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Isolation and characterization of *Microbulbifer* species 6532A degrading seaweed thalli to single cell detritus particles

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Abstract To reduce the volume of seaweed wastes and extract polysaccharides, seaweed-degrading bacteria were isolated from drifting macroalgae harvested along the coast of Toyama Bay, Japan. Sixty-four bacterial isolates were capable of degrading "Wakame" (*Undaria pinnatifida*) thallus fragments into single cell detritus (SCD) particles. Amongst these, strain 6532A was the most active degrader of thallus fragments, and was capable of degrading thallus fragments to SCD particles within a day. Although the sequence similarity of the 16S rRNA gene of strain 6532A was 100% similar to that of *Microbulbifer elongatus* JAMB-A7, several distinct differences were observed between strains, including motility, morphology, and utilization of D-arabinose and gelatin. Consequently, strain 6532A was classified as a new *Microbulbifer* strain, and was designated *Microbulbifer* sp. 6532A. Strain 6532A was capable of degrading both alginate and cellulose in the culture medium, zymogram analysis of which revealed the presence of multiple alginate lyases and cellulases. To the best of our knowledge, this is the first study to directly demonstrate the existence of these enzymes in *Microbulbifer* species. Shotgun cloning and sequencing of the alginate lyase gene in 6532A revealed a 1,074-bp open reading frame, which was designated *algMsp*. The reading frame encoded a PL family seven enzyme composed of 358 amino acids (38,181 Da). With a similarity of 74.2%, the deduced amino acid sequence was most similar to a *Saccharophagus* enzyme (*alg 7C*). These findings suggest that *algMsp* in strain 6532A is a novel alginate lyase gene.

Keywords Seaweed · Degradation · Alginate lyase · Cellulase · *Microbulbifer*

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Introduction

Marine macroalgae, which can broadly be divided into green, brown, and red algae, play an important role in the ecosystem. Photosynthetic biomass production by seaweeds in coastal waters is extremely high; for example, the total production of seaweeds in Japan exceeds 450,000 t year^{-1} (Ministry of Agriculture Forestry and Fisheries 2008). Seaweeds synthesize a wide variety of polysaccharides for use as cell wall components or to store nutrients, and many of these compounds are utilized by humans as food, fertilizer, industrial products, medical products,

cosmetics, and for producing biofuel (Jensen 1993; Tseng 2001; Qin et al. 2005; Ross et al. 2008). However, considerable amounts of seaweed wastes are also produced during these manufacturing processes. In addition, disposal of the increased amounts of drifting seaweed debris due to seaweed farming and eutrophication of the marine environment has become a problem (Morand and Merceron 2005; Liu et al. 2009). Since these wastes or debris consist of thallus fragments, stipes, and holdfasts—most of which is generally discarded in landfills—considerable interest exists in their efficient utilization.

Although numerous studies have reported the ability of bacteria to degrade seaweed polysaccharides, such as alginate (Wong et al. 2000), laminarin (Alderkamp et al. 2007), fucoidan (Holtkamp et al. 2009), cellulose (Taylor et al. 2006), agar (Fu and Kim 2010), carrageenan (Michel et al. 2006), and starch (Horikoshi 1999), none of these reports examined the degradation of seaweed thalli by bacteria. Of the studies that have specifically examined the bacterial degradation of seaweed thalli and thallus fragments (Uchida and Nakayama 1993; Uchida 1995, 1996; Camacho et al. 2004), few have identified the specific enzymes responsible for the degradation of polysaccharides in seaweeds.

This study therefore attempted to isolate marine bacteria capable of degrading seaweed thalli and the polysaccharides they contain. Seaweed-degrading bacteria were isolated from drifting thalli under the assumption that indigenous bacteria are likely to adhere to macroalgal substrates drifting in the sea. Characterization of one such bacterial strain, *Microbulbifer* sp. 6532A, revealed that it was able to degrade both fragments and polysaccharides of "Wakame" (*Undaria pinnatifida*) thalli, as well as alginate, and cellulose. In addition, this strain could potentially be applied to reducing the volume of seaweed wastes, producing functional materials from seaweeds, and as a source of a new alginate lyase gene.

Materials and methods

Isolation and cultivation of bacteria

Drifting seaweeds were collected along the coastal zone of Toyama Bay, Japan. Approximately 10 g (wet weight) of collected material was added to 100 ml of artificial seawater (ASW) culture medium (800 ml ASW, 1 g NH_4NO_3, 0.02 g K_2HPO_4, 0.5 g yeast extract, 200 ml distilled water (DW); pH 7.8) prepared using a slight modification of Higashihara et al. (1978). The mixture was incubated on a shaker for 2 days at 30°C. Marine Art SF-1 (Tomita Pharmaceutical Co. Ltd., Tokushima, Japan) was used as the ASW in the ASW culture medium. After incubation, a 50 μl aliquot of the culture medium was spread on ASW agar (1.5%, w/v) plates containing 10 mg ml^{-1} Wakame (*U. pinnatifida*) powder (Wakamidori; Riken Vitamin Co. Ltd., Tokyo, Japan). The resulting colonies were isolated and transferred to ASW liquid medium containing 5 mg ml^{-1} Wakame thallus flakes (No. 15, DR-1; Riken Vitamin Co. Ltd.) and incubated on a shaker for 2–7 days. Bacterial clones that degraded the Wakame thallus flakes were preserved on ASW agar slants and used in this study.

Particle-size distribution of degraded seaweed

The size-frequency distribution of the Wakame thallus debris degraded by the bacteria was determined by milling dried Wakame thallus flakes into fine particles. These particles were then passed through a sieve to collect particles between 300 and 710 μm in diameter. These milled and sieved particles were suspended in 100 ml ASW medium to create a 1% (w/v) mixture, which was then autoclaved for 15 min at 121°C. Subsequently, 1 ml of culture containing pure strain 6532A at a cell density of 10^7 CFU ml^{-1} was added to the suspension, which was then incubated at 30°C with shaking at 200 rpm (Bioshaker BR-180LF; Taitec Corp., Saitama, Japan). Aliquots were removed at 0, 12, 18, 24, and 48 h after bacterial inoculation and the size distribution of the degraded samples was determined using a laser scattering particle size distribution analyzer (LA-920, Horiba Ltd., Kyoto, Japan). The size range of particles was 0.02–2,000 μm.

