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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Characterization of a novel alginate lyase Alg0392 with organic solvent-tolerance from *Alteromonas* sp. A1-6

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Abstract

Enzymatic depolymerization of seaweed polysaccharides aroused great interest in the production of functional oligosaccharides and fermentable sugars. Alginate lyase Alg0392, a potential novel member of the polysaccharide lyase PL17 family, was cloned from *Alteromonas* sp. A1-6. The enzymatic properties, kinetic parameters, and hydrolytic products of Alg0392 were systematically characterized. Especially, the recombinant enzyme Alg0392 showed excellent tolerance to organic reagents. When treated with 5 mmol/L of TritonX-100 or 20%(v/v) of methanol, its relative enzyme activity could be maintained at more than 70%. The recombinant enzyme has a substrate preference for poly (β-D-mannuronic acid). The products of alginate hydrolysis catalyzed by Alg0392 are mainly monosaccharides, disaccharides, and trisaccharides. The products generated by the degradation of polymannuronic acid (polyM) are mainly monosaccharides. So Alg0392 is a polymannuronate cleaving enzyme. It has excellent organic solvent-tolerance and possesses both endo- and exo-glycosidase activities towards alginate. These unique properties make the recombinant enzyme Alg0392 more advantageous for the future industrial production of biofuels and the preparation of alginate oligosaccharides.

Key points

- *Alg0392 is a bifunctional alginate lyase with exolytic and endolytic cleavage activity.*
- *Alg0392 exhibits excellent organic solvent tolerance.*
- *The enzymatic hydrolysates of Alg0392 exhibit antioxidant activity.*

Keywords Alginate lyase with exolytic and endolytic cleavage activity · Heterologous expression · Enzymatic properties · Polymannuronic acid preference · Organic solvent-tolerant

Introduction

Brown algae are a highly productive and widely distributed multifunctional seaweed, which is one of the important raw materials for the production of biofuels. Alginate is an important component of the cell wall in brown algae and some red algae (Li et al. 2023a). It is a linear acidic polysaccharide which consists of α-L-guluronic acid (G) and β-D-mannuronic acid (M) linked by a 1,4-glycosidic bond in an alternating building block arrangement (Xu et al. 2018). In terms of the content of the two monosaccharides in the alginate, it can be categorized into three types: polymannuronic acid (polyM), polyguluronic acid (polyG), and hybrid (polyMG) alginate. The type of block and molecular size distribution affect the gel-forming ability and viscosity of polysaccharides (Zhang et al. 2021). The alginate with different molecular weights formed by these two monomers in different occupancy ratios and arrangements is used in different applications. Alginate and its lysates were widely used as a biomaterial in the field of bioengineering and medicine due to their favorable properties. They also were used as a thickener and emulsifier in the food industry. Especially,

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they have attracted much attention in biofuel development and biochemical uses recently (Cragg et al. 2015; Hehemann et al. 2014; Martin et al. 2014; Zhu et al. 2017). However, the properties of alginate such as its high degree of polymerization, high molecular mass, and high viscosity are often the main factors limiting its potential applications (Yang et al. 2021). Therefore, scientists often use alginate lyases to depolymerize it, obtaining smaller molecular weight hydrolysis products (alginate oligosaccharides) for application. To further promote the development of brown algae resources and develop high value-added brown algae oligosaccharide products, it is necessary to continuously explore the excellent enzyme-producing strains and gene resources of enzymes.

Alginate oligosaccharides (AOS), which are degradation products of alginate, have received increasing attention due to their low molecular weight, good water solubility, easy absorption by the organism, and good bioactivity. It has been found that oligosaccharides exist in major biomolecules, participate in different life metabolic activities, and also exhibit special biological activities that alginate does not possess, including antitumor (Han et al. 2019), antioxidant (Zhou et al. 2015), antibacterial (Tøndervik et al. 2014), antihypertensive (Terakado et al. 2012), hypoglycemic (Zhang et al. 2008), and neuroprotective effects (Eftekharzadeh et al. 2010), etc. According to the composition of glucuronides, AOS are classified as mannuronic acid oligosaccharides (MAOS), glucuronic acid oligosaccharides (GAOS), and heterozygous acid oligosaccharides (HAOS) (Lu et al. 2022). Both MAOS and GAOS are oligosaccharides composed of one type of monosaccharide molecule. AOS with different glucuronic acid compositions exhibit different activities (Huang et al. 2024). In addition, oligosaccharides with different degrees of polymerization (DP) have different functions. AOS with a DP of 3–6 induce cytokine synthesis and regulate blood glucose and lipids, while AOS with a DP of 2–10 inhibit the growth of human prostate cancer cells (Guo et al. 2024). Currently, alginate oligosaccharides are prepared by three main methods: chemical, physical degradation, and biodegradation/biocatalysis. Chemical and physical degradation methods are the traditional preparation methods, which have many disadvantages, such as high cost, poor product recovery, and easy generation of by-products. In contrast, the biodegradation/biocatalysis method, which has high catalytic efficiency, strong substrate specificity, and is more safe and environmentally friendly, has become the main method for alginate oligosaccharides preparation.

Alginate lyases are polysaccharide lytic enzymes that degrade alginate by β-eliminating the 1, 4 glycosidic bond between C4 and C5 at the nonreducing end, thus producing unsaturated alginate oligosaccharides as the main products (Sun et al. 2020). Based on their preference for different substrates, they are classified into three groups: polyguluronic acid (PolyG)-specific ligases, polymannuronic acid (PolyM)-specific ligases, and bifunctional lyases (Zhu and Yin 2015). Hundreds of alginate lyases have been categorized in the CAZy database (http://www.cazy.org/) into PL5, 6, 7, 14, 15, 17, 18, 32, 34, 36 and 39 families (Duan et al. 2009; Sawabe et al. 2001). Most of the characterized enzymes have endoglycosidase activity, and fewer exoglycosidase alginate lyases have been reported, of which most of the lysases with exoglycosidase activity that have been reported belong to the PL15 and PL17 families (Yang et al. 2021). The complete saccharification of alginate requires the joint action of both endoglycosidases and exoglycosidases (Amtzen et al. 2021). Some studies on the efficient degradation of alginate using the synergistic action of alginate lyase have been widely noticed (Badur et al. 2015). The bifunctional alginate lyase with both endo- and exo-activities is more advantageous in hydrolyzing alginate for the preparation of high-value-added alginate oligosaccharides. In this study, we predicted and cloned the alginate-degrading gene *alg0392* from an alginate-degrading lyase bacterium *Alteromonas* sp. A1-6 and carried out a more systematic study on the enzymatic properties of the recombinant enzyme. The enzyme possessed endo/exo activities at the same time and it was a kind of bifunctional lyase enzyme. This study could provide a theoretical basis for the better development of the brown algae resources. It also provides a new enzyme for the preparation of alginate oligosaccharides.

Materials and methods

Bacterial strain, plasmids, chemical reagents, and culture medium

Alteromonas sp. A1-6 (Li et al. 2023b) was originally isolated using *Ulva prolifera* polysaccharide as the only carbon source. It was also able to grow with alginate as the only carbon source. It was cultured in an artificial seawater containing 4 g/L of alginate as a carbon source. Artificial seawater comprises (per liter): 20 g NaCl, 3.6 g $MgSO_4{\cdot}7H_2O$, 3 g $MgCl_2{\cdot}6H_2O$, 1 g $(NH4)_2SO_4$, 0.5 g KCl, 0. 42 g KH_2PO_4, 0.3 g $CaCl_2{,}{\cdot}2H_2O$, 0.2 g $NaHCO_3$, 0.05 g KBr, 0.02 g $SrCl_2{\cdot}6H_2O$, and 0.01 g $(NH_4)Fe(SO_4){\cdot}6H_2O$ (Batani et al. 2019).

TransStart FastPfu DNA Polymerase was purchased from TransGen Biotech Co, Ltd. One-step seamless cloning kit was purchased from Vazyme Biotech Co., Ltd. PolyG, PolyM, alginate oligosaccharides, and monosaccharides were purchased from Qingdao BZ Oligo Biotech Co., Ltd.

All other chemicals for buffers and medium preparation were of analytical reagent grade.

Determination of growth curve and enzyme production of bacterium A1-6

The single colony of bacterium A1-6 was inoculated into 5 mL of artificial seawater medium containing 4 g/L of alginate as carbon sources. The culture was incubated at 28 °C until OD_{600} reached 0.3–0.4, and then 0.5 mL culture was inoculated into 50 mL of artificial seawater with 4 g/L of alginate. The samples were taken to measure their OD_{600} at an interval of 24 h. At the same time, the enzyme activity of alginate lyase was determined in the fermentation solution using the DNS method (Sumner and Graham 1921).

The expression level of gene alg0392 was validated by quantitative real-time PCR

The single colony of bacterium A1-6 was inoculated in 10 mL of artificial seawater medium with 2 mM of alginate or succinate (control group) as carbon sources. The inoculants were cultured on a gyratory shaker (28 °C, 160 rpm) for 6 days. RNA extraction was carried out using Trizol solution. The concentration of RNA was determined by denovix DS-11 + Ultra-Micro Spectrophotometer (USA), 1 μg of total RNA was reverse transcribed with a PrimeScript RT kit after trace amounts of residual contaminating genomic DNA was treated and eliminated with gDNA eraser (Takara, Dalian, China). The cDNA was synthesized with the extracted RNA as a template after it was monitored with the reverse transcriptase for quantitative real-time PCR. Quantitative real-time PCR was performed using the 16S rRNA gene as the internal reference gene (all primers are listed in Table 1). qRT-PCR assays were performed on Bio-Rad CFX96 Touch with a final volume of 25 μL containing 12.5 μL of 2× SYBR Green qPCR mix (ToroGreen® qPCR mix, China), 1 μL of cDNA template, and 1 μL of each primer and 9.5 μL ddH_2O. The reaction program for PCR amplification of *alg0392* (Accession number: PV021574) was as follows: denaturation at 95 °C for 3 min; 40 cycles (denaturation at 95 °C for 15 s, annealing at 56 °C for 20 s, and extension at 72 °C for 30 s); and a final extension at 72 °C for 5 min. The relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). PCR products were analyzed by agarose gel electrophoresis for non-specific amplification.

