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Purification and characterisation of a bifunctional alginate lyase from novel *Isoptericola halotolerans* CGMCC 5336

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ABSTRACT

A novel halophilic alginate-degrading microorganism was isolated from rotten seaweed and identified as *Isoptericola halotolerans* CGMCC5336. The lyase from the strain was purified to homogeneity by combining of ammonium sulfate fractionation and anion-exchange chromatography with a specific activity of 8409.19 U/ml and a recovery of 25.07%. This enzyme was a monomer with a molecular mass of approximately 28 kDa. The optimal temperature and pH were 50 °C and pH 7.0, respectively. The lyase maintained stability at neutral pH (7.0–8.0) and temperatures below 50 °C. Metal ions including Na^+, Mg^{2+}, Mn^{2+}, and Ca^{2+} notably increased the activity of the enzyme. With sodium alginate as the substrate, the K_m and V_{max} were 0.26 mg/ml and 1.31 mg/ml min, respectively. The alginate lyase had substrate specificity for polyguluronate and polymannuronate units in alginate molecules, indicating its bifunctionality. These excellent characteristics demonstrated the potential applications in alginate oligosaccharides production with low polymerisation degrees.

1. Introduction

Alginate is a linear acidic polysaccharide consisting of 1,4-linked β-D-mannuronate (M) and its C5 epimer α-L-guluronate (G). These uronate residues are randomly arranged as homopolymeric G blocks [poly (G)] and homopolymeric M blocks [poly (M)], and heteropolymeric MG blocks [poly (MG)] (Gacesa, 1992). Alginate oligosaccharides are depolymerization products of alginate by alginate lyases or physicochemical method. They have attracted considerable attention because of their applications in the food industry as growth promoters for plants (Hu, Jiang, Hueymin, Liu, & Guan, 2004) and as therapeutic agents [i.e., anticoagulants, tumour inhibitors and suppression of IgE (An et al., 2009; Iwamoto et al., 2005; Tusi, Khalaj, Ashabi, Kiaei, & Khodagholi, 2011)]. They are also involved in the reduction of blood sugar and lipids (Zhang, Zhou, Jia, Zhang, & Gu, 2004). Therefore, alginate lyases for mild degradation have been the focus of different research groups.

Alginate lyases can catalyse alginate degradation through the β-elimination mechanism, with unsaturated uronic acid products at the non-reducing terminus (Linhardt, Galliher, & Cooney, 1987). Based on the substrate specificities, alginate lyases are classified into mannuronate lyase (EC 4.2.2.3) and guluronate lyase (EC 4.2.2.11) that preferentially break up the M- and G-rich alginates, respectively. Alginate lyases specific to G or M blocks are monofunctional, whereas those specific to MG blocks are bifunctional (Tondervik et al., 2010).

So far, alginate lyases have been widely isolated and studied from various sources including marine molluscs (Sim et al., 2012; Suzuki, Suzuki, Inoue, & Ojima, 2006), seaweeds (An et al., 2008), marine bacteria (Kobayashi, Uchimura, Miyazaki, Nogi, & Horikoshi, 2009; Wakabayashi et al., 2012), marine fungi (Schaumann & Weide, 1990), some bacteriophages (Bartell, Orr, & Lam, 1966) and viruses (Suda, Tanji, Hori, & Unno, 1999). They have been used not only in the production of alginate oligosaccharides, but also in the elucidation of alginate fine structures, cell wall development of *Fucus* (Matsubara, Kawada, Iwasaki, Oda, & Muramatsu) and protoplast production of red and brown algae (Inoue, Mashino, Kodama, & Ojima, 2011). Recent investigations have illustrated the potential application of alginate lyases in cystic fibrosis management in conjugation with other chemotherapeutics (Li, Dong, et al., 2011; Li, Guan, Jiang, & Hao, 2011; Li, Jiang, Guan, Wang, & Guo, 2011) and seaweed waste management (Xiao, Han, Yang, Lu, & Yu, 2006).

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Present studies on alginate lyase are still being developed. Poor sources, narrow substrate specificity and low enzyme activity limit the generation of novel alginate oligosaccharides. Therefore, various isolates with high enzyme activity and wide substrate specificity should be screened to expand the potential applications of alginate lyases.

In this paper, a new alginate lyase-producing strain was isolated and identified as *Isoptericola halotolerans* CGMCC 5336. After the enzyme was purified, its characteristics in terms of enzyme activity were analysed under different temperature and pH conditions. This study is the first to report on a bifunctional alginate lyase derived from the genus *Isoptericola*.

2. Materials and methods

2.1. Microorganism

The alginate lyase-producing bacterial strain used in this study was isolated from decomposed seaweed via an enrichment procedure. Rotting seaweed samples were collected from an industrial production area of sodium alginate in Lianyungang City, China.