Morphological observations and physiological analysis

The morphology and motility of the isolated bacterial samples were initially observed under a phase contrast microscope (BX51; Olympus Optical Co.

Ltd., Tokyo, Japan). The cells were then negatively stained with 1% (w/v) phosphotungstic acid and observed under a JEM-100SX electron microscope (JEOL, Tokyo, Japan). Micrographs were taken at an accelerating voltage of 80 kV. Bacterial physiological analysis was performed using the API 20NE system (bioMérieux, Marcy, l'Etoile, France). Catalase activity was confirmed by placing several drops of 3% H_2O_2 directly onto 2-day-old colonies growing on the agar medium. A positive reaction was indicated by the evolution of gas bubbles from the colonies. Oxidase activity was assayed using a cytochrome oxidase test strip (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) and 2-day-old colonies growing on agar medium; colonies that were positive for the oxidase test turned deep violet color within 30 s.

Polysaccharide degradation

Strain 6532A was cultured in 400 ml ASW containing 1% (w/v) Wakame thallus fragments for 2 days at 30°C. After centrifugation at $12,000 \times g$ for 15 min at 4°C, the enzyme activities of the supernatants were determined by degradation of the polysaccharides alginate, cellulose, fucoidan, laminarin, and agar. A reaction mixture containing 100 μl of the supernatant and 300 μl of 0.5% (w/v) substrate in 20 mmol l^{-1} Tris–HCl (pH 8.0) was incubated at 30°C for 1 h. The reducing sugars released were measured as D-glucose reduction equivalents using the Somogyi–Nelson method (Somogyi 1952). One unit of enzyme activity was defined as the production of 1.0 μg ml^{-1} h^{-1} of reducing sugars (glucose equivalents) (Uchida 1995).

Zymogram analysis

After centrifugation at $12,000 \times g$ for 15 min, the culture supernatant (described above) was fractionated with ammonium sulfate (50% saturation) overnight at 4°C. The precipitates were collected by centrifugation at $15,000 \times g$ for 30 min, and resuspended in 4 ml of 20 mmol l^{-1} Tris–HCl (pH 8.0). This solution was dialyzed against the same buffer for 18 h at 4°C and stored as a concentrated culture medium at −80°C until further use. This procedure increased the protein concentration by approximately 25-fold and the alginate lyase activity by approximately 23-fold. These concentrated culture media

samples were then denatured by heating at 95°C for 2 min in 1% (w/v) sodium dodecyl sulfate (SDS) and 5% (v/v) β-mercaptoethanol. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 7.5% acrylamide gels (Laemmli 1970) and the gels were stained for proteins using Quick Blue Staining Solution (Bio Dynamics Laboratory Inc., Tokyo, Japan). The molecular mass was estimated using a mixture of low- and high-range molecular mass standards (Bio-Rad Laboratories, Inc., Tokyo, Japan), which was included myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

Alginate lyase was detected in zymograms using the method of Pecina and Paneque (1994). After SDS-PAGE, a gel containing 0.1% (w/v) sodium alginate was washed once for 15 min in DW at room temperature, and renatured at 4°C in casein buffer (50 mmol l^{-1} Tris–HCl buffer, 1% [w/v] casein, 2 mmol l^{-1} EDTA, and 0.01% NaN_3; pH 8.2) for three 30-min periods with gentle shaking. The gel was then incubated in 10 mmol l^{-1} phosphate buffer (pH 7.5) containing 0.2 mol l^{-1} NaCl, 0.2 mol l^{-1} KCl, and 0.01% NaN_3 for 1 h at room temperature with gentle shaking. The gel was treated by gently immersing it with a solution of 10% (w/v) cetylpyridinium chloride for 20 min at room temperature. Alginate lyase activity was then visualized as a clear zone in the opaque gel matrix.

To detect cellulase activity, SDS polyacrylamide gels containing 0.1% (w/v) carboxymethyl (CM) cellulose (Sigma, St. Louis, MO) were used. Following electrophoresis, the gels were washed with DW for 15 min and incubated for 10 h at room temperature in 0.1 mol l^{-1} succinic acid buffer (pH 5.8). The gels were then submerged in a 0.1% (w/v) Congo red solution for 10 min and washed with 1 mol l^{-1} NaCl until clearance bands appeared (Schwarz et al. 1987).

Genetic and phylogenetic analyses

Bacterial colonies were picked from the agar plates and suspended in sterile DW. Samples were then boiled for 5 min to release DNA into the DW, and the 16S rRNA gene of the bacterial isolates was then amplified by polymerase chain reaction (PCR) using

the eubacterial primers 27f (5′-AGAGTTTGATC CTGGCTCAG-3′) and 1525r (5′-AAAGGAGGT GATCCAGCC-3′). PCR was performed with Takara Ex Taq Hot Start version Kit (Takara Bio Inc., Shiga, Japan) and each PCR sample contained $1 \times$ Ex Taq buffer, $200 \ \mu mol \ l^{-1}$ dNTP mix, $0.25 \ \mu mol \ l^{-1}$ primer 27f, $0.25 \ \mu mol \ l^{-1}$ primer 1525r, 0.5 U Ex Taq HS (Taq DNA polymerase). PCR reactions were performed using a thermal cycler (Takara PCR Thermal Cycler Dice, Takara Bio Inc.), with 3 min initial denaturation at 94°C, followed by 35 cycles of amplification (denaturation for 60 s at 94°C, annealing for 60 s at 60°C, and elongation for 60 s at 72°C), followed by a final 3 min terminal elongation step at 72°C and cooling at 4°C. The amplified products were purified using a QIAquick PCR purification kit (Qiagen, Tokyo, Japan) and directly sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI Prism 3130xl genetic analyzer (Applied Biosystems). Sequence homology searches were performed using the BLAST program and sequences on the NCBI website (http://www.ncbi.nlm.nih.gov). A 1,491-bp nucleotide sequence of the 16S rRNA gene was used for phylogenetic analysis, with the neighbor-joining algorithm used to construct a phylogenetic tree (Saitou and Nei 1987). The nucleotide sequence of the 16S rRNA gene from *Microbulbifer* sp. 6532A was deposited in GenBank under accession number AB553869.

