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Characterization of a new alginate lyase from newly isolated *Flavobacterium* sp. S20

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Abstract Alginate lyase is a promising biocatalyst because of its application in saccharification of alginate for the production of biochemicals and renewable biofuels. This study described the isolation of a new alginate metabolizing bacterium, *Flavobacterium* sp. S20, from sludge samples and the characterization of its alginate lyase Alg2A. The alginate lyase gene, *alg2A*, was obtained by constructing and screening the genomic library of the strain S20 and overexpressed in *Escherichia coli*. Substrate specificity assays indicated Alg2A preferred poly-α-L-guluronate as a substrate over poly-β-D-mannuronate. In the saccharification process of a high content (10 %, w/v) of sodium alginate, the recombinant alginate lyase Alg2A yielded 152 of mM the reducing sugars after 69 h of reaction, and the amounts of oligosaccharides with a different degree of polymerization (DP) generated by Alg2A gradually accumulated without significant variation in the distribution of oligosaccharide compositions. These results indicated that Alg2A possessed high enzymatic capability for saccharifying the alginate, which could be used in saccharifying the alginate biomass prior to the main fermentation process for biofuels. In addition, Alg2A had a different endolytic reaction mode from both the two commercial alginate lyases and other alginate lyases from polysaccharide lyase family 7 owing to high yields of penta-, hex-, and hepta-saccharides in the hydrolysis products of Alg2A. Thus, Alg2A could be a good tool for the large-scale preparation of alginate oligosaccharides with high DP.

Keywords *Flavobacterium* · Alginate lyase · Saccharification · Large-scale preparation · Alginate oligosaccharides

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Introduction

To solve the controversy on the sustainability of land-based fuels, biofuels produced from algal biomass have been proposed as a sustainable third-generation biofuel [30]. Although macroalgae do not generally produce lipids like microalgae, promising work was attempted to ferment laminarin and mannitol of brown seaweeds for bioethanol production [1, 12]. Recently, the utilization of alginate as a potential source of fermentable sugars for bioethanol production is also getting attention because of its abundant biomass in brown seaweeds [18]. Alginate, a structural component in brown seaweeds, is a linear polysaccharide

composed of β-D-mannuronic acid (M) and α-L-guluronic acid (G), and the two monomers that were linked together by α-/β-1,4-linkages arranged in three types of block structures: and poly-α-L-guluronate [poly (G)], poly-β-D-mannuronate [poly (M)], and heteropolymeric [poly (MG)] regions [8, 10]. However, existing ethanologenic microbes, such as *Saccharomyces cerevisiae* and *Zymomonas mobilis*, cannot directly ferment alginate into ethanol because they can neither degrade alginate nor convert the alginate-derived sugars into ethanol [31]. The realization of converting alginate into fuels requires two processes: (1) alginates are depolymerized efficiently into oligomeric or monomeric sugars; (2) metabolically engineered microorganisms assimilate alginate degradation products into biofuels [36].

Alginate lyases, a member of the polysaccharide lyases (PL), can cleave the glycosidic bond of alginate though the β-elimination mechanism, generating a saturated uronate at the new reducing end and an unsaturated 4-deoxy-L-erythro-hex-4-enopyranosyluronic acid at the new non-reducing end [37]. Owing to their substrate specificity, the enzymes are classified into poly (β-D-mannuronate) lyase (EC 4.2.2.3) and poly (α-L-guluronate) lyase (EC 4.2.2.11) [17], and they are also grouped into seven polysaccharide lyase families including PL-5, PL-6, PL-7, PL-14, PL-15, PL-17, and PL-18 in the Carbohydrate Active Enzyme (CAZy) database (http://www.cazy.org/) based on their primary structures. Microbial alginate lyases were well used in analysis of the alginate structure [27], extraction of the algal protoplasts [5], and biofilm degradation of mucoid *Pseudomonas aeruginosa* [2], while only a little attention focused on the use of alginate lyases for efficient saccharification of alginate. For example, Choi et al. [7] reported that the saccharification of alginate by means of culturing *Pseudoalteromonas agarovorans* CHO-12 with 15 g l^{-1} (w/v) sodium alginate, with a yield of 15.0 g l^{-1} (w/v) sugar. Alternatively, around 3.3 mg ml^{-1} unsaturated monosaccharides were obtained from 1 % (w/v) alginate using the recombinant exolytic oligoalginate lyase from *Sphingomonas* sp. MJ-3 [28].

However, for the saccharification process, operating degradation with high substrate concentrations will increase the product concentration, which facilitates the downstream processing and product recovery [15]. Therefore, searching for more alginate metabolizing microorganisms to evaluate the potential of new alginate lyases for efficient depolymerization of a high content of alginate would be an important prerequisite step towards fermentation of alginate with commercial yields. In this study, a new alginate lyase (Alg2A) was cloned from a newly isolated alginate metabolizing bacterium, *Flavobacterium* sp. S20. Compared to the commercial alginate lyases, the recombinant alginate lyase Alg2A had high capability for saccharifying the sodium alginate, and released high amounts of penta-, hex-, and hepta-saccharides from sodium alginate.

Materials and methods

Materials

Sodium alginate from brown algae (viscosity, 250 cP) and alginate lyase from *Sphingobacterium multivorum* were purchased from Sigma-Aldrich, St. Louis, MO, USA. Another alginate lyase was purchased from Nagase & Co., Ltd., Japan. Restriction endonucleases were purchased from Takara Biotechnology Co., Ltd., Dalian, China.