Bioinformatics analysis of alginate lyase Alg0392

The N-terminal signal peptide of Alg0392 was predicted with Signal-6.0. The theoretical isoelectric point and molecular weight of the protein were predicted using ProtParam in ExPAsy. The phylogenetic tree was constructed by the software MEGA 11 (version 4.0) using the neighbour-joining (NJ) method. The structural domains of alginate lyase Alg0392 were aligned with sequences in the NCBI Conserved Domain database for further confirmation. Alg0392 was subjected to multiple sequence analysis on DNAMan software with four alginate lyases from the PL17 family that have been fully resolved, namely AlgL17 from *Microbulbifer* sp. ALW1 (ATG71374.1), AlgSH17 from *Microbulbifer* sp. SH-1 (QIJ31368.1), Alg17 C from *Saccharophagus degradans* 2–40 (Q21 FJ0.1), and OalS17 from *Shewanella* sp. Kz7 (AHW45238.1).

Cloning of alginate lyase gene alg0392

The 2166 bp target gene without the signal peptide sequence was amplified with PCR using the genome of *Alteromonas* sp. A1-6 as a template. The PCR product was ligated into the pET28a to construct a recombinant plasmid and then transformed into *Escherichia coli* DH5α. The recombinants were verified by PCR amplification. The recombinant plasmid was transformed into *E. coli* BL21(DE3) receptor cells before DNA sequencing.

A single colony of *Alteromonas* sp. A1-6 was picked and suspended in 50 μL of sterile water, then heated at 100 °C for

Table 1 Primers used in this study

Primer name	Sequence (5′−3′)	Annealing temperature (°C)
Orf0392-F (RTFQ PCR)	AATCGCCCACAATACCCTCT	56
Orf0392-R (RTFQ PCR)	TTCACCCGCAATATGTCCAA	
Eub341 F	CCTACGGGAGGCAGCAG	56
Eub534R	ATTACCGCGGCTGCTGGC	
Orf0392-F	AGGAGATATACCATGAATAATCACCCGAACCTTATTCTC	57
Orf0392-R	TGCGGCCGCAAGCTTCTGATAATAGTACGGGCCTGAC	
pET28a-F	CATGGTATATCTCCTTCTTAAACAAAATTAT	62
pET28a-R	AAGCTTGCGGCCGCACTC	

10 min, and centrifuged at 12,000 rpm for 10 min after rapid cooling on ice. The supernatant obtained from the lysate of *Alteromonas* sp. A1-6 was used as a template for PCR amplification of the genes of interest. The PCR amplification system was as follows: 10 μL of 5× PCR Buffer, 1 μL of pF, 1 μL of pR1, 0.5 μL of fast pfu enzyme, 4 μL of dNTPs (2.5 mmol/L each), and 23.5 μL of ddH_2O. PCR amplification conditions were as follows: denaturation at 95 °C for 5 min; 30 cycles (denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 2.5 min), and a final extension at 72 °C for 8 min. The vector pET28a was linearized with PCR. The PCR amplification system was as follows: 10 μL of 5× PCR Buffer, 0.5 μL of pET28a, 1 μL of pET28a-F, 1 μL of pET28a-R, 0.5 μL of fast pfu enzyme, 4 μL of dNTPs (2.5 mmol/L each), and 33 μL of ddH_2O. PCR amplification conditions: pre-denaturation at 95 °C for 5 min, denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s, extension at 72 °C for 3 min, cycling for 30 times, and a final extension at 72 °C for 8 min. PCR amplification products of the target gene and the linearized fragments of the vector were subjected to gel electrophoresis and recovered from the gel.

Construction of recombinant expression vector

The target gene amplification product was ligated with the linearized vector using the One-Step Seamless Cloning kit according to the manufacturer's protocol. The enzyme reaction system was as follows: target gene fragment 3 μL, linear vector 2 μL, 2× One Step Cloning Mix 5 μL. The ligation mixture was incubated at 50 °C for 20 min before it was transformed into *E. coli* DH5α. The positive clones were screened with colony PCR. The DNA sequence of the cloned interest gene was determined by Sangon Biotech (Shanghai, China). The correct recombinant plasmid was designated as pET28a-*alg0392*. It was transformed into *E. coli* BL21(DE3) for the gene expression.

Induced expression and purification of recombinant enzyme Alg0392

A single colony of *E. coli* BL21 (DE3) (pET28a-*alg0392*) was inoculated in 5 mL LB medium supplemented with 50 μg/mL kanamycin and cultured on a gyratory shaker (180 rpm, 37 °C) overnight. Five milliliters of overnight culture was transferred to a 500 mL LB medium containing 50 mg/mL kanamycin. When OD_{600} of the culture reached 0.5–0.6, isopropyl-β-D-thiogalactoside (IPTG) was added at a final concentration of 0.1 mmol/L. The expression was induced for 16 h at 16 °C. The bacterial cells were collected by centrifugation at 8000 rpm. The bacteria precipitate was washed twice with PBS buffer (50 mmol/L PBS, pH 7.4), resuspended in the same buffer, and then ultrasonically crushed at 400 W for 30 min with the working/gap time of 2 s/3 s. The lysate was centrifuged to get the crude enzyme solution. The recombinant enzyme in the supernatant was purified by Ni–NTA agarose affinity chromatography. The target protein was detected and analyzed by dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Protein content was determined by Bradford's method (Inoue et al. 2001) using bovine serum albumin as the standard.

Determination of enzyme activity and enzyme kinetic parameters

The DNS method was used to determine the amount of reducing sugars generated. Two hundred microliters of 4 mg/mL alginate solution (dissolved in 50 mmoL/L Na_2HPO_4-NaH_2PO_4 buffer, pH 6.0), mixed with 1 μL of enzyme solution and incubated at 30 °C for 5 min, then DNS was added to terminate the reaction. The color was developed in a boiling water bath for 5 min and then quickly cooled to room temperature in ice water. The supernatants were taken to determine the value at OD_{540}. The inactivated enzyme solution was added to the control group, and three replicates were conducted for both the control and experimental groups during the enzyme activity assay. One enzyme activity unit was defined as the amount of enzyme required to degrade alginate to produce 1 μmol of reducing sugar per minute at 30 °C.

The enzyme activities of the recombinant enzyme Alg0392 were determined at different substrate concentrations (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5 mg/mL). The maximum reaction rate (V_{max}), the value of the Michaelis–Menten constant (K_m), the value of the catalytic constant (K_{cat}), and the value of K_{cat}/K_m were calculated for Alg0392 using Origin2021 to fit the relationship between the substrate concentration and the reaction rate with substrate concentration [S] as the horizontal coordinate and [V] as the vertical coordinate.

Analysis of enzymatic properties

The optimum pH and pH stability of Alg0392

Buffers of different pH (50 mmoL/L acetic acid-sodium acetate buffer, pH 4.0 ~ 6.0; 50 mmoL/L Na_2HPO_4-NaH_2PO_4 buffer, pH6.0 ~ 8.0; 50 mmoL/LTris-HCl buffer, pH8.0 ~ 9.0; 50 mmoL/L glycine–NaOH buffer, 9.0 ~ 10.0) were prepared. The enzyme activity of alginate lyase Alg0392 was determined at different pH according to the above enzyme

activity assay method. The relative enzyme activity of the recombinant enzyme at different pH was calculated by taking the highest enzyme activity measured as 100%.

The enzyme solution was incubated with buffers of different pH for 1 h, and then the relative enzyme activity of recombinant enzyme treated with buffers of different pH was calculated against that of the untreated enzyme solution as 100%. The pH stability of the recombinant enzyme was determined.

The optimum temperature and temperature stability of Alg0392

The enzyme activity of alginate lyase Alg0392 was determined in the temperature range of 0 ~ 90 ℃ using alginate dissolved in 50 mmoL/L Na_2HPO_4-NaH_2PO_4 buffer (pH = 6.0) as the substrate. The highest enzyme activity was taken as 100%, and the relative enzyme activity of the recombinant enzyme at different temperatures was determined.

The relative enzyme activity of the recombinant enzyme treated at various temperatures for 30 minutes was calculated by setting the activity of the untreated enzyme (control group) as 100%. The temperature stability of the recombinant enzyme was subsequently determined.

Effect of metal ions on enzyme activity

One mmol/L or five mmol/L metal ions were added into the above reaction system for the determination of enzyme activity, and the enzyme activity after 5 min reaction under the optimal reaction condition was determined. An equal volume of 50 mmoL/L Na_2HPO_4-NaH_2PO_4 buffer (pH = 6.0) substituted for metal ions solution was added to the reaction system as the control group. The relative enzyme activity of recombinant enzyme after the addition of different concentrations of ions was calculated with that of the control group as 100%.

Effect of organic reagents on enzyme activity

One mmol/L (or 5% (v/v), where the concentrations of methanol, ethanol, isopropanol, and DMSO were 5% (v/v), and the concentration of the other reagents was 1 mmol/L) and 5 mmol/L (or 20% (v/v) of organic reagents were added into the above reaction system. The enzyme activity after the reaction for 5 min under the optimal reaction conditions was determined. An equal volume of 50 mmoL/L Na_2HPO_4-NaH_2PO_4 buffer (pH = 6.0) substituted for organic reagents was added in the control group, and the relative activities of the recombinant enzyme after the addition of different concentrations of organic reagents were calculated with that of the control group as 100%.

Effect of different concentrations of NaCl on enzyme activity

NaCl was added to the enzyme activity assay system described above to achieve final concentrations ranging from 0 to 600 mmol/L. The enzyme activity measured without NaCl was set as 100%, and the effect of different concentrations of NaCl on the activity of the recombinant enzyme Alg0392 was analyzed.

Substrate specificity analysis

Four mg/mL of alginate, PolyG, and PolyM solution was prepared, respectively. The different substrates were mixed with the purified enzyme individually and reacted for 5 min under the optimal reaction conditions. The enzyme activity was measured. The preference of recombinant enzyme for different substrates was analyzed, with alginate activity defined as 100%.