Cloning and sequencing of the alginate lyase gene

Genomic DNA was isolated from cells grown in a marine broth (Marine Broth 2216; Becton, Dickinson and Company, MD, USA) medium using a phenol–chloroform extraction. Genomic DNA was partially digested with *Sau*3AI (Nippon Gene, Tokyo, Japan) and the resulting DNA fragments were electrophoresed on 0.8% agarose gels. Thereafter, the 2–10 kbp fragments were recovered and ligated into pUC118 that had been digested with *Bam*HI (Nippon Gene) and dephosphorylated with bacterial alkaline phosphatase. Finally, the ligation products were introduced into *Escherichia coli* DH5α (Nippon Gene). To screen for alginate lyase activity, the transformants were grown on Luria–Bertani agar supplemented with 0.1% sodium alginate, ampicillin (50 $\mu g \ ml^{-1}$), isopropyl-β-D-thiogalactopyranoside (IPTG) (50 μl

of 0.1 mol l^{-1} stock per plate), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (10 μl of 4% stock per plate). A layer of soft agar (0.2% sodium alginate, 0.2 mol l^{-1} NaCl, 200 m mol l^{-1} phosphate buffer, and 0.8% agar, pH 7.5) was then poured over the agar plate as described by Kobayashi et al. (2009) with minor modifications. The agar plates were then incubated at 37°C for 24 h before 0.1% cetylpyridinium chloride solution was spread on the surface of the soft agar. White colonies with clear halos were then selected, and recombinant plasmids were extracted using a PureYield Plasmid Miniprep System (Promega, Madison, WI, USA). The inserted DNA fragments were amplified by PCR using universal M13 primers targeting both ends of the cloning vector and DNA sequencing of the inserted DNA fragments was performed as described above. Nucleotide and deduced amino acid sequence analysis, open reading frame (ORF) searches, multiple alignment, and molecular-mass and isoelectric-point calculations were all performed using Genetyx software (Ver.8, Genetyx, Tokyo, Japan). A database homology search was then performed using the BLAST program hosted on the NCBI website. The DNA sequence data of the alginate lyase gene (*algMsp*) was deposited in the GenBank database (Accession number AB603802).

Results

Degradation of Wakame thallus fragments

Of the bacteria that were isolated from the drifting thalli, 64 strains were capable of degrading Wakame thallus fragments into finer particles. Among these strains, strain 6532A degraded the thallus substrates most rapidly, and into the finest particles (Fig. 1). Among the Wakame thallus fragments that were not inoculated, all remained intact (Fig. 1a, c) and abundant multi-cellular sheets were observed under a microscope (Fig. 1d). After incubation with strain 6532A for 2 days, most of the Wakame thallus fragments had been degraded (Fig. 1b, e) producing numerous, fine, rectangular-shaped algal particles in the medium (Fig. 1f, g). Each of these algal particles measured approximately $10.3 \times 5.7 \times 5.1 \ \mu m$ (on average). In addition to WET-SEM observations of Wakame thalli (data not shown), these findings
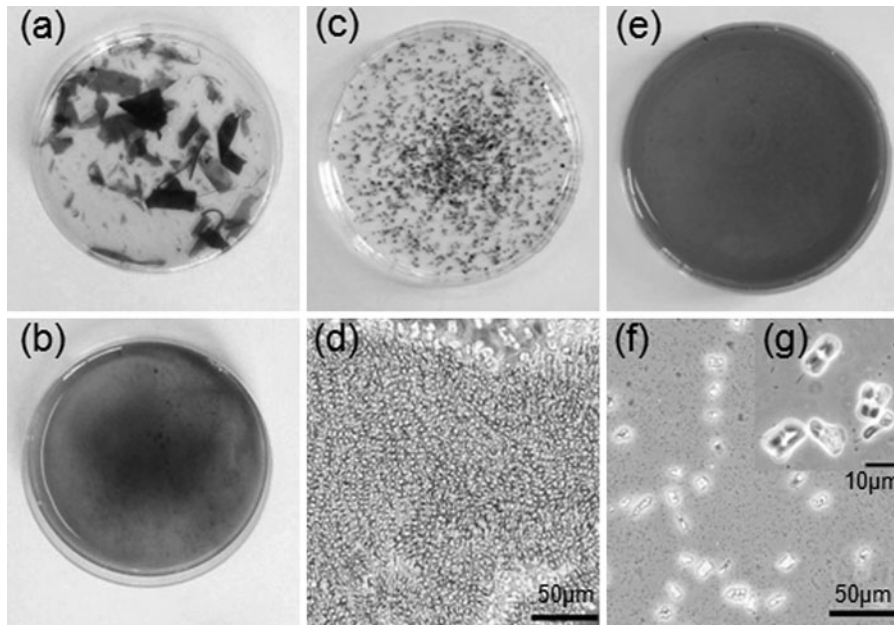
Fig. 1 Degradation of Wakame (*U. pinnatifida*) thallus fragments by bacterial strain 6532A. Uninoculated (**a**, **c**, **d**) and inoculated (**b**, **e–g**) samples are shown **c** Milled and sieved Wakame thallus flakes used for the experiment shown in

Fig. 2. **d** Enlarged view of flake. In inoculated samples, thallus fragments were degraded into fine particles (single cell detritus) (**f**, **g**); in the uninoculated samples, fragments remained largely intact

suggested that these algal particles consisted primarily of Wakame thallus cortical cells, referred to in previous studies as single cell detritus (SCD) particles (Uchida 1996; Camacho et al. 2004). Based on these initial observations, strain 6532A was selected for further physiological and biochemical analysis.

Figure 2b shows the size-frequency distribution of milled and sieved Wakame thallus fragments after varying periods of incubation with 6532A. The most frequently encountered peak diameter of kelp plants was initially approximately 700 μm (0 h), but after 18 h of incubation with strain 6532A, the most frequently encountered particle diameter changed markedly, decreasing to around 10.0 μm. After 24 h, there was one major peak with an average particle diameter of 9.9 μm, which decreased to approximately 7.8 μm after 48 h. Particles <10 μm in diameter (SCD) accounted for >70% of all particles. These changes were not observed in samples that were not inoculated with strain 6532A (Fig. 2a).