2.2. Media and culture conditions

All chemicals were of reagent grade. Sodium alginate minimal medium (Li, Dong, et al., 2011; Li, Guan, et al., 2011; Li, Jiang, et al., 2011) containing 5.0 g/l sodium alginate, 5.0 g/l $(NH_4)_2SO_4$, 2.0 g/l K_2HPO_4, 30.0 g/l NaCl, 1.0 g/l $MgSO_4{\cdot}7H_2O$ and 0.01 g/l $FeSO_4{\cdot}7H_2O$ at pH 7.0 was used for the bacterium isolation. An optimised fermentation medium for growth enrichment was composed of 6.0 g/l sodium alginate, 5.0 g/l tryptone, 2.5 g/l yeast extract, 25.0 g/l NaCl, 2 mM $MgSO_4$, 0.5 mM $CaCl_2$, 1 mM KH_2PO_4, 0.2 mM $FeSO_4$, and 0.3 mM $MnSO_4$ at pH 8.0. Solid medium was prepared by adding 20 g/l of agar to the above medium. Bacteria were aerobically cultured in the 250 ml shake flasks containing 20 ml liquid medium at 25 °C and 200 rpm, which was a result of optimisation.

2.3. Strain isolation and identification

The preliminary screening for the alginate-degrading microorganism was conducted as follows: A 1 g sample of decomposed seaweed sample was suspended in 10 ml minimal medium and 1 ml suspension samples were transferred in a test tube with 10 ml minimal medium for enrichment. The inoculated test tubes were cultivated at 25 °C for 48 h with continuous shaking at 200 rpm. Microorganisms that could grow on a minimal medium using sodium alginate as the sole carbon source were accumulated by subculturing, which was repeated more than thrice every two days. The accumulated cultures were subsequently diluted and spread on minimal medium plates containing 2% agar for isolation until pure cultures were obtained.

The re-screening process was conducted as follows. Strains with clear hydrolytic zones were selected and incubated aerobically in a fermentation medium under the same conditions as above. Furthermore, the activity of alginate lyase was determined by 3,5-dinitrosalicylic acid (DNS) colorimetry (Preiss & Ashwell, 1962). Among the isolates, the most active strain WX was selected for further studies.

Bacterial identification was performed based on 16S rDNA gene sequence, which was amplified using polymerase chain reaction (PCR) with the following universal primers: GAGAGTTTGATCCTGGCT CAG (P_0) and CTACGGCTACCTTGTTACGA (P_6) (Weisburg, Barns, Pelletier, & Lane, 1991). The PCR conditions were as following: initial denaturation at 95 °C for 10 min followed by 30 cycles of denaturation (45 s, 95 °C), annealing (45 s, 56 °C), extension (60 s, 72 °C) and final extension of 72 °C for 10 min. Moreover, 16S rDNA was sequenced by Sangon Biotech (Shanghai) and compared using the Basic Local Alignment Search Tool (BLAST) at the National Centre for Biotechnology Information databases (NCBI) and registered at the GeneBank database.

2.4. Enzyme purification

All the purification procedures were carried out at 4 °C.

Step 1: Ammonium sulfate precipitation

A 1 L aliquot of the supernatant separated from bacteria cells were obtained via centrifugation at 6000 rpm for 30 min. Solid ammonium sulfate was added to the crude enzyme solution to obtain 50% to 70% saturation. The resultant precipitate was centrifuged at 6000 rpm for 30 min, dissolved in 50 ml sodium phosphate buffer (PB, pH 7.0) and then dialyzed overnight against a large volume of the same buffer.

Step 2: Anion-exchange chromatography

The crude enzyme solution from step 1 was filtered through a 0.45 μm filter membrane to remove any undissolved substances. The concentrated crude enzyme solution was then applied to an anion-exchange column of Q-Sepharose Fast Flow (2.6 cm × 30 cm, Pharmacia), which had been equilibrated with 50 mM PB (pH 7.0) in advance. The absorbed proteins were eluted with a gradient elution at 0.3 M NaCl followed by a linear gradient of 0.3 M to 1 M NaCl at a low flow rate of 1 ml/min. The eluted fractions were collected every 5 ml and assayed for the enzyme activity. Main active fractions were pooled and lyophilised. The dried powder dissolved in a certain PB (pH 7.0) was used as a purified enzyme for further experiments.

2.5. Assay for alginate lyase activity and protein concentration

Alginate lyase activity was determined through 3,5-dinitrosalicylic acid (DNS) colorimetry. Briefly, 1 ml of the reaction mixture containing 900 μl of 1% sodium alginate (dissolved in 50 mM PB at pH 7.0) and 100 μl of the prepared enzyme was incubated at 40 °C for 15 min. The activity was measured by monitoring the increased absorbance of the reaction products (oligosaccharides) at 520 nm. One unit of alginate lyase activity (U) was defined as the amount of enzyme required to generate 1 μg glucuronic acid per min. Protein concentration was determined through Bradford's method using bovine serum albumin as the standard (Marion, 1976).