Isolation of alginate-degrading bacteria

Sludge samples that were collected from a seaweed factory in Hainan Province, China, were diluted successively with sterilized water and then spread on alginate medium plates containing (w/v) 0.7 % sodium alginate, 0.5 % $(NH_4)_2SO_4$, 0.5 % NaCl, 0.1 % KH_2PO_4, 0.05 % $MgSO_4{\cdot}7H_2O$, 0.02 % $CaCl_2$ and 0.002 % $FeSO_4{\cdot}7H_2O$ (pH 7.2). The plates were incubated at 30 °C for 36 h. The colonies were streaked out from the plates and inoculated in liquid alginate medium consisting of (w/v) 1 % sodium alginate, 0.1 % peptone, 0.1 % yeast extract, and the same mineral salts described above. After being incubated 2 days at 30 °C with shaking (150 rpm), the supernatants were collected by centrifugation at 12,000 rpm, 4 °C, for 5 min to determine the alginate lyase activity. The alginate lyase activity was measured using 0.5 % (w/v) sodium alginate according to method of dinitrosalicylic acid [24]. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of the reducing sugars (glucose equivalent) per minute under the optimal conditions.

Phylogenetic analysis of 16S rRNA gene

The genomic DNA of strain S20 was extracted by a bacterial genomic DNA extract kit (Tiangen, China). The 16S rRNA gene was amplified according to the method described by Kim et al. [16]. The evolutionary distance was estimated by the maximum composite likelihood (MCL) method and the phylogenetic tree was inferred through the neighbor-joining algorithm using MEGA version 5 [32].

Cloning and recombinant expression of the alginate lyase from *Flavobacterium* sp. S20

Flavobacterium sp. S20 genomic DNA was partially digested by *Sau*3AI and the fragments from 2 to 6 kb were

recovered and inserted into the pGEM-11Zf (+) (Promega, Madison, WI, USA). Screening for alginate lyase active clones was conducted as previously described [11]. Briefly, library clones were cultured in 96-well plates containing LB-medium supplemented with 100 μg ml^{-1} ampicillin overnight at 37 °C. The cells were collected and then ruptured by freeze–thaw for three cycles. The lysates were spotted on the 1.5 % (w/v) agar plates containing 1.5 % (w/v) sodium alginate. After incubation at 30 °C for 16 h, the plates were poured with 1.0 M $CaCl_2$ and positive clones with clearing zone were selected and sequenced. The open reading frames were analyzed by Vector NTI Suite (Invitrogen, Carlsbad, CA, USA), and homology searches were performed in GenBank using the BLAST alignment algorithm. Domain architecture analysis was carried out using the online facilities of the Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de/).

The *alg2A* gene was amplified by PCR using the primers Alg2AF (5′-CCCCATATGCAGGATAAAAAATCAAAAAGCAAAACTG-3′) and Alg2AR (5′-CCCCTCGAGATGAGTAACTTGTAAAGAATAT-3′). The amplified products were digested with *Nde*I and *Xho*I and then ligated with *Nde*I- and *Xho*I-digested pET21a (Novagen, USA). The resultant plasmid was designated as pET21a-Alg2A and transformed into *Escherichia coli* BL21 (DE3). Transformants harboring the pET21a-Alg2A were grown in LB medium supplemented with ampicillin (100 μg ml^{-1}) at 37 °C until OD_{600} reached 0.6–0.8. Expression of Alg2A was then induced with 0.1 mM isopropyl-3-D-1-thiogalactoside (IPTG) at 30 °C for 5 h. The cells were collected by centrifugation and subsequently suspended in lysis buffer (20 mM Tris–HCl, 50 mM NaCl, pH 7.4), and then subjected to sonication. Recombinant Alg2A was purified using Ni–NTA resin (Novagen, USA) according to the method described by Chang et al. [6] and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [20]. Protein concentrations were determined by the protein quantitative analysis kit (Shenergy Biocolor, China).

Characterization of the recombinant alginate lyase

The pH effect on Alg2A activity was determined by incubating the enzyme in 50 mM Tris–HCl buffer with the pH range from 6 to 10 at an interval of 0.5-pH unit at 40 °C for 10 min. The effect of temperature on enzymatic activity was determined at temperatures ranging from 20 to 70 °C for 10 min in 50 mM Tris–HCl buffer (pH 8.5).

Assays of the pH stability were performed by measuring the residual activities after the Alg2A was treated at 30 °C for 24 h in the following buffers: 50 mM phosphate-citrate (pH 4.0–5.0) and 50 mM Tris–HCl (pH 6.0–10.0). The thermostability of Alg2A was evaluated by measuring the residual enzyme activities after the enzyme was incubated in 50 mM Tris–HCl buffer (pH 8.5) at different temperatures ranging from 20 to 50 °C for 1 h.

The effects of metal ions on Alg2A were carried out by measuring the alginate lyase activities in present of 5 mM KCl, NaCl, $CaCl_2$, $MgCl_2$, $FeSO_4$, $CoCl_2$, $ZnCl_2$, $CuCl_2$, or $MnCl_2$. The activity assayed without adding metal ions (control) was defined as 100 %.

Substrate specificity of Alg2A was investigated using 0.5 % (w/v) sodium alginate (M, 77 %; G, 23 %), poly-β-D-mannuronate [poly(M); M, 97 %; G, 3 %], and poly-α-L-guluronate [poly(G); M, 23 %; G, 77 %] in 50 mM Tris–HCl buffer (pH 8.5) at 30 °C. Poly (M) and poly (G) were prepared from sodium alginate according to the method of Haug et al. [9]. Percentages of mannuronic acid and guluronic acid were estimated by the method reported previously [35]. Reactions were initiated by adding appropriate enzymes, and the amounts of new unsaturated uronic acid yielded were monitored by recording the absorbance of the reaction mixture at 235 nm [29].