Examination of the relationship between cell growth and the degradation of Wakame thallus fragments into SCD particles revealed that degradation occurred rapidly during the late logarithmic

growth phase (0.5–1 day after inoculation; Fig. 3). In addition, the morphology of 6532A also changed during cell growth. Specifically, most of the 6532A cells were rod-shaped during the logarithmic growth phase (0.5 day after inoculation) when they measured approximately 3.0 × 0.6 μm (average length × width, respectively; Figs. 3, 4a, c). However, after the early stationary phase (1.5 days after inoculation), the proportion of rod-shaped cells decreased markedly (from 95% to <20%), as coccoid cells appeared and increased (Figs. 3, 4b, d, e). The rod-shaped cells were motile and had 1–6 flagella per cell, and plural flagella were apparently peritrichous (Fig. 4a). Coccoid cells had flagella during the early stationary phase, but became non-motile and lost their flagella over time. When coccoid cells were resuspended in a fresh medium, they regained the rod-like shape and the motility within a day (data not shown).

Identification of bacterial strain 6532A

The 16S rRNA gene of strain 6532A was amplified by PCR and the resulting 1,491 bp were sequenced. Homology searches revealed that strain 6532A was

Fig. 2 Particle-size
distributions of Wakame
thallus fragments following
degradation by strain
6532A. **a** Uninoculated and
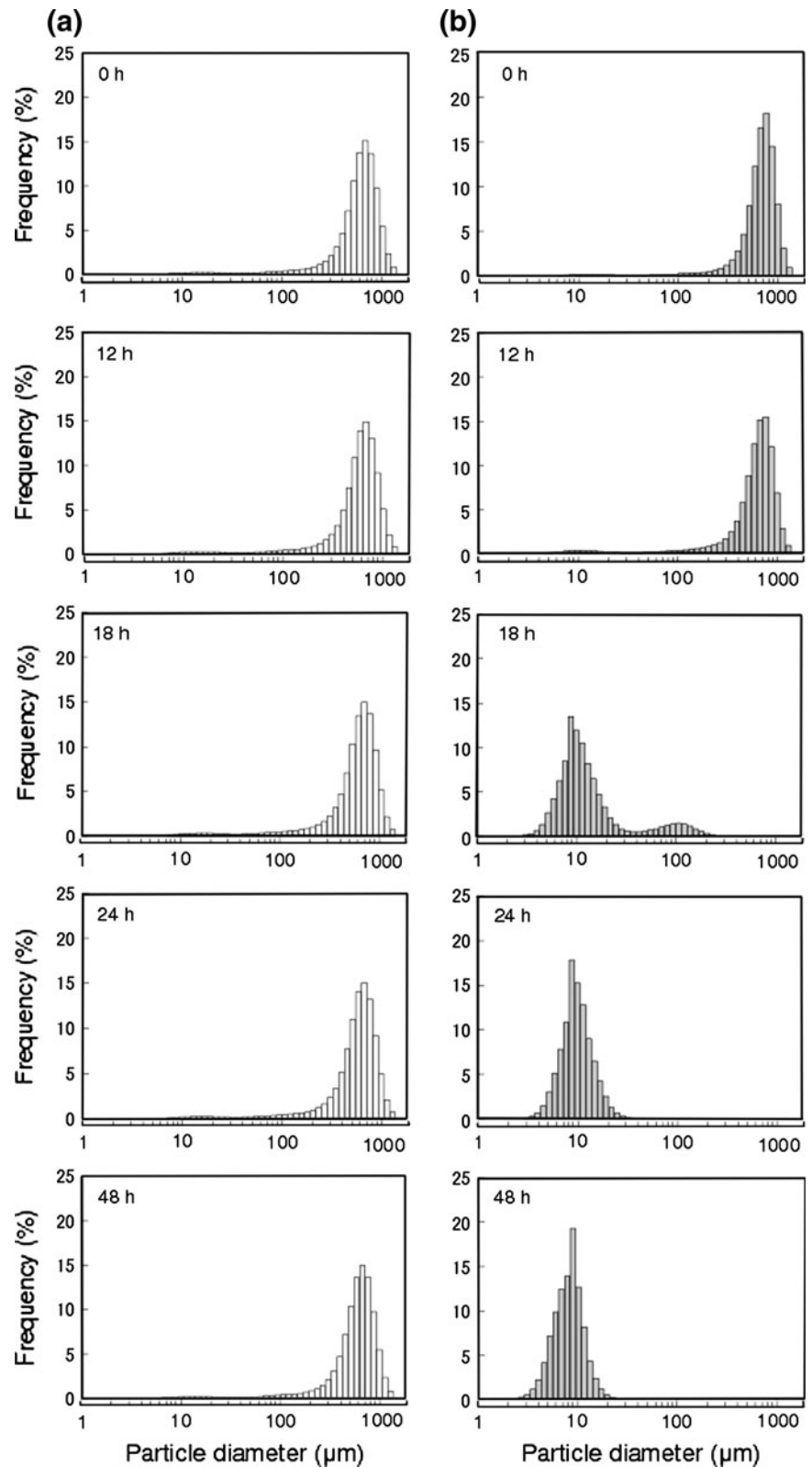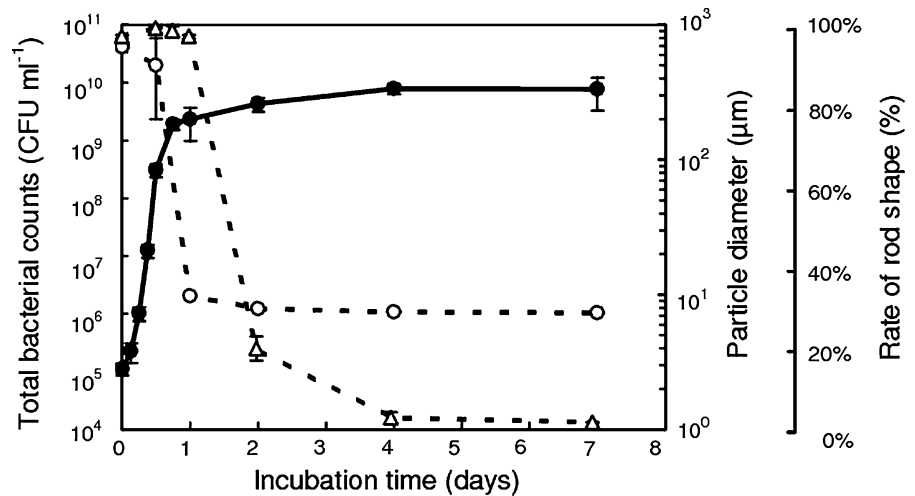**b** inoculated cultures