2.6. Gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out on 10% poly-acrylamide gel with 0.1% SDS according to method described by Laemmli (1970). The gel was stained with Coomassie Brilliant Blue R-250 followed by destaining with methanol–acetic acid (2:1, v/v). The unstained protein molecular weight marker (Fermentas, China) was served as the molecular mass marker.

2.7. Characterisation of alginate lyase

2.7.1. Effects of temperature and pH on alginate lyase activity and stability

The effects of pH on the enzyme activity were evaluated by incubating the purified enzyme in PB at pH 5.5 to 9.5 under the standard assay conditions. The pH stability depended on the residual enzyme activity when the enzyme was conserved in different pH buffers at 4 °C for 12 h in advance. Meanwhile, the effects of temperature (25–65 °C) on the purified alginate lyase were investigated at pH

7.0. The thermal stability of the enzyme was determined under the standard assay conditions after incubating purified enzyme solutions at 25–65 °C for 30 min. Furthermore, residual activities were measured after incubation of the enzyme at 40, 50, 60 °C for different time periods (30, 60, 90, 120, 150, 180 min) at pH 7.0.

2.7.2. Effects of NaCl concentration and metal ions on alginate lyase activity

The effects of NaCl on alginate lyase activity were investigated at different concentrations between 1% and 10% under standard test conditions. The enzyme activity without NaCl served as the control. The influences of different metal ions on the enzyme activity were performed by incubating the enzyme in 50 mM PB (pH 7.0) at 4 °C for 30 min in the presence of various metal compounds at a concentration of 5 mM. Further residual activities were then assayed. The mixture without any metal ion was used as the control with the corresponding enzyme activity designated as 100%.

2.7.3. Determination of kinetic parameters

The kinetic constants of the purified enzyme were determined by measuring the enzyme activity at different sodium alginate concentrations. The K_m and V_{max} values were estimated using linear regression plots of Lineweaver and Burk (Lineweaver & Burke, 1934).

2.7.4. Thin-layer chromatography (TLC)

The reaction mixture containing 1 ml 1.0% sodium alginate and 1 ml enzyme was incubated at 30 °C for 24 h. A 5 μl aliquot of the reaction products (unsaturated oligosaccharides) was subjected to Thin-layer chromatography (TLC) using a solvent system of 1-butanol:acetic acid:water (3:2:2, v/v). Plots in TLC plates were visualised by heating at 120 °C for 5 min after spraying with 10% (v/v) sulfuric acid in ethanol (Ma, Chi, Li, & Wu, 2008).

2.7.5. Substrate specificity

The purified enzyme was reacted with sodium alginate and two types of homopolymeric substrates [poly (M) and poly (G)] to evaluate the substrate specificity. Poly (M) and poly (G) homopolymeric blocks were prepared via two cycles of HCl-hydrolysis according to the procedure of Haug, Larsen, and Smidsrod (1967). The assay of enzyme activity was the same as previously described. For the two types of homopolymeric substrates, the reaction was carried out by replacing sodium alginate with M or G block of 1% concentration.

2.7.6. Identification of peptide mass fingerprinting

The identification of internal peptide sequence was carried out through mass spectrometry. The purified protein in sodium dodecyl sulfate–polyacrylamide gel electrophoresis was cut out. After destaining (100 mM NH_4HCO_3 and 30% acetonitrile) and washing, the dried gel pieces were treated with trypsin (50 μg/ml) in 50 mM NH_4HCO_3 buffer (pH 8.0) at 37 °C overnight. Considering the different molecular masses with different time-of-flight detections, these peptide fragments were analysed using an Applied Biosystem reflectron time-of-flight mass spectrometer and identified by comparison with various protein identification databases.

3. Results and discussion

3.1. Isolation of alginate-degrading microorganism

Approximately 40 isolates on the minimal medium supplemented with 0.5% (w/v) sodium alginate as the sole carbon source were screened initially based on the appearance of clearing hydrolytic zone and alginate lyase activities from fermentation culture. The strain with the most remarkable activity was selected and designated as WX for further studies. BLAST analysis on sequence

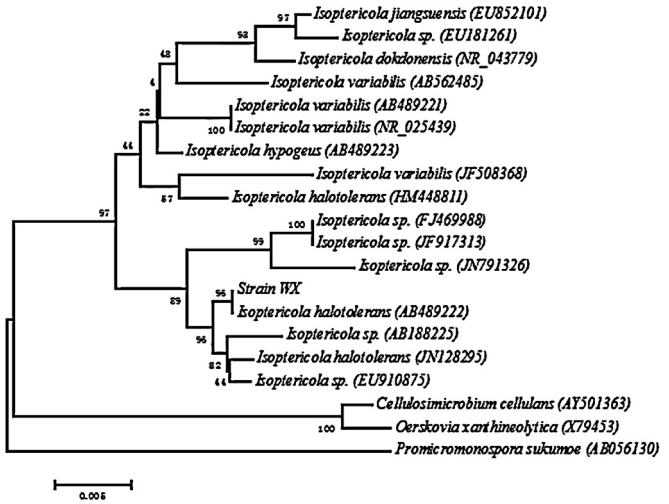

Fig. 1. Phylogenetic dendrogram obtained by distance matrix analysis of 16S rDNA gene sequences, reflected the relationship of the strain CGMCC 5336. Bootstrap percentages (based on 1000 replications) are shown at branch points. *Promicromonospora sukumoe* was used as the outgroup.