Analysis of hydrolysis products of alginate lyase

Five microliters of the enzyme (30.557 ± 0.553U/mg) solution were added to 200 μL of 4 mg/mL of alginate, PolyG, and PolyM, respectively, after the catalytic reaction for different times. The supernatant was centrifuged for spot sampling. The degradation products of alginate, PolyG, and PolyM by the recombinant enzyme for 30 min or overnight were analyzed by thin layer chromatography (TLC). The spreading agent was a mixture of n-butanol: formic acid: water = 4:6:1. The silica gel plate was dried with the blower after the spreading. Ten percent ethanol sulfate was evenly sprayed on the silica gel plate, and the color development was carried out at 100 °C for 30 min. The samples were analyzed by liquid chromatography and mass spectrometry using an Agilent LC–MS system equipped with a Poroshell 120 EC-C18 column (2.1 × 150 mm, 2.7 μm). The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (acetonitrile). A gradient elution was employed with a flow rate of 0.3 mL/min as follows: 0–2 min, 95% solvent A and 5% solvent B; 2–5 min, solvent A decreased from 95 to 50%, and solvent B increased from 5 to 50%; 5–12 min, solvent A decreased from 50 to 5%, and solvent B increased from 50 to 95%; 12–15 min, solvent A returned from 5 to 95%, and solvent B decreased from 95 to 5%. The column temperature was maintained at 40 °C, and the injection volume was set to 3 μL. Mass spectrometric analysis was conducted using a SCIEX QTRAP 6500 + system in negative electrospray ionization mode (ESI-negative). The instrument parameters were configured as follows: capillary voltage, 3.0

kV; entrance potential, 10 V; declustering potential (DP), 100 V; ion source temperature, 550 °C; curtain gas flow rate, 35 L/h; ion source gas 1 and 2 flow rates, 50 L/h each; collision energy, 35 eV; and mass range (m/z), 100–1200.

Verification of the antioxidant activity of alginate oligosaccharides

The experiment utilized ultrapure water to prepare a 4 mg/mL alginate solution, which was sterilized prior to use. Subsequently, 50 µL of purified enzyme solution was added to 1 mL of the alginate solution, followed by incubation at 30 °C and 200 rpm on a shaking incubator. Samples were collected at 1 h and 24 h to determine the concentration of reducing sugars and the antioxidant activity of alginate oligosaccharides. Each experimental group was conducted in triplicate to ensure data reliability. The antioxidant activity was evaluated using the ferric-reducing ability of plasma (FRA) assay. The FRA working solution (900 µL) consisted of 750 µL TPTZ dilution solution, 75 µL TPTZ solution, and 75 µL detection buffer. The reaction system for antioxidant measurement included 900 µL of FRA working solution, 30 µL of the test sample, and 90 µL of distilled water. The mixture was thoroughly homogenized and allowed to react at room temperature for 10 min before the antioxidant activity was assessed. The amount of reducing sugars produced was quantified simultaneously using the DNS method.

Results

Growth curve and enzyme production of bacterium A1-6

As shown in Fig. 1, bacterium A1-6 was in the lag phase from 0 to 48 h in the medium with alginate as the only carbon source, with a slower growth rate and less enzyme production. In the period from 72 to 144 h, the bacterium A1-6 entered into the logarithmic phase, and the enzyme production increased gradually. The biomass reached the maximum after 168 h incubation, and the growth of the strain entered the stationary phase. The biomass and enzyme production began to decline in the period of 216 h with the depletion of the substrate. It can be inferred that bacterium A1-6 can utilize alginate to grow and produce the enzyme that can decompose alginate.

The expression level of gene alg0392

Alteromonas sp. A1-6 was originally an *Ulva prolifera* polysaccharide degrading bacteria (Li et al. 2023b). When performing genomic information data mining on *Alteromonas* sp. A1-6, we found that gene *alg0392* was probably an alginate lyase gene. In order to further verify whether this gene is an alginate degradation-related gene, we carried out a qRT-PCR experiment using cDNA as the template and the 16S

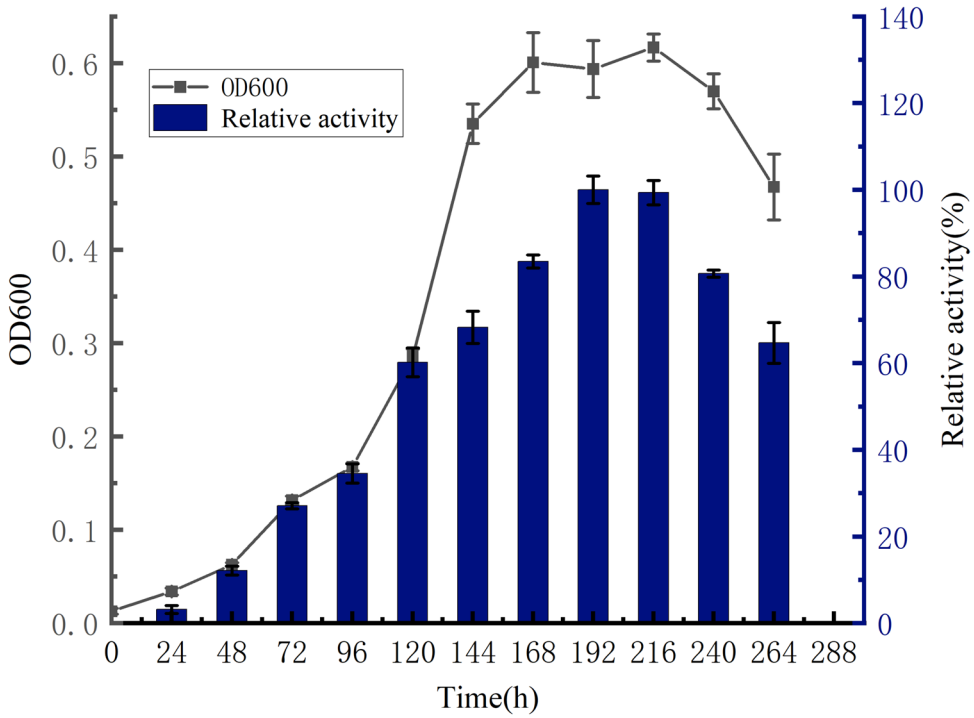

Fig. 1 Growth curve and enzyme production

rRNA gene as the internal reference. It can be seen that the expression level of gene *alg0392* was significantly increased after the induction with alginate compared with that of the succinate control group, so it was initially inferred that the gene *alg0392* was an alginate degradation-related gene.

Bioinformatics analysis of alginate lyase Alg0392

The length of gene *alg0392* is 2214 bp, encoding 737 amino acids, and there is a signal peptide consisting of 15 amino acids at the N-terminal end of Alg0392 predicted with Signal-6.0. The molecular weight of the protein is predicted to be 83 KDa, and the theoretical isoelectric point (pI) is 4.89 using ExPAsy. The sequence of Alg0392 was aligned with those of alginate cleavage enzymes that had been deposited and annotated in the CAZy database. It was found that Alg0392 shared the highest identity (37.75%) with the alginate lyase from *Saccharophagus degradans* (Accession number: Q21 FJ0.1). From the phylogenetic tree, Alg0392 belonged to the alginate lyase of the PL17 family (Fig. 2A). Multiple alignment analysis of the structural conserved domain showed that there were two structural domains in Alg0392, one is the N-terminal Alg Lyase superfamily domain and the other is the C-terminal HeparII/III domain, which is in line with alginate lyases of the reported PL17 family. Multiple sequence comparisons show that Alg0392 also has conserved sequence residues common to other alginate lyases of the PL17 family. The multiple sequence alignment of Alg0392 with alginate lyases which have available structures showed that the catalytic residues (His190, Tyr264, and Tyr456) and substrate-binding residues (Gln134, Asn137 Asn189, Tyr263, Arg266, Tyr267, His419, Arg444, and Glu670) were conserved in the Alg0392 (As shown in Fig. 2B) (Park et al. 2014; Wang et al. 2018). Therefore, it is inferred that Alg0392 belongs to the PL17 family.

Gene expression and purification of recombinant enzyme Alg0392

The sequencing results were consistent with the sequence of the target gene, which indicated that recombinant plasmid pET28a-*alg0392* was successfully constructed. The recombinant bacterium BL21 (pET28a-*alg0392*) successfully expressed the target protein Alg0392 by the induction of IPTG at low temperature. The cells of BL21 (pET28a-*alg0392*) were collected by centrifugation, crushed, and then centrifuged to obtain the crude enzyme solution. The enzyme was purified by Ni–NTA affinity chromatography. The target protein was eluted under 80 mM imidazole. The purified protein was detected by SDS-PAGE and the size of the bands matched the expected molecular weight of the target protein (Fig. 3).

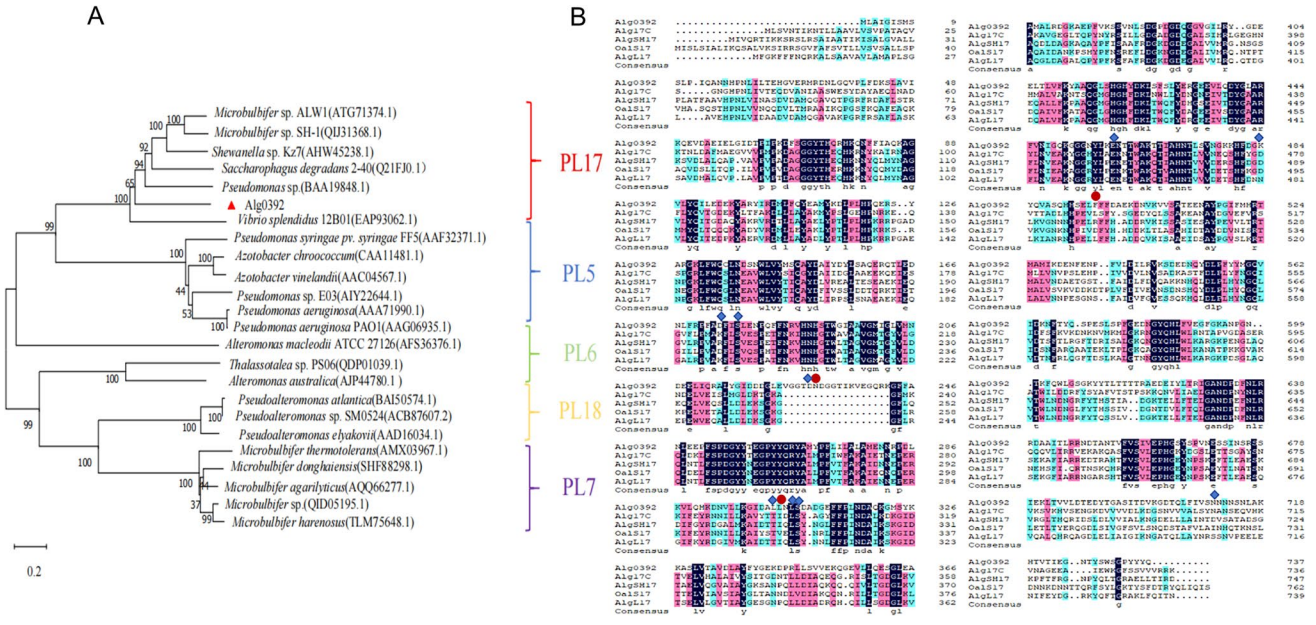

Fig. 2 Sequence and phylogenetic analysis of Alg0392. **A** The phylogenetic tree of alginate lyase Alg0392. **B** Sequence and phylogenetic analysis of Alg0392. Identical amino acid sequences are indicated in dark blue. Brick red circles (His190, Tyr264, and Tyr456) and light blue diamonds (Gln134, Asn137, Asn189, Tyr263, Arg266, Tyr267, His419, Arg444, and Glu670) indicate the proposed catalytic sites and substrate interaction sites of Alg0392, respectively