Michaelis–Menten constants of Alg2A for the sodium alginate substrate were determined according to the Lineweaver–Burk method [21]. Reactions were carried out in 50 mM Tris–HCl buffer at optimal conditions for 10 min, with different substrate concentrations ranging from 1 to 8 mg ml^{-1}.

Enzymatic depolymerization of sodium alginate

Four alginate lyases, including the crude alginate lyase from Sigma (AlgS), the crude alginate lyase from Nagase (AlgN), the crude alginate lyase from *Flavobacterium* sp. S20 (AlgF) and the recombinant Alg2A were used for the depolymerization of sodium alginate (Bright Moon Seaweed Group Co. Ltd., China). AlgF was prepared as follows: strain S20 were cultured in liquid alginate medium at 37 °C for 48 h, and the cells were collected and ruptured by sonication. About 5 U of alginate lyases was diluted in 50 ml of 20 mM Tris–HCl buffer (pH 8.5 for Alg2A and AlgF, pH 6.3 for AlgN, and pH 6.0 for AlgS) supplemented with 0.01 % (w/v) NaN_3 and 40 μg ml^{-1} tetracycline. After adding 5 g of sodium alginate, enzymatic degradations were initiated and maintained at 30 °C, 220 rpm, and aliquot samples were taken at 2, 4, 8, 20, 32, 44, and 69 h to determine the amount of reducing sugars released and oligosaccharide composition by the dinitrosalicylic acid method [24]. For thin layer chromatography (TLC) assay, the samples were desalted using anion exchange resins (Lianguan Biological Chemical Co., Ltd., China) and spotted on the silica gel 60 F_{254} (Merck, Germany). The plates were subsequently developed with a solvent system of 1-butanol/formic acid/water (4:6:1, v/v). Oligosaccharide

products were visualized by heating TLC plates at 110 °C for 5 min after spraying with 10 % (v/v) sulfuric acid in ethanol [16].

To profile the oligosaccharide composition by electrospray ionization mass spectrometry (ESI–MS), hydrolysates taken at 69 h were loaded onto a microcrystalline cellulose column followed by a cation exchange column to remove proteins and salts, and then concentrated, dried, and dissolved in 1 ml of methanol. After centrifugation, 2 µl of the supernatant was loop-injected to an LTQ XL linear ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The oligosaccharides were detected in a positive-ion mode using the following settings: ion source voltage, 4.5 kV; capillary temperature, 275–300 °C; tube lens, 250 V; sheath gas, 30 arbitrary units (AU); scanning the mass range, 150–2,000 *m/z*.

Nucleotide sequence accession number

16S rDNA sequence of *Flavobacterium* sp. S20 and the nucleotide sequence of *alg2A* were submitted to the GenBank database under accession numbers JQ886462 and JF412659, respectively.

Results and discussion

Isolation of strain S20 and phylogenetic analysis

By using the alginate as the sole carbon source, ten alginate metabolizing strains were obtained. Among these strains, the one designated as S20 showed the highest productivity of alginate lyase, with 0.37 Uml^{-1} extracellular alginate lyase and 1.34 Uml^{-1} intracellular alginate lyase activity after 48-h fermentation in liquid alginate medium. Analysis of the 16S rDNA on sequence similarity revealed that the closest relatives of the strain S20 were *Flavobacterium omnivorum* AS 1.2747 (NR_025202.1) (97 %) and *Flavobacterium fryxellicola* LMG 22022 (NR_042332.1) (97 %). In the neighbor-joining phylogenetic tree, the strain S20 robustly grouped within the genus *Flavobacterium* (Fig. 1a). Consequently, the strain S20 was designated as *Flavobacterium* sp. S20 and it has been deposited at the China General Microbiological Culture Collection Center (CGMCC) under accession number 5026. In addition, the genus *Flavobacterium* has shown good ability in degrading complex polysaccharides, including alginate [3], but the alginate lyase gene from the genus *Flavobacterium* is still unidentified.

Cloning and recombinant expression of the *alg2A* gene

With the objective to clone the alginate lyase gene, the genomic library of the strain S20 was constructed and screened for alginate lyase active clones. As a result, one positive clone, which produced a hydrolysis halo, was obtained and a putative alginate lyase gene, designated as *alg2A*, was identified by sequencing. The *alg2A* gene is 867-bp in length encoding a protein of 288 amino acids with a deduced molecular mass of 32,915.46 Da and shows the highest sequence identity (71 %) with the Lacal 1753 gene, a putative alginate lyase from *Lacinutrix* sp. 5H-3-7-4 (CP002825.1). However, the amino acid sequence of Alg2A has the highest identity (62 %) with an alginate lyase from *Flavobacteriaceae* bacterium S85 (ZP_09498948.1). The domain architecture analysis grouped Alg2A into polysaccharide lyase (PL) family 7 with a predicted signal peptide from Met^{1} to Ala^{22} and an alginate lyase 2 superfamily domain from Ile^{34} to His^{288}.

Alg2A was subsequently heterologously expressed in *E. coli* BL21 (DE3) as a fusion protein with a C-terminal 6 × His tag. After 5 h of IPTG induction, the Alg2A product in recombinant *E. coli* BL21 (DE3) reached 19.6 Uml^{-1}, 11-fold increase to that of *Flavobacterium* sp. S20. Recombinant Alg2A was purified by Ni–NTA chromatography and showed a single band around 33 kDa in SDS-PAGE gel (Fig. 1b), which is consistent with the deduced molecular mass (31,420.50 Da) of the 6 × His-tagged mature enzyme. Overall, 5.74-fold purification was achieved with 58 % yield in single step of Ni–NTA chromatography, and the activity reached 365.38 Uml^{-1} (Table 1).