Fig. 3 Changes in cell morphology and degradation of Wakame thallus fragments associated with growth of strain 6532A in ASW medium. *Filled circle* Cell growth curve, *open circle* average particle size of degraded samples, and *open triangle* rate of rod-shaped cells. Values are means ± SE, n = 3

most closely related to *Microbulbifer elongatus* JAMB-A7 (AB107975; 100% similarity), *Microbulbifer* sp. CMC-5 (EU121671; 99.8% similarity), and *M. elongatus* strain DSM 6810 (AF500006; 99.5% similarity). Since strain 6532A grouped with genus *Microbulbifer* (Fig. 5), strain 6532A was designated as *Microbulbifer* sp. 6532A.

Table 1 compares *Microbulbifer* sp. 6532A and other reported *Microbulbifer* strains (Yoon et al. 2003; Ohta et al. 2004; Jonnadula et al. 2009).

Although *Microbulbifer* sp. 6532A had a sequence similarity of 100% with *M. elongatus* type strain JAMB-A7, several important differences were observed. *Microbulbifer* sp. 6532A was isolated from drifting seaweeds that were collected along the coastline of Toyama Bay in the Sea of Japan (East Sea), whereas *M. elongatus* JAMB-A7 was isolated from sediments in Sagami Bay in the Pacific Ocean at a depth of 1,174 m (Ohta et al. 2004). Strain 6532A had flagella (Fig. 4a) and was motile, while strain
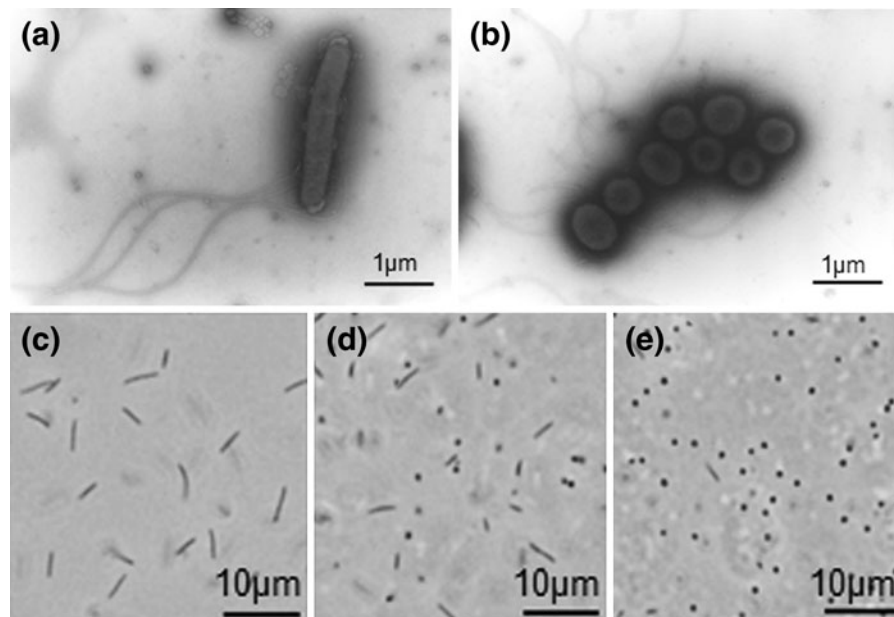

Fig. 4 Morphology of strain 6532A cells. Cells changed from being (**a**, **c**) rod-shaped to (**b**, **e**) coccoid-shaped depending on growth phase (**c**, **d**, **e**). **a**, **c** Logarithmic growth phase (0.5 day); **d** early stationary phase (1.5 days); **b**, **e** mid-to-late stationary phase (4 days). **a**, **b** Electron and **c**, **d**, **e** phase contrast micrographs

Fig. 5 Phylogenetic tree based on the partial 16S rRNA gene sequence of *Microbulbifer* sp. 6532A and related species. Numbers adjacent to branch nodes indicate the bootstrap values (%) of 1000 replicates
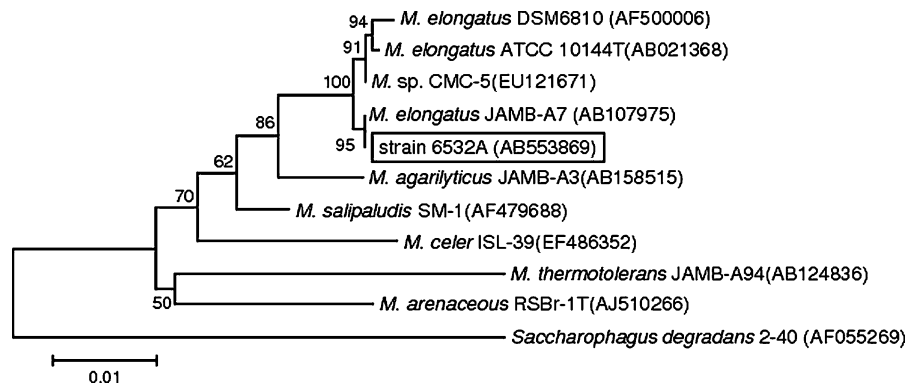
JAMB-A7 was non-motile. Furthermore, in contrast to strain JAMB-A7, the morphology of strain 6532A was observed to change between rod and coccoid shapes (Figs. 3, 4) and it was unable utilize D-arabinose or gelatin. The existence of two morphological types has also been reported in *M. elongatus* strain DSM 6810, which is motile (Yoon et al. 2003). However, unlike DSM 6810, strain 6532A cannot produce H$_2$S or utilize D-arabinose or gelatin (Table 1).

