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Preparation of Alginate Oligosaccharides from *Laminaria japonica* Biomass by a Novel Biofunctional Alginate Lyase with pH and Salt Tolerance

Lei Song [1], Yan-Feng Guo [1], Yi-Lei Wang [1], Chuan-Bao Wang [1], Jing-Hua Liu [1], Guo-Qing Meng [1,*] and Zhi-Peng Wang [2,*]

[1] College of Agriculture and Bioengineering, Heze University, Heze 274015, China; songlei@hezeu.edu.cn (L.S.); guoyanfeng@hezeu.edu.cn (Y.-F.G.); wangyi-lei@hezeu.edu.cn (Y.-L.W.); wangchuanbao@hezeu.edu.cn (C.-B.W.); liujinghua@hezeu.edu.cn (J.-H.L.)
[2] School of Marine Science and Engineering, Qingdao Agricultural University, Qingdao 266109, China
* Correspondence: mengguoqing@hezeu.edu.cn (G.-Q.M.); wangzp@qau.edu.cn (Z.-P.W.)

Abstract: Enzymatic alginate oligosaccharides were proved with various biological activities. Developing robust alginate lyase with high production is essential for its industrial application. In this study, a novel alginate lyase gene, AL07, was successfully screened and expressed in Pichia pastoris, and its characteristics were characterized. The secreted alginate lyase has a molecular weight of approximately 40.0 kDa and an activity of 45.4 U/mL. AL07 exhibits superior biological activity at pH 7.0 and 40 °C, with a preference for polyG substrates. Notably, the enzyme exhibits more than 60% relative activity over a pH range of 5.0 to 10.0. AL07 is also independent of ions and exhibits salt tolerance properties. The main degradation product of AL07 is DP2 oligosaccharide. Using AL07-based enzyme cocktail, *Laminaria japonica* can be degraded within 120 min. Therefore, AL07 has been demonstrated to be an efficient tool for the preparation of alginate oligosaccharides and the degradation of *L. japonica*.

Keywords: alginate lyase; pH stability; NaCl-independent; alginate oligosaccharides; *Laminaria japonica*

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1. Introduction

Alginate is a naturally occurring anionic polysaccharide, consisting mainly of two distinct uronic acids, including α-L-gulonuronic (G) and β-D-mannosyluronic acid (M), which are linked via 1,4-glucoside bonds to form three types of blocks: homomeric M block, homomeric G block, and heteromeric MG block [1]. Typically, alginate is extracted from brown algae, the dominant structure of the cell wall polymer, which accounts for approximately 20% of the algae dry weight (*w*/*w*) [2]. Due to the favorable gelation, chelate alloy properties, thickening and stability, it has been extensive applied in many fields, primarily in the printing, food, chemical, textile, and medical industries [3–5]. Furthermore, as a linear heteropolymer, alginate could be hydrolyzed into alginate oligosaccharides (AOS) by alginate lyases through β-elimination to cleave the glycosidic bond [6]. Owing to its significant role in several important biological processes, such as antioxidant activity, anti-inflammatory activity, prebiotic, and antibacterial activity, AOS has gained considerable attention, and is widely applied in the agriculture, food, and medicine fields [7]. For example, with a low M/G ratio and polymerization degree, AOS can induce cytokine synthesis, and significantly inhibit the pancreatic lipase, as well as the growth of prostate cancer cells [8–10]. As the principal component in propylene glycol alginate sodium sulfate, it is helpful for the prevention of ischemic cardio-cerebrovascular diseases and HPL (hyperlipidemia) [11]. In agriculture, it plays a positive role in promoting lettuce seedling growth [12]. Therefore, seeking an efficient way to produce alginate oligosaccharides is of considerable significance.

Thus far, alginate lyases have been identified from many sources, including fungi, marine bacteria, viruses, marine mollusks, algae, etc. [13]. According to the CAZY database, alginate lyases are classified into polysaccharide lyase (PL) families by analysis of their similarity sequences [14]. Furthermore, depending on substrate specificities, alginate lyases can be distributed into three types: poly G lyases (EC 4.2.2.11), poly M lyases, (EC 4.2.2.3), poly MG lyases (EC 4.2.2.) [15]. PL5, PL14, and PL17 are poly M lyases; PL7, PL15, and PL18 show poly M and poly G specificity; only PL6 is a poly G lyase.

The yield of alginate lyase from the traditional channels is low. To reduce the cost of alginate lyase and AOS, heterologous expression is the most effective method to increase the yield of enzyme. Many alginate lyases isolated from *Vibrio*, *Thalassomonas*, *Alteromonas*, *Gilvimarinus agarilyticus*, *Pseudoalteromona*, *Flavobacterium*, *Bacillus*, and *Nitratirupto* have been successfully expressed in several hosts [16–21]. In addition, recent reports on the successful heterologous expression of alginate lyase continue to emerge. AlyC8 from marine alginate degrading strain *Vibrio* sp. C42 was successfully expressed in *E. coli* [22]. The alginate lyase Aly01 of PL7 family from *Vibrio albicans* SK42.001 has been extracellularly expressed in *E. coli* [23]. Based on the simplicity of purification, the alginate lyase genes also have been expressed in a yeast host. With the extracellular location of the recombinant enzyme, the application of the recombinant alginate lyase has been enhanced.

Laminaria japonica is the most widely cultivated commercial edible brown seaweed around the world, especially in East Asia. Alginate accounts for approximately 22–44% of the dry cells weight of *L. japonica*. In China, *L. japonica* is the main source for alginate production. *L. japonica* was also developed as an important source for preparing both AOS and an AOS based biostimulant. In this study, a novel alginate lyase gene AL07 has been successfully cloned, and then expressed as well as characterized in *P. pastoris*. The activity of recombinant AL07 achieved 45.4 U/mL, with an Mw of approximately 40 kDa. The temperature and pH properties were also investigated. AL07 showed ions-independent and salt tolerance. AL07 based enzyme cocktail can efficiently hydrolyze alginate in *L. japonica*.

2. Results and Discussion

2.1. Sequence Analysis

The alginate lyase encoding gene, AL07, oriented from *Vibrio* sp. has been cloned, in addition to having been obtained by an analysis of its genomic sequence. The putative gene possesses an open reading frame (ORF) with a length of 1071 bp, and contains 356 amino acid residues. Further analysis by a bioinformatics method indicates that the isoelectric point (pI) and theoretical molecular weight (Mw) of AL07 are 6.05 and 40.3 kD, respectively. According to the signal peptide analysis by SignalP 5.0 and searching in NCBI CDD, AL07 is a novel member of polysaccharide lyase (PL) family 7, containing a 26-residue signal peptide.

In order to determine the attribution of a recombinant enzyme, the phylogenetic trees were created based on the sequences of reported alginate lyases and AL07. Figure 1 clearly shows that AL07 is assembled in the same branch with reported alginate lyases from *Vibrio* sp. (Alyw201), and apparently belongs to the PL7 family [24]. For further verification of conserved amino acids, AL07 and seven reported distinctive PL7 family alginate lyases were analyzed by multiple sequence alignment. The seven representative enzymes contain alginate lyases from *Vibrio* sp. (accession number: ASA33935.1); *Vibrio halioticoli*. AlyVGI (Accession number: AAF22512.1); *Pseudoalteromonas* sp. CY24 (accession number: ACM89454.1); *Vibrio* sp. W2 (accession number: Alwy201); *Agarivorans* sp. L11 (accession number: AJO61885.1); *Klebsiella pneumoniae* (accession number: AAA25049.1); and *Saccharophagus degradans* (accession number: ABD81807.1) [25–28]. As the results show in Figure 2, AL07 contains "RTELREMLR", "QIH", and "MYFKAG" conserved regions, which typically belong to the PL7 family. The conserved regions have been verified as being of value in the substrate preferences of alginate lyases [29]. Commonly, the "QIH" motif shows preferences for polyG, while the QVH motif is more prone to degrade polyM

blocks [30–32]. Thus, AL07 alginate lyase containing the "QIH" conservative region shows a preference for the polyG block.