Biochemical properties of the recombinant Alg2A

The optimal pH of the recombinant Alg2A was pH 8.5, and the enzyme retained over 60 % activities after incubation at pHs ranging from 5.0 to 10.0 for 24 h, but lost about 80 % of its activity after incubated at pH 4.0 for 24 h (Fig. 2a, b). The optimal temperature of the recombinant Alg2A was 45 °C (Fig. 2c). Thermostability analysis indicated Alg2A was a mesophilic enzyme, and it was stable below 45 °C (Fig. 2d). The *Km* value and *Vmax* for Alg2A was 3.33 mg ml^{-1} and 1,666.7 µmol min^{-1} mg^{-1}, while the turnover number *kcat* and the catalytic efficiency constant *kcat/Km* values were 872.8 s^{-1} and 262. 1 mg^{-1} ml s^{-1}, respectively.

Previous studies indicated Na^{+} and K^{+} were important activators for some bacterial alginate lyases, and the divalent cations, particularly Ca^{2+}, Mg^{2+}, and Mn^{2+}, had enhancing effects on the alginate lyases from *Corynebacterium* sp. and *Pseudoalteromonas elyakovi* (Table 2). In case of Alg2A, K^{+} and Na^{+} exhibited the similar activation effects, with approximately 48 and 19 % increase of activity, respectively. The divalent ions, such as Fe^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, or Mn^{2+}, caused an almost complete inhibition of enzyme activity, while the same concentration of Ca^{2+} or Mg^{2+} gave about 61 % or 22 % inhibition, respectively.

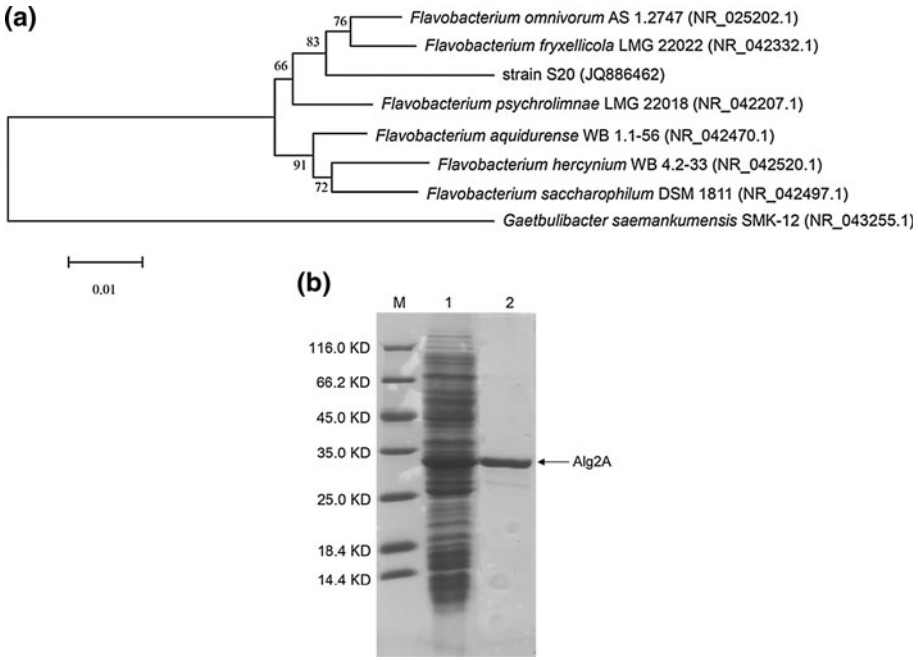

Fig. 1 The phylogenetic analysis of strain S20 and electrophoretic profile of Alg2A. **a** The phylogenetic tree. Closely related strains including *Flavobacterium omnivorum* AS 1.2747 (NR_025202.1), *Flavobacterium fryxellicola* LMG 22022 (NR_042332.1), *Flavobacterium psychrolimnae* LMG 22018 (NR_042207.1), *Flavobacterium aquidurense* WB 1.1–56 (NR_042470.1), *Flavobacterium hercynium* WB 4.2–33 (NR_042520.1), *Flavobacterium saccharophilum* DSM 1811 (NR_042497.1) and out-group strain *Gaetbulibacter saemankumensis* strain SMK-12 (NR_043255.1) were used to construct the neighbor-joining tree. The bootstrap test of the neighbor-joining tree was performed with 500 replications. The *scale bar* indicates 1 base change per 100 nucleotides. **b** Sodium dodecyl sulfate polyacrylamide gel electrophoresis of Alg2A. *Lane M*, protein marker (Fermentas, Canada); *lane 1*, *E. coli* BL21 (DE3) cell extract; *lane 2*, purified Alg2A. The *arrow* indicates the position of Alg2A

Table 1 Purification summary of recombinant Alg2A

Purification step	Total protein (mg)	Volume (ml)	Total activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
Crude enzyme	15.44	50	980	63.6	1	100
Ni–NTA chromatography	1.56	1.5	570	365.38	5.74	58

Substrate specificity of Alg2A was investigated by measuring the OD_{235} values of the unsaturated uronic acids that were generated from the oligomers via a β-elimination reaction. As shown in Fig. 3, the OD_{235} increased more rapid in the poly (G) reaction mixture than in both sodium alginate and poly (M) mixture, indicating Alg2A preferred to depolymerize poly(G) and the enzyme might be a member of poly (G) lyase (EC 4.2.2.11). The preference of the recombinant Alg2A suggested it can be used for the production of guluronate oligosaccharides from poly (G) blocks, and preparation of poly (M) blocks from sodium alginates via degrading the poly (G) and poly (MG) blocks [26].