similarity revealed that strain WX was located in the same clade as *I. halotolerans* (AB489222) with 100% homology (Fig. 1). Therefore the strain WX was identified as *I. halotolerans* and named as *I. halotolerans* WX. The 16S rDNA sequence was registered at the GenBank database with accession No. JQ288840. This novel isolated strain was deposited for the meantime at China General Microbiological Culture Collection Centre (No.CGMCC 5336). To the best of our knowledge, this study is the first to report on a bifunctional alginate lyase derived from the genus *Isoptericola*. Moreover, lyase activity was not detected in the non-sodium alginate medium under the same conditions, which demonstrated that the enzyme was inducible. This result was typical for marine bacteria.

3.2. Purification of the alginate lyase

The crude alginate lyase was purified using two simple steps of ammonium sulfate fractionation and anionic exchange chromatography of Q-Sepharose Fast Flow. The protein concentration was 7-fold while subjected to ammonium sulfate fractionation which could contribute in dissolving other nonspecific proteins and eliminating bulks of nucleic acids and acidic carbohydrates (Izydorczyk & Biliaderis, 1996). The fraction precipitates after dialysis had low viscosity and high concentration. Furthermore, the specific protein was separated sufficiently via Q-Sepharose Fast Flow (Fig. 2). One single sharp peak displayed the highest specific activity of alginate lyase, whereas fractions from other elution peaks did not exhibited enzyme activity. The specific activity of the desired lyase finally reached up to 8409.19 U/ml, which was nearly 2-fold than the initial stage. The enzyme was purified with a recovery of approximately 25.87% compared with the crude enzyme (Table 1). The latter fraction showed a single band with 28 kDa on SDS–PAGE followed by Coomassie Blue staining (Fig. 3). Thus these purified fractions were pooled for further studies.

The low recovery of alginate lyase could be attributed to the strong binding capacities between the substrate and alginate. Only a few lyases are known to be purified to homogeneity (Wong, Preston, & Schiller, 2000). However, the alginate lyase from *I. halotolerans* CGMCC5336 with high enzyme activity processes the superiority of simple purification accompanied by with a small quantity of nonspecific proteins, thus offering potential possibilities for large-scale industrialisation.

Table 1
Purification of the alginate lyase from *I. halotolerans* CGMCC 5336.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification Fold (%)	Yield
Liquid supernatant	72.00	345,850.49	4803.48	1.00	100
Ammonium sulfate	24.89	135,331.2	4985.90	1.04	39.13
Fractionation Q-sepharose FF	10.64	89,473.76	8409.19	1.75	25.87

Alginate lyase activity was determined by 3,5-dinitrosalicylic acid (DNS) colorimetry as described in the text. One unit of enzyme activity (U/ml) was defined as the amount of enzyme required to generate 1 μg glucuronic acid per min.

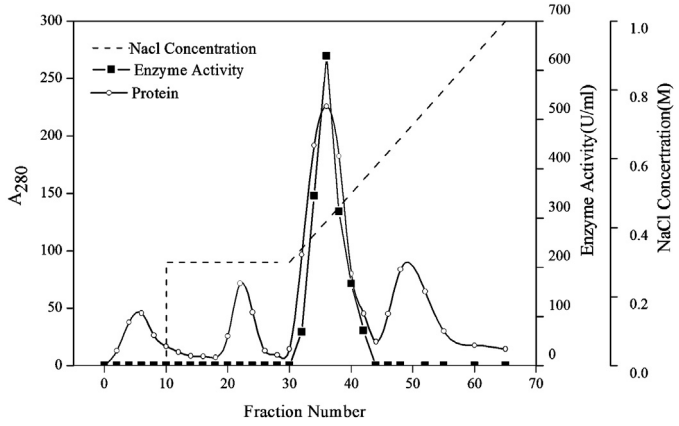

Fig. 2. Purification by anionic exchange chromatography (Q-Sepharose Fast Flow) of the alginate lyase from *I. halotolerans* CGMCC 5336.

3.3. Properties of alginate lyase from I. halotolerans CGMCC 5336

3.3.1. Molecular mass determination

The protein pattern after each purification procedure is shown in the SDS–PAGE (Fig. 3). The single band after the final procedure indicated that the alginate lyase was composed of one polypeptide chain with an approximate molecular mass of 28 kDa. The different lyases produced by marine isolates differs in size from 24 kDa to 110 kDa (Wong et al., 2000). The lyase from *I. halotolerans* CGMCC5336 corresponded with the range. However, Li et al. has recently reported a monomeric alginate lyase with a molecular weight of 23 kDa (Li, Dong, et al., 2011; Li, Guan, et al., 2011; Li, Jiang, et al., 2011).