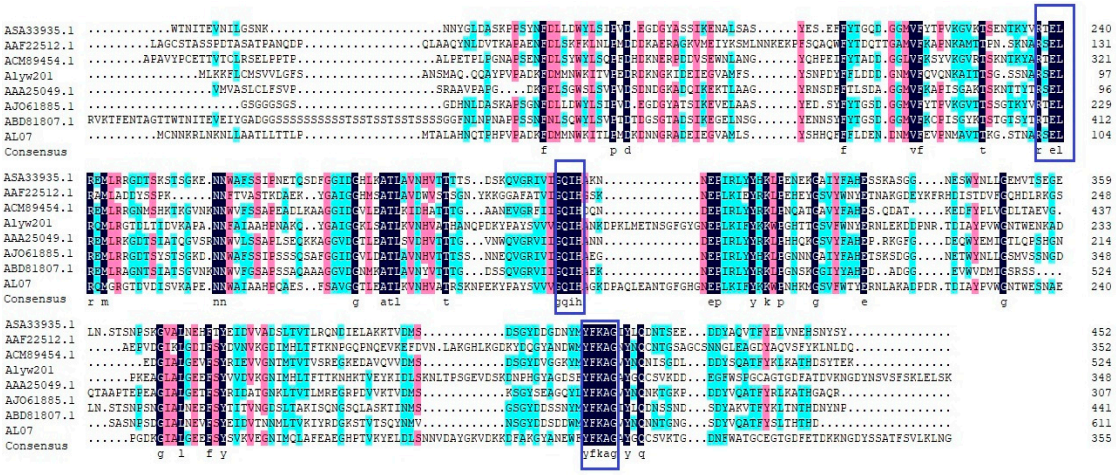

Figure 1. Sequence comparative analysis of AL07 with seven relevant alginate lyases. The conserved amino acid regions are listed in the blue block.

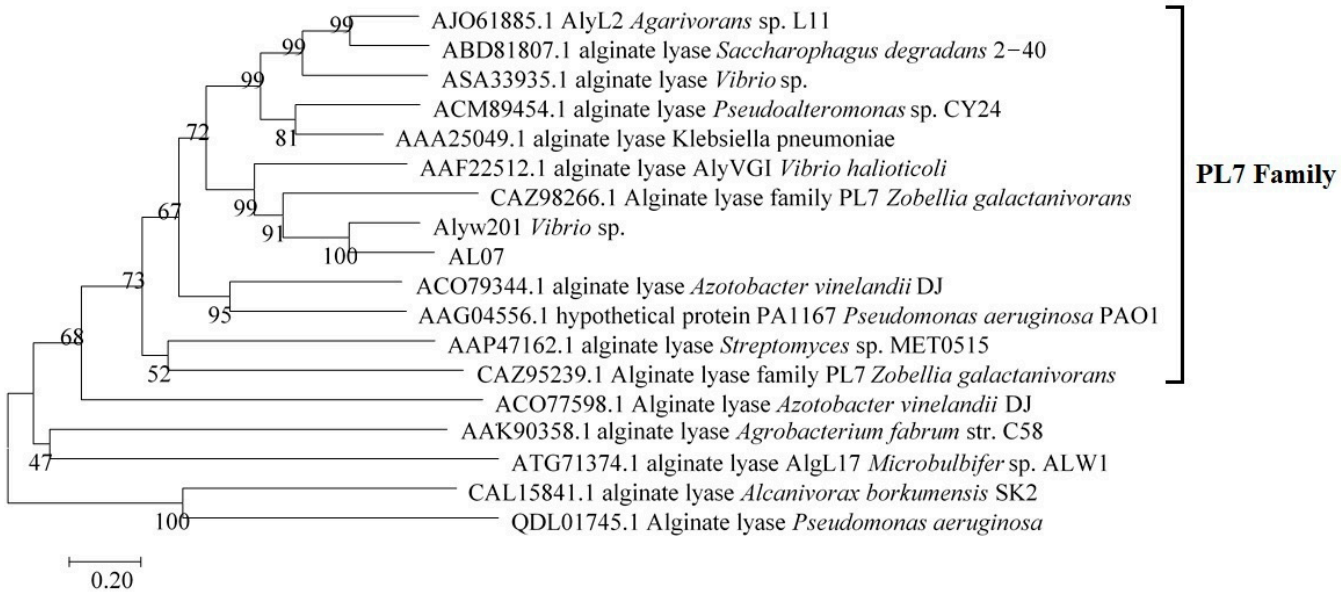

Figure 2. The phylogenetic tree, constructed according to the sequences of AL07 and other reported alginate lyases.

2.2. Expression and Purification of AL07

In most of the previous studies, alginate lyase coding genes were expressed in *E. coli*. However, this technology has many shortcomings, such as weak secretion capacity, generation of pyrogen and endotoxin [33]. In present study, the gene of AL07 has been successfully recombined and expressed in a secured heterologous host with highly extracellular secretion ability, *P. pastoris* [29,34]. After incubation in BMMY culture medium for 120 h with methanol induction, the alginate lyases activity is determined as 45.4 U/mL; much higher than that of the wild *Vibrio* strain. As shown in Table 1, several other alginate lyases are also successfully expressed in yeast hosts. The activity of AL07 is obviously higher than that of cAlyM, which is also expressed in *P. pastoris*, but lower than that of rSAGL. With the same promoter from the plasmid pPIC9K and the same host strain, the differences of the activities were attributed to the characteristics of each alginate lyase protein. It is also demonstrated that some alginate lyases are expressed in *Yarrowia lipolytica* host with high

activities. To gain a higher activity of AL07, the gene can be expressed in *Y. lipolytica* in the follow-up study. It is difficult to directly compare the specific activity of AL07 with many reported alginate lyase, owing to the differences in the definition of enzyme activity units. Compared with alginate lyase with the same enzyme activity unit definition, the specific activity of AL07 is significantly superior to that of many reported alginate lyases.

Table 1. Comparison of the activities of recombinant alginate lyases in *P. pastoris* and *Y. lipolytica*.

Name	Source	Production (U/mL)	Hosts	Reference
cAlyM	*Microbulbifer* sp.	33.82	*P. pastoris*	[35]
rSAGL	*Flavobacterium* sp.	93.5	*P. pastoris*	[36]
AlyL1	*Agarivorans* sp.	8.7	*Y. lipolytica*	[37]
Alyw201	*Vibrio* sp.	64.2	*Y. lipolytica*	[24]
Alyw202	*Vibrio* sp.	102.4	*Y. lipolytica*	[38]
AlyS02	*Flavobacterium* sp.	36.8	*Y. lipolytica*	[39]
AL07	*Vibrio* sp.	45.4	*P. pastoris*	This study

The recombinant protein is separated and purified using Ni-NTA column chromatography, and further analyzed via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Figure 3a, a clear band appeared on the lane indicating that the Mw of AL07 is nearly 40 kD, which corresponds with the theoretical Mw (40.3 kDa). The specific activity of AL07 is determined as 256.2 U/mg. The enzyme activity of AL07 toward polyG, polyM and sodium alginate show the enzyme a bifunctional alginate lyase. As shown in Figure 3b, the relative activities of AL07 are higher for polyG (156.4%) than that of sodium alginate (100%) and polyM (76.34%). Therefore, AL07 can be considered a novel alginate lyase that is more particular to polyG. The conserved amino acids "QIH" make alginate lyases, including AL07, prefer polyG as their substrate. Alginate lyase AlgNJ-04 also has the same "QIH". The Km values of AlgNJ-04 for sodium alginate and polyG were lower than that of polyM, showing a polyG preference [32].