Saccharifying alginate using alginate lyases

Since high product concentration after the saccharification of polysaccharides is preferable for the fermentation process [15], the yields of the reducing sugars released from high initial alginate concentrations by the recombinant Alg2A was assessed by comparing with that released by two commercial alginate lyases and the crude alginate lyase complex from *Flavobacterium* sp. S20. To circumvent the limitation that the thick colloids formed by a high concentration of sodium alginate limited the access of the alginate lyases to the sodium alginate substrates, the dry sodium alginate was added to the diluted alginate lyases solutions, and then the enzymes were dispersed into the colloids depending on the water absorbability of the sodium alginate. During the initial phase of hydrolysis (0–20 h), the rate of degrading sodium alginate by Alg2A was similar to that of AlgN, lower than that of AlgS, and higher than that of AlgF (Fig. 4). However, after hydrolysis for 69 h, there was no statistically significant difference in the yield of oligosaccharides released by all these alginate

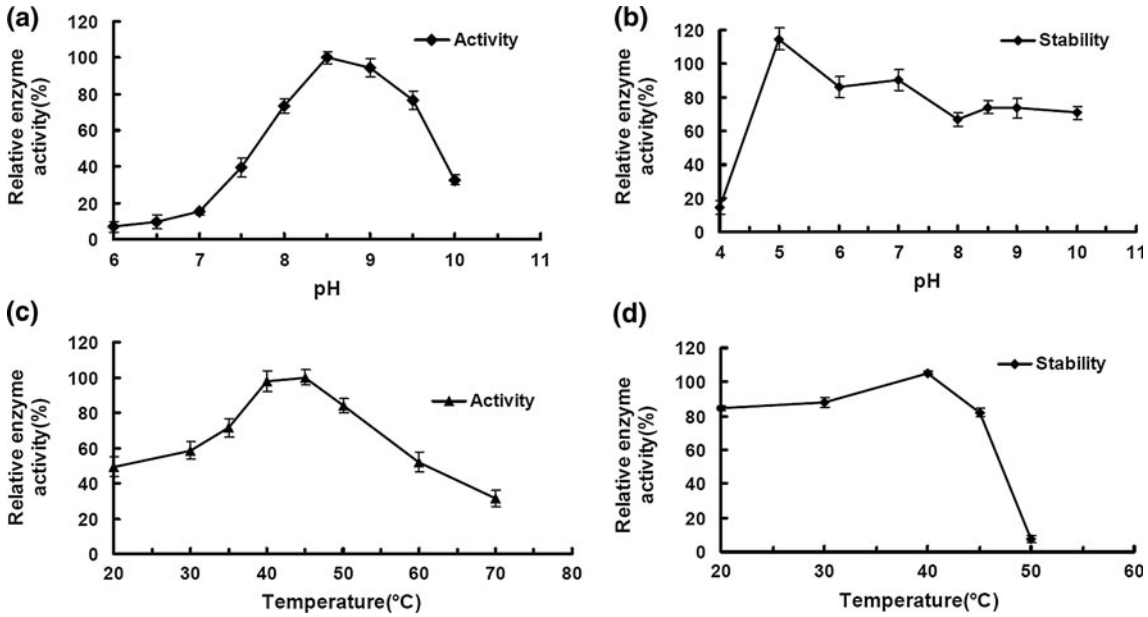

Fig. 2 Enzymatic profiles of the recombinant alginate lyase Alg2A. **a** The optimal pH of Alg2A. Reactions were conducted at 40 °C for 10 min in 50 mM Tris–HCl buffer with the pH range from 6 to 10. Activity at pH 8.5 was taken as 100 %. **b** The pH stability of Alg2A. After preincubation of the enzyme in buffers at various pHs at 30 °C for 24 h, the residual enzyme activities were measured in 50 mM Tris–HCl buffer (pH 8.5) at 45 °C. The initial activity was taken as 100 %. **c** The optimal temperature of Alg2A. Reactions were conducted for 10 min at different temperatures in 50 mM Tris–HCl (pH 8.5). Activity at 45 °C was taken as 100 %. **d** Thermal stability. After preincubation of the enzyme at different temperatures for 1 h, the remaining activity was measured in 50 mM Tris–HCl buffer (pH 8.5) at 45 °C. The initial activity was taken as 100 %. Each value represents the mean of three replicates ± standard deviation

Table 2 Comparison of the properties of Alg2A with those of some alginate lyases from different microorganisms

Enzyme	Source	Substrate specificity	End product[a]	Cations activators	Cations inhibitors	PL family	Reference
Alg2A	*Flavobacterium* sp. S20	polyG	DP 5, 6, and 7	K^+ and Na^+	Ca^{2+}, Mg^{2+}, Fe^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, and Mn^{2+}	7	In this study
alyPEEC	*Pseudoalteromonas elyakovi*	polyM and polyG	DP > 4	Na^+, K^+, Mg^{2+}, Mn^{2+}, Ba^{2+}, Ca^{2+}	Zn^{2+}, Ag^+, and Co^{2+}	18	[22]
alyPG	*Corynebacterium* sp. ALY-1	polyG	N.D.	Ca^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}	Hg^{2+}, Cu^{2+}, Fe^{2+}, Al^{3+}	7	[23]
A1-II	*Sphingomonas* sp. A1	polyG	DP2, 3 and 4	N.D.	N.D.	7	[39]
PA1167	*Pseudomonas aeruginosa* PAO1	poly(MG)	DP2, 3 and 4	N.A.	N.A.	7	[38]
Alg7D	*Saccharophagus degradans* 2–40	PolyM and polyG	DP2, 3, 4 and 5	Na^+	Ca^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+} and Mn^{2+}	7	[19]

ND not determined, *NA* not available, *DP* different degree of polymerization

[a] Sodium alginate was used as the substrate

lyases, with the approximate total reducing sugars (140–160 mM).

