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Original Article

Isolation and identification of a novel alginate-degrading bacterium, *Ochrobactrum* sp.

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Abstract

An alginate-degrading bacterium, identified as *Ochrobactrum* sp. on the basis of 16S rDNA gene sequencing, was isolated from brown algal samples collected from the waters in close vicinity to the Dongtou Isles in the East China Sea. The strain, designated WZUH09-1, is a short rod, gram-negative, obligatory aerobic, grows under the following conditions: 5-40°C, pH 3-9, and 0-2 times of the seawater concentration, and is able to depolymerize alginates with higher enzyme activity than that of others reported so far.

Keywords: *Ochrobacterium*, alginate, identification, degradation

1. Introduction

Alginates occur in brown algae and can also be produced by some bacteria, *e.g.*, *Azotobacter vinelandii* and *Pseudomonas aeruginosa* (Rehm and Valla, 1997), which are linear unbranched polymers containing randomly organized blocks of homopolymeric sequences of either β-(1→4)-linked D-mannuronic acid residues (polyM) or α-(1→4)-linked L-guluronic acid residues (polyG) separated by heteropolymeric sequences of polyMG arranged in a nearly alternating fashion with a wide range of molecular weights. Alginates have widespread application owing to their ability to form viscous solutions and to form gels with calcium ions, gelling at far lower concentrations than gelatin. Depolymerized alginates and their derivatives have been widely used as releasing agents in pharmacy (Ci *et al*., 1999), additives in food industry (Johnson *et al*., 1997), and growth-promoters in agriculture (Kupper *et al*., 2001). They also exhibit high tumour inhibition against solid Sarcoma 180 (Hu *et al*., 2004), and have found important application in tissue engineering (Kataoka *et al*., 2004). Alginate oligosaccharide fragments can be produced by either enzymatic digestion or controlled acid hydrolysis. Controlled acid hydrolysis results in random cleavage along the polysaccharide chains and produces oligosaccharide fragments with unmodified hexuronic acid residues at both termini. Enzymatic degradation has recently attracted public attention because it expands the potential application of these oligosaccharides (Hu *et al*., 2006). The enzymes that depolymerize alginates are classified as either mannuronate (EC 4.2.2.3) or guluronate lyases (EC 4.2.2.11) display variable specificities for the homopolymeric and heteropolymeric sequences. They cleave a hexose-l,4-α- or β-uronic acid sequence by β-elimination of the 4-*O*-linked glycosidic bond and formation of a double bond between C-4 and C-5, resulting in an unsaturated sugar residue at the nonreducing terminus (Nakada and Sweeny, 1967). The unsaturated sugar has an important impact on some bioactivities of the oligomers (Li *et al*., 2003). Alginate-degrading enzymes mainly occur in marine bacteria and fungi (Wong *et al*., 2000), such as *Agarbacterium alginicums* (William and Eagon, 1962), *Alginovibrio aquatilis* (Gacesa,

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1992), *Alteromonas* sp. (Iwamoto *et al*., 2001), *Azotobacter vinelandii* (Davidson and Lawson, 1977), *Bacillus circulans* (Hansen *et al*., 1984), *Dendryphiella salina* (Shimokawa *et al*., 1997), *Enterobacter cloacae* (Nibu *et al*., 1995), *Flavobacterium multivolum* (Takeuchi *et al*., 1994), *Klebsiella aerogenes* (Boyd and Turvey, 1977), *Klebsiella pneumoniae* (Ostgaard *et al*., 1993), *Pseudomonas aeruginosa* (Linker and Evans, 1984), and *Vibrio* spp. (Li *et al*., 2003; Chao *et al*., 1992a; 1992b; 1992c).

To prepare the alginate-degrading enzymes on a commercial scale, many attempts have been made to select superior producer strains to improve the yield and quality (Hu *et al*., 2006). Our study was initiated to expand the knowledge of marine bacteria that decompose alginates. We describe here a novel alginate-degrading bacterium belonging to *Ochrobactrum*, a genus that has not been reported previously to have this activity, and this preliminary characteristics.

