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Isolation and Characterization of a Novel Agarase-Producing *Pseudoalteromonas* spp. Bacterium from the Guts of Spiny Turban Shells

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An agar-degrading bacterium was isolated from the guts of spiny turban shells. It was identified as a *Pseudoalteromonas* species and named *Pseudoalteromonas* sp. JYBCL 1. The viscosity of the inoculated agar medium decreased by more than 60% after 20 h cultivation. The agarase produced by the isolate had optimal activities at 35°C and pH 7. The enzyme had extremely strong resistance to ionic stress compared with other known agarases. Its molecular mass was estimated at about 60 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The agarase could saccharify *Gelidium amansii* directly, with an efficiency about half that compared with agar saccharification.

Keywords: Agarase, *Pseudoalteromonas*, isolation, saccharification, *Gelidium amansii*

Agar is a marine polysaccharide that is a major component of red algae. Agar is composed of agarose and agaropectin. Agarose, which is the linear polysaccharidic portion of agar, is made up of repeating units, D-galactose, and 3,6-anhydro-L-galactopyranose [7]. Several bacterial species are known to utilize agar, including *Agarivorans* sp. [5, 8], *Alteromonas* sp. [18], *Cytophaga* sp. [23, 25], *Pseudomonas* sp. [6, 9], and *Pseudoalteromonas* sp. [2, 14, 15, 20]. Numerous agar-degrading bacteria produce agarase extracellularly, whereas some produce it intracellularly. Most agar-degrading bacteria were isolated from marine environments [1, 8, 12, 14, 21, 23], with a few isolated from soil [10, 11, 20] and fresh water [19, 25].

Agarase is an enzyme that cleaves agarooligosaccharides into smaller units. Although many studies have been aimed at finding and characterizing new agarases, there are few published reports of agarase with respect to biological pretreatment enzymes. Structural stability is an important factor for the use of an enzyme in the pretreatment process. In this study, a new agarase-producing bacterium, which secretes an extracellular agarase with strong stability against ionic stress, was isolated. In addition, its potential as a biological catalyst to degrade *Gelidium amansii* directly was described. This property is noteworthy, because direct saccharification of *G. amansii* without the pretreatment step to extract agarose or agar would reduce the time and costs involved when *G. amansii* is utilized as biomass.

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An agar-degrading bacterium was isolated from the guts of spiny turban shells. The medium was composed of yeast extract 0.5 g/l, NaCl 5.0 g/l, $(NH_4)_2SO_4$ 1.0 g/l, K_2HPO_4 2.0 g/l, $CaCl_2 \cdot 2H_2O$ 0.02 g/l, $MgCl_2 \cdot 6H_2O$ 0.2 g/l, and agar 1.0 g/l. Lugol's solution was poured onto solid media to confirm the existence of extracellular agarase.

Chromosomal DNA extracted from the isolate was used as a template for the amplification of 16S rRNA of the isolate by polymerase chain reaction, and its base sequence was determined. The result was compared with the GenBank database using the BLASTN program, followed by a homology comparison study with the Clustal W and Mega 4 programs.

Crude agarase solution was prepared. The 12-h-cultured medium was centrifuged at 4°C and 7,000 ×*g* for 30 min; 70% (w/v) ammonium sulfate was gradually added to the supernatant and dissolved spontaneously. It was then centrifuged at 4°C and 14,500 ×*g* for 30 min. Thereafter, the supernatant was removed, and an appropriate amount of 20 mM Tris-Cl buffer was added, followed by dialysis.

The reaction mixture, containing the crude agarase solution and 0.25% agarose in 20 mM Tris-Cl buffer (pH 8), was incubated at 25°C and 120 rpm. After incubation, 100 µl of galactose standard and 1 ml of DNS solution were added to 100 µl of sample supernatant. The sample–DNS mixtures were then incubated at 99°C for 10 min and cooled to room temperature. The optical density of the mixture was measured by a UV/Vis spectrophotometer (Shimadzu) at 546 nm.

The medium used to assess the change in viscosity was the same as that described above, except that 2 g/l agar was