To analyze the oligosaccharide compositions of hydrolysis products released by different alginate lyases, samples taken at 4, 8, 20, 32, and 69 h were subjected to TLC assay. Figure 5 showed the TLC plates that represented the migration of oligosaccharides with different DP generated by with different alginate lyases after serial reaction times. As shown in Fig. 5, after 4 h of reaction, except in the case of AlgF (Fig. 5d), faint spots that represented to smaller oligosaccharides were observed in the lanes of AlgS, AlgN, and Alg2A (Fig. 5a, b, c). Moreover, the amount of low DP oligosaccharides in the AlgS digested mixture was higher than either AlgN or Alg2A as to the TLC results, which

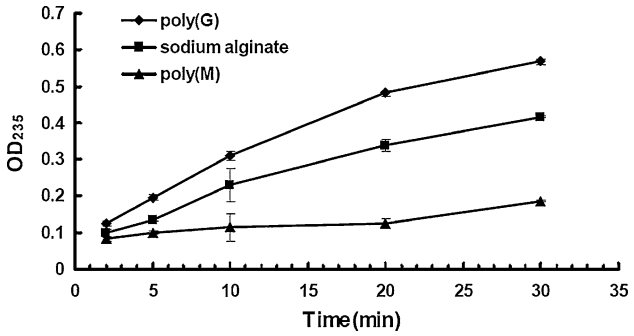

Fig. 3 Substrate specificity of the recombinant Alg2A. Reactions were conducted at 30 °C in a mixture consisting of 50 mM Tris–HCl buffer (pH 8.5), purified Alg2A, and the following various substrates (0.5 %, w/v); poly-α-L-guluronate (*closed rhombuses*), sodium alginate (*closed squares*), poly-β-D-mannuronate (*closed triangles*). The unsaturated uronic acids released were measured by recording the absorbance at 235 nm. All data are represented as the mean of three replicates ± standard deviation

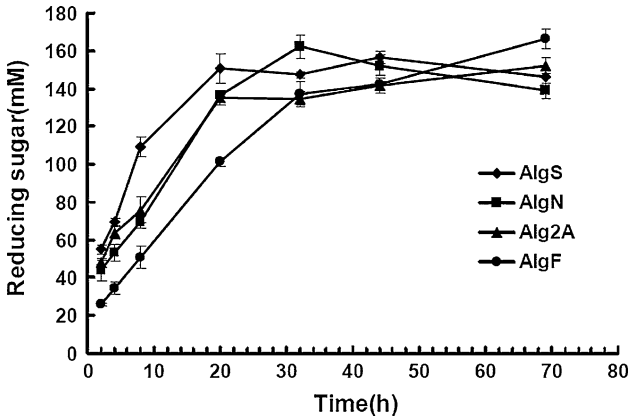

Fig. 4 Saccharification of a high content of alginate by alginate lyases. The courses of depolymerization were monitored by measuring the amount of total reducing sugars. Alginate lyase (*Sigma*) (*closed rhombuses*); alginate lyase (Nagase) (*closed squares*); Alg2A (*closed triangles*) and crude alginate lyase from strain S20 (*closed circles*). Enzymatic assays in this experiment were conducted in triplicate

were consistent with the above assay for the total reducing sugars. For the two commercial enzymes AlgS and AlgN, both disaccharide and trisaccharide gradually accumulated owing to the breakdown of high DP oligomers with prolonging the hydrolysis, and became the predominant oligosaccharides after 20 h of reaction (Fig. 5a, b). However, the hydrolysis products of both Alg2A and AlgF exhibited a distinct distribution of oligosaccharide compositions, with higher yields of unsaturated oligosaccharides with DP of 5–7 compared to either AlgS or AlgN, and the pentasaccharide was the predominant oligosaccharide after 69 h of reaction (Fig. 5c, d). Additionally, the amounts of oligosaccharides with different DP generated by Alg2A gradually accumulated without significant variation in the distribution of oligosaccharides compositions during hydrolysis process, while interestingly, in the case of AlgF, monosaccharide was found in the hydrolysates and there was a slight decrease in the amount of oligosaccharides with DP > 7 after 32 h of reaction.

To further confirm the DP of the unsaturated oligosaccharide and oligosaccharide compositions, samples taken at 69 h were also analyzed using ESI–MS. As shown in Fig. 6, under the positive mode, ion peaks that emerged at *m/z* 375, 551, 727, 903, 1,079, 1,255, 1,431, 1,607, and 1,783 over the *m/z* range of 150–2,000 represented the mono-dehydrated sodium adducts of a series from disaccharide and deca-saccharide ($[Hex_{2-10}\text{-}H_2O + Na]^+$) [25]. In the case of two commercial enzymes, the predominant ion peaks were observed at 551 and 727 *m/z*, which represent trisaccharides and tetrasaccharides (Fig. 6a, b). For recombinant Alg2A, the predominant ion peaks observed at 727, 903, and 1,079 *m/z* can be attributed to tetrasaccharide, pentasaccharide, and hexasaccharide (Fig. 6c), while in the hydrolysates produced by crude AlgF, an additional intense ion peak of trisaccharide adduct was observed as well as ion peaks of tetrasaccharide, pentasaccharide, and hexasaccharide (Fig. 6d). Interestingly, Fig. 6 showed that the ion peak of tetrasaccharide was the most intense in all cases and the intense for tetrasaccharide and hexasaccharides were the same in the case of Alg2A. However, TLC results indicated that disaccharide and trisaccharide were the predominant oligosaccharides for the enzymes AlgS and AlgN, while the pentasaccharide was the predominant oligosaccharide for the enzymes Alg2A and AlgF. Considering that the different ionization efficiencies of the constituents in a mixture and the analyte's uneven distribution in ESI droplets can affect the final ion yields [34], it was speculated that unsaturated uronic acid oligosaccharide with DP 4 was more readily ionized in the positive mode of ESI–MS than the other uronic acid oligosaccharides, resulting in the high ion yields of tetrasaccharide.