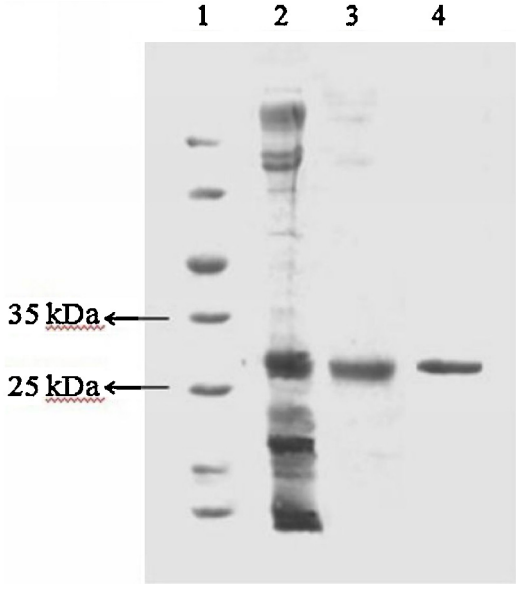

Fig. 3. SDS–PAGE analysis of purified enzyme from *I. halotolerans* CGMCC 5336. Lane 1, molecular mass marker; lane 2, culture fluid; lane 3, after ammonium sulfate fractionation; lane 4, after Q-Sepharose.

3.3.2. Effects of temperature and pH on alginate lyase activity and stability

The maximum enzyme activity of the lyase was observed at 50 °C and the enzyme was stable below 40 °C (Fig. 4A). The lyase possessed approximately 50% activity after incubation at 45 °C for 30 min and was gradually inactivated as temperature increased. Further studies on thermal stability at 40, 50 and 60 °C with different time periods (Fig. 4B) demonstrated that the enzyme retained more than 60% activity after incubation at 40 °C for 3 h. This finding suggests that that the enzyme had a favourable thermal stability up to 40 °C. This stability may be related to the marine environment where *I. halotolerans* CGMCC 5336 was isolated.

Optimal temperature of the alginate lyases from marine bacterial differ significantly. The optimal temperature of the enzyme isolated from *I. halotolerans* CGMCC 5336 was higher than that from *Gracilibacillus* A7 and *Agarivorans* sp. (Kobayashi et al., 2009; Tang, Zhou, Chu, & Nagata, 2011), but similar to that from *Sphingomonas* sp. MJ-3 (Park, Kam, Lee, & Kim, 2012). However, the alginate lyase from *Sphingomonas* sp. showed a higher optimal temperature of 70 °C but was unstable (Yonemoto et al., 1993).

The optimum pH for the enzyme activity was 7.0 and retained more than 60% activity at a broad pH range of pH 5.5 to 8.5 after incubation for 12 h. However, the lyase was the mostly stable at pH 7.5. The half inactivation was found at pH 9.5. The pH stability manifested that the alginate lyase was applied at nearby neutral pH conditions. The structural changes in the lyase resulting in enzyme activity drop might be attributed to deflecting acid or alkali and high temperature (Fig. 4C). Previous studies reported that the optimal pH for most of alginate lyases from marine bacteria were between 7.0 and 8.5. No dissimilarity was observed in the alginase from *I. halotolerans* CGMCC 5336.

3.3.3. Effects of NaCl concentration and metal ions

The effect of NaCl on the enzyme activity was shown in Fig. 5A. The activity reached maximum at 4% NaCl, boosted around 25% compared to the control without NaCl, suggesting that NaCl acted as a strong activator for this alginate lyase. This result is also found in a few marine bacteria except for a much higher optimal concentration of NaCl (0.6 M) (Hu, Jiang, & HWang, 2006; Tang, Taniguchi, Chu, Zhou, & Nagata, 2009). Nakada and Sweeny (1967) had postulated that this high ionic strength might be required to maintain the uronic acid units at a minimal interunit period for proper fitting of the enzyme. These enzymatic promoting effects of high NaCl levels may be partly caused by the removal of bound water from sodium alginate molecules or by the effects of charges in forming the alginate-enzyme complex formation (Kitamikado, Tseng, Yamaguchi, & Nakamura, 1992).

Besides, the activity of alginate lyase from *I. halotolerans* CGMCC 5336 cultured in the fermentation medium without NaCl was not detected. However, the purified enzyme possessed activity without NaCl. Thus, NaCl is speculated to be necessary for the biosynthesis of alginate lyase from *I. halotolerans* CGMCC 5336. Further investigations are required to reveal the role of NaCl in synthetic pathways.

