microplate reader (Benchmark, BioRad, Hercules, CA, U.S.A.) and translated to μmol of pNP using a calibration curve prepared under the same conditions. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of pNP per min at 30°C under the above assay conditions [5, 9].

Analytical Method

Protein concentration was determined by the Bradford method using a Bradford reagents kit (BioRad, Hercules, CA, U.S.A.) and bovine serum albumin as a standard [2]. The detection and identification of gellan-degrading products were carried out by TLC analysis. Two μl of the reaction mixture was spotted onto a Silica gel 60 F_{254} plate and developed with a solvent system of *n*-butanol/acetic acid/water (5:4:1, v/v/v) in a TLC developing tank. Ascending development was repeated twice at room temperature. The plate was allowed to air-dry in a hood, and then soaked rapidly into 1 l of methanol solution containing 20% (v/v) sulfuric acid and 3 g of naphtol. The plate was dried and placed in a 110°C oven for 10 min to visualize the spots.

The molecular mass of major gellan-degraded product was measured with QUATTRO LC Triple Quadrupole Tandem Mass Spectrometry at NCIRF (National Center for Inter-University Research Facilities, Busan, Korea). To this end, 0.05% gellan in 50 mM HEPES buffer (pH 7.5) was incubated with enzyme at 30°C for 3 h. The reaction mixture was frozen and thawed at room temperature, and then centrifuged at 8,000 ×*g* for 20 min to remove unreacted gellan. The resultant supernatant was frozen and thawed again as above, and the clear supernatant obtained after centrifugation. The supernatant was confirmed by the TLC method and the sample was applied to the MS. After the first mass spectrometry, MS-MS spectrometry of 645 m/z peak (expected as gellan-degraded product) was performed to confirm the molecular mass of the gellan-degraded product.

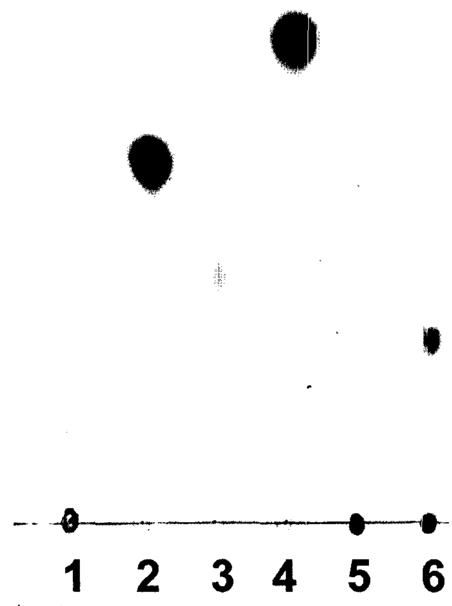

Fig. 1. TLC profile of the gellan degradation product after 48 h incubation.
Lane 1, gellan (0.5%); lane 2, glucose (1%); lane 3, glucuronic acid (1%); lane 4, rhamnose (1%); lanes 5–6, gellan degradation product after 12 and 48 h incubation.

Results and Discussion

Screening and Isolation of a Novel Gellan-Degrading Microorganism

Soil samples were directly inoculated into 0.2% gellan medium and then aerobically cultured for seven days. It was not easy to confirm the growth of the microorganisms because of the turbidity of the soil samples. The growth of microorganisms was confirmed with optical microscopy. An aliquot of initial culture was transferred to 0.5% liquid gellan medium and incubated for 48 h. TLC analysis of the culture fluid was performed to examine the gellan-degrading activity. On a TLC plate, spots of gellan-degraded product were distinguished from the components of gellan polymer, such as glucose, glucuronic acid, and rhamnose (Fig. 1). On gellan plates, the microorganisms completely liquefied the plates after prolonged incubation. The microbial cells initially formed a colony on the surface of the plates, and then some of them sank into the plates, making a pit (Fig. 2A). Strong pit-forming colonies were picked up and smeared by streaking on sterile solid 1% glucose medium, for the isolation of gellan-degrading microorganism, because the gellan-degrading microorganisms formed watery colonies during incubation and thus mixed with other colonies easily making it difficult to isolate a single colony