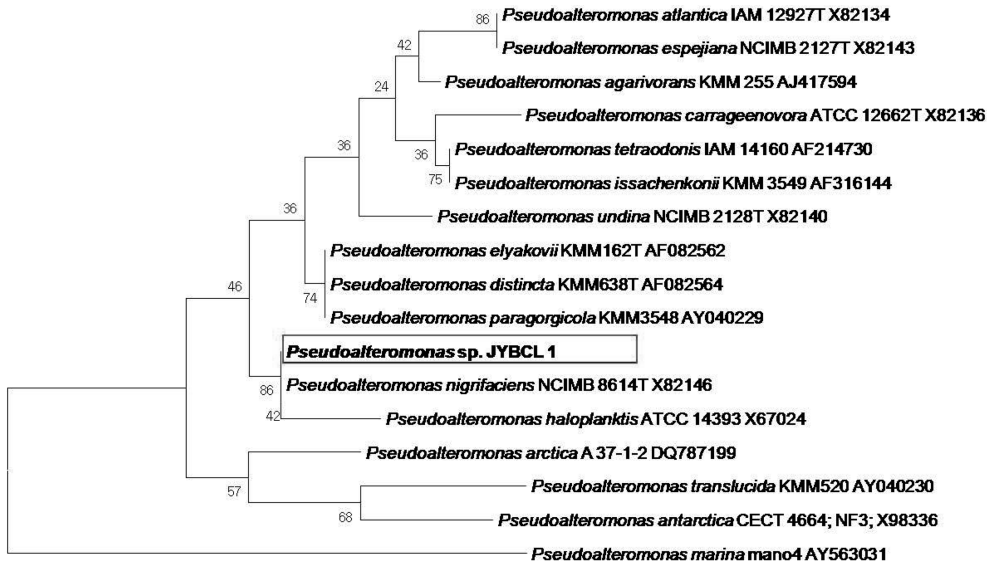

Fig. 1. Phylogenetic tree of the isolate.
Neighbor-joining analysis of the isolate indicated that strain JYBCL 1 was positioned among species of the genus *Pseudoalteromonas*.

added. It was measured using a DV-II+Pro Viscometer (Brookfield).

Enzyme–substrate mixtures were incubated at 15, 25, 35, 45, and 55°C at pH 8 for temperature optimization, followed by pH 4, 5, 6, 7, 8, and 9 at 35°C for pH optimization. To measure the stability of the agarase against ionic stress, enzyme–substrate mixtures were incubated with varying NaCl concentrations. Saccharification of several substrates, including agarose, agar, and *G. amansii*, was performed at the determined optimal conditions.

One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis and zymography were performed. Samples were loaded on a 10% polyacrylamide gel. Protein bands were visualized by the silver staining procedure. For zymogram analysis, the electrophoresized gel was placed on an agar gel and incubated in an appropriate buffer for 12 h. The agar gel was stained with Lugol's iodine after incubation.

The isolate secreted agarase extracellularly and showed a deep halo around its colony. According to the 16S rRNA homology comparison study, it was revealed that the isolate belongs to the genus *Pseudoalteromonas*. Several species, including *P. nigrifaciens*, *P. haloplanktis*, and *P. elyakovii*, showed extremely high identity with the isolate in terms of 16S rRNA sequence, and the isolate was named *Pseudoalteromonas* sp. JYBCL 1 (Fig. 1).

The viscosity of the inoculated medium was measured by a viscometer. It could not be measured precisely around or above 50cP, because the medium contained a large amount of irregular agar gel. However, below 30cP, the measured value was stable, and the viscosity of the medium decreased by about 60% after 20 h of cultivation (Fig. 2).

According to the temperature optimization test, the optimum temperature was 35°C (Fig. 3A). Activity decreased drastically as temperature increased. The enzyme was most activate at pH 7 and lost its activity in acidic conditions

Table 1. Reducing sugar released from different substrates.

Substrate	Reducing sugar released (μM)	Relative activity[a] (%)
Agarose	1.378	157
Agar	0.875	100
Gelidium amansii	0.408	47

[a]Relative activity was calculated assuming that the amount of reducing sugar released with agar was 100%.

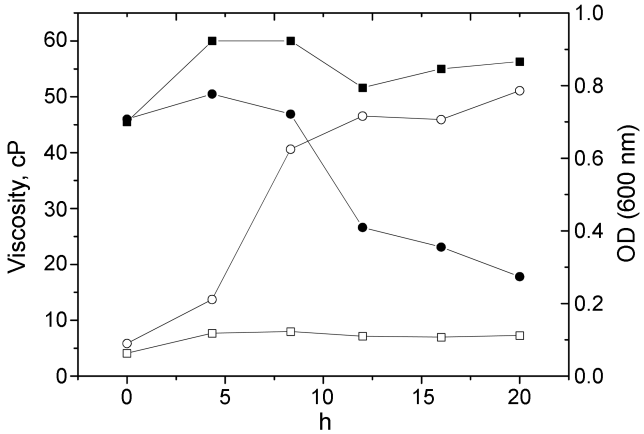

Fig. 2. Viscosity changes of the inoculated medium.
Inoculated medium and control were incubated together. ■, Control viscosity; ●, sample viscosity; □, control optical density (OD); ○, sample OD.