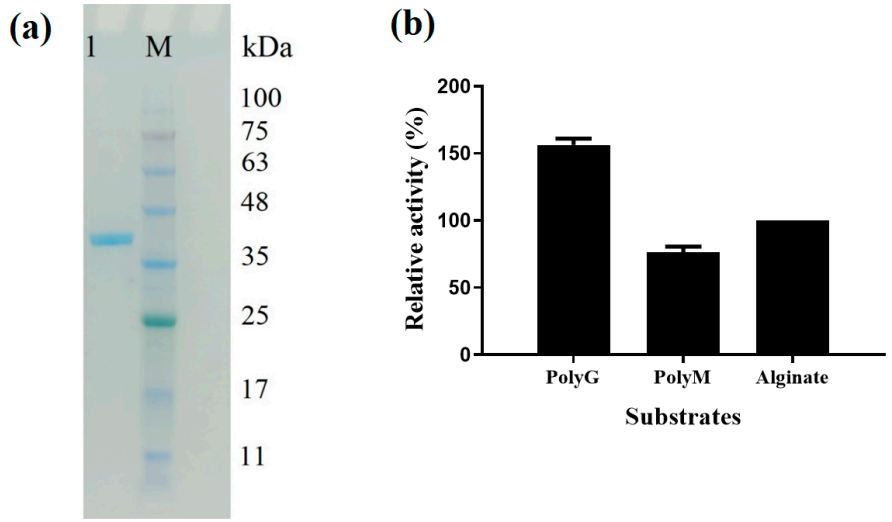

Figure 3. (**a**) Analysis of AL07 by SDS-PAGE. Lane M, prestained protein ladder; lane 1, purified AL07. (**b**) Substrate specificity analysis of AL07.

As two typical cases, the specific activities of AlyH1 and Aly7B-Wf from *Vibrio furnisii* H1 and *Wenyingzhuangia fucanilytica* are merely 2.40 and 23.24 U/mg, respectively [40,41]. The specific activity of cAylM expressed in *E. coli* is as high as about 1300 U/mg, which is significantly superior to that expressed in *P. pastoris*, and may be due to the glycosylation modification of enzyme [42]. Interestingly, some specific alginate lyases with strict or special substrate specificity are conducive to the analysis of substrate sequence and the

directed production of alginate oligosaccharides. It is more notable that alginate lyases with strict substrate specificity can be applied as an outstanding collaborative instrument for the production of target oligosaccharides with specific functions [43]. A polyG-enriched alginate originating from *Thalassotalea crassostreae*, abbreviated as TcAlg1, displays characteristic exo-type activities [44]. Two polyM restriction specific lyases—HdAlex and HdAly, produced by *Haliotis discus hannai*—reveal external and internal degradation activities. HdAlex can release the dimer from the reducing end via degradation of the DP3-4 generated by HdAly from alginate. Applying this technique, G-rich alginate oligosaccharides were successfully prepared [45]. Therefore, AL07 has broad application prospects in the field of functional oligosaccharides preparation.

2.3. Temperature Properties of AL07

The effect of temperature on catalyzing activity has been investigated and applied to the purified AL07. As revealed in Figure 4a, AL07 shows the highest catalytic activity at 40 °C; it exceeds 80% of relative activity, which is detected at 35–55 °C. Even at 60 °C, it still possesses more than 60% relative activity. During the thermal stability study of AL07, the enzyme activity without 10 h incubation is considered as 100%. The present alginate lyase displays favorable stability below 35 °C, while the activity decreases sharply over 40 °C and even disappeared above 50 °C (Figure 4b). It was reported that the highest activity of most alginate lyases occurs at about 40 °C [25–27,46], while the alginate lyase adapted to cold environment shows the highest catalytic activity at lower than 35 °C (Table 1). There are relatively few reports about thermophilic alginate lyase. The alginate lyase from *Nitratiruptor* sp. SB155-2 has the highest activity at 70 °C, but it loses 80% of initial activity at 50 °C after an incubation period of 16 h [17]. The optimal catalytic temperature of recombinant alginate lyase from *Saccharophagus degradans* is 50 °C, but after incubation at 30 °C for 0.5 h, its initial activity is significantly reduced to 42% of the initial activity [47]. Two kinds of alginate lyases originating from *Rhodothermus marinus*, named AlyRm4 (PL17) and AlyRm3 (PL39), display the most superior activity at 81 and 75 °C, respectively, which make it one of the most heat-resistant alginate lyases reported so far [48]. From the above test results, it can be seen that AL07 alginate lyase possesses a certain heat resistance and thermal stability.

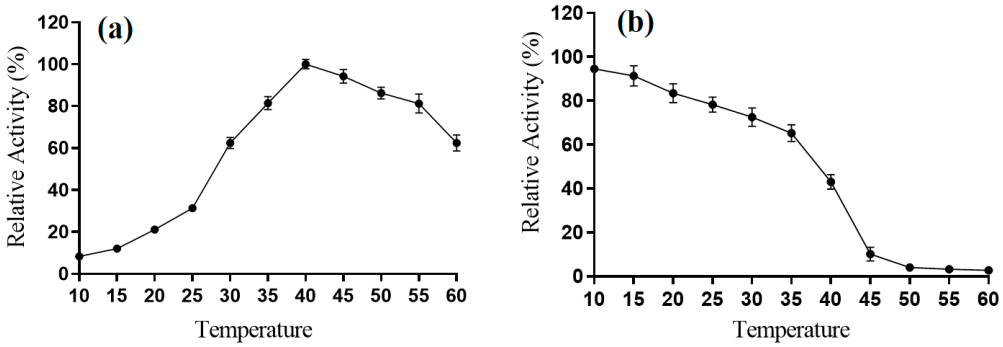

Figure 4. Effect of temperature on the activity (**a**) and stability (**b**) of AL07. Data are given as means ± standard deviation, $n = 3$.