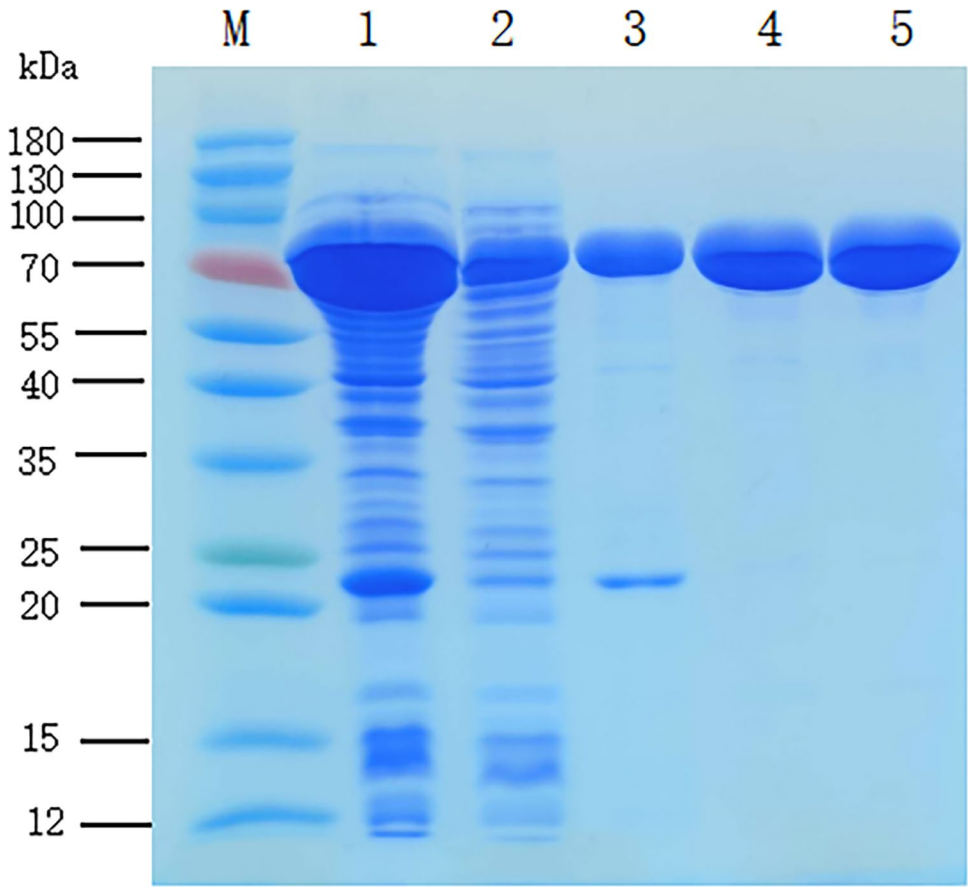

Fig. 3 SDS-PAGE of recombinant alginate lyase. M, protein marker; 1: crude enzyme solution. 2: flow-through fluid. 3: 20 mM imidazole eluting protein. 4: 40 mM imidazole eluting protein. 5: 80 mM imidazole eluting protein

Determination of enzyme kinetic parameters

The enzyme-catalyzed reaction rate was determined under optimal reaction conditions using different concentrations of alginate as substrate. The K_m and V_{max} values for the recombinant Alg0392 against alginate are 3.772 mg/mL and 0.102 μmol/min, respectively. K_{cat} was 87.775 s^{-1}. The calculated K_{cat}/K_m was 23.271 mL/mg·s.

Analysis of enzymatic properties

Optimum pH and pH stability

The enzyme activity of the recombinant enzyme Alg0392 was determined at different pH, and the results showed that the optimum pH of the recombinant enzyme Alg0392 was 6.0 (Fig. 4A). The enzyme activity of the residual recombinant enzyme was determined after incubation at different pH for 1 h, and the results showed that the recombinant enzyme activity could be maintained at more than 90% within the range of pH 6.0–9.0, and above 50% of its activity was maintained in the range of 9.0–10.0, which indicated that the enzyme had good pH stability (Fig. 4B).

Optimum temperature and temperature stability

The optimum temperature of recombinant enzyme Alg0392 was 30 °C (Fig. 4C). The residual enzyme activity of recombinant enzyme was determined after the incubation at different temperatures for 30 min. The results showed that the relative enzyme activity of recombinant enzyme could be maintained at above 90% within the temperature range of 5 ~ 30 °C, and the recombinant enzyme still had 80% relative enzyme activity under the condition of 30 ~ 40 °C; however, the enzyme activity decreased obviously when the temperature was elevated to 45 °C. The recombinant enzyme was completely inactivated after exceeding 50 °C (Fig. 4D).

Effect of metal ions on the enzyme activity

Different metal ions were added to determine their effects on the enzyme activity of the purified enzyme Alg0392. At the low concentration of 1 mmol/L, Mn^{2+}, Mg^{2+}, Ca^{2+}, and EDTA did not affect on the activity of Alg0392, and the relative enzyme activity was maintained at more than 80%. Fe^{2+}, Zn^{2+}, and Ni^{2+} had less effect on the recombinant enzyme, and the relative enzyme activity was maintained at more than 70%, while Co^{2+} and Cu^{2+} had obvious inhibitory

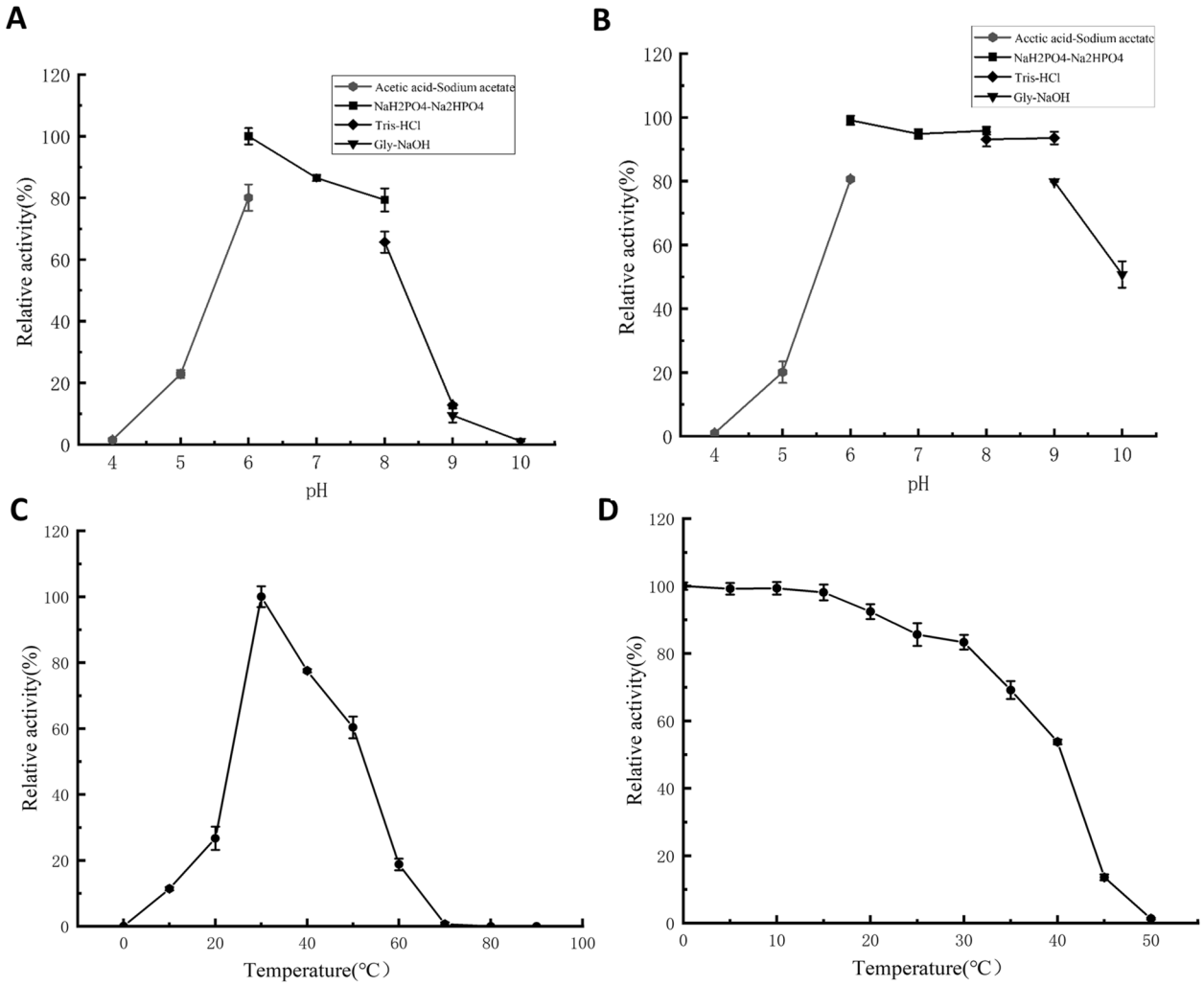

Fig. 4 **A** The optimal pH of Alg0392. **B** The pH stability of Alg0392. **C** The optimal temperature of Alg0392. **D** The temperature stability of Alg0392

effect on it. At the high concentration of 5 mmol/L, the relative enzyme activity was still maintained at about 90% with Ca^{2+}. When treated with Mg^{2+}, Ni^{2+}, Zn^{2+}, and EDTA, respectively, the relative enzyme activities were above 50%, while the recombinant enzyme was almost inactivated under the effect of Co^{2+} and Cu^{2+} (Fig. 5).