In depolymerization of a high content of sodium alginate, Alg2A generated the equal total molar amounts of oligosaccharides, but the mounts of oligosaccharides with DP of 5–10 were higher than both of the two commercial enzymes (Figs. 5, 6). When an exolytic alginate lyase was simultaneously added in saccharification process, it is possible that the cooperative actions of Alg2A and the exolytic alginate lyase would give the high yield of fermentable sugars [18, 28]. Hence, Alg2A seems to be a good candidate for efficiently saccharifying the alginate biomass in a pre-treatment process prior to the fermentation for biofuels production. Furthermore, TLC assays

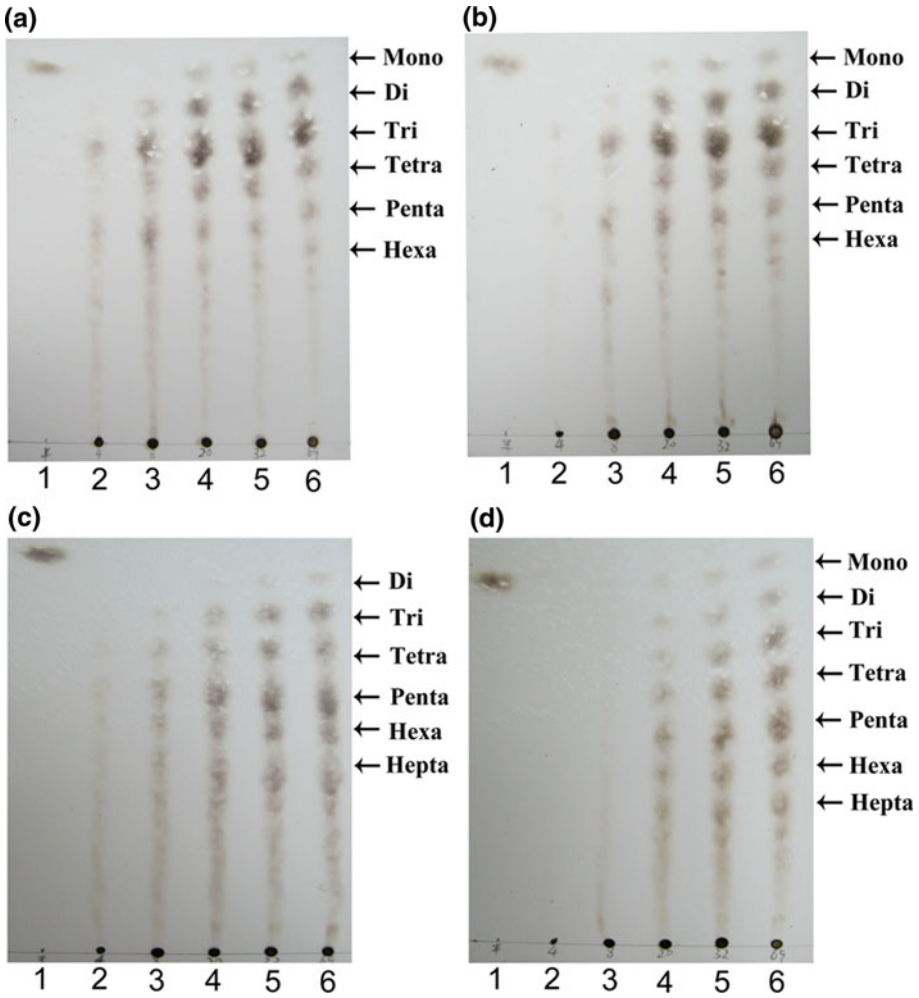

Fig. 5 TLC analysis of the oligosaccharides from the alginate lyase hydrolysates; 10 % (w/v) sodium alginate was incubated respectively with 5 U of AlgS (**a**), AlgN (**b**), Alg2A (**c**) or AlgF (**d**) at 30 °C, 220 rpm. Aliquot samples were taken at the times indicated below and then spotted on the TLC plates. *Lane 1* Galacturonic acid, *lane 2* 4 h, *lane 3* 8 h, *lane 4* 20 h, *lane 5* 32 h; *lane 6* 69 h. Mono, monosaccharide; di, disaccharide; tri, trisaccharide; tetra, tetrasaccharide; penta, pentasaccharide; hexa, hexasaccharide; hepta, heptasaccharide

showed that the crude alginate lyase from *Flavobacterium* sp. S20 degraded alginate oligosaccharides with DP > 7 and generated monosaccharides, suggesting another type of alginate lyase(s) produced by *Flavobacterium* sp. S20 were involved in the breakdown of high DP oligosaccharides. Recently, the second alginate lyase of *Flavobacterium* sp. S20 was also cloned and expressed in *E. coli*, and the properties of the enzyme are currently being assessed. These data will provide a better understanding of alginate metabolism in *Flavobacterium* sp. S20 and may be used to develop the metabolically engineered microbes for the production of biochemicals and renewable biofuels.