2.4. pH Properties of AL07

The influence of different pH conditions on catalyzing activity of AL07 has been further investigated, with the consequences shown in Figure 5a. As displayed in Figure 5a, AL07 displays superior activity at pH 7.0. In particular, over 60% of activity is obtained in the wide pH range of 5.0–11.0. As for the study results of AL07′s pH stability, as shown in Figure 5b, an excess of 40% of the activity is still retained within the detection range of pH 3.0–10.0, and has excellent stability in an alkaline environment. In general, the alginate lyases from bacteria are liable to perform catalytic hydrolysis reactions in neutral, as well as narrow pH ranges [21,24,49,50]. AlyM2 alginate lyase from *Pseudomonas arctica*

M9 shows the superior activity at pH 8.0, and has a relative activity of more than 50% at pH 7.0–9.0, while the enzyme activity drops rapidly to 18.2% at pH 10.0 [51]. Alginate lyases AlyC8 and AlyC8-CD1 derived from *Vibrio* sp. C42 indicate the superior activity at pH 9.0 and 8.0, respectively, while maintaining the highest activity of more than 40% only in the narrow pH range of 8.0 to 9.0 [22]. Alginate lyase AlgA of PL5 family from marine bacteria *Pesuaomonas* sp. E03 has the highest catalytic activity at pH 8.0, and shows more than 60% of the maximum activity in the narrow range of pH 7.0 and 9.0 [52]. In order to adapt to the marine environment, most marine derived alginate lyases from different families display more than 50% of the maximum activity between pH 7.0 and 9.0 [22]. In addition, relative to the alginate lyases reported in Table 2, AL07 shows superior pH stability than most alginate lyases, and is catalytically active over a wider pH range. For example, A9m from *Vibrio* sp. Shows the highest activity at the neutral pH of 7.5 [31], and the narrow range of pH stability is 7.0–10.0. The pH stability scopes of AlgNJU-03 and TsAly6A are 6.0–9.0 and 6.6–8.95 [26,53], respectively. the pH stable ranges of Alyw201 and Algb were both 4–10, close to that of AL07. Indeed, AL07 and the reported pH-stable mesophilic alginate lyase AlgNJ-04 manifest similar pH stabilization properties [32]. The unique pH stability of alginate lyase AL07 can promote its application in the preparation of various bioactive substances in brown algae.

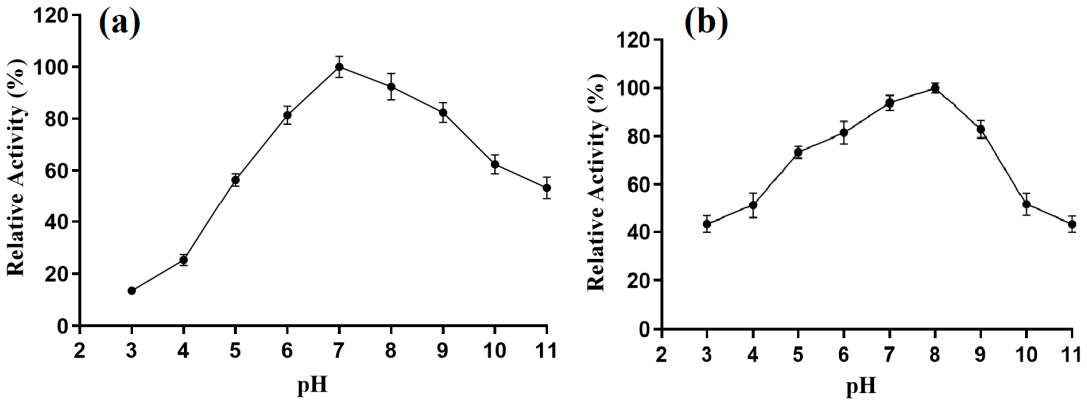

Figure 5. Effect of pH on the activity (**a**) and stability (**b**) of AL07. Data are expressed as mean ± standard deviation, $n = 3$.

Table 2. Comparison of the properties of AL07 with other alginate lyases.

Name	Source	Optimal pH/ Temperature (°C)	pH Stable Range	Product (DP)	Reference
Alyw201	*Vibrio* sp. W2	8.0/35	4–10	2–6	[22]
AlgNJU-03	*Vibrio* sp. NJU-03	7.0/30	6.0–9.0	2–5	[26]
A9m	*Vibrio* sp. A9mT	7.5/30	7–10	-	[29]
Algb	*Vibrio* sp. W13	8.0/30	4–10	2–5	[33]
AlyPM	*Pseudoalteromonas* sp.	8.0/35	-	1	[16]
AlyGC	*Glaciecola chathamensis*	7.0/30	-	1	[42]
TsAly6A	*Thalassomonas* sp.	8.0/35	6.6–8.95	2–3	[41]
TsAly7B	*Thalassomonas* sp.	8.0/20	7.3–8.6	2–3	[21]
ZH0-IV	*Sphingomonas* sp.	7.5/35	6.0–9.0	1	[43]
AL07	*Vibrio* sp.	7.0/40	3.0–10.0	2	This study

2.5. Effects of Ions on AL07

The resistance of AL07 to metal ions is displayed in Figure 6a. As shown in the figure, except for Ba^{2+}, other metal ions cannot effectively inhibit the enzyme activity of AL07 at the low concentration of 1 mM. Interestingly, the activity is activated by some metal ions. For example, the relative activity of AL07 containing Mn^{2+} is approximately 158.8% of the activity for the reaction system without any ions. Moreover, in the case of SDS and EDTA at 1 mM, the relative activities decrease to 86.3% and 75.2%, respectively. At

the concentration of 10 mM, Ba^{2+}, Ca^{2+}, SDS, and EDTA show significant inhibition of the activity of AL07, while Co^{2+} also displays certain stimulation with relative activity of 163.8%. Therefore, the above analysis results indicate that AL07 exhibits favorable metal ion tolerance, which will be conducive to industrial applications, and can run the catalytic process in complex environments.

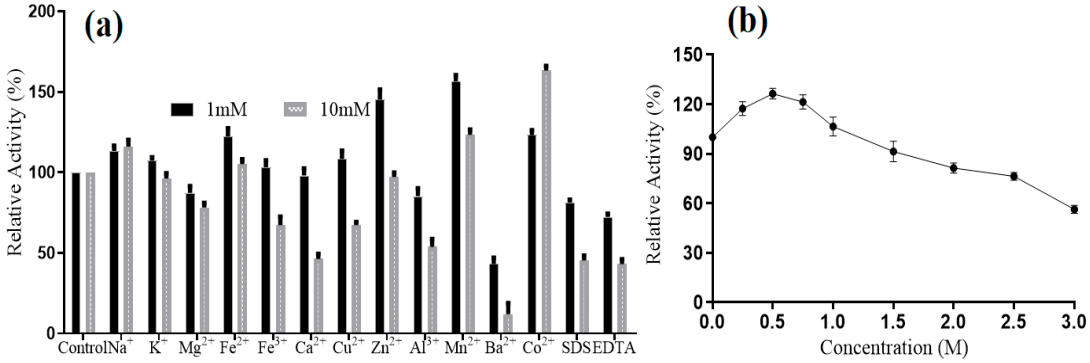

Figure 6. (**a**) Effects of metal ions, EDTA, and SDS on the activity of AL07. (**b**) Effects of NaCl on the activity of Al07. Data are shown as means ± SD (n = 3).

In this work, the influence of NaCl on the catalytic activity of AL07 has been further studied, with the consequences shown in Figure 6b. It can be seen from the figure that NaCl hardly activates the enzyme activity of AL07, and slightly inhibits the activity of present enzyme at concentrations of 0.5 to 3.0 M, where the highest activity of 128.5% is reached at 0.5 M. For many reported alginate lyases, a specific concentration of NaCl is necessary for the activation of alginate lyase. For instance, the activity of Aly08 from *Vibrio* sp. SY01 is about eight times higher at 0.3 M NaCl than it is in the absence of NaCl [53]. Similarly, the activity of AlgM4 has also been increased approximately seven times at 1 M NaCl [54]. On the contrary, the activities of AL07 are not dependent on the presence of NaCl. Owing to its superior salt tolerance, AL07 alginate lyase can degrade alginates in a variety of specific processing processes.