2. Materials and Methods

2.1 Isolation of alginate-degrading bacteria

Blades of brown algae were collected from the waters in close vicinity to the Dongtou Isles (27°40' - 28°02' N, 120°56' - 122°17' E) in the East China Sea and returned to the laboratory for isolation within the day of collection. The collected blades were cut into discs (1.5 cm in diameter) and rinsed three times with sterile seawater. The cut blades were submerged into 50 mL of a basal medium and shaken at 180-200 rpm at 25-28°C for 3 days. The basal medium contained alginate (10 g L^{-1}), NaCl (30 g L^{-1}), $(NH_4)_2SO_4$ (5 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (1 g L^{-1}), K_2HPO_4 (2 g L^{-1}), and $FeSO_4$ (0.01 g L^{-1}) at pH 7.5. 5 mL of the culture broths were transferred into 50 mL of the fresh basal medium and repeatedly incubated under the same conditions until the culture broths showed obvious turbidity. The turbid culture broths after dilution with sterile water were plated on agar plates comprising the same ingredients as the basal medium and kept at 25-28°C for 2 days. The colonies that were able to decompose the surroundings or occurred in the clear zones were transferred to the fresh agar plates and subsequently purified. Stock cultures are maintained on agar slopes containing alginate (10 g L^{-1}), peptone (5 g L^{-1}), yeast extract (1 g L^{-1}), and NaCl (15 g L^{-1}) at pH 7.5.

2.2 Selection of alginate-degrading enzyme producer

The pure isolates were placed in 50 mL of the basal medium which contained 5 g L^{-1} of alginate other than 10 g L^{-1} and shaken at 180-200 rpm at 25-28°C. 5 mL of the 24-hour old culture broths were used as initial inoculum for batch fermentation to produce alginate-degrading enzyme and the batch fermentation was run for 48 hours under the same conditions as the inoculum preparation. The culture broths were centrifuged at 5000 rpm for 10 min and the alginate-degrading enzyme activity of the resultant supernatants was determined. The strain exhibiting the highest activity was selected.

2.3 Determination of alginate-degrading enzyme activity

The assay for alginate-degrading enzyme activity was conducted as follows. In each assay, reaction products were confirmed to increase proportionally with time and enzyme concentration in the reaction mixture (Li *et al*., 2003; Shin *et al*., 2006). 0.1 mL of the supernatants was added into 1 mL of 0.3% alginate in 0.2 M phosphate buffer (pH 7.0) and then kept at 40°C for 20 min. Quantitative estimates of the alginate degradation were made by measuring the increase in absorbance at 235 nm, because 4-deoxy-L-*erythro*-hex-4-ene pyranosyl uronic acid at the nonreducing terminus of the resultant oligomers formed by β-elimination provides a UV chromophore (232 nm). One unit of enzyme activity causes an increase of 0.1 optical density unit per min.

2.4 Phylogenetic analysis

The 16S rDNA gene was amplified and sequenced in the China Center of Industrial Culture Collection (Beijing, China). Forward and reverse primers were 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-ACGGCTACCTTGTTACGACT-3', respectively. PCR amplifications were performed with the following cycling program: denaturation for 6 min at 94°C, 30 amplification cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C, with a 10 min extended elongation step. The sequences were compared to 16S rDNA gene sequences in the public database using the BlastN program (Altschul *et al*., 1997). Related sequences were obtained from the GenBank database (National Center for Biotechnology Information, U.S. National Library of Medicine) by using the BLAST search program. Phylogenetic analysis was carried out according to Kimura's two-parameter and neighbor-joining method (Saitou and Nei, 1987) using the RDP Phylip Interface (Maidak *et al*., 2001).