Detection of enzyme activities

When *Microbulbifer* sp. 6532A cells were cultured in ASW medium containing 1% (w/v) Wakame thallus fragments, the concentration of reducing sugars increased rapidly 0.5–1 day after inoculation, before decreasing gradually thereafter (Fig. 6). Enzyme activities in the culture supernatant of strain 6532A were therefore tested against several polysaccharides, viz. alginate, cellulose, fucoidan, laminarin, and agar (Fig. 6). Alginate lyase demonstrated the highest levels of activity (114.8 U over 2 days of culture). Cellulase had the next highest levels of activity (55.7 U), followed by fucosidase (7.1 U), laminarase (0.9 U), and agarase (0.8 U). Zymogram analysis indicated that the concentrated culture supernatant had at least four active polypeptides for alginate (140.3, 120.6, 86.5, and 38.3 kDa) and five active polypeptides for cellulose (138.2, 120.1, 101.8, 76.3, and 52.9 kDa; Fig. 7).

Nucleotide and deduced amino acid sequences of *algMsp*

The alginate lyase gene was cloned from a genomic DNA fragment of *Microbulbifer* sp. 6532A by the shotgun method. The gene contained one ORF of 1074 bp, and was designated *algMsp* (Fig. 8). The *algMsp* sequence started with a GTG codon and terminated at a TAA codon, and the G + C content of the ORF was 53.7%. The deduced product of the *algMsp* gene is a protein of 358 amino acids with an estimated molecular mass of 38,181 Da, a pI of 4.52, and a repetitive sequence rich in serine. At 74.2% (in 279 amino acids overlap sequence), the amino acid sequence of the *algMsp* gene product was most similar to that of a putative alginate lyase (ABD81738; *alg 7C*) from *Saccharophagus degradans* 2–40, followed by alginate lyases from *S. degradans* 2–40 (ABD81807), *Vibrio harveyi* 1DA3 (EEZ88534) and *Polaribacter* sp. MED152 (EAQ42287) with similarities of 71.0, 64.0, and 63.1% respectively.

Discussion

This study successfully reported the isolation and characterization of *Microbulbifer* strain 6532A. The strain was capable of rapidly degrading Wakame thallus fragments, as well as alginate and cellulose. To the best of our knowledge, this is the first report to directly show the degrading properties of *Microbulbifer* sp. by demonstrating the degradation of seaweed thallus fragments into SCD particles.

The sequence similarity of the 16S rRNA gene sequences from *Microbulbifer* sp. 6532A was 100% similar to *M. elongatus* type strain JAMB-A7. However, several differences were observed between *Microbulbifer* sp. 6532A and *M. elongatus* JAMB-A7, including motility, morphology, and utilization of D-arabinose and gelatin. Consequently, *Microbulbifer*

Table 1 Characteristics of *Microbulbifer* sp. 6532A (AB553869) and other *Microbulbifer* species: *M. elongatus* JAMB-A7 (AB107975), *Microbulbifer* sp. CMC-5(EU121671) and *M. elongatus* DSM6810 (NR025246)

Characteristics	6532A[a]	JAMB-A7[b]	CMC-5[c]	DSM6810[d]
Source	Drifting in Toyama Bay, Japan	Sediment in Sagami Bay, Japan	Decomposing seaweed	ND
Gram stain	Negative	Negative	Negative	Negative
Cell morphology	Rods or Cocci	Rods	Rods	Rods or Cocci
Motility	+	−	−	+
Catalase	+	+	+	+
Oxidase	+	+	+	ND
Urease	−	ND	−	−
H_2S production	−	−	−	+
Indole production	−	−	ND	−
Nitrate reduction	−	−	ND	−
Acid production from				
D-arabinose	−	+	+	+
D-fructose	+	+	−	+
D-galactose	+	+	+	+
D-glucose	+	+	+	+
Polysaccharide degradation				
Gelatin	−	+	+	+
Agar	+	+	+	+
Alginic acid	+	ND	+	+
Cellulose	+	ND	+	+
Fucoidan	−	ND	ND	ND
Laminarin	−	ND	ND	ND
Growth temperature (°C)	20–40	10–43	30	25–30
NaCl concentration for growth	3–7%	0.3–11.0%	2–8%	2–3%
16S rRNA homology		100% (1491/1491)	99.8% (1488/1491)	99.5% (1484/1491)

ND Not described; +, positive reaction; −, negative reaction

[a] Present study, [b] Ohta et al. 2004, [c] Jonnadula et al. 2009, and [d] Yoon et al. 2003

sp. 6532A and *M. elongatus* JAMB-A7 should be regarded as two different strains. Furthermore, strain 6532A and *M. elongatus* JAMB-A7 were isolated from different sites and depths, and it has been suggested that the genus *Microbulbifer* has a wide geographical and topographical distribution in marine environments (Jonnadula et al. 2009; Nishijima et al. 2009).

When strain 6532A cells were cultured in the presence of Wakame thallus fragments, alginate lyase and cellulase activities in the culture medium increased after 1 day, reaching a plateau after 2 days; these elevated activities were maintained for the next