2.6. End Products of AL07

The enzymatic hydrolysates products of AL07 have been analyzed via thin-layer chromatography (TLC) assay. As displayed in Figure 7a, the migration rate of these spots corresponding with an alginate oligosaccharides (DP2-DP6) marker indicating that AL07 is an efficient tool in producing alginate oligosaccharides, and the main product is disaccharide (DP2). The main products of disaccharides were also verified by ESI-MS (Figure 7b). Owing to the fact that alginate oligosaccharides are widely applied in the beverage manufacturing, paper making, and food processing fields, especially in recent years, alginate oligosaccharides display broad application prospects in the field of medicine, and thus enzymatic preparation of single homogeneous oligosaccharides has received continuous attention. Nevertheless, most reported degradation products of alginate lyase are concentrated in the mixture of DP2-DP6 [50,54]. For instance, the degradation product of alginate lyase produced by *Vibrio* sp. W2 is DP2-DP6 [46]. In addition, AlgNJU-03 generated from *Vibrio* sp. takes DP2-DP5 as the main products [28]. Similarly, the main degradation product of AL07 is disaccharide, which is conducive to the efficient production of functional alginate oligosaccharides, displaying potential medicinal prospects.

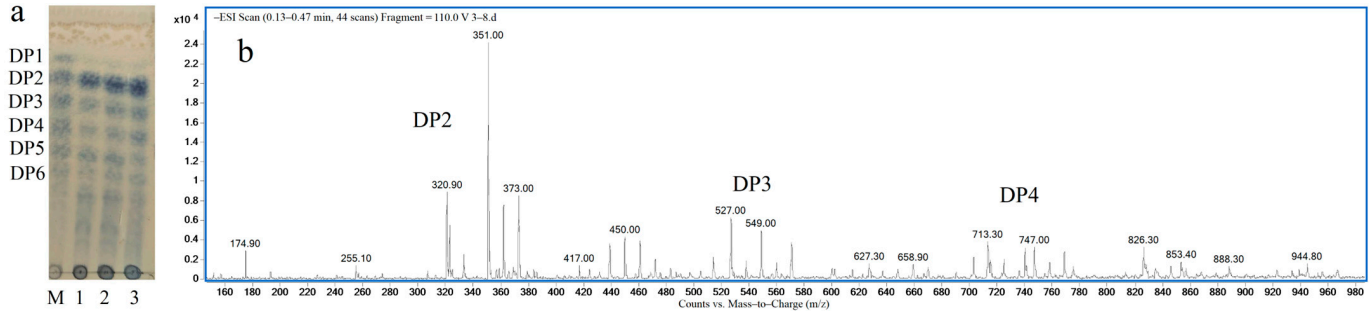

Figure 7. (**a**) TLC analysis of the hydrolysis products of AL07. Lane M, standard alginate oligosaccharides; lanes 1–3: hydrolytic products of AL07. (**b**) Analysis of degradation products of AL07 by ESI-MS.

2.7. AOS Yiled of AL07 from L. japonica

Based on the analysis of the composition of *L. japonica*, the powder used in this study consisted of 23.2% (*w*/*w*) alginate. In the cell wall of *L. japonica*, there is another linear polysaccharide: cellulose. Thus, to degrade *L. japonica*, cellulase and alginate lyase are all indispensable. Using the enzyme cocktail prepared in this study, the viscosity of *L. japonica* liquid dramatically decreased, with the increase of OD_{235}. After a 120 min reaction, the remaining viscosity is 4.6%, and the absorbance at 235 nm is stable (4.81). Although the viscosity cannot be zero for the existence of AOS, the stable absorbance at 235 nm demonstrates that the alginate was completely hydrolyzed, with cellulose converting into glucose (Figure 8). The final AOS concentration was detected as 25.3 g/L, with a yield of 0.92 g/g from alginate in *L. japonica*. The reduced AOS was attributed to the conversion of monosaccharide enzymatically from alginate. Thus, AL07 has proved an efficient tool for degrading *L. japonica*.

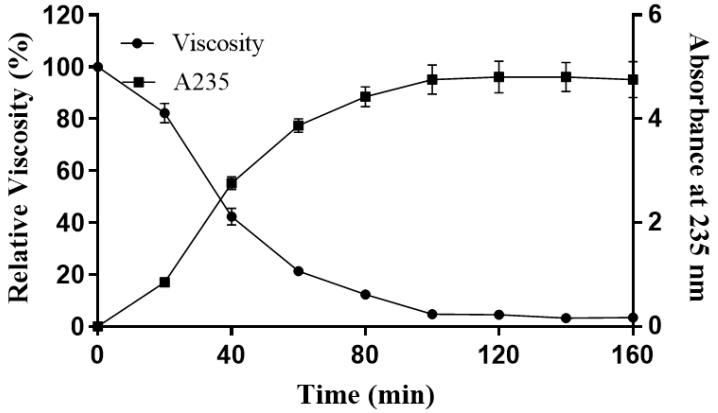

Figure 8. Viscosity and absorbance changes during the *L. japonica* degradation process.

3. Materials and Methods

3.1. Strain Screening and Gene Cloning

The 2216E plates were adopted to isolate bacteria from seawater samples [30]. The isolated strains were cultivated at 25 °C on modified 2216E plates, with alginate as the sole carbon source, to screen potential alginate lyase-producing strains. To complete the strain identification, PCR amplification was performed on the 16S rDNA of strain, using universal primers 27F and 1492R. The 16S rDNA was then subjected to sequencing and BLAST alignment. In order to identify the genetic code of the alginate lyase from strain *Vibrio* sp. SL01, Novogene was applied to annotate and sequence the genomic DNA of the current initiate lyase. To obtain the annotation information about the sugar-active enzymes, the gene sequence was compared with the carbohydrate-active enzymes (CAZy) database.

3.2. Activity Determination

The alginate with 98% purity and 240 mPa·s viscosity was purchased from the Bright Moon Seaweed Group. PolyM and PolyG polymers with 95% purity were purchased from Bozhihuili company. To quantify the activity of the alginate lyase activity, the increase of the absorbance at 235 nm caused by the formation of unsaturated double bonds was adopted and measured by a spectrophotometer. Firstly, the alginate substrate was prepared in phosphate buffer (20 mM), with a pH of 7.6 and a concentration of 0.5% (w/v). The substrate solution (900 µL) was preheated at 40 °C, and then 100 µL of enzyme solution was added to maintain for 10 min. The reaction mixture was boiled for 10 min to terminate the determination, the reaction mixture using boiled enzyme solution was set as the control. The activity unit (U) was defined by the specific amount of the enzyme, which can catalyze the reaction with an increase of 0.1 in the absorbance at 235 nm in one minute. PolyM blocks and PolyG blocks were prepared in phosphate buffer (20 mM) with a pH of 7.6, as the reaction substrates to study of substrate specificities.

3.3. Sequence Analysis

The open reading frame (ORF) of AL07 was confirmed by the ORF finder program (https://www.ncbi.nlm.nih.gov/orffinder/, accessed on 11 August 2022), and a signal peptide of AL07 was identified applying the Signal 5.0 server (http://www.dtu.dk/services/SignalP/, accessed on 15 August 2022). Domain analysis of AL07 was predicted via a search of the NCBI Conserved Domain Database (CDD) (https://www.ncbi.nlm.nih.gov/cdd, accessed on 16 August 2022). The Mw/pI online tool was applied to forecast the theoretical molecular weight (Mw), as well as isoelectric point (pI) (https://web.expasy.org/compute_pi/, accessed on 20 August 2022). DNAMAN 6.0 was used to complete multiple sequence alignment between AL07 and other alginate lyases of PL7 family.