Effect of organic compounds on the enzyme activity

Different organic compounds were applied to determine their effects on the activity of Alg0392. The analysis showed that at both low and high concentrations, Dithiothreitol (DTT) and β-Mercaptoethanol (β-Met) had a more obvious stimulation effect on the enzyme activity of Alg0392, which ultimately enhanced the recombinant enzyme activity by nearly 10%. The recombinant enzyme activity was largely unaffected by treatment with 1 mmol/L guanidine hydrochloride, TritonX-100 or 5% (v/v) methanol, ethanol, isopropanol, and DMSO, and its relative enzyme activity was basically maintained at more than 70%. When treated with 5 mmol/L of TritonX-100, 20% (v/v) methanol, or DMSO, the relative enzyme activity of Alg0392 could also be maintained at more than 70%. The relative enzyme activity of Alg0392 was maintained at 51% or more than 45% in the presence of high concentrations of 20% (v/v) ethanol or isopropanol (Fig. 6).

Effect of NaCl concentration on enzyme activity

As shown in Fig. 7, the recombinant enzyme Alg0392 exhibited alginate lyase activity in the absence of NaCl, whereas the addition of low concentrations of NaCl (0–400 mM) enhanced its activity. The enzyme exhibited maximum activity in the presence of 100 mmol/L NaCl, which increased the enzyme activity by 181.26%. It is noteworthy that the

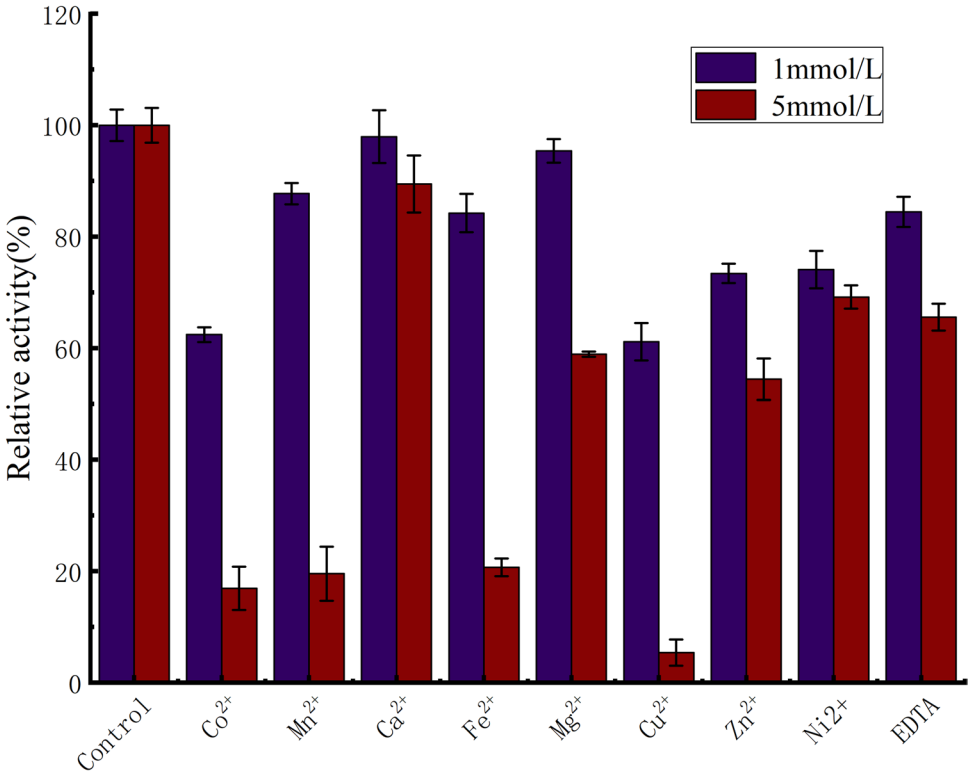

Fig. 5 Effects of metal ions on the activity of Alg0392

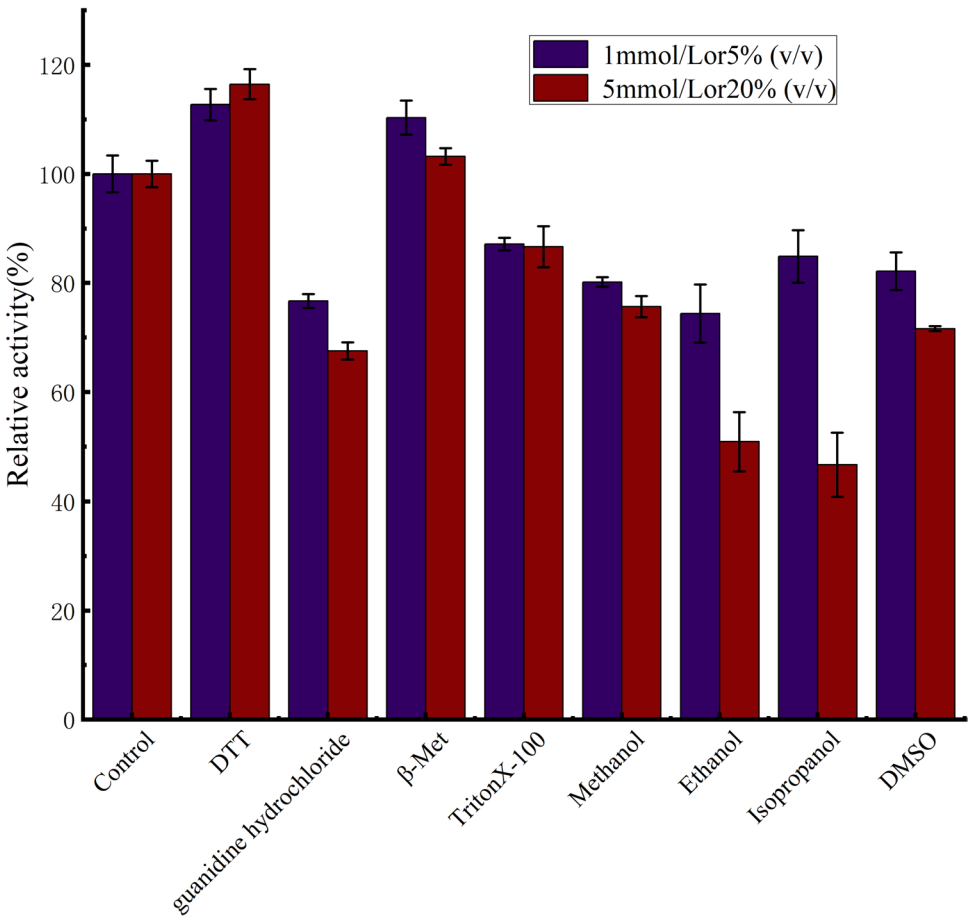

Fig. 6 Effects of organic reagents on the activity of the recombinant enzyme Alg0392. The concentration of methanol, ethanol, isopropanol, and n-butanol is in mg/mL, and the concentration of other reagents is in mmol/L

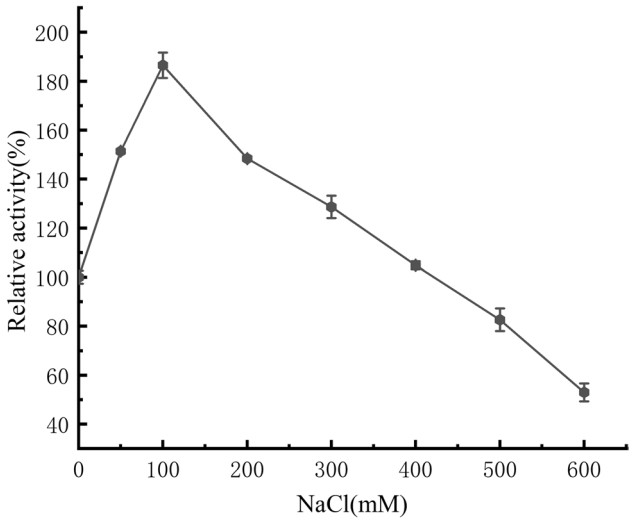

Fig. 7 Effect of NaCl on the activity of the recombinant enzyme Alg0392

recombinant enzyme activity was inhibited when the NaCl concentration was higher than 400 mM (Fig. 7).

Substrate specificity analysis

The enzyme activity of Alg0392 was determined under different substrates to investigate its substrate preference. The recombinant enzyme showed high degradation efficiency for alginate and polyM with a higher specific activity of 48.152 ± 1.175 U/mg for PolyM and 30.557 ± 0.553 U/mg for alginate, respectively, in contrast to polyG with a lower activity of 11.902 ± 0.205 U/mg, which indicated that the enzyme was a polymannuronide cleavage enzyme with specificity for PolyM.

Analysis of hydrolysis products of Alg0392

The components of hydrolysis products generated by the action of recombinant enzyme in each substrate were analyzed using thin-layer chromatography. When the reaction time was 30 min, the products of alginate generated by recombinant enzyme degradation were mainly disaccharides, trisaccharides, and some oligosaccharides with DP 4 ~ 6. While products generated by the degradation of PolyM were monosaccharides, disaccharides, and trisaccharides. When the recombinant enzyme reacted with each substrate overnight, the degradation products of alginate generated by the recombinant enzyme were mainly monosaccharides, disaccharides, and trisaccharides. All the products generated by the degradation of PolyM were converted into monosaccharides. In the experimental group where PolyG was used as a substrate, no obvious substrate was generated, which was presumed to be due to the low degradation activity of the enzyme on PolyG. In order to further determine the components of the hydrolysis products of alginate lyase, the degradation products of alginate, PolyM, and PolyG were determined and analyzed using LC–MS. As shown by the mass spectrometry results (Fig. 8), the enzyme degraded the above three substrates to produce monosaccharides and a small amount of oligosaccharide with DP2-6, with the highest amount of monosaccharide, and in addition to the production of saturated monosaccharides, there was also the production of unsaturated monosaccharides, 4-deoxy-L-erythro-5-hexoseulose uronic acid (DEH) and therefore it was inferred that Alg0392 was an exonuclease. However, at the same time, these results showed that a small amount of oligosaccharide was indeed generated, indicating that the recombinant enzyme has endonuclease activity. The recombinant enzyme is a PolyM-preferring alginate-cleaving enzyme with both endo- and exo-cleavage properties.