3. Results and Discussion

The selected strain is designated WZUH09-1. Cells are short rods, measuring 0.4-0.6 μm in width and 0.6-0.9 μm in length, gram-negative, obligatory aerobic, grow under the conditions: 5-40°C, pH 3-9, and 0-2 times of the seawater concentration. After growth on agar plates comprising the same ingredients as the basal medium at 25°C for 24 h, the bacterial colonies are typically circular, entire, low convex, opaque, mucoid, and about 1 mm in diameter. No colony pigment development is observed. The full details of the phenotypic characteristics of the strain WZUH09-1 are given in Table 1.

Most of the reported alginate-degrading enzyme producers are marine microorganisms active in algal cell wall decomposition. Curiously, decomposition of alginate by

Table 1. Phenotypic characteristics of strain WZUH09-1

Characteristics	Strain WZUH09-1
Morphology	short rods
Gram-reaction	-
Aerobic growth	+
Anaerobic growth	-
Optimum pH	6-7
Optimum temperature	25-28°C
Oxidase	+
Catalase	+
Indole production	-
Hydrolysis of	
gelatin	-
starch	-
Substrate utilization	
glucose	+
fructose	+
sucrose	+
maltose	-
Tolerance to salinity	+ (up to 2 times of the seawater concentration)

microorganisms appears to be performed almost entirely by gram-negative bacteria, although few if any gram-positive bacteria have been identified as producers of alginate-degrading enzyme (Hansen *et al*., 1984).

The taxonomic identity of the strain WZUH09-1 was ascertained by 16S rDNA gene sequencing. The sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number EF465412.

The phylogenetic tree for the strain WZUH09-1 and *Ochrobactrum* spp. and related genera is showed as Figure 1. The 16S rDNA gene of the strain WZUH09-1 showed the closest match to that of *O. grignonense* with a homology of 98.8%. The similarities to *Ochrobactrum anthropi*, *Ochrobactrum cytisi* and *Ochrobactrum lupine* are all 98.1%. Therefore, it could be identified as *Ochrobactrum* sp.

Ochrobactrum anthropi was originally classified at the Centers for Disease Control and Prevention, U.S.A. as Group Vd and was believed to have many similarities to the genus *Achromobacter.* However, Holmes *et al.* (1988) proposed the classification of the genus *Ochrobactrum* and transferred Group Vd to *O. anthropi*, the sole and type species. To date, *O. intermedium* (Velasco *et al*., 1998), *O. grignonense* (Lebuhn *et al*., 2000), *O. tritici* (Lebuhn *et al*., 2000), *O. gallinifaecis* (Kampfer *et al*., 2003), *O. lupini* (Trujillo *et al*., 2005), *O. oryzae* (Tripathi *et al*., 2006), *O. cytisi* (Zurdo-Pineiro *et al*., 2007), and *O. pseudintermedium* (Teyssier *et al*., 2007) have been reported as species belonging to this genus.

Much of what is currently known about the genus *Ochrobactrum* is based on its emergence as an opportunistic human pathogen. Other information is very limited (Yuan *et al*., 2005) perhaps due to the fact that they have only been

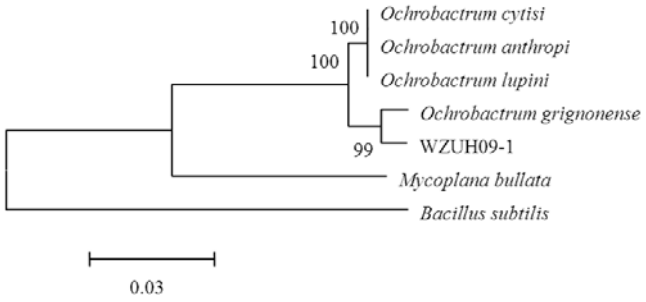

Figure 1. Phylogenetic tree based on 16S rDNA gene sequencing showing the relationships between the alginate-degrading strain WZUH09-1, *Ochrobactrum spp.* and related genera. Their GenBank accession numbers for the bacteria in the tree are: *Ochrobactrum grignonense*, AJ242581; *Ochrobactrum anthropi*, DQ468351; *Ochrobactrum cytisi*, AY776289; *Ochrobactrum lupini*, AY457038; *Mycoplana bullata*, D12785, and *Bacillus subtilis*, AB 271744. The scale bar corresponds to 0.03 estimated nucleotide substitution per sequence position.

