

Article

# Cloning and Characterization of an Alginate Lyase from Marine *Vibrio*. sp. QD-5

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**Abstract:** The string of bacteria, *Vibrio*. sp. QD-5 utilizing alginate, was separated from rotten kelp. The results of genome sequencing showed that the strain QD-5 contained four alginate lyase genes. One of the alginate genes Aly-IV was cloned and linked to the plasmid pET-22b (+). The heterologous expressed alginate lyase aly-IV was characterized, which possessed the theoretical molecular mass of 62 kDa, and theoretical isoelectric point (pI) of 5.12. - The enzyme aly-IV was purified and the activity reached 1256.78 U/mg, with optimal temperature of 35 °C and pH value of 8.9. Nurtured in the temperature below 25 °C for 30 minutes, the activity was almost stable. The result also suggested that the activity of enzyme was strongly affected by - NaCl whose optimal concentration was 15 mM in a lab environment. The TLC and ESI-TOF-MS analysis suggested that the enzyme aly-IV could degrade sodium alginate and polyG in endo-lytic type, producing monomer, dimer and trimer. So, aly-IV can also be widely applied to make large scale preparation of alginate oligosaccharides with low degree of polymerization (DP).

**Keywords:** alginate lyase; *Vibrio* sp.QD-5; alginate

## 1. Introduction

Alginates are unbranched polymer- comprised of  $\alpha$ -L-guluronic acid (G) and its C5 epimer  $\beta$ -D-mannuronic acid (M). The monomers can form the homopolymeric polyG, polyM, and heteropolymeric polyMG in alginates by the different arrangement [1]. Because of the distinctive chemical and physical properties,, alginates were widely used in many fields [2], such as ingredients, stabilizers and thickeners in the food and beverage industry [3]. In addition, alginates could also function as a thickening agent in fabric printing, adhesive agent and filler in paper industries, stabilizer cum suspending agent in paints, a novel way of ceramic shaping in gelcasting, and biocatalysts in water treatments [4]. In the healthcare and pharmaceutical industry, alginates were also a growing importance.. Alginates were involved in tablet making to improve bioadhesive property for buccal adhesive tablets [5]. Alginate (10–100 kDa) could potentially combat hypercholesterolemia and diabetes mellitus [6]. Due to the strong gelation and less easily absorbed in vivo, , the application of alginates was largely limited in medicine [7]. The research revealed that the oligosaccharides from alginates are easily dissolved, highly stable and nontoxic. They could be used to resist virus, restrain the growth of some bacteria and promote the growth of plants [8, 9].

Alginate lyases have been separated from various sources, including marine algae, marine mollusks, marine bacteria, soil bacteria, fungi and viruses [10]. These lyases were used to break the glycoside bond in alginate through  $\beta$ -elimination mechanism to form a double band between C4 and C5 at the non-reducing end of the degrading product. [10]. According to their different substrate specificities, alginate lyases were categorized into M-specific lyase (EC 4.2.2.3), G-specific lyase (EC 4.2.2.11). . In terms of mode action, the alginate lyases were also classified into endolytic

and exolytic enzyme [10]. So far, various alginate lyases have been cloned and characterized [11]. Based on their primary structures, the alginate lyases were also grouped into seven polysaccharide lyase families in the Carbohydrate Active Enzyme (CAZy) database (<http://www.cazy.org/>), such as PL-5, PL-6, PL-7, PL-14, PL-15, PL-17, and PL-18 [12]. The problem concerning the crystal structures of various alginate lyases were solved, A  $\beta$ -sandwich-like fold structure with a large active cleft covered by 2 short flexible loops was shared in PL7 alginate lyases [10]. Now, alginate lyases have been widely used for different purposes, such as analysis of the alginate structure [13], extraction of the algal protoplasts [14], and biofilm degradation of mucoid *Pseudomonas aeruginosa* [15].

In this study, an alginate lyase-producing bacteria *Vibrio* sp. QD-5 was isolated from rotten kelp. An alginate lyase gene (Aly-IV) was cloned from this strain and expressed in *Escherichia coli* BL21 (DE3). Biochemical properties of the alginate lyase (aly-IV) were characterized after purification. The reaction mode and final degradation products of the recombinant enzyme were also analyzed.