Oligosaccharides derived from enzymatic degradation of alginate have fascinated researchers alike owing to their biological activities and application in therapeutics and biotechnology [33], and alginate oligosaccharides with high degree of polymerization (DP) seem to show better bioactivities. Alginate oligosaccharides (DP 3–6) promoted root growth of lettuce seedlings and alginate oligosaccharides (DP 6–8) showed the maximal elicitor activity on soybean cotyledons and the antibacterial activities against *Pseudomonas aeruginosa* [4, 14]. Unsaturated alginate oligosaccharides (DP 3–9) increased tumor necrosis factor-α (TNF-α) secretion in mouse macrophage cell line RAW264.7, whereas saturated alginate oligosaccharides with same DP prepared by the chemical acid hydrolysis showed fairly less effect on the production of TNF-α [13]. In order to obtain high DP oligosaccharides, the partial hydrolysis of alginate is generally carried out via controlling the ratio of enzyme to substrate and enzymatic hydrolysis time in laboratory scale, but in this manner, the hydrolysis rate of alginate is low, and additional costs are needed for the fast inactivation of the enzymes when in a large-scale process. Interestingly, Alg2A released high amounts of penta-, hex-, and hepta-saccharides from the sodium alginate, and the amount of oligosaccharides with DP > 7 did not decrease along with the hydrolysis. This degradation product pattern was different with the previously reported endolytic alginate lyases from PL-7 family, despite their diverse substrate specificities (Table 2). Therefore, the unique endolytic reaction mode of Alg2A give

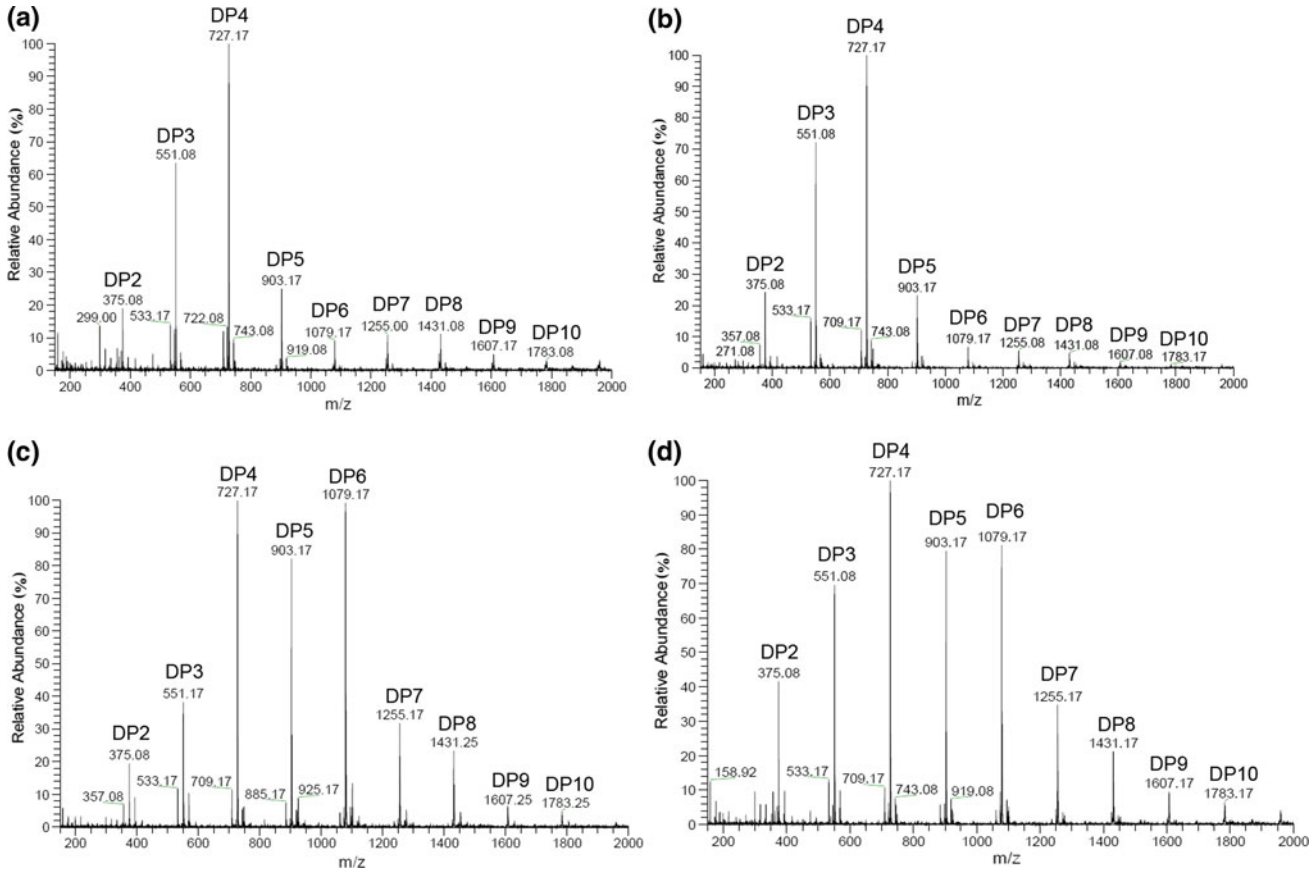

Fig. 6 ESI-MS spectrums of 69 h hydrolysates of AlgS (**a**), AlgN (**b**), Alg2A (**c**), and AlgF (**d**). DP indicates the degree of polymerization of oligosaccharides from the alginate lyase hydrolysates

it a distinct advantage, which facilitates high yields of uronic acid oligosaccharides with high DP in the large-scale process.

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