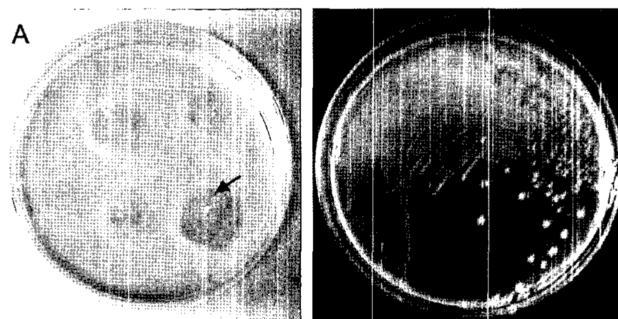

Fig. 2. Morphology of gellan-degrading microorganism in gellan (**A**) and nutrient agar (**B**) media.
Arrow shows the pit formation.

in the gellan medium. The single isolate on glucose medium was reconfirmed by streaking on nutrient agar medium, and a gellan-degrading isolate was finally obtained and named as YJ-1 (Fig. 2B).

Identification of Gellan-Degrading Microorganism

For the identification of the gellan-degrading isolate YJ-1, 16S rDNA sequencing was carried out. The PCR product amplified by universal 16S rDNA primers was sequenced and compared with the BLAST program. The isolate YJ-1 showed high identity to that of *Bacillus* sp. GL1 (96.4%), *Paenibacillus* sp. JL-02 (95.5%), and *Paenibacillus hongkongensis* (94.9%). *Bacillus* sp. GL1 is known to be a gellan-degrading bacterium [5, 6]. The API 50CHB/20E kit, which is used to differentiate *Bacillus* species, was selected for the biochemical identification of the isolate. The data of API 50CHB/20E kit were read in the database (APILAB) and the isolate YJ-1 showed 84.8% homology with *Bacillus circulans*. Combined with the results of the 16S rDNA sequence and API 50CHB/20E kit, the isolate YJ-1 was classified to be of *Bacillus* species. The phylogenetic tree was drawn to confirm the relationship with other *Bacillus* species (Fig. 3). Therefore, the novel gellan-degrading microorganism was named as *Bacillus* sp. YJ-1.

Optimal Culture Condition of *Bacillus* sp. YJ-1 for Gellan Degradation

To determine the optimal culture condition for gellan degradation, the culture temperature (20–45°C), pH (6.5–8.5), and nitrogen sources were examined. The gellan-degrading activity was evaluated based on the production of degradation products and cell growth. The growth rate of *Bacillus* sp. YJ-1 was highest at 37°C, and The TLC analysis of the culture supernatant also confirmed the highest gellan-degrading activity in proportion to cell growth by showing a strong degradation spot (data not shown). The gellan-depolymerized spot began to be observed after 6 h and increased up to 12–15 h and then noticeably decreased after 24 h incubation in spite of the continuous increase of the cell density. The effect of culture pH was examined after the cell was incubated at 37°C for 12 h. The intensity of gellan-depolymerized spots at a given pH was not significantly different except at pH 8.5. At pH 8.5, the cells did not grow very well. Among various nitrogen sources, yeast extract showed the highest gellan-degrading activity. The gellan-depolymerized product spot on TLC was strong with yeast extract at the concentrations of 0.1–0.5% compared with the others at the early period of 6–10 h. After prolonged incubation, the gellan-depolymerized spot was gradually weaker.