3.4. Heterologous Expression and Purification of AL07

The gene of AL07 was connected to the expression vector pPIC9K with his-Tag, and then the recombinant plasmid after linearization by SacI was integrated into the strain *P. pastoris* using the electroporation method. The recombinant strain was incubated in YPD medium 28 °C for 24 h, which contained 2% glucose, 2% peptone, and 1% yeast extract. The cells were then collected by centrifugation, and transferred to BMMY medium (Buffered Methanol-Complex Medium) at 28 °C for 120 h. A total of 1% (v/v) was added into the medium every 24 h. and the cultured supernatant was acquired via centrifugation at 4 °C, 12,000× g for 15 min and loaded onto a Ni-NTA sepharose column (GE Healthcare, Stanford), using the AKTA150 automatic intelligent protein purification system. HiTrap™ desalting column was used to further purify the enzyme. The purity and Mw of AL07 was determined via SDS-PAGE on the 10% (w/v) resolving gel. Additionally, the recombinant protein concentration was assayed with a protein quantitative analysis kit. Furthermore, sodium alginate, polyG and polyM were applied for activity assay to reveal the substrate specificity of AL07.

3.5. Effects of Temperature and pH on AL07

The concentration of AL07 was prepared as 100 U/mL. To examine the superior reaction temperature of AL07, alginate lyase activity was measured in a 10 mM citric glycine-NaOH medium (pH 8.0) within a range of 10 °C to 60 °C. The residual activity was measured at 40 °C for detection of the thermostability, after AL07 was cultured for 10 h at 10 °C to 60 °C. The measurements were carried out in triplicate. The superior pH conditions for AL07 activity were measured in the Britton–Robinson (B-R) medium (40 mM phosphoric acid, 40 mM acetic acid, and 40 mM boric acid, regulated to different pH conditions and applying 0.1 M NaOH) within the pH range of 3–11 at the optimum temperature (40 °C). After incubating alginate lyase in a buffer solution with various pH values at 40 °C for 12 h, the residual activity of AL07 was measured to confirm the pH

stability. The buffer solution applied in the present study included NaOH-glycine buffer (pH 8.0–11.0, 50 mM), Tris-HCl buffer (pH 7.0–9.0, 50 mM), phosphate buffer (pH 6.0–8.0, 50 mM), and citric acid-Na_2HPO_4 buffer (pH 4.0–7.0, 50 mM). The measurements were carried out in triplicate.

3.6. Effects of Ions and NaCl on AL07

To inspect the effects of metal ions, as well as NaCl, on enzymatic activity, alginate lyase activity was determined in the presence of various metal ions with a concentration of 1 mM and 10 mM. The enzymatic activity was monitored in the different concentrations of NaCl from 0 to 3.0 M at 40 °C. As a control, the original alginate without extra substance was designated as 100%. The measurements were carried out in triplicate.

3.7. Analysis of End Products from Alginate Degraded by AL07

To confirm the degradation products of the recombinant enzyme AL07, 9 mL of 0.5% sodium alginate solution, combined with 10 mL of purified AL07 (0.5 mg), was incubated at 45 °C for 40 min. The samples were prepared for monitoring at an ultraviolet absorption of 235 nm after reaction for 40 min. When the absorption value was stable, the degradation products were identified by applying a thin layer chromatography (TLC) plate with acetic-acid/water/butanol ($v/v/v$, 1:1:2.), and then thanol/sulfuric acid solution (v/v, 4:1) was used to complete the color development after heating the plate for 30 min at 80 °C. The degraded products were desalted and investigated by ESI-MS to determine the degree of polymerization (DP).

3.8. Degrading L. japonica using AL07 Based Enzyme Cocktail

The cultured *L. japonica* was purchased from Rongcheng, Shandong Provance. The seaweed was washed using distilled water, sundried for 10 days and milled. The final *L. japonica* powder was kept with a particle size below 40-mesh. The dried material consisted of 8.3% protein, 12.6% cellulose, 27.4% alginate, and 6.4% fucoidan. To complete the degradation, 10 g dried *L. japonica* powder was first swelled in 100 mL distilled water at 40 °C for 8 h, and the pH was adjusted to 5.0. Afterwards, 5 mL AL07 solution (100 U/mL) was mixed with 5 mL commercial and composite cellulase Celluclast 1.5 L, forming the enzyme cocktail. Then, a 4 mL enzyme cocktail was added into the swelled *L. japonica* and kept at 40 °C for 160 min, with a 1000 rpm-agitation. Every 40 min, a 3 mL sample was taken to measure the viscosity and absorbance at 235 nm. An Ostwald viscometer was used to measure the viscosity of the mixture.

4. Conclusions

The results showed that the present enzyme AL07 possessed high activity and stability. Specifically, the optimum temperature of AL07 was 40 °C, and it showed more than 80% relative activity at 35–55 °C. Additionally, the present enzyme displayed more than 60% relative activity in the wide pH range of 5.0 to 10.0. AL07 also possessed the properties of being ion-independent and salt tolerant. Oligosaccharide of DP2 was the final degradation product. *L. japonica* can be degraded in 120 min using an AL07 based enzyme cocktail. Owing to the unique stability characteristics, AL07 could become an effective tool for potential industrial applications.