Enzymatic hydrolysates possess a certain level of antioxidant capacity

The concentration of reducing sugars produced after 1 h of reaction was 0.545 mg/mL, and the total antioxidant capacity of the enzymatic hydrolysate was 1.227 mmol/g. After 24 h of reaction, the concentration of reducing sugars increased to 0.674 mg/mL, and the total antioxidant capacity of the enzymatic hydrolysate reached 2.547 mmol/g. These experimental results preliminarily demonstrate that enzymatic hydrolysates possess a certain level of antioxidant capacity.

Discussion

Alteromonas sp. A1-6 was originally an *Ulva prolifera* polysaccharide degrading bacteria (Li et al. 2023b), and it was found to be able to grow with alginate as the only carbon source. Since *Ulva prolifera* polysaccharide and alginate are algae-derived polysaccharides with certain similarities, this bacterium was cultured with alginate as the only carbon source to detect its growth. As a naturally abundant polymer, alginate serves dual roles as a good biomaterial and a raw material used in the production of biofuel ethanol. The depolymerization of high-MW alginate into low-MW alginate oligosaccharides (AOS) is critical to recovering alginate for biofuel production. Alginate oligosaccharides (AOS) of different molecular weights and compositions also have various biological properties. Compared with GAOS, MAOS has the potential to protect the nervous system and to prevent and treat neurodegenerative diseases. Derivatives of MAOS also exhibit good antiviral, hypoglycemic, hypolipidemic, and immunomodulatory activities (Huang et al. 2024). Alginate lyase can hydrolyze alginate into small molecules of unsaturated alginate oligosaccharides. In this study, a novel

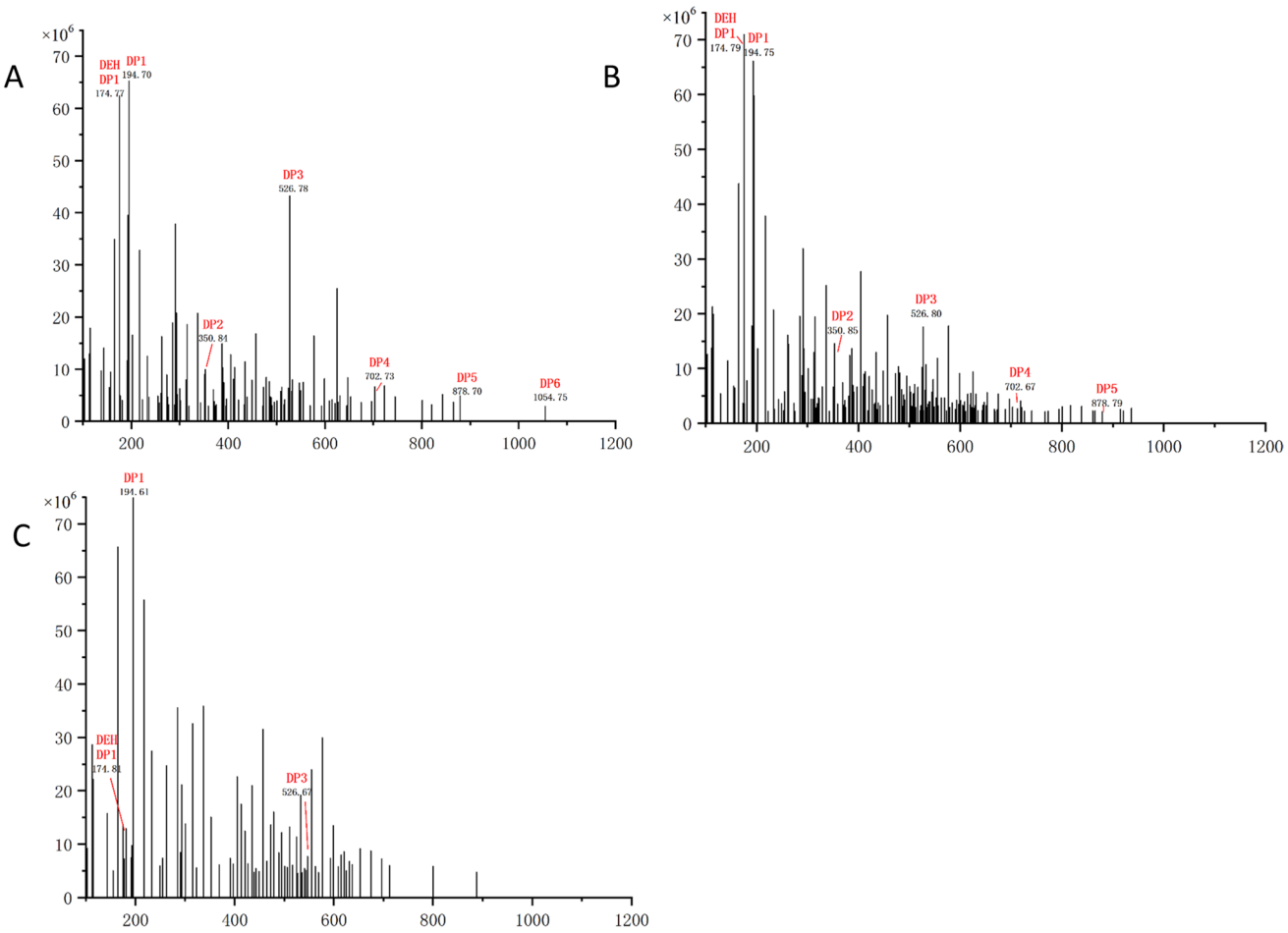

Fig. 8 **A** LC–MS analysis of hydrolysis products of alginate overnight reaction. **B** LC–MS analysis of hydrolysis products of PolyM overnight reaction. **C** LC–MS analysis of hydrolysis products of PolyG overnight reaction

alginate lyase with exolytic and endolytic cleavage activity was identified from *Alteromonas* sp. A1-6, which belongs to the PL17 family.

Previous studies indicate the PL17 family of alginate lyases are all PolyM-preferring, with low degradation activity for PolyG, such as AlgSH17 from *Microbulbifer* sp. SH-1 (Wang et al. 2021; Yang et al. 2021), and AlgL17 from *Microbulbifer* sp. ALW1 (Jiang et al. 2019). Alginate lyase with specificity for PolyM is more advantageous in the preparation of MAOS (Stender et al. 2019). It has been verified that recombinant enzyme Alg0392 is a polymannuronic acid lyase specific for PolyM by substrate specificity analysis, and its degradation ability for PolyG is lower. In the analysis experiments of the hydrolysis products of alginate lyase, it can be found that the products with different polymerization degrees can be obtained by different times of reaction, and different MAOS products can be obtained through reaction time modulation in the industrial production process. This gives it a greater potential for application in the production of MAOS and its derivatives. It also provides new possibilities for the production of natural antioxidants as neuroprotective drugs.

The growing interest in alginate oligosaccharides has driven the increasing research on alginate oligosaccharides, and more and more alginate-cleaving enzymes have been characterized. Among the reported alginate lyases, most of them are enzymes with endolytic cleavage activity. Few studies have characterized alginate lyases possessing dual exolytic/endolytic activities. The identification of exoglycosidase alginate lyases and bifunctional enzymes is still limited (Yang et al. 2021). Most of the existent enzymes with exoglycosidase activity belong to the PL15 and PL17 families. The recombinant enzyme Alg0392 was analyzed as an alginate lyase belonging to the PL17 family. At present, most of the identified PL17 family alginate lyases are exoglycosidase (exo-alginate lyase), which degrade alginate and mainly release monosaccharides. For example, OalV17 from *Vibrio* sp. SY01 (Li et al. 2020) and TcAlg1 from *T. crassostreae* (Wan et al. 2018) are exo-type alginate lyases of the PL17 family. Unlike most PL17-family alginate lyases,

recombinant enzyme Alg0392 degraded alginate and PolyM to generate end products that were mainly monosaccharides and a small amount of oligosaccharide with DP2-6, whereas the degradation of polyG produced end products that were mainly monosaccharides and a small amount of disaccharide and trisaccharide. Generally, exolytic alginate lyases could not generate oligomer intermediates, because they released monomers one by one from the end of the alginate polysaccharide chain (Chu et al. 2020; Tang et al. 2020). LC–MS analysis revealed that Alg0392 generates both monosaccharides and oligosaccharides (DP2-6), demonstrating its unique bifunctionality that possesses exolytic and endolytic cleavage activities. This property is similar to two other enzymes of the PL17 family, AlgSH17 (Yang et al. 2021) and Aly23 (Tang et al. 2022). Both enzymes have a PolyM preference. The products of hydrolysis of alginate by AlgSH17 consisted mainly of monosaccharides and a small amount of DP 2–6 oligosaccharides, and the products of degradation of alginate by Aly23 were monosaccharides, disaccharides, and trisaccharides, which are similar to those of Alg0392. The efficient degradation of alginate relies on the synergistic action of alginate lyases with exolytic and endolytic cleavage activity, which is a unique property exhibited by the recombinant enzyme Alg0392. Furthermore, the high salt environment of the ocean makes alginate lyases derived from marine bacteria usually salt-activated or salt-tolerant, so that the recombinase enzyme activity was slightly activated at low concentrations of NaCl. Analysis of the antioxidant activity of alginate hydrolysis products confirms that the products generated by Alg0392 exhibit notable antioxidant properties. This provides a solid theoretical basis for developing alginate oligosaccharides as natural antioxidants and underscores the enzyme's significant potential in alginate oligosaccharide production.