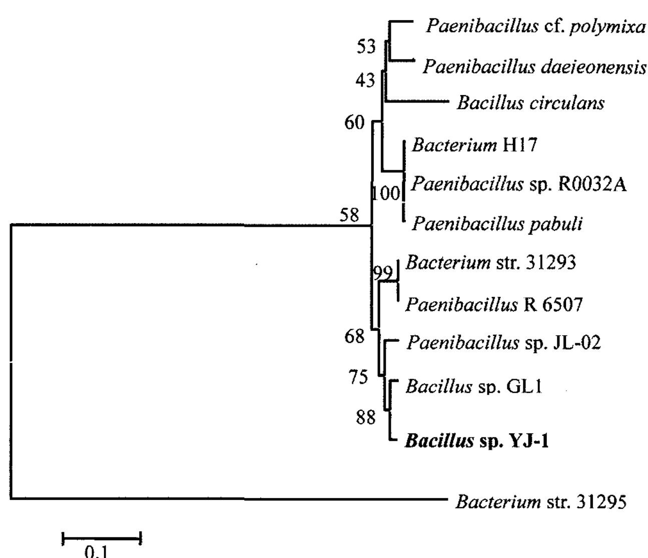

Fig. 3. Phylogenetic tree of *Bacillus* sp. YJ-1 with related species. Neighbor-joining tree based on 16S rDNA sequences shows the positions of strain *Bacillus* sp. YJ-1, *Bacillus* species, *Paenibacillus* species, and representatives of some other taxa. Branch lengths are proportional to the number of nucleotide differences. The marker bar denotes the relative branch length. Bootstrap values, expressed as percentages of 1,000 replications, are given at branch points.

Previous studies of microbial gellan-degrading enzymes had suggested that gellan depolymerization was started to produce the unsaturated tetrasaccharide [Δ4,5-glucuronic acid-(1→4)-β-D-glucose-(1→4)-α-L-rhamnose-(1→3)-β-D-glucose] unit by gellan lyase, and then further metabolized by other enzymes [6]. They confirmed the formation of the unsaturated oligosaccharide by the measurement of the absorbance at 235 nm, where the double bond of compound could be detected. When we measured the gellan degradation product by TLC and the absorbance at 235 nm of the culture supernatant from *Bacillus* sp. YJ-1, the TLC pattern and the absorbance data showed good correlation, in which the product was increased at the early period and then gradually decreased as incubation time was prolonged (Fig. 4). Therefore, the major degradation product was assumed to be the unsaturated tetrasaccharide, which was observed by another group [6]. As summarized with the absorbance data, and the results of TLC analysis and cell growth, it is thought that *Bacillus* sp. YJ-1 initially degrades the gellan polymer into unsaturated oligosaccharides, and then the oligosaccharides are taken up by the cell through a certain transport mechanism or degraded by other extracellular enzymes.

Identification of Gellan-Degrading Product from *Bacillus* sp. YJ-1

Under the optimum culture condition determined above, *Bacillus* YJ-1 degraded gellan efficiently and the culture supernatant showed a single spot on TLC plate, which is thought to be an oligosaccharide product. The molecular weight of the gellan-depolymerization product was examined by mass spectrometry. The molecular weight of 646 was obtained from a 645 m/z ion corresponding to the

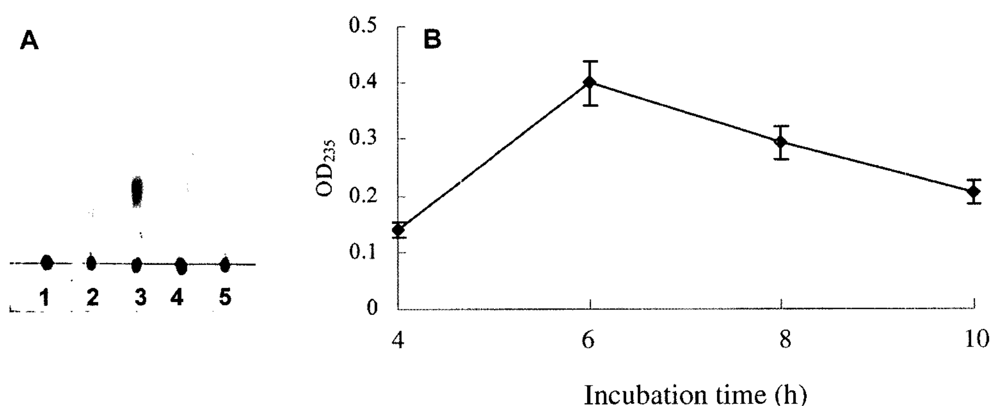

Fig. 4. Time courses of the formation of gellan-degradation product confirmed by TLC (**A**) and double-bond formation at 235 nm (**B**).
Lane 1, 0 h; lane 2, 4 h; lane 3, 6 h; lane 4, 8 h; lane 5, 10 h.

deprotonated ion $[M-H]^-$ in the negative mode (data not shown). The value was consistent with the theoretical one of an unsaturated tetrasaccharide [Δ4,5-glucuronic acid-(1→4)-β-D-glucose-(1→4)-α-L-rhamnose-(1→3)-β-D-glucose], which is the dehydrated form of a gellan repeating unit. The molecular weight of the product was further confirmed by MS-MS result showing a 645 m/z ion (Fig. 5). Therefore, it was confirmed that the major gellan-degrading enzyme of *Bacillus* sp. YJ-1 is indeed a gellan lyase.