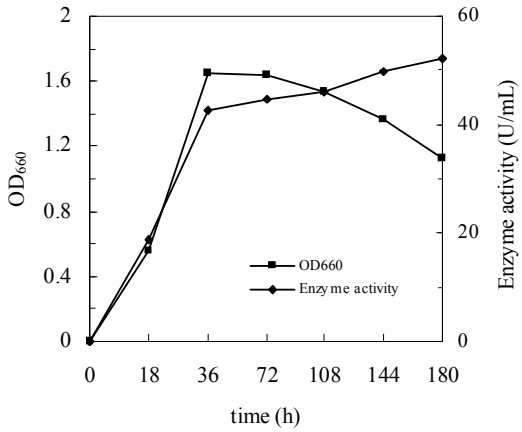

Figure 2. Time courses for cell growth of strain WZUH09-1 and alginate-degrading enzyme activity change in culture broth. Cells were grown in a medium at 180-200 rpm at 25-28°C. The medium contained alginate (5 g L^{-1}), NaCl (30 g L^{-1}), $(NH_4)_2SO_4$ (5 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (1 g L^{-1}), K_2HPO_4 (2 g L^{-1}), and $FeSO_4$ (0.01 g L^{-1}) at pH 7.5. Cell growth was estimated by optical density at 660 nm (OD_{660}). One unit of enzyme activity causes an increase of 0.1 optical density unit per min at 235 nm. All experiments were performed twice and data represent the average of duplicate samples taken on each occasion.

described since 1988. The genus *Ochrobactrum* have potential applications for bioremediation. *Ochrobactrum* spp. demonstrate the abiliby to degrade phenol (El-Sayer *et al*., 2003), dichlovos (Zhang *et al*., 2006), atrazine (Laura *et al*., 1996), halobenzoate (Bongkeuu *et al*., 2000), and 4-chloro-2-methylphenol (Lechner *et al*., 1995), to reduce toxic hexavalent chromium (Sultan and Hasnain, 2006; 2007), and to adsorb heavy metal ions (Ozdemir *et al*., 2003). Several enzymes have been identified from the genus *Ochrobactrum*, including D-stereospecific amino acid amidase (Asano *et al*., 1989; Komeda *et al*., 2003), aminopeptidase (Bompard-

Gilles *et al*., 2000), and glutathione *S*-transferase (Favaloro *et al*., 1998; Tamburro *et al*., 2004). They can also produce biosurfactant (Wasko and Bratt, 1991) and degrade nicotine (Yuan *et al*., 2005). There has been no evidence regarding alginate-degrading activity within the genus *Ochrobactrum*, before we describe here the strain WZUH09-1.

To clarify the level of alginate degradation, the cell growth during the batch fermentation of the strain WZUH09-1 to produce alginate-degrading enzyme and the change of alginate-degrading enzyme activity in the supernatants of the culture broth were investigated. The time course is shown in Figure 2. Cell growth was estimated by optical density at 660 nm (OD_{660}).

The biomass reached maximum and the alginate-degrading enzyme activity was 42.5 U/mL after cultivation for 36 h. During the logarithmic phase of growth the enzyme activity showed a rapid increase. It continued to increase until it achieved approximately 50 U/mL during the stationary and decline phase. The enzyme thus appeared to be found in two pools during growth: one extracellular (present in the culture broth during the logarithmic phase) and the other one cell associated (showing a slow dissociation from cell debris during the stationary and decline phase).

Compared to *Vibrio* sp. QY102 that requires 3 days to achieve 37.8 U/mL of enzyme activity, which was obviously higher than that of others reported so far (Li *et al*., 2003), the strain WZUH09-1 may be an efficient alginate-degrading enzyme producer.

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