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References

1. Gacesa, P. Alginates. *Carbohyd. Polym.* **1988**, *8*, 161–182. [CrossRef]
2. Zhu, B.; Yin, H. Alginate lyase: Review of major sources and classification, properties, structure-function analysis and applications. *Bioengineered* **2015**, *6*, 125–131. [CrossRef] [PubMed]
3. Smidsrod, O.; Haug, A.; Larsen, B.R. The influence of pH on the rate of hydrolysis of acidic polysaccharides. *Acta Chem. Scand.* **1966**, *20*, 1026–1034. [CrossRef]
4. Reddy, B.N.; Rauta, P.R.; Lakshmi, V.V.; Sreenivasa, S. Development, formulation, and evaluation of sodium alginate-g-poly (acryl amide-co-acrylic acid/cloiste-30b)/agnps hydrogel composites and their applications in paclitaxel drug delivery and anticancer activity. *Int. J. Appl. Pharm.* **2018**, *10*, 141–150. [CrossRef]
5. Draget, K.I.; Skjak-Bræk, G.; Stokke, B.T. Similarities and differences between alginic acid gels and ionically crosslinked alginate gels. *Food Hydrocolloid.* **2006**, *20*, 170–175. [CrossRef]
6. Wong, T.Y.; Preston, L.A.; Schiller, N.L. Alginate lyase: Review of major sources and enzyme characteristics, structure-function analysis, biological roles, and applications. *Annu. Rev. Microbiol.* **2000**, *54*, 289. [CrossRef] [PubMed]
7. Xing, M.; Cao, Q.; Wang, Y.; Xiao, H.; Zhao, J.; Zhang, Q.; Ji, A.; Song, S. Advances in research on the bioactivity of alginate oligosaccharides. *Mar. Drugs* **2020**, *18*, 144. [CrossRef]
8. Wilcox, M.D.; Brownlee, I.A.; Richardson, J.C.; Dettmar, P.W.; Pearson, J.P. The modulation of pancreatic lipase activity by alginates. *Food Chem.* **2014**, *146*, 479–484. [CrossRef]
9. Fang, W.; Bi, D.; Zheng, R.; Cai, N.; Xu, H.; Zhou, R.; Lu, J.; Wan, M.; Xu, X. Identification and activation of TLR4-mediated signalling pathways by alginate-derived guluronate oligosaccharide in RAW264. 7 macrophages. *Sci. Rep.* **2017**, *7*, 1663. [CrossRef]
10. Han, Y.; Zhang, L.; Yu, X.; Wang, S.; Xu, C.; Yin, H.; Wang, S. Retracted article: Alginate oligosaccharide attenuates α2, 6-sialylation modification to inhibit prostate cancer cell growth via the Hippo/YAP pathway. *Cell Death. Dis.* **2019**, *10*, 374. [CrossRef]
11. Xin, M.; Ren, L.; Sun, Y.; Li, H.; Guan, H.S.; He, X.X.; Li, C.X. Anticoagulant and antithrombotic activities of low-molecular-weight propylene glycol alginate sodium sulfate (PSS). *Eur. J. Med. Chem.* **2016**, *114*, 33–40. [CrossRef]
12. Iwasaki, K.; Matsubara, Y. Purification of alginate oligosaccharides with root growth-promoting activity toward lettuce. *Biosci. Biotech. Biochem.* **2000**, *64*, 1067–1070. [CrossRef] [PubMed]
13. Inoue, A.; Ojima, T. Alginate-modifying enzymes. *Nippon. Suisan. Gakk.* **2017**, *83*, 827. [CrossRef]
14. Cantarel, B.L.; Coutinho, P.M.; Rancurel, C.; Bernard, T.; Lombard, V.; Henrissat, B. The carbohydrate-active enzymes database: An expert resource for glycogenomics. *Nucleic Acids Res.* **2009**, *37*, 233–238. [CrossRef] [PubMed]
15. Yang, M.; Yu, Y.; Yang, S.; Shi, X.; Mou, H.; Li, L. Expression and characterization of a new polyG-specific alginate lyase from marine bacterium *Microbulbifer* sp. Q7. *Front. Microbiol.* **2018**, *9*, 2894. [CrossRef]
16. Chen, X.L.; Dong, S.; Xu, F.; Dong, F.; Li, P.Y.; Zhang, X.Y.; Zhou, B.C.; Zhang, Y.Z.; Xie, B.B. Characterization of a new cold-adapted and salt-activated polysaccharide lyase family 7 alginate lyase from *Pseudoalteromonas* sp. SM0524. *Front. Microbiol.* **2016**, *7*, 1120. [CrossRef]
17. Inoue, A.; Anraku, M.; Nakagawa, S.; Ojima, T. Discovery of a novel alginate lyase from *Nitratiruptor* sp. SB155-2 thriving at deep-sea hydrothermal vents and identification of the residues responsible for its heat stability. *J. Biol. Chem.* **2016**, *291*, 15551–15563. [CrossRef]
18. Jagtap, S.S.; Hehemann, J.H.; Polz, M.F.; Lee, J.K.; Zhao, H. Comparative biochemical characterization of three exolytic oligoalginate lyases from *Vibrio splendidus* reveals complementary substrate scope, temperature, and pH adaptations. *Appl. Environ. Microb.* **2014**, *80*, 4207–4214. [CrossRef]
19. Schiller, N.; Monday, S.; Boyd, C.; Keen, N.; Ohman, D. Characterization of the *Pseudomonas aeruginosa* alginate lyase gene (algL): Cloning, sequencing, and expression in *E. coli*. *J. Bacteriol.* **1993**, *175*, 4780–4789. [CrossRef]
20. Thomas, F.; Lundqvist, L.C.; Jam, M.; Jeudy, A.; Barbeyron, T.; Sandström, C.; Michel, G.; Czjzek, M. Comparative characterization of two marine alginate lyases from *Zobellia galactanivorans* reveals distinct modes of action and exquisite adaptation to their natural substrate. *J. Biol. Chem.* **2013**, *288*, 23021–23037. [CrossRef]
21. Zhang, Z.; Tang, L.; Bao, M.; Liu, Z.; Yu, W.; Han, F. Functional characterization of carbohydrate-binding modules in a new alginate lyase, TsAly7B, from *Thalassomonas* sp. LD5. *Mar. Drugs* **2019**, *18*, 25. [CrossRef]
22. Sun, X.M.; Xue, Z.; Sun, M.L.; Zhang, Y.; Fu, H.H.; Zhang, Y.Q.; Wang, P. Characterization of a novel alginate lyasewith two alginate lyase domains from the marine bacterium *Vibrio* sp. C42. *Mar. Drugs* **2022**, *20*, 746. [CrossRef] [PubMed]
23. Meng, Q.; Tian, X.Y.; Jiang, B.; Zhou, L.C.; Cheng, J.; Zhang, C.T. Characterization and enhanced extracellular overexpression of a new salt-activated alginate lyase. *J. Sci. Food. Agric.* **2021**, *101*, 5154–5162. [CrossRef] [PubMed]
24. Wang, Z.P.; Cao, M.; Li, B.; Ji, X.F.; Zhang, X.Y.; Zhang, Y.Q.; Wang, H.Y. Cloning, secretory expression and characterization of a unique pH-stable and cold-adapted alginate lyase. *Mar. Drugs* **2020**, *18*, 189. [CrossRef] [PubMed]