Especially, the recombinant enzyme Alg0392 has excellent tolerance to organic reagents, a property that few enzymes have been reported to have. Currently, the production process for preparing alginate oligosaccharides from brown algae often involves the use of organic reagents such as methanol, ethanol, or isopropanol during the pre-treatment stage, which can affect the enzymes used in the subsequent enzymatic hydrolysis step. Utilizing alginate lyases that are tolerant to organic solvents offers certain advantages. Therefore, the stability of enzymes in the presence of organic reagents is a crucial characteristic for their industrial applications. Additionally, when hydrolyzing alginate to produce monosaccharides for ethanol production via microbial fermentation, it is also necessary to consider the organic solvent tolerance of the alginate lyases used. The currently reported organic solvent-tolerant alginate lytic enzyme MtAl138 (Jeong et al. 2021) derived from *Microbulbifer thermotolerans* DAU221 has only 9% and 25% enzyme activity in the presence of 20% (v/v) dimethyl sulfoxide or ethanol. Whereas the recombinant enzyme Alg0392 had 76% and 51% enzyme activity in 20% (v/v) dimethyl sulfoxide, or 20% (v/v) ethanol, respectively. MtA1138 exhibits a preference for polyG and has an optimal pH of 7.0. However, its relative enzymatic activity drops below 10% when the pH is lower than 6.0 or exceeds 8.0. In contrast, Alg0392 maintains over 80% of its relative activity within the pH range of 6.0–8.0, demonstrating a broader pH stability compared to MtA1138. Although the alginate lyase MAAL1 (Yan et al. 2024), derived from *Microbulbifer* sp. QZHA1, also exhibits tolerance to organic solvents. In the presence of 20% (v/v) methanol, ethanol, or isopropanol, its relative enzymatic activity remains above 80%, demonstrating superior solvent-tolerance compared to Alg0392. However, its specific activity is relatively low, measuring only 4.30 U/mg. The alginate lyase VRALG1 (Li et al. 2024), which is derived from *Vibrio* sp. DS32, had only 50% enzyme activity remaining after incubation with 5% (v/v) isopropanol, and in isopropanol solutions under the same conditions (Table 2); however, the enzyme activity of Alg0392 was always maintained above 80%. In addition, the enzyme activity of the recombinant enzyme Alg0392 increased in both high and low concentrations of DTT and β-mercaptoethanol solutions. This series of enzymatic properties makes the recombinant enzyme Alg0392 more advantageous for the industrial preparation of alginate oligosaccharides.

Compared with the reported exolytic alginate lyases, the recombinant enzyme Alg0392 exhibits superior industrial applicability. Its uniqueness lies in its bifunctional activity, possessing both endolytic and exolytic capabilities, which enables it to efficiently degrade alginate and produce unsaturated monosaccharide DEH. DEH can be further fermented into ethanol for biofuel production, while also generating oligosaccharides with antioxidant activity. In industrial applications, the synergistic action of endolytic and exolytic enzymes is commonly employed to enhance alginate degradation efficiency. For example, Wang et al. (2014) successfully produced high-purity DEH using the endolytic enzyme Alg7D and the exolytic enzyme Alg17 C. Alg17 C (PL17 family) exhibits an optimal pH of 6.0 and retains more than 60% of its relative activity within the pH range of 5.0–7.0, but its activity drops to only 10% at pH 8.0. In contrast, Alg0392 maintains high stability across a broader pH range (6.0–8.0), consistently retaining over 80% of its activity, indicating greater adaptability. Furthermore, kinetic analysis shows that Alg17 C shows a K_m of 35.2 mg/mL and V_{max} of 41.7 U/mg, whereas Alg0392 demonstrates lower K_m (3.772 mg/mL) and higher V_{max} (0.102 μmol/min is equivalent to 73.91 U/mg) suggesting that Alg0392 exhibits higher catalytic efficiency and stronger substrate affinity, making it more suitable for efficient alginate degradation. Additionally, Santos et al. (2013) constructed a recombinant *Escherichia coli* system capable of directly utilizing brown algae for ethanol production via metabolic engineering coupled with recombinant enzymes. This system includes the endolytic enzymes AlyA, AlyB, AlyD, and AlyE, as well as the exolytic enzymes OalA, OalB, and

Table 2 Characteristics of alginate lyases

Name	Source	PL family	Substrate specificity	Organic solvent tolerance		Specific activity (U/mg)	Reference
				Organic reagent at low concentration	Organic reagent at high concentration		
Alg0392	*Alteromonas* sp. A1-6	17	polyM	Enzyme activity in different reagents (5% (v/v)): Methanol, isopropanol, and DMSO activity all above 80%, ethanol activity above 70%	1. Enzyme activity in different reagents (20% (v/v)): Methanol and DMSO activity above 70%, ethanol (51%); isopropanol (45%) 2. Enzyme activity in different reagents (5 mmol/L): DTT and β-mercaptoethanol (above 100%)	30.56	This study
MtAl138	*Microbulbifer thermotolerans* DAU221	7	polyG		1. Enzyme activity in different reagents (20% (v/v)): Ethanol (25%); DMSO (9%); isopropanol (79%) 2. Enzyme activity in different reagents (5 mmol/L): DTT (90%); β-mercaptoethanol (69%)	609.00	(Jeong et al. 2021)
VRALG1	*Vibrio* sp. DS32	7	polyG	Enzyme activity in different reagents (50 mg/mL): Ethanol (25%); isopropanol (50%); Triton X-100 (20%)	Enzyme activity in different reagents (5 mmol/L): DTT (60%)	5.87	(Li et al. 2024)
AlyE2	*Vibrio alginolyticus*	7	polyG		Enzyme activity in different reagents (20% (v/v)): Ethanol (70%); isopropanol (30%)	-	(Zheng et al. 2022)
MAAL1	*Microbulbifer* sp. QZH A1	7	polyM		Enzyme activity in different reagents (20% (v/v)): Methanol, ethanol, isopropanol, and DMSO all above 80%	4.30	(Yan et al. 2024)

OalC. Among them, OalA, OalB, and OalC (all belonging to the PL17 family) exhibit specific activities of 29.0 U/mg, 20.0 U/mg, and 21.0 U/mg, respectively, all of which are lower than that of Alg0392, further demonstrating that Alg0392 is a promising enzyme for biofuel production. However, due to various factors, alginate lyases currently applied in industrial production remain limited. Therefore, future research should focus on protein engineering to modify Alg0392, optimizing its enzyme activity, stability, and substrate specificity to enhance its potential for industrial-scale alginate degradation and biofuel production.

Author contribution Y. D. conceived and designed research. L. T., L. J., and D. M. conducted experiments. C. J., G. X., and S. F. contributed new reagents or analytical tools. L. T., G. X., and H. Z. analyzed data. Y. D. and L. T. wrote the manuscript. All authors read and approved the manuscript.

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Data availability *Alg0392* was deposited to GenBank under the accession number PV021574. All data generated or analyzed during this study are included in this manuscript.

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

References

Amtzen MO, Pedersen B, Klau LJ, Stokke R, Oftebro M, Antonsen SG, Fredriksen L, Sletta H, Aarstad OA, Aachmann FL, Horn SJ, Eijsink VGH (2021) Alginate degradation: insights obtained through characterization of a thermophilic exolytic alginate lyase. Appl Environ Microbiol 87(6):e02399-e2420. https://doi.org/10.1128/aem.02399-20

Badur AH, Jagtap SS, Yalamanchili G, Lee JK, Zhao H, Rao CV (2015) Alginate lyases from alginate-degrading *Vibrio splendidus* 12B01 are endolytic. Appl Environ Microbiol 81(5):1865–1873. https://doi.org/10.1128/aem.03460-14

Batani G, Bayer K, Böge J, Hentschel U, Thomas T (2019) Fluorescence in situ hybridization (FISH) and cell sorting of living bacteria. Sci Rep 9(1):18618. https://doi.org/10.1038/s41598-019-55049-2

Chu YJ, Kim HS, Kim MS, Lee EY, Kim HS (2020) Functional characterization of a novel oligoalginate lyase of *Stenotrophomonas maltophilia* KJ-2 using site-specific mutation reveals bifunctional mode of action, possessing both endolytic and exolytic degradation activity toward alginate in seaweed biomass. Front Mar Sci 7:420. https://doi.org/10.3389/fmars.2020.00420

Cragg SM, Beckham GT, Bruce NC, Bugg TDH, Distel DL, Dupree P, Etxabe AG, Goodell BS, Jellison J, McGeehan JE, McQueen-Mason SJ, Schnorr K, Walton PH, Watts JEM, Zimmer M (2015) Lignocellulose degradation mechanisms across the tree of life. Curr Opin Chem Biol 29:108–119. https://doi.org/10.1016/j.cbpa.2015.10.018

Duan G, Han F, Yu W (2009) Cloning, sequence analysis, and expression of gene alyPI encoding an alginate lyase from marine bacterium Pseudoalteromonas sp. CY24. Can J Microbiol 55(9):1113–1118. https://doi.org/10.1139/w09-051

Eftekharzadeh B, Khodagholi F, Abdi A, Maghsoudi N (2010) Alginate protects NT2 neurons against H_2O_2-induced neurotoxicity. Carbohyd Polym 79(4):1063–1072. https://doi.org/10.1016/j.carbpol.2009.10.040

Guo Q, Dan M, Zheng Y, Zhao G, Wang D (2024) Construction and characterization of a novel fusion alginate lyase with endolytic and exolytic cleavage activity for industrial preparation of alginate oligosaccharides. Food Chem 453:139695. https://doi.org/10.1016/j.foodchem.2024.139695

Han Y, Zhang L, Yu X, Wang S, Xu C, Yin H, Wang S (2019) Alginate oligosaccharide attenuates α2,6-sialylation modification to inhibit prostate cancer cell growth via the Hippo/YAP pathway. Cell Death Dis 10(5):374. https://doi.org/10.1038/s41419-019-1560-y

Hehemann JH, Boraston AB, Czjzek M (2014) A sweet new wave: structures and mechanisms of enzymes that digest polysaccharides from marine algae. Curr Opin Struct Biol 28:77–86. https://doi.org/10.1016/j.sbi.2014.07.009

Huang YT, Liang QP, Gao XY (2024) Research progress on preparation and functions of alginate oligosaccharides with different structures. Food Sci 45(01):313–325. https://doi.org/10.7506/spkx1002-6630-20230112-096