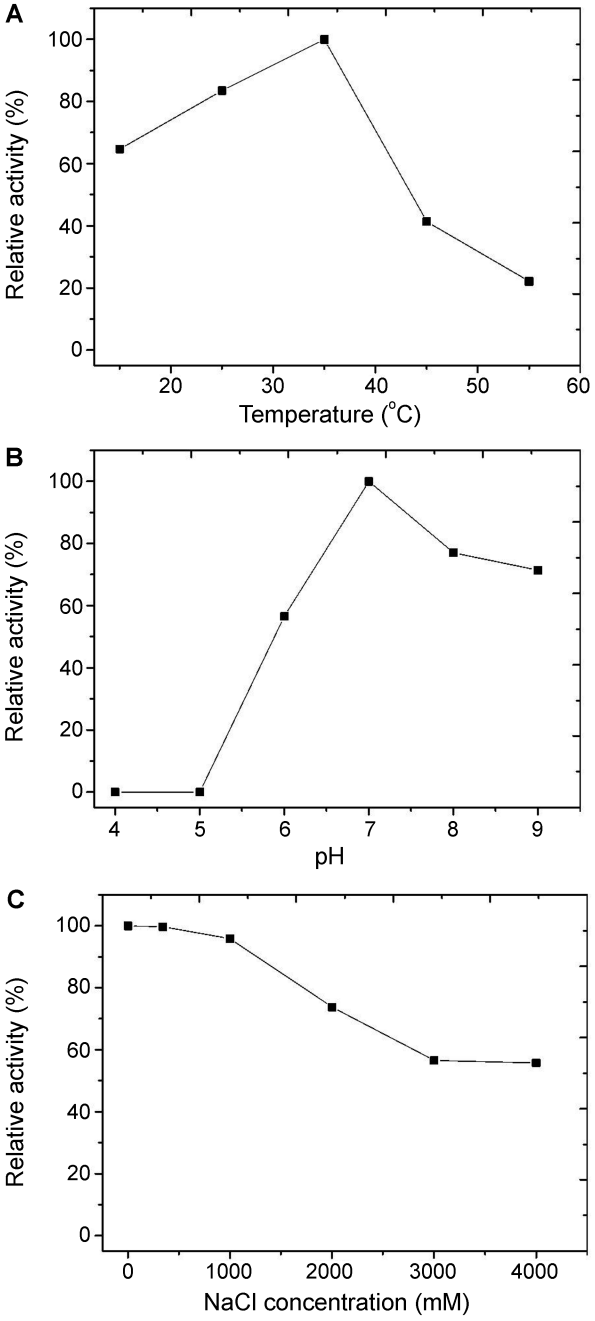

Fig. 3. Temperature, pH optimization, and NaCl tolerance tests. Enzyme activity was expressed in terms of relative activity. For each test, the point where the amount of reducing sugar released was maximum was considered as 100%. **A**. Temperature optimization; **B**. pH optimization; **C**. NaCl tolerance test.

(Fig. 3B). In addition, the enzyme showed extremely high resistance against ionic stress. According to previous studies, most agarases completely lose their activity at an NaCl concentration of 500 mM [3, 4, 12, 23]. However, the agarase examined in this study showed almost the same activity in both the 0 mM and 1,000 mM NaCl solutions, and even maintained more than half of its maximum activity at an NaCl concentration of 4,000 mM (Fig. 3C). This result is remarkable, because the agarase showed greater tolerance than most reported agarases. For example, Leon *et al*. [12] reported an agarase maintaining its activity about 50% at 0.1 mM NaCl and so did Wang *et al*. [24] that lost its activity at higher than 2% NaCl. In addition, it showed even better tolerance than an agarase, maintaining its activity about 80% at 1 M NaCl, that previously received attention for its tolerance to ionic stress; this finding indicates that the enzyme examined here has a fairly stable structure [3].

All substrates, including agarose, agar, and *G. amansii*, produced agarooligosaccharides with reducing ends when they were mixed with crude agarase solution. Agarose was the most degraded substrate among the substrates. It is because agarose is a linear polymer of comparatively pure and fine galactan, so that cleavage by agarase can occur more easily in agarose than the others. The saccharification efficiency of the *G. amansii*–agarase mixture was about half that of the agar–agarase mixture. This result is remarkable, because it signifies the possibility of saccharifying *G. amansii* directly, without extraction processes, which are expensive and time-consuming, to obtain agar or agarose.

An agarase band was detected by zymogram analysis. This finding signifies that there was only one agarase secreted into the medium. The molecular mass of the enzyme was estimated to be above 55 kDa and below 72 kDa. Most of the reported extracellular agarases from the genus *Pseudoalteromonas*, including *P. gracilis* B9 (30 kDa) [20], *Pseudoalteromonas* sp. CY24 (50.4 kDa) [13], *Pseudoalteromonas* sp. AG52 (33 kDa) [16], *Pseudoalteromonas* sp. AG4 (30 kDa) [17], and *P. antarctica* N-1 (33 kDa) [26], are smaller than that of the agarase identified in this study.

We isolated an agar-digesting bacterium that produces an extracellular agarase, which we named *Pseudoalteromonas* sp. JYBCL 1. Agarase enzyme activity was optimal at 35°C and pH 7. The enzyme showed extremely high tolerance against ionic stress, which signifies that its structure was quite stable against the ionic effect of the reaction mixture. The agarase could saccharify *G. amansii* directly, without the need for a pretreatment process. This result is of particular value, because direct saccharification of raw algae may simplify the pretreatment process, thereby reducing the time, processes, and costs involved. Future studies will focus on the identification, cloning, and purification of the isolated agarase.

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