Enzymes Involved in Gellan Degradation

Three enzymes associated with gellan degradation (gellan lyase, β-D-glucosidase, and α-L-rhamnosidase) have been known to be induced by gellan-containing medium [5]. To examine the gellan depolymerization route of *Bacillus* sp. YJ-1, cells were grown in the 0.5% gellan and 0.5% glucose medium, respectively. Three enzyme activities in both the intracellular and the extracellular fractions were determined (Table 1). The gellan lyase activity was higher on the gellan medium than on glucose medium. Little enzyme activity was detected when the gellan was replaced with glucose. Cells did not grow well on the glucose medium. On the other hand, β-D-glucosidase and α-L-rhamnosidase activities were detected in extracellular and intracellular fractions. These activities were much higher in cells grown with gellan than those with glucose. These results agree

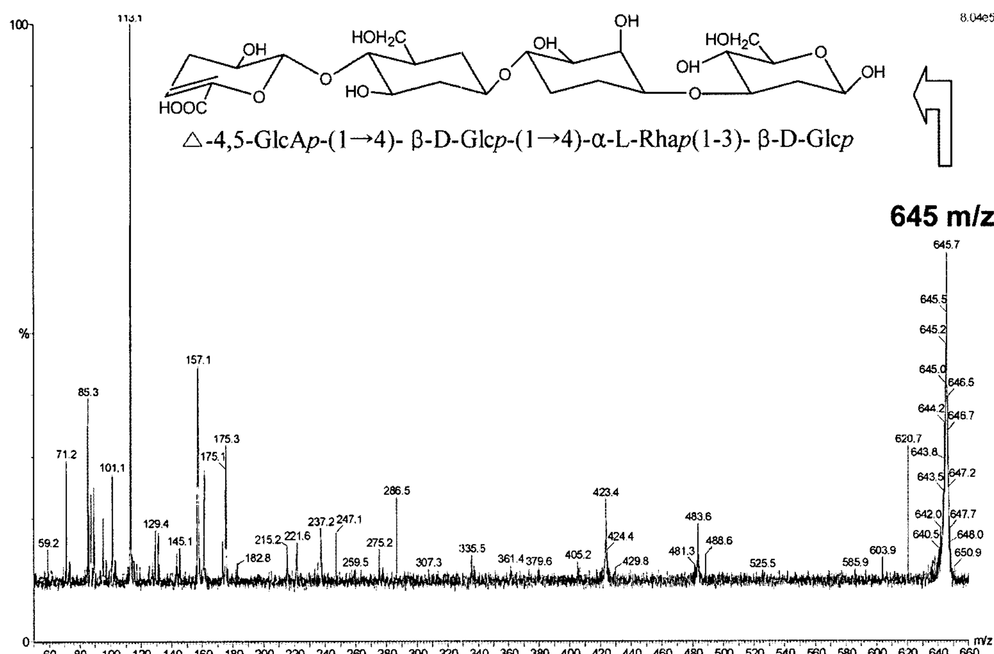

Fig. 5. Mass-Mass spectrum of the gellan depolymerization product.
The mass of gellan-degrading product was measured with QUATTRO LC Triple Quadrupole Tandem Mass Spectrometry with negative mode $[M-H]^-$.

Table 1. Specific activity of gellan-degrading enzymes in *Bacillus* sp. YJ-1

Enzyme	Specific activity (U/mg)			
	Glucose medium		Gellan medium	
	Extracellular	Intracellular	Extracellular	Intracellular
Gellan lyase[1]	8.1	0.1	96.5	14.7
β-D-Glucosidase	ND[2]	112.9	131.2	920.2
α-L-Rhamnosidase	ND	14.8	ND	248.8

[1]Gellan lyase activity was determined by the TBA method.
[2]Not detected.

well with the result that gellan-degrading enzymes were inducible in the presence of gellan, and that β-D-glucosidase and α-L-rhamnosidase were expressed at higher levels in order to utilize efficiently the gellan-depolymerization product.