Inoue M, Tajima K, Mizutani M, Iwata H, Iwase T, Miura S, Hirose K, Hamajima N, Tominaga S (2001) Regular consumption of green tea and the risk of breast cancer recurrence: follow-up study from the Hospital-based Epidemiologic Research Program at Aichi Cancer Center (HERPACC). Japan Cancer Lett 167(2):175–182. https://doi.org/10.1016/s0304-3835(01)00486-4

Jeong HR, Yoo JS, Choi YL, Jang YS, Lee YS (2021) Characterization of an organic solvent-tolerant polysaccharide lyase from *Microbulbifer thermotolerans* DAU221. Int J Biol Macromol 169:452–462. https://doi.org/10.1016/j.ijbiomac.2020.12.138

Jiang Z, Guo Y, Wang X, Li H, Ni H, Li L, Xiao A, Zhu Y (2019) Molecular cloning and characterization of AlgL17, a new exo-oligoalginate lyase from *Microbulbifer sp. ALW1*. Protein Expr Purif 161:17–27. https://doi.org/10.1016/j.pep.2019.03.015

Li S, Wang L, Jung S, Lee BS, He N, Lee MS (2020) Biochemical characterization of a new oligoalginate lyase and its biotechnological application in *Laminaria japonica* degradation. Front Microbiol 11:316. https://doi.org/10.3389/fmicb.2020.00316

Li L, Zhu B, Yao Z, Jiang J (2023a) Directed preparation, structure-activity relationship and applications of alginate oligosaccharides

with specific structures: a systematic review. Food Res Int 170:112990. https://doi.org/10.1016/j.foodres.2023.112990
Li XY, Wang JH, Zhen L, Xu Z, Dai JC, Yan DZ, Chen J (2023) Screening of Ulva prolifera polysaccharide-degrading bacteria and analysis of its carbon source utilization spectrum. J Guangxi Univ Sci Technol 34(03):123–131. https://doi.org/10.16375/j.cnki.cn45-1395/t.2023.03.017
Li HM, Shao ZZ, Lu Y, Yang JK, Zhou MX (2024) Heterologous expression and enzymatic properties of the alginate lyase gene vralg1 from Vibrio sp. DS32. J Appl Oceanogr 43(02):255–265. https://doi.org/10.27776/d.cnki.gwhgy.2023.000029
Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T) Method. Methods 25(4):402–408. https://doi.org/10.1006/meth.2001.1262
Lu S, Na K, Wei J, Zhang L, Guo X (2022) Alginate oligosaccharides: the structure-function relationships and the directional preparation for application. Carbohydr Polym 284:119225. https://doi.org/10.1016/j.carbpol.2022.119225
Martin M, Portetelle D, Michel G, Vandenbol M (2014) Microorganisms living on macroalgae: diversity, interactions, and biotechnological applications. Appl Microbiol Biot 98(7):2917–2935. https://doi.org/10.1007/s00253-014-5557-2
Park D, Jagtap S, Nair SK (2014) Structure of a PL17 family alginate lyase demonstrates functional similarities among exotype depolymerases. J Biol Chem 289(12):8645–8655. https://doi.org/10.1074/jbc.M113.531111
Santos CN, Regitsky DD, Yoshikuni Y (2013) Implementation of stable and complex biological systems through recombinase-assisted genome engineering. Nat Commun 4:2503. https://doi.org/10.1038/ncomms3503
Sawabe T, Takahashi H, Ezura Y, Gacesa P (2001) Cloning, sequence analysis and expression of *Pseudoalteromonas elyakovii* IAM 14594 gene (alyPEEC) encoding the extracellular alginate lyase. Carbohydr Res 335(1):11–21. https://doi.org/10.1016/s0008-6215(01)00198-7
Stender EG, Andersen CD, Fredslund F, Holck J, Solberg A, Teze D, Peters GH, Christensen BE, Aachmann FL, Welner DH (2019) Structural and functional aspects of mannuronic acid–specific PL6 alginate lyase from the human gut microbe Bacteroides cellulosilyticus. J Biol Chem 294(47):17915–17930. https://doi.org/10.1074/jbc.RA119.010206
Sumner JB, Graham VA (1921) Dinitrosalicylic acid: a reagent for the estimation of sugar in normal and diabetic urine. J Biol Chem 47(1):5–9. https://doi.org/10.1016/S0021-9258(18)86093-8
Sun H, Gao L, Xue C, Mao X (2020) Marine-polysaccharide degrading enzymes: status and prospects. Compr Rev Food Sci Food Saf 19(6):2767–2796. https://doi.org/10.1111/1541-4337.12630
Tang L, Wang Y, Gao S, Wu H, Wang D, Yu W, Han F (2020) Biochemical characteristics and molecular mechanism of an exo-type alginate lyase VxAly7D and its use for the preparation of unsaturated monosaccharides. Biotechnol Biofuels 13:99. https://doi.org/10.1186/s13068-020-01738-4
Tang X, Jiao C, Wei Y, Zhuang XY, Xiao Q, Chen J, Chen FQ, Yang QM, Weng HF, Fang BS, Zhang YH, Xiao AF (2022) Biochemical characterization and cold-adaption mechanism of a PL-17 family alginate lyase Aly23 from marine bacterium Pseudoalteromonas sp. ASY5 and its application for oligosaccharides production. Mar Drugs 20(2):126. https://doi.org/10.3390/md20020126
Terakado S, Ueno M, Tamura Y, Toda N, Yoshinaga M, Otsuka K, Numabe A, Kawabata Y, Murota I, Sato N, Uehara Y (2012) Sodium alginate oligosaccharides attenuate hypertension and associated kidney damage in Dahl salt-sensitive rats fed a high-salt diet. Clin Exp Hypertens 34(2):99–106. https://doi.org/10.3109/10641963.2011.618196
Tøndervik A, Sletta H, Klinkenberg G, Emanuel C, Powell LC, Pritchard MF, Khan S, Craine KM, Onsøyen E, Rye PD, Wright C, Thomas DW, Hill KE (2014) Alginate oligosaccharides inhibit fungal cell growth and potentiate the activity of antifungals against *Candida* and *Aspergillus* spp. PLoS One 9(11):e112518. https://doi.org/10.1371/journal.pone.0112518
Wan J, Zhang J, Chen D, Yu B, Mao X, Zheng P, Yu J, Luo J, He J (2018) Alginate oligosaccharide-induced intestinal morphology, barrier function and epithelium apoptosis modifications have beneficial effects on the growth performance of weaned pigs. J Anim Sci Biotechnol 9:58. https://doi.org/10.1186/s40104-018-0273-x
Wang DM, Kim HT, Yun EJ, Kim DH, Park Y-C, Woo HC, Kim KH (2014) Optimal production of 4-deoxy-L-erythro-5-hexoseulose uronic acid from alginate for brown macro algae saccharification by combining endo-and exo-type alginate lyases. Bioprocess Biosyst Eng 37:2105–2111. https://doi.org/10.1007/s00449-014-1188-3
Wang D, Aarstad OA, Li J, McKee LS, Sætrom GI, Vyas A, Srivastava V, Aachmann FL, Bulone V, Hsieh YS (2018) Preparation of 4-Deoxy-L-erythro-5-hexoseulose uronic acid (DEH) and guluronic acid rich alginate using a unique exo-alginate lyase from *Thalassotalea crassostreae*. J Agric Food Chem 66(6):1435–1443. https://doi.org/10.1021/acs.jafc.7b05751
Wang B, Dong S, Li FL, Ma XQ (2021) Structural basis for the exolytic activity of polysaccharide lyase family 6 alginate lyase BcAlyPL6 from human gut microbe *Bacteroides clarus*. Biochem Biophys Res Commun 547:111–117. https://doi.org/10.1016/j.bbrc.2021.02.040
Xu F, Wang P, Zhang YZ, Chen XL (2018) Diversity of three-dimensional structures and catalytic mechanisms of alginate lyases. Appl Environ Microbiol 84(3):e02040-e2117. https://doi.org/10.1128/aem.02040-17
Yan KL, Shao ZZ, Wang WP, Xie YZ, Zhou MX (2024) Enzymatic properties of alginate lyase MAAL1 from Microbulbifer sp. QZHA1. J Appl Oceanogr 43(02):266–275. https://doi.org/10.3969/J.ISSN.2095-4972.20230224001
Yang J, Cui D, Ma S, Chen W, Chen D, Shen H (2021) Characterization of a novel PL 17 family alginate lyase with exolytic and endolytic cleavage activity from marine bacterium *Microbulbifer* sp. SH-1. Int J Biol Macromol 169:551–563. https://doi.org/10.1016/j.ijbiomac.2020.12.196
Zhang D, Fujii I, Lin C, Ito K, Guan H, Zhao J, Shinohara M, Matsukura M (2008) The stimulatory activities of polysaccharide compounds derived from algae extracts on insulin secretion in vitro. Biol Pharm Bull 31(5):921–924. https://doi.org/10.1248/bpb.31.921
Zhang L, Li X, Zhang X, Li Y, Wang L (2021) Bacterial alginate metabolism: an important pathway for bioconversion of brown algae. Biotechnol Biofuels 14(1):158. https://doi.org/10.1186/s13068-021-02007-8
Zheng Z, Zhang HX, Liu ZW, Lv QQ (2022) Enzymatic characterization of alginate lyase AlyE2. Period Ocean Univ China 52(12):44–50. https://doi.org/10.16441/j.cnki.hdxb.20210333
Zhou R, Shi XY, Bi DC, Fang WS, Wei GB, Xu X (2015) Alginate-derived oligosaccharide inhibits neuroinflammation and promotes microglial phagocytosis of β-amyloid. Mar Drugs 13(9):5828–5846. https://doi.org/10.3390/md13095828
Zhu BW, Yin H (2015) Alginate lyase: review of major sources and classification, properties, structure-function analysis and applications. Bioengineered 6(3):125–131. https://doi.org/10.1080/21655979.2015.1030543
Zhu YT, Thomas F, Larocque R, Li N, Duffieux D, Cladière L, Souchaud F, Michel G, McBride MJ (2017) Genetic analyses unravel the crucial role of a horizontally acquired alginate lyase for brown algal biomass degradation by *Zobellia galactanivorans*. Environ Microbiol 19(6):2164–2181. https://doi.org/10.1111/1462-2920.13699