7 days. These results suggest that the alginate lyase and cellulases produced by strain 6532A were extremely stable, and/or that this strain was able to produce and secrete these enzymes for extended periods. Moreover, since the production of reducing sugars and the degradation of Wakame thallus fragments to SCD particles both peaked at the same time, the pericellular matrix containing alginate and cellulose was likely to have been degraded by these enzymes for almost 1 day. Therefore, the reducing sugars produced during incubation were probably utilized by the bacteria to support cell growth and related metabolic activities. Thus, the cells of strain
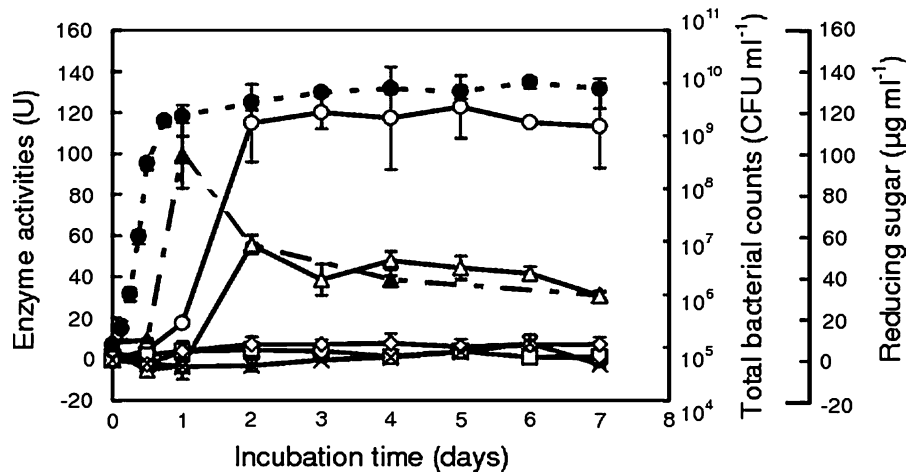
Fig. 6 Extracellular polysaccharide-degrading activities associated with cell growth. *Filled circle* overall growth rate, *filled triangle* quantities of reducing sugars in the culture supernatants. Polysaccharidases, *open circle* alginate lyase, *open triangle* CM-cellulase, *open square* agarase, *open diamond* fucosidase, and *times* laminarinase activities were measured at specific times and are expressed in units (U). Units were defined as the production of 1 μg ml^{-1} h^{-1} of reducing sugar (glucose equivalents). Values are means ± SE, $n = 3$

Fig. 7 Results of SDS-PAGE and zymogram analysis of alginate lyase and CM-cellulase. *Lanes 1, 4* molecular weight markers. *Lanes 2, 3, 5, 6* concentrated proteins in culture supernatant after precipitation with 50% ammonium sulfate. *Lanes 1, 2, 4, 5* coomassie blue-stained proteins. *Lane 3* active staining for alginate lyase. *Lane 6* active staining for CM-cellulase
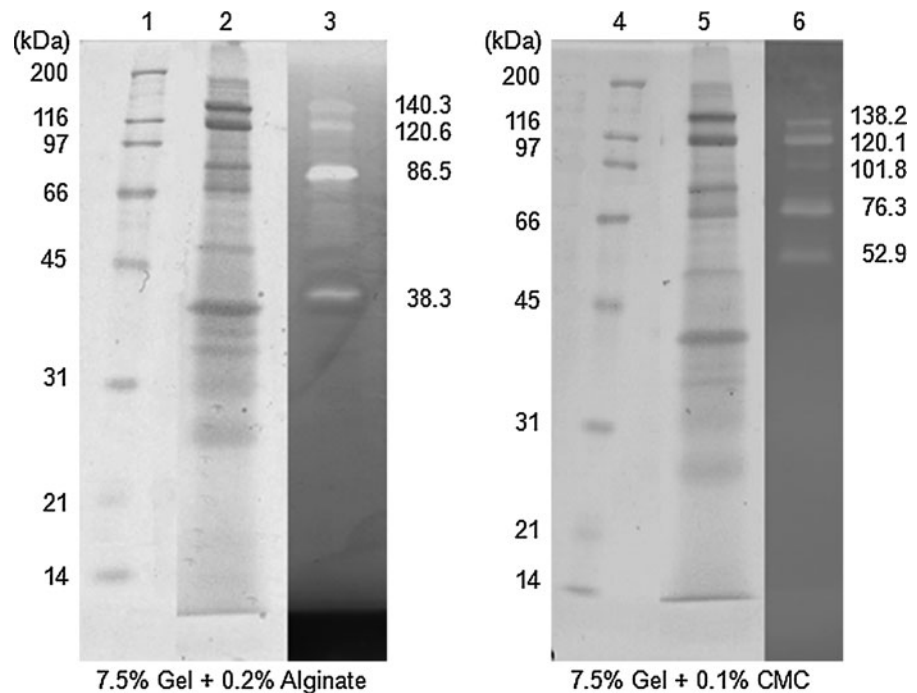
6532A, or the enzymes released by this strain, may prove useful in reducing the volume of seaweed wastes and for producing small functional oligosaccharides or monosaccharides that have been demonstrated to enhance bifidobacterial growth, germination, and shoot elongation in higher plants, as well as possibly having antitumor effects (Wong et al. 2000). The SCD particles produced by strain 6532A also have potential in the development of aquaculture diets, as reported by Uchida et al. (1997) and Camacho et al. (2004). Indeed, our preliminary experiments showed that the larvae of sea urchins (*Anthocidaris crassispina*, *Hemicentrotus pulcherrimus*) and sea cucumbers (*Stichopus japonicus*) feed on these particles.

Fig. 8 Nucleotide sequence and deduced amino acid sequence of the gene, *algMsp*, encoding alginate lyase. Numbering of nucleotides and amino acids (*bold*) is shown at *right*. Amino acid sequences that are highly conserved among G-block-degrading enzymes in PL family-7 are *boxed* and identical amino acid residues are shown in *bold*