In conclusion, we found a new microorganism that can degrade gellan and confirmed the enzymes involved in gellan degradation. The new microorganism and its enzymes might be useful for the modification of high molecular weight gellan, which has a variety of industrial application such as a thickener and gelling agent, a solidifying agent, and for replacing agar in media for microbial growth. The control of the viscosity of gellan may result in the finding of extended areas for industrial applications, particularly in the food industry. Although chemical methods to produce low-molecular and low-viscous oligosaccharide have been developed, the enzymatic method can produce these products that are more appropriate for industrial purposes. The purification of each enzyme is now under progress.

Acknowledgment

This work was supported by grant R01-2002-000-00207-0 from the Basic Research Program of the Korea Science & Engineering Foundation.

REFERENCES

1. Allen, F. L., G. H. Best, and T. A. Lindroth. 1990. Welan gum in cement compositions. US Patent 4,963,668.
2. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
3. Giavasis, I., L. M. Harvey, and B. McNeil. 2000. Gellan gum. *Crit. Rev. Biotechnol.* **20:** 177–211.
4. Harris, J. E. 1985. Gelrite as an agar substitute for the cultivation of *Methanobacterium* and *Methanobrevibacter* species. *Appl. Environ. Microbiol.* **50:** 1107–1109.
5. Hashimoto, W., T. Inose, H. Nakajima, N. Sato, S. Kimura, and K. Murata. 1996. Purification and characterization of microbial gellan lyase. *Appl. Environ. Microbiol.* **62:** 1475–1477.
6. Hashimoto, W., K. Maesaka, N. Sato, S. Kimura, K. Yamamoto, H. Kumagai, and K. Murata. 1997. Microbial system for polysaccharide depolymerization: Enzymatic route for gellan depolymerization by *Bacillus* sp. GL1. *Arch. Biochem. Biophys.* **339:** 17–23.
7. Jansson, P. E., B. Lindberg, and P. A. Sandford. 1983. Structural studies of gellan gum, an extracellular polysaccharide elaborated by *Pseudomonas elodea. Carbohydr. Res.* **124:** 135–139.
8. Kang, K. S., G. T. Veeder, P. J. Mirrasoul, T. Kaneko, and I. W. Cottrell. 1982. Agar-like polysaccharide produced by a *Pseudomonas* species: Production and basic properties. *Appl. Environ. Microbiol.* **43:** 1086–1091.
9. Kennedy, L. and I. Sutherland. 1994. Gellan lyases - novel polysaccharide lyases. *Microbiology* **140:** 3007–3013.
10. Kennedy, L. and I. Sutherland. 1996. Polysaccharide lyases from gellan-producing *Sphingomonas* spp. *Microbiology* **142:** 867–872.
11. Mikolajczak, M. J., L. Thorne, T. J. Pollock, and R. W. Armentrout. 1994. Sphinganase, a new endoglycanase that cleaves specific members of the gellan family of polysaccharides. *Appl. Environ. Microbiol.* **60:** 402–407.
12. O'Neil, M. A., R. R. Selvendran, and V. J. Morris. 1983. Structure of the acidic extracellular gelling polysaccharide produced by *Pseudomonas elodea. Carbohydr. Res.* **124:** 123–133.
13. Rodrigues, R. C. and R. C. Tait. 1988. *Recombinant DNA Techniques: An Introduction.* Benjamin/Cummins Publishing Co. Inc., pp. 162–163.
14. Sanderson, G. R. 1990. Gellan gum, pp. 201–232. *In* Harris, P. (ed.). *Food Gels.* Elsevier Science Publishing, New York.
15. Sanderson, G. R. and R. C. Clark. 1983. Laboratory-produced microbial polysaccharide has many potential food applications as a gelling, stabilizing and texturizing agent. *Food Technol.* **37:** 63–70.
16. Sutherland, I. W. 1992. The role of acylation in exopolysaccharides including those for food use. *Food Biotechnol.* **6:** 75–86.
17. Weissbach, A. and J. Huritz. 1958. The formation of 2-keto-3-deoxyheptonic acid in extracts of *Escherichia coli* B. *J. Biol. Chem.* **234:**705–709.