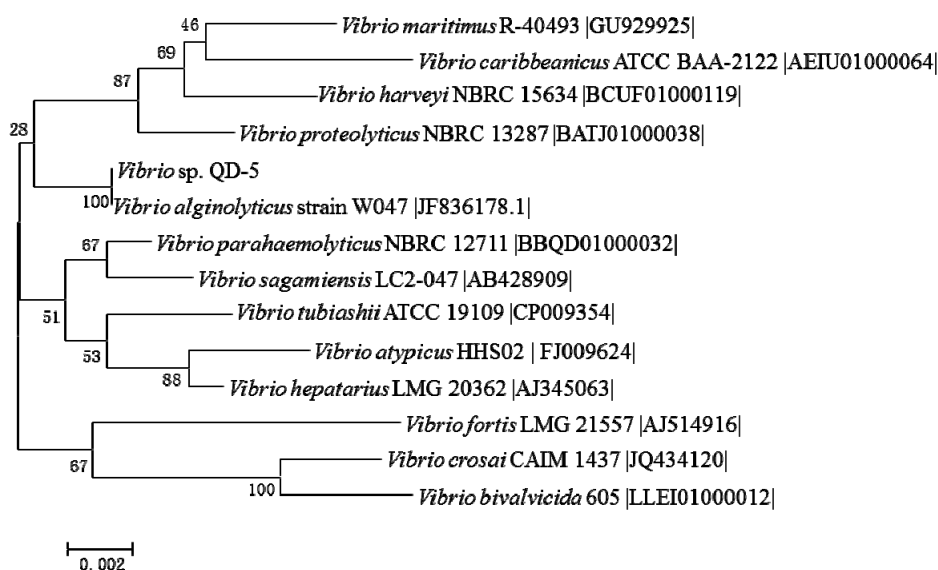
## 2. Results and Discussion

### 2.1. Isolation of alginate-degrading bacteria and phylogenetic analysis

The rotten kelp samples were spread on the alginate plates. After being incubated at 28 °C for 72 h, several hollows emerged (Figure 1A). The colonies digging holes on the plates were picked up and purified three times on the same medium, for their potential ability of alginate-degrading. In this article, QD-5 was selected for the following researches. The 16S rRNA gene of strain QD-5 was sequenced and submitted to Genbank (accession number PRJNA382465). A 1437 bp fragment of the gene was aligned with other strains. The closest strain was *Vibrio alginolyticus* strain W047 (JF836178.1). Combined with the phylogenetic position (Figure 1B), QD-5 was named *Vibrio* sp. QD-5 (Figure 1).



**Figure 1A.** Hollow zones on the plate. The colonies digging holes on the plates had the potential of digest alginate.



**Figure 1B.** Phylogenetic analysis of strain QD-5. Phylogenetic tree was constructed through the neighbor-joining algorithm using MEGA6.0, and the evolutionary distance was estimated by the maximum composite likelihood (MCL) method [16].

## 2.2. Information about the Aly-IV gene and the deduced amino acid sequence

Using the sequenced genomic data extracted from an Illumina HiSeq sequencing run, the draft genome of the *Vibrio* sp. QD-5 was constructed. It consists of two circular chromosomes whose sizes are at about 3.3 M bp and about 1.8 M bp (Figure 2A). The genome sequence has been submitted to Genbank (accession number PRJNA382465). The cluster of alginate lyase genes located on the chromosome 2 was found (Figure 2B). One of the genes, Aly-IV, was amplified by PCR. It encoded a predicted protein with 560 amino acids including a putative signal peptide sequence of 22 amino acid residues (Met<sup>1</sup> to Ala<sup>22</sup>) (Figure 3). The molecular mass of the aly-IV was approximately 62 kDa. The theoretical isoelectric point (pI) was 5.12. Conserved domain analysis of the putative protein revealed that the protein was classified into F5/8 type C and Alginate lyase2 domain. F5/8 type C domain in aly-IV was from Thr<sup>77</sