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ORIGINAL ARTICLE

Isolation and characterization of alginate-degrading bacteria for disposal of seaweed wastes

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Abstract

Aims: Isolation of novel alginate degrading bacteria for the disposal of seaweed waste in composting process.

Methods and Results: Decomposition of alginate polymers was checked by the 3,5-dinitrosalicylic acid (DNS) method for reducing sugar, and absorbance at 235 nm for unsaturated sugar. A bacterium A7 was isolated from wakame compost and confirmed to belong to the genus *Gracilibacillus* by partial 16S rDNA analysis. The optimum condition for the growth of A7 in a medium containing 5 g l^{-1} of sodium alginate is as follows: pH, 8·5–9·5; NaCl, 0·5 mol l^{-1}; temperature, 30°C and polypeptone as nutrient content, 2–5 g l^{-1}. In a laboratory-scale composting experiment, the alginate content in wakame compost decreased to 14·3% after 72 h of composting from an initial value of 36%, indicating the effectiveness of alginate decomposition of A7 in wakame composting.

Conclusions: The bacterium A7 was found to be alginate lyase-producing in genus *Gracilibacillus* and effective in degrading alginate to oligosaccharides in wakame during composting process.

Significance and Impact of the Study: Development of new methods for the disposal of marine wastes and production of functional products.

Introduction

The amount of seaweed wastes has increased in recent years due to two reasons: one is the culturing of seaweed as an industrial resource; another is the eutrophication of seawater resulting in a rapid growth of seaweeds. One of the main organic components in seaweed is alginate, the content of which is as high as 50% in species such as wakame (*Undaria pinnatifida*) (Skriptsova *et al.* 2004). The disposal and utilization of seaweed wastes are important for preservation of the marine environment and recycling of organic substances. However, the degradation of alginate by general micro-organisms is not easy, mainly due to its complicated molecular structure. Thus, isolation of specific micro-organisms and using them to degrade alginate is indispensable, which is important for the effective disposal of seaweed wastes.

Alginate is a linear copolymer of β-1,4-D-mannuronic acid and α-1,4-L-guluronic acid with the residues organized in blocks of polymannuronate (MM) and polyguluronate (GG), as well as heteropolymeric sequences of both uronic acids (MG) (Moen and Ostgaard 1997). Alginate can be degraded by radiation or thermal treatment (Said and Hassan 1993; Nagasawa *et al.* 2000). Biological degradation, on the other hand, is generally conducted by an alginate lyase that acts on the 4-O-linked glycosidic linkage of alginate (Iwamoto *et al.* 2001). Alginate lyases have been isolated from a wide range of organisms, including algae, marine invertebrates, and marine and terrestrial micro-organisms (Wong *et al.* 2000). Most alginate degrading bacteria were originally found to be closely associated with marine algae or mollusks. The research until now is mainly concentrated on the characterization of alginate lyase and corresponding DNA genes

in the alginate degrading micro-organisms (Schaumann and Weide 1990; Matsubara *et al.* 2000; Hashimoto *et al.* 2005). Moen *et al.* (1997) reported that microbial degradation of alginate in *Laminaria hyperborea* stipes by the mixed microbial community. However, there has been no report on the isolation of alginate-degrading bacteria and their direct application to the disposal of seaweed wastes.

Products from the microbial degradation of alginate are polysaccharides with a different length of carbon chain. It has been proved that the alginate polysaccharides are effective in promoting the growth of plant roots (Iwasaki and Matsubara 2000; Cao *et al.* 2007). This indicates that the product after disposal of alginate-containing seaweeds such as wakame could possibly be used as a fertilizer in agriculture. The direct application of alginate degrading bacteria instead of alginate lyase is preferable in the field disposal of seaweed waste to achieve this.

In this research, a bacterium with the ability to degrade alginate was isolated from wakame compost and its properties were characterized so that it could be used for the disposal of seaweed waste in the future.

Materials and methods

The wakame compost was prepared as described previously (Tang *et al.* 2007). For the isolation of alginate degrading bacteria, the compost sample was diluted successively and then spread on an agar plate (15 g l^{-1} of agar) containing the following components: 0·5 g polypeptone, 0·3 g yeast extract, 2 g sodium alginate, 2 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 0·5 g $MgSO_4.7H_2O$ in 1000 ml water. Its pH was adjusted to 8·5 with NaOH. The bacteria in the agar plate were further checked in a liquid media containing the same components described above except that the alginate content was 5 g l^{-1}.

A 16S rDNA analysis was carried out to identify the species of the isolated bacteria. The bacteria were first incubated for 24 h, and then DNA extraction was carried out using a Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI). The primers used to amplify the 16S rDNA fragment were EUB27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and EUB 533r (5′-TTACCGCGGCKGCTGRCAC-3′). PCR was carried out by using a GeneAmp PCR System 9700 (Applied Biosystem, Foster City, CA, USA) with the following conditions for amplification: initial denaturation at 94°C for 5 min, 35 cycles of 0·5 min of denaturation at 94°C, 0·5 min of annealing at 55°C, and 1 min of extension at 72°C, and then final extension at 72°C for 10 min. A phylogenetic dendrogram was constructed using the 'ClustalW Submission Form' (http://www.ebi.ac.uk/clustalw/). Evolutionary distances were calculated using the method of Jukes and Cantor (Jukes and Cantor 1969) and the topology was inferred using the 'neighbor-joining' method (Saitou and Nei 1987).

The reducing sugar was detected using the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959). The supernant of the culture medium was mixed with DNS solution and boiled for 5 min. Absorbance of the reaction solution was analysed at 540 nm after 1:10 dilution with distilled water, and the result was expressed as mg glucose ml^{-1} culture. The production of unsaturated sugar through β-elimination reaction by alginate lyase was determined by measuring the absorbance at 235 nm (Iwamoto *et al.* 2001).

The composting of wakame by strain A7 was conducted in a composting system which consisted of a 5 l ice box with insulation film outside to maintain the temperature as well as aeration of 0·5 l min^{-1} from bottom of the container. Details of the composting process and analysis of alginate content were reported in a previous study (Tang *et al.* 2007). Two grams of a dry compost sample was first extracted with 100 ml distilled water under stirring for 3 h. After filtration through a layer of cloth, 50 ml of 3% Na_2CO_3 was added to the algal fronds and extraction was carried out at 75°C for 1 h under stirring. This process was repeated twice and the alginate solution was filtered through a layer of cloth. The alginate was then precipitated and cleaned according to the procedure of Nishide *et al.* (1987). The result was expressed as a percentage of the dry weight compost.

The GenBank accession number of the sequence reported in this paper is EU262659.

Results

Isolation and identification of effective bacteria A7

In the primary screening, four strains of bacteria from a total of 56 strains were isolated based on high alginate degradation ability. All of the four bacteria designated to A7, N7, N10 and N14 were Gram-positive, long rod-shaped and had a white colony colour on the agar plate. Every bacterium was unable to grow in the liquid medium without alginate. Changes in reducing sugars and unsaturated polysaccharides produced by the different bacteria in the medium containing 5 g l^{-1} alginate during incubation were shown in Fig. 1. The reducing sugars and unsaturated sugars were low for the control using a marine bacterium AW4 which was isolated in a previous research (Tang *et al.* 2008). Four kinds of alginate-degrading bacteria, A7, N7, N10 and N14, on the other hand, showed an increase in both reducing and unsaturated sugars to the maximum values within 72–96 h of incubation, respectively. Among them, A7 showed the most rapid degradation of alginate and was selected for further study.

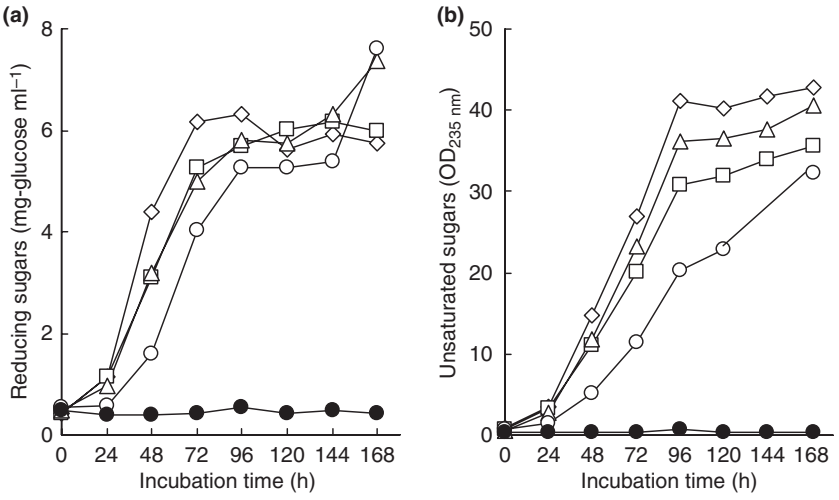

Figure 1 Changes in reducing sugars (a) and unsaturated sugars (b) by four different kinds of alginate-degrading bacteria in comparison with the marine bacterium AW4. The strains A7, N7, N10, N14, and AW4 were grown in the following medium: 0·5 g polypeptone, 0·3 g yeast extract, 5 g sodium alginate, 2 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 0·5 g $MgSO_4{\cdot}7H_2O$ in 1000 ml water, pH 8·5, NaCl 0·5 g l^{-1}. (◇) A7; (□) N7; (Δ) N10; (○) N14; (●) AW4.

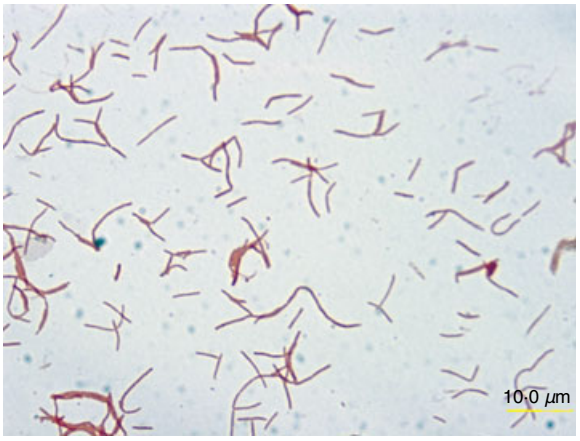

Figure 2 Picture of strain A7 under a microscope.

A7 was long rod-shaped, 5–10 μm in length and filament-forming (Fig. 2). Colonies of strain A7 were circular, creamy white, nontransparent and about 2 mm in diameter on the alginate-agar medium after 5 days of incubation at 30°C. Partial 16S rDNA analysis suggested that A7 was most closely related to the genus *Gracilibacillus halotolerans* (99% similarity), which was first isolated and named by Waino *et al.* (1999). A phylogenetic tree based on partial 16S rRNA gene showed the relation of strain A7 and other related species (Fig. 3). In addition to *G. halotolerans*, other related bacteria showing a close similarity in Genbank were found in the *Halobacillus* and *Virgibacillus* genus.

Characterization of A7

The growth of strain A7 in a liquid medium consisting of low nutrients such as 0·1 g polypeptone and 0·06 g yeast

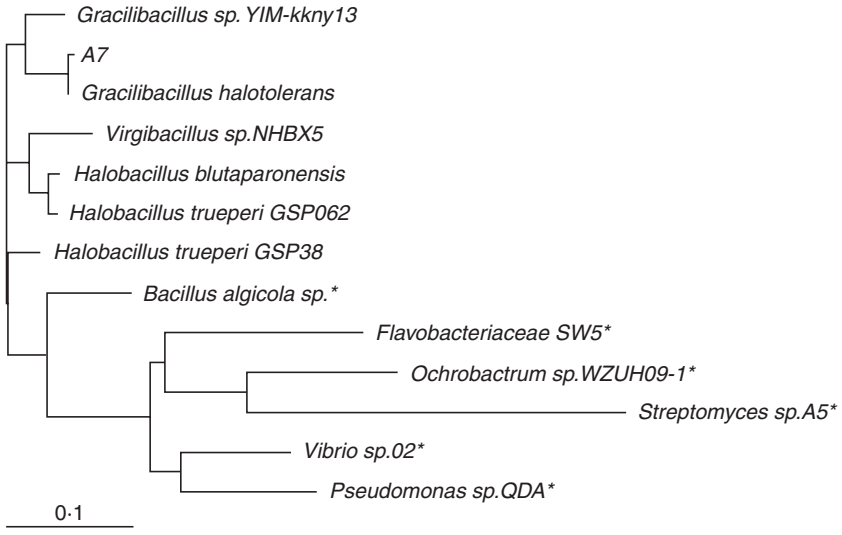

Figure 3 Phylogenetic tree based on a partial 16S rRNA gene of strain A7 and other related species. Evolutionary distances were calculated using the method of Jukes and Cantor (1969) and the topology was inferred using the 'neighbor-joining' method. Scale bar, 10 % sequence divergence.

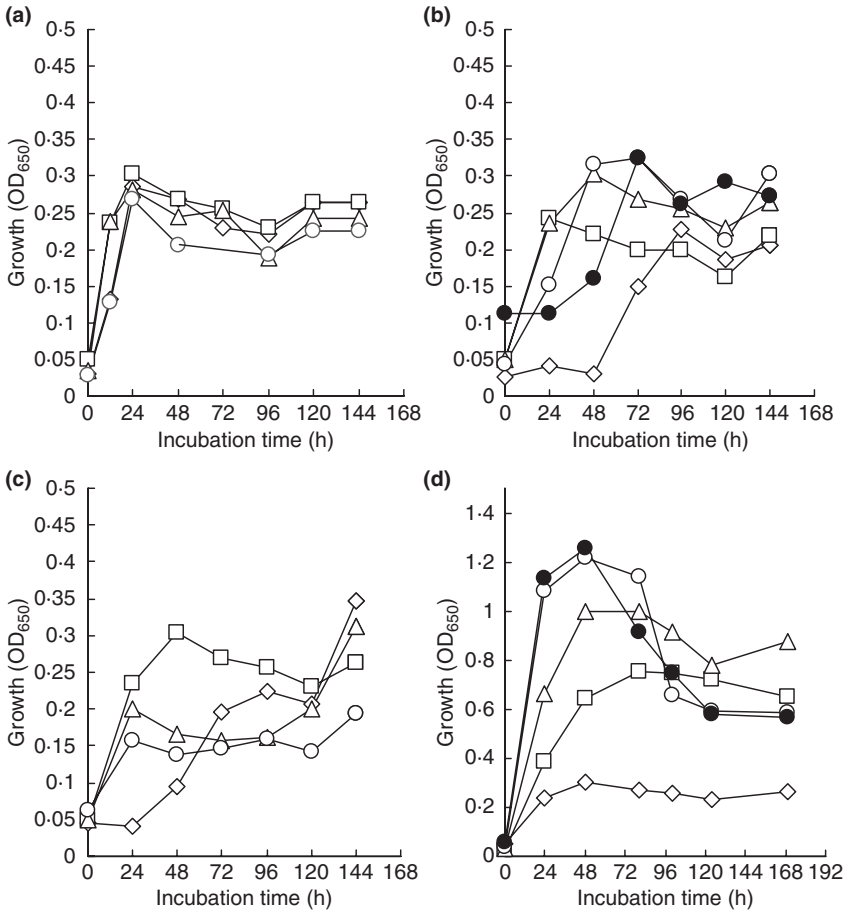

Figure 4 Growth of strain A7 under different conditions. (a) pH: (◇), 7·5; (□), 8·5; (△), 9·5; (○),10; incubation in the presence of 0·5 mol l^{-1} NaCl, 30°C, 0·1 g l^{-1} polypeptone. (b) salinity: (◇), 0 mol l^{-1} NaCl; (□), 0·2 mol l^{-1} NaCl; (△), 0·5 mol l^{-1} NaCl; (○), 1 mol l^{-1} NaCl; (●), 2 mol l^{-1} NaCl; incubation at pH 8·5, 30°C, 0·1 g l^{-1} polypeptone. (c) temperature: (◇), 20°C; (□), 30°C; (△), 40°C; (○), 45°C; incubation at pH8·5, 0·5 mol l^{-1} NaCl, 0·1 g l^{-1} polypeptone). (d) nutrient content of polypeptone: (◇), 0·1 g l^{-1}; (□), 0·5 g l^{-1}; (△), 1 g l^{-1}; (○), 2 g l^{-1}; (●), 5 g l^{-1}; incubation at pH 8·5, 30°C, 0·5 mol l^{-1} NaCl. Alginate content was fixed at 5 g l^{-1}. Yeast extract was also added at a ratio of 3 : 5 with polypeptone.

extract in 1 l media was characterized under different pH values, NaCl concentrations, temperature and nutrient contents (Fig. 4). Rapid growth of A7 was found within 24–48 h of incubation; cell turbidity at OD_{650} then remained at a high value or decreased slightly. As the initial time, before 48 h, is most important for the growth of A7, the effect of incubation conditions was characterized based on the data before or around 48 h. Across the different pH values studied (7·5–10), rapid growth was observed at pH 8·5–9·5, suggesting that an alkaline environment is favourable for strain A7 although the effect of pH is not so different at the later period of incubation. The bacterium grew well in the presence of 0·5–2 mol l^{-1} NaCl, while no growth was found in the liquid medium without the addition of NaCl before 48 h of incubation. When the NaCl concentration was >0·5 mol l^{-1}, the growth rate of A7 was reduced. Under different temperatures (20, 30, 40 and 45°C) 30°C showed the highest value of cell growth at 48 h of incubation although a sharp increase of growth within 24 h was observed at 45°C. The A7 bacterium showed a much slower growth rate at 20°C. The results suggested that the strain A7 can grow at higher temperatures, which is one of the important characteristics for waste disposal. Turbidity at 650 nm after 48 h of incubation increased with the increase of nutrient content. However, there is not much difference in the polypeptone medium between 2 and 5 g l^{-1}. The result suggests that the optimum conditions for the growth of A7 in the medium containing 5 g l^{-1} of sodium alginate is as follows; pH 8·5–9·5, NaCl 0·5 mol l^{-1}, temperature 30°C and nutrient content of 2–5 g l^{-1} polypeptone.

Decomposition of alginate by strain A7 in composting process

Change of alginate content and absorbance of compost water extract (1 : 9) at 235 nm during the composting of wakame as inoculated by strain A7 is shown in Fig. 5. The initial value of alginate content in wakame compost was about 36%, which decreased to 14·3% after 72 h of composting and remained almost unchanged after that. At the same time, absorbance at 235 nm, the indicator of unsaturated sugars, increased fourfold after 72 h of

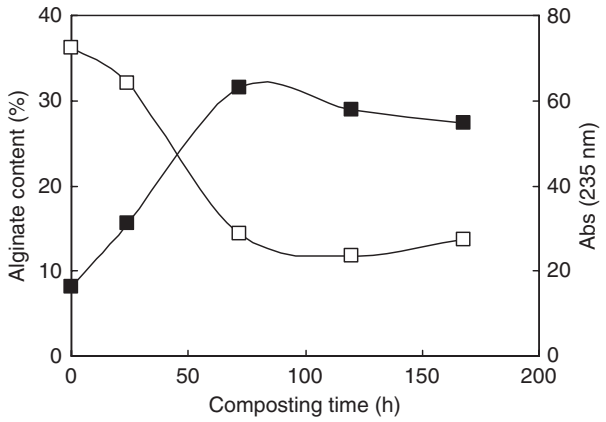

Figure 5 Change of alginate content and absorbance at 235 nm during composting of wakame as inoculated by strain A7. (□) Alginate content, (■) absorbance (235 nm).

composting compared with the value at 0 h. The degradation rate was much higher than in our previous study using another bacterium, *Bacillus* sp. HR6, for inoculating wakame composting (Tang *et al.* 2007). The result suggested that strain A7 is effective in decomposing alginate in the composting of alginate-containing wastes.

Discussion

Alginate-degrading bacteria play an important role in the nutrient-recycling system in a marine environment. However, application of this kind of bacteria to waste control in the marine environment has not been well studied, probably due to the little information that exists on the isolation of special alginate degrading bacteria. Some of the alginate-degrading bacteria reported in GenBank, *Flavobacteriaceae bacterium* SW5, *Ochrobactrum* sp. WZUH09-1, *Vibrio* sp. O2, *Streptomyces* sp. A5, *Pseudomonas* sp. QDA and *Bacillus algicola*, were also compared in a phylogentic tree with A7, in which a low similarity, <90%, was observed. Some of the above alginate-degrading bacteria such as *Vibrio* sp. O2 (Kawamoto *et al.* 2006) and *Bacillus algicola* (Ivanova *et al.* 2004) have been studied in detail. It should be noted that it is the first time that an alginate-degrading bacterium has been reported from the genus *Gracilibacillus*.

The growth of *Gracilibacillus* sp. A7 is favourable in normal nutrient conditions with an alkaline pH at moderate temperatures, where the alginate is indispensable for the growth of A7. This suggests that A7 can grow and proliferate in the composting process of seaweeds containing alginate. Previous research (Tang *et al.* 2008) also proved that inoculated bacteria generally proliferate and predominate in the initial period of composting and then other bacteria increase and a mixed microbial community subsequently appears over the composting period. The degradation of alginate takes place at a rate of 60% within 72 h in composting process, indicating that most of alginate can be degraded within a normal composting period of a few weeks or months. From the increase in unsaturated sugar during the composting process, it is clear that the mechanism of alginate degradation is a β-elimination reaction during composting of wakame using A7, although further study is needed to clarify the detailed mechanism of the degradation process.

The genus *Gracilibacillus* is closely related to *Bacillus*, which is known to be widely distributed in the natural environment and some extreme living conditions (Waino *et al.* 1999). The novel bacterium A7 is thought to be widely applied for disposal of seaweed waste and for producing valuable substances such as polysaccharides through the degradation of alginate in seaweed. As compared to the mixed culture, the degradation process using a pure culture of A7 is favourable for producing valuable substances from seaweed waste. However, application of A7 in the field disposal of seaweed waste requires further understanding of its properties.

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