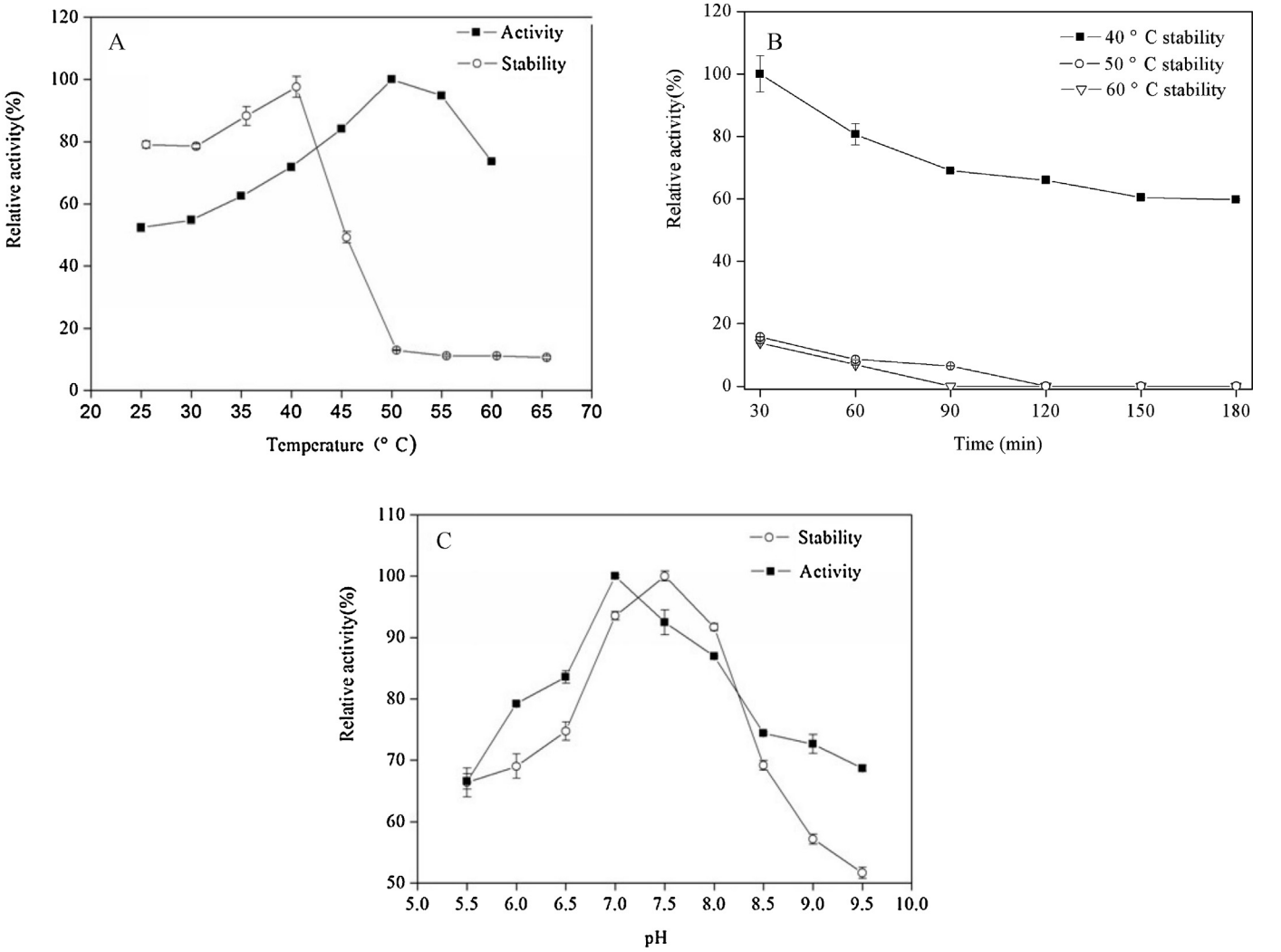

Fig. 4. Effects of temperature and pH on stability and activity of the alginate lyase derived from *I. halotolerans* CGMCC 5336. (A) Temperature profiles at different temperatures (25–65 °C). The activity was measured by the method of 3,5-dinitrosalicylic acid (DNS) colorimetry at 50 ml PB (pH 7.0). (B) Thermostability of the enzyme at 40, 50 and 60 °C for different time periods. (C) pH profiles. pH was determined at 40 °C in PB ranging from pH 5.5 to 9.5.