25. Li, S.; Yang, X.; Bao, M.; Wu, Y.; Yu, W.; Han, F.; Sauer, M. Family 13 carbohydrate-binding module of alginate lyase from *Agarivorans* sp. L11 enhances its catalytic efficiency and thermostability, and alters its substrate preference and product distribution. *FEMS Microbiol. Lett.* **2015**, *362*, 5882. [CrossRef] [PubMed]
26. Kim, H.T.; Ko, H.J.; Kim, N.; Kim, D.; Lee, D.; Choi, I.G.; Woo, H.C.; Kim, M.D.; Kim, K.H. Characterization of a recombinant endo-type alginate lyase (Alg7D) from *Saccharophagus degradans*. *Biotechnol. Lett.* **2012**, *34*, 1087–1092. [CrossRef]
27. Yan, J.; Chen, P.; Zeng, Y.; Men, Y.; Mu, S.; Zhu, Y.; Chen, Y.; Sun, Y. The characterization and modification of a novel bifunctional and robust alginate lyase derived from *Marinimicrobium* sp. H1. *Mar. Drugs* **2019**, *17*, 545. [CrossRef]
28. Zhu, B.; Sun, Y.; Ni, F.; Ning, L.; Yao, Z. Characterization of a new endo-type alginate lyase from *Vibrio* sp. NJU-03. *Int. J. Biol. Macromol.* **2018**, *108*, 1140–1147. [CrossRef]
29. Zhang, P.; Wang, Z.P.; Sheng, J.; Zheng, Y.; Ji, X.F.; Zhou, H.X.; Liu, X.Y.; Chi, Z.M. High and efficient isomaltulose production using an engineered *Yarrowia lipolytica* strain. *Bioresour. Technol.* **2018**, *265*, 577–580. [CrossRef]
30. Zhuang, J.; Zhang, K.; Liu, X.; Liu, W.; Lyu, Q.; Ji, A. Characterization of a novel polyM-preferred alginate lyase from marine *Vibrio splendidus* OU02. *Mar. Drugs* **2018**, *16*, 295. [CrossRef]
31. Uchimura, K.; Miyazaki, M.; Nogi, Y.; Kobayashi, T.; Horikoshi, K. Cloning and sequencing of alginate lyase genes from deep-sea strains of *Vibrio* and *Agarivorans* and characterization of a new *Vibrio* enzyme. *Mar. Biotechnol.* **2010**, *12*, 526–533. [CrossRef] [PubMed]
32. Zhu, B.; Ni, F.; Ning, L.; Sun, Y.; Yao, Z. Cloning and characterization of a new pH-stable alginate lyase with high salt tolerance from marine *Vibrio* sp. NJ-04. *Int. J. Biol. Macromol.* **2018**, *115*, 1063–1070. [CrossRef] [PubMed]
33. Miyamoto, T.; Okano, S.; Kasai, N. Inactivation of *E. coli* endotoxin by soft hydrothermal processing. *Appl. Environ. Microb.* **2009**, *75*, 5058–5063. [CrossRef] [PubMed]
34. Madzak, C. *Yarrowia lipolytica*: Recent achievements in heterologous protein expression and pathway engineering. *Appl. Microbiol. Biot.* **2015**, *99*, 4559–4577. [CrossRef]
35. Yang, S.X.; Liu, Z.M.; Fu, X.D.; Zhu, C.L.; Kong, Q.; Yang, M.; Mou, H.J. Expression and characterization of an alginate lyase and its thermostable mutant in *Pichia pastoris*. *Mar. Drugs* **2020**, *18*, 305. [CrossRef]
36. Li, S.Y.; Wang, Z.P.; Wang, L.N.; Peng, J.X.; Wang, Y.N.; Han, Y.T.; Zhao, S.F. Combined enzymatic hydrolysis and selective fermentation for green production of alginate oligosaccharides from *Laminaria japonica*. *Bioresour. Technol.* **2019**, *281*, 84–89. [CrossRef]
37. Li, H.; Wang, S.; Zhang, Y.; Chen, L. High-level expression of a thermally stable alginate lyase using *Pichia pastoris*, characterization and application in producing brown alginate oligosaccharide. *Mar. Drugs* **2018**, *16*, 158. [CrossRef]
38. Ma, Y.; Li, J.; Zhang, X.Y.; Ni, H.D.; Wang, F.B.; Wang, H.Y.; Wang, Z.P. Characterization of a new intracellular alginate lyase with metal ions-tolerant and ph-stable properties. *Mar. Drugs* **2020**, *18*, 416. [CrossRef]
39. Zhou, H.X.; Xu, S.S.; Yin, X.J.; Wang, F.L.; Li, Y. Characterization of a new bifunctional and cold-adapted polysaccharide lyase (pl) family 7 alginate lyase from *Flavobacterium* sp. *Mar. Drugs* **2020**, *18*, 388. [CrossRef]
40. Zhu, X.; Li, X.; Shi, H.; Zhou, J.; Tan, Z.; Yuan, M.; Yao, P.; Liu, X. Characterization of a novel alginate lyase from marine bacterium *Vibrio furnissii* H1. *Mar. Drugs* **2018**, *16*, 30. [CrossRef]
41. Pei, X.; Chang, Y.; Shen, J. Cloning, expression and characterization of an endo-acting bifunctional alginate lyase of marine bacterium *Wenyingzhuangia fucanilytica*. *Protein Expr. Purif.* **2019**, *154*, 44–51. [CrossRef] [PubMed]
42. Yang, M.; Li, N.N.; Yang, S.X.; Yu, Y.; Han, Z.L.; Li, L.; Mou, H.J. Study on expression and action mode of recombinant alginate lyases based on conserved domains reconstruction. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 807–817. [CrossRef] [PubMed]
43. Cheng, D.Y.; Jiang, C.C.; Xu, J.C.; Liu, Z.; Mao, X.Z. Characteristics and applications of alginate lyases: A review. *Int. J. Biol. Macromol.* **2020**, *164*, 1304–1320. [CrossRef] [PubMed]
44. Wang, D.; Aarstad, O.A.; Li, J.; McKee, L.S.; Saetrom, G.I.; Vyas, A.; Srivastava, V.; Aachmann, F.L.; Bulone, V.; Hsieh, Y.S. 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.* **2018**, *66*, 1435–1443. [CrossRef]
45. Suzuki, H.; Suzuki, K.; Inoue, A.; Ojima, T. A novel oligoalginate lyase from abalone, *Haliotis discus hannai*, that releases disaccharide from alginate polymer in an exolytic manner. *Carbohyd. Res.* **2006**, *341*, 1809–1819. [CrossRef]
46. Zhu, B.; Tan, H.; Qin, Y.; Xu, Q.; Du, Y.; Yin, H. Characterization of a new endo-type alginate lyase from *Vibrio* sp. W13. *Int. J. Biol. Macromol.* **2015**, *75*, 330–337. [CrossRef]
47. Han, W.; Gu, J.; Cheng, Y.; Liu, H.; Li, Y.; Li, F. Novel alginate lyase (aly5) from a polysaccharide-degrading marine bacterium, *Flammeovirga* sp. strain my04: Effects of module truncation on biochemical. characteristics, alginate degradation patterns, and oligosaccharide-yielding properties. *Appl. Environ. Microb.* **2015**, *82*, 364. [CrossRef]
48. Dobruchowska, J.M.; Bjornsdottir, B.; Fridjonsson, O.H.; Altenbuchner, J.; Watzlawick, H.; Gerwig, G.J.; Dijkhuizen, L.; Kamerling, J.P.; Hreggvidsson, G.O. Enzymatic depolymerization of alginate by two novel thermostable alginate lyases from Rhodothermus marinus. *Front. Plant. Sci.* **2022**, *13*, 981602. [CrossRef]
49. Wang, Y.; Chen, X.; Bi, X.; Ren, Y.; Han, Q.; Zhou, Y.; Han, Y.; Yao, R.; Li, S. Characterization of an alkaline alginate lyase with pH-stable and thermo-tolerance property. *Mar. Drugs* **2019**, *17*, 308. [CrossRef]
50. Xue, Z.; Sun, X.M.; Chen, C.; Zhang, X.Y.; Chen, X.L.; Zhang, Y.Z.; Fan, S.J.; Xu, F. A novel alginate lyase: Identification, characterization, and potential application in alginate trisaccharide preparation. *Mar. Drugs* **2022**, *20*, 159. [CrossRef]

51. Zhu, B.W.; Huang, L.S.; Tan, H.D.; Qin, Y.Q.; Du, Y.G.; Yin, H. Characterization of a new endo-type polyM-specifific alginate lyase from *Pseudomonas* sp. *Biotechnol. Lett.* **2015**, *37*, 409–415. [CrossRef] [PubMed]
52. Gao, S.; Zhang, Z.; Li, S.; Su, H.; Tang, L.; Tan, Y.; Yu, W.; Han, F. Characterization of a new endo-type polysaccharide lyase (PL) family 6 alginate lyase with cold-adapted and metal ions-resisted property. *Int. J. Biol. Macromol.* **2018**, *120*, 729–735. [CrossRef] [PubMed]
53. Huang, G.; Wen, S.; Liao, S.; Wang, Q.; Pan, S.; Zhang, R.; Lei, F.; Liao, W.; Feng, J.; Huang, S. Characterization of a bifunctional alginate lyase as a new member of the polysaccharide lyase family 17 from a marine strain BP-2. *Biotechnol. Lett.* **2019**, *41*, 1187–1200. [CrossRef]
54. Xu, F.; Dong, F.; Wang, P.; Cao, H.Y.; Li, C.Y.; Li, P.Y.; Pang, X.H.; Zhang, Y.Z.; Chen, X.L. Novel molecular insights into the catalytic mechanism of marine bacterial alginate lyase AlyGC from polysaccharide lyase family 6. *J. Biol. Chem.* **2017**, *292*, 4457–4468. [CrossRef] [PubMed]