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GTGTCTATCTGGAAACTTCACGCGGTATCGAAGCCAGCTCTCTGCCGAATTTTATTCGCC    60
 V   S   I   W   K   L   H   A   V   S   K   P   A   L   C   R   I   L   F   A    20
AGTATTTTCAGTTTAGGCTTAGCCGGTTGCGGCAGTGACAACTCTGAGCCGAATCACACC   120
 S   I   F   S   L   G   L   A   G   C   G   S   D   N   S   E   P   N   H   T    40
GACGCCGGAGCGAGTAGTTCGTCTTCCAGCTCATCATCAGGGTCTTCTTCGAGCTCTTCT   180
 D   A   G   A   S   S   S   S   S   S   S   S   G   S   S   S   S   S   S   S    60
TCAGGTTCGTCATCCAGCTCCAGTGGTTCTGGTAGCGGTTCAGGTAGTGGGCTGGACCCA   240
 S   G   S   S   S   S   S   G   S   G   S   G   S   G   S   G   L   D   P    80
ATGCTCCCGCCCTCCAGCAATTTTGACCTGGCGGCCTGGTATCTCAGTGTACCGACTGAC   300
 M   L   P   P   S   S   N   F   D   L   A   A   W   Y   L   S   V   P   T   D   100
GATGACGGCAATGGACGCGCCGACTCTATCTACGAAGCTGAATTGAACAGCGGCTACGGA   360
 D   D   G   N   G   R   A   D   S   I   Y   E   A   E   L   N   S   G   Y   G   120
AACAGTAATTATTTCTACACCGGCGAAGATGGCGGTATGGTATTCCGCTGCCCCATCGCC   420
 N   S   N   Y   F   Y   T   G   E   D   G   G   M   V   F   R   C   P   I   A   140
GGCTTCAAAACCTCCACCAATACCTCATATACCCGCACCGAATTGCGCGGCATGCTGCGC   480
 G   F   K   T   S   T   N   T   S   Y   T  |R   T   E   L|  R   G   M   L   R   160
CGTGGCGATACCAGTATCAGCACGCAAGGAGTAAACAAGAACAACTGGGTATTCAGCTCC   540
 R   G   D   T   S   I   S   T   Q   G   V   N   K   N   N   W   V   F   S   S   180
GCTCCCATCGCTGCCCGTGAAGCCGCCGGTGGCGTAGATGGCGTGCTGCGTGCAACCCTC   600
 A   P   I   A   A   R   E   A   A   G   G   V   D   G   V   L   R   A   T   L   200
GCTGTCAACCACGTCACGACAACCGGGGACAGCGGCCAGACAGGCCGCGTCATCGTCGGC   660
 A   V   N   H   V   T   T   T   G   D   S   G   Q   T   G   R   V   I   V   G   220
CAGATCCACGCCAACGACGACGAACCTCTGCGCCTCTACTACCGCAAACTGCCAGACAAC   720
|Q   I   H|  A   N   D   D   E   P   L   R   L   Y   Y   R   K   L   P   D   N   240
AGCAAGGGCAGTATCTACATCGCTCACGAAATCAAAGGCGGTGATGACACCTGGTACGAA   780
 S   K   G   S   I   Y   I   A   H   E   I   K   G   G   D   D   T   W   Y   E   260
ATGATCGGCAGCCGCTCCAGTAGCGCATCAAATCCGGAAGATGGAATCGCGCTGAATGAA   840
 M   I   G   S   R   S   S   S   A   S   N   P   E   D   G   I   A   L   N   E   280
ATTTTCAGTTATGAAATCAGGGTCGAGGGCAACACACTCACCGTCACTATTTTCCGCGAA   900
 I   F   S   Y   E   I   R   V   E   G   N   T   L   T   V   T   I   F   R   E   300
GGGAAAGACGATGTGATTCAGATGGTCGATATGAGTGAAAGCGGCTATGACACCGAGGAT   960
 G   K   D   D   V   I   Q   M   V   D   M   S   E   S   G   Y   D   T   E   D   320
CAATATATGTACTTCAAGGCTGGGGTGTATAACCAGAATAATAGCGGGGATGACAGCGAC  1020
 Q   Y   M  |Y   F   K   A   G   V   Y   N   Q|  N   N   S   G   D   D   S   D   340
TATGTGCAGGCGACTTTTTATGCACTTGAGAACAGCCACACTGAATACGAAGACTAA     1077
 Y   V   Q   A   T   F   Y   A   L   E   N   S   H   T   E   Y   E   D   *      358
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Zymogram analysis showed that strain 6532A possesses several catalytically active polypeptides that are capable of degrading alginate and cellulose, suggesting the existence of complex enzymatic systems for the efficient degradation of alginate and cellulose. While several studies have reported the existence of alginate lyases and cellulases in other *Microbulbifer* species (Yoon et al. 2003; Jonnadula et al. 2009; Nishijima et al. 2009), the activities of alginate lyase or cellulase were either inferred by the production of reducing sugars or by the use of a halo assays on agar plates. However, this is likely the first study to directly demonstrate the existence of these enzymes and estimate their molecular masses in *Microbulbifer* species.

The alginate lyase gene, *algMsp*, was cloned and identified. This alginate lyase is considered to belong to PL family-7, because the obtained amino acid sequence contained three regions typically conserved in the PL family-7: RTEL, QIH, and YFKAGVYNQ (Uchimura et al. 2009) (http://www.cazy.org/Polysaccharide-Lyases.html). These regions are considered to be involved in the catalytic domain (Osawa et al. 2005). The repetitive serine-rich sequence in this protein may act as a flexible linker between catalytic and binding domains (Howard et al. 2004). Although the 16S rRNA gene of strain 6532A had a sequence similarity that was 100% similar to *M. elongatus* JAMB-A7, the sequence of the alginate lyase gene in JAMB-A7 has not yet been published to date. The degree of similarity observed between the alginate lyase genes of *S. degradans* 2–40, *V. harveyi* 1DA3, and *Polaribacter* sp. MED152 suggest that *algMsp* is a new alginate lyase gene. Indeed, it is possible that the product of this gene may have been the alginate lyase band

with an approximate molecular weight of 38.3 kDa in the zymogram (Fig. 7).

As the cell density of *Microbulbifer* sp. 6532A increased, the shape of the cells changed from being rod- to coccoid-shaped and cell motility was lost. When the coccoid cells were resuspended in fresh culture medium, they reverted to being rod-shaped. Nishijima et al. (2009) also reported this rod-coccus cell cycle in other non-motile *Microbulbifer* species (e.g. *M. variabilis* and *M. epialgicus*). However, since these morphological changes in strain 6532A were observed in cultures without Wakame thallus fragments, it is possible that the observed changes in morphology were not caused by the degradation products of Wakame thallus fragments. Indeed, these morphological changes may have arisen in response to changes in gene expression through, for example, quorum sensing (Miller and Bassler 2001; Whitehead et al. 2001).

Strain 6532A may therefore be well suited to the degradation and reduction of algal wastes and the production of functional oligo- or mono-saccharides for use as fertilizer, industrial products, medical products, cosmetics, and biofuels. However, further investigations are required in order to evaluate the characteristics and activities of the enzymes involved in these processes.

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