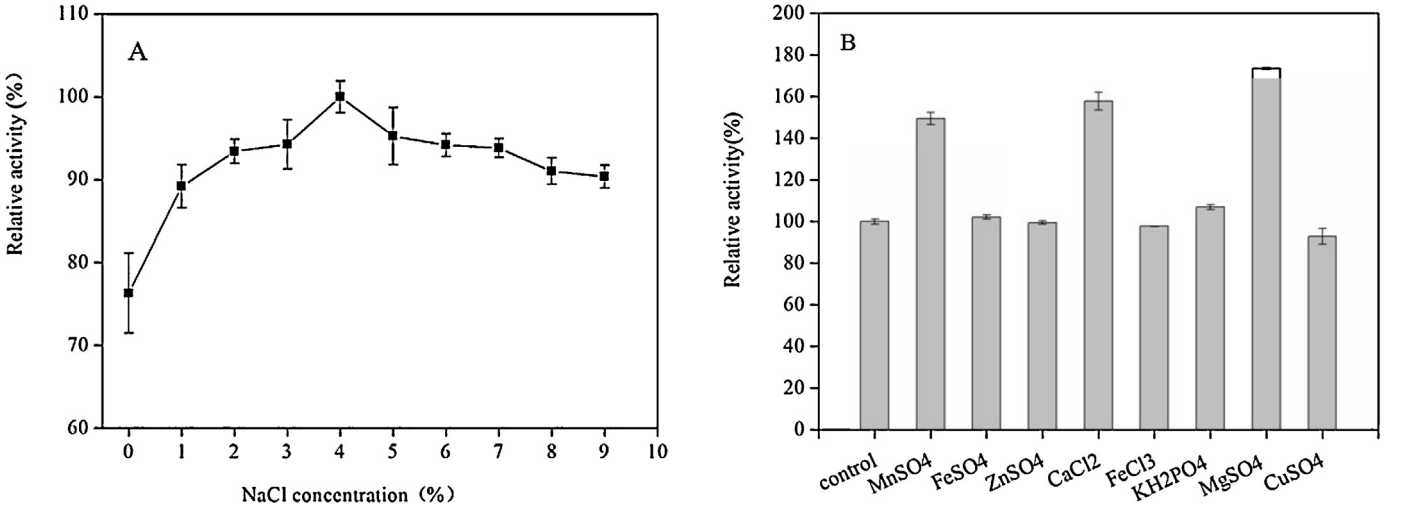

Fig. 5. Effects of metal ions and NaCl on activity of the alginate lyase derived from *I. halotolerans* CGMCC 5336. The enzyme activity was determined in the reaction mixture as described at 4 °C for 30 min in the presence of various metal ions. (A) Influences of the concentration of NaCl profiles were shown as above. The activity of the control was relatively taken as 100%. (B) The enzyme activity without metal ions served as the control.

Table 2
Sequence of internal peptides of alginate lyase from *I. halotolerans* CGMCC 5336.

Accession number	Organism	Sequence	Identity (%)
ADI04722.1	*Streptomyces bingchenggensis* BCW-1	94AAVDGVTTGGSSYPR108	100
YP_003512095.1	*Stackebrandtia nassauensis* DSM 44728	88SAVDGVTTGGSKYPR102	93
YP_003379401.1	*Kribbella flavida* DSM 17836	234AAVNGVTTSGNYPR248	80
NP_627698.1	*Streptomyces coelicolor* A3(2)	93SAVNGVTTSGSSYAR107	79

Analysis of mass spectrometry was performed though MALDI-TOF and the identification of proteins were compared in various protein identification databases.

The effects of monovalent and divalent metal ions on enzyme activity were performed in absence of NaCl at a concentration of 5 mM (Fig. 5B). Among the metal ions investigated, K^+, Mg^{2+}, Mn^{2+}, Ca^{2+} and Fe^{2+} displayed activating effects. The divalent metal ion Mg^{2+} showed the most stimulating effect with 173.50% of relative activity followed by Ca^{2+} with 157.69%. The importance of Mg^{2+} and Ca^{2+} for some lyases from marine bacteria such as *Azotobacter vinelandii* (Gimmestad et al., 2009) and *Streptomyces* A5 (Cao et al., 2007) has also been reported. Surprisingly, Mn^{2+} could significantly enhance the activity of the purified enzyme activity, which was scanty report. By contrast, cations such as Zn^{2+}, Fe^{3+} and Cu^{2+} had slight inhibitory effects.

Several studies have reported on the activation of alginate lyase by monovalent and divalent metal ions from marine bacteria (Matsushima et al., 2010; Wang et al., 2006). These reports may reflect the physiological adaptation capability of the alginate lyases to sea water environment (Rahman, Inoue, Tanaka, & Ojima, 2010). Meanwhile, metal ions including Na^+, K^+, Mg^{2+}, Mn^{2+} and Ca^{2+} are believed to protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzyme at high temperatures (Kumar & Takagi, 1999). Favorov, Vozhova, Denisenko, and Elyakova (1979) revealed that cations might decrease the surface density of the substrate charge and weaken the ionic interactions between alginate and the enzyme.

3.3.4. Kinetic parameters

The Lineweaver–Burke graph demonstrated the K_m and V_{max} of values were 0.26 mg/ml and 1.31 mg/ml min, respectively. These values suggest that the enzyme had high affinity and catalytic efficiency to the substrate of sodium alginate. The K_m values of another bifunctional alginate lyase from *Pseudoalteromonas* sp. SM0524 were 1.086, 0.465 and 2.751 mg/ml depending on different substrates (Li, Dong, et al., 2011; Li, Guan, et al., 2011; Li, Jiang, et al., 2011). Compared with other alginate lyases, this enzyme had a lower K_m value and a higher V_{max} value, indicating its superior property.

3.3.5. Substrate specificity

The substrate specificity of the alginate lyase was characterised using both qualitative and quantitative assays for catalytic activity. The results suggested that the alginate lyase purified from *I. halotolerans* CGMCC 5336 was active in degrading both poly (M) and poly (G) simultaneously. Moreover, it prefers poly (G) over poly (M). Previous studies reported that only a few strains produce bifunctional alginate lyase. This type of lyase assists in the recovery of sensible pathogenic bacteria to antibiotic by degrading the biomembranes of alginate polysaccharides (Song, Yu, Han, Han, & Li, 2003). Moreover, no activity was found when the sodium alginate was substituted by several polysaccharides such as pectin, xanthan, guar gum, arabic gum, sesbania gum, guar gum and carrageenan. This finding proves that the alginate lyase was strongly specific to the substrate of sodium alginate.

3.3.6. TLC of depolymerisation products

Fractionations of each component of the sodium alginate depolymerization products were carried out using TLC. The results on TLC plates clearly indicated that penta-, tetra-, tri- and di-alginate oligosaccharides were generated during hydrolysis reaction (Fig. 6). Trisaccharide as major degradation products along with various sizes of intermediary oligosaccharides. The commercial enzyme originated from *Flavobacterium* sp. preferred poly(α-L-guluronate) over poly(β-D-mannuronate) and degraded alginate into penta- to hepta-oligosaccharides (Sim et al., 2012). This indicates that the alginate lyase may be a useful enzyme for producing lower molecular weight alginate products than the commercial enzyme.

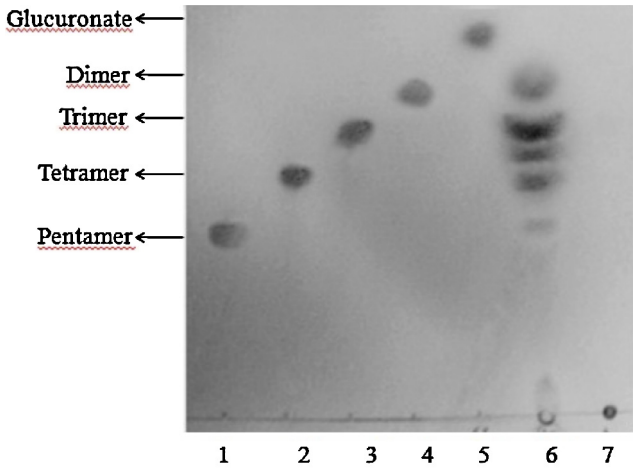

Fig. 6. TLC of the hydrolysis products of sodium alginate. Lane 1: Pentamer; Lane 2: Tetramer; Lane 3: Trime; Lane 4: Dimer; Lane 5: Glucuronate; Lane 6: Hydrolyzed sodium alginate (1.0%); Lane 7: Sodium alginate.

3.3.7. Determination of partial amino-acid sequences

The identification of homologous protein was determined by comparing short amino acid sequences using BLAST at the NCBI database (Table 2). The internal amino acids of the bifunctional enzyme from *I. halotolerans* CGMCC 5336 were AAVDGVTTGGSSYPR, which were completely identical to those of polyguluronate lyase from *Streptomyces bingchenggensis* BCW-1. Meanwhile, the molecular mass of the bifunctional enzyme (28 kDa) was close to that of polyguluronate lyase (Wang et al., 2010). However, a discrepancy in substrate specificity was found between the two lyases.

To resolve the above controversy, methods like site-directed mutagenesis and chemical-modification could be applied successfully for further studies targeting the significant amino acids responsible for the substrate specificity.

4. Conclusion

In view of present studies, the halophilic strain of *I. halotolerans* CGMCC 5336 isolated from the rotten seaweed showed a tremendous superiority in degrading alginate. Reports on alginate lyase produced by this species are lacking. The enzyme's excellent characteristics such as high specific activity (8409.19 U/mg), a broad pH range for enzyme activity, thermal stability, and specificity for poly

(M) and poly (G) open potential applications for large-scale industrialisation. The results of the present study may serve as a basis for further studies with large-scale fermentation. Meanwhile, Ca^{2+} had a distinct effect on promoting the enzyme activity and Zn^{2+} hardly served as an inhibitor. This finding indicates the potential application of alginate lyases from *I. halotolerans* as an adjuvant therapeutic agent for treating diseases associated with *Pseudomonas aeruginosa* infection. The characterisation of the bifunctional alginate lyase from *I. halotolerans* CGMCC 5336 suggests that this enzyme may produce abundant alginate oligosaccharides with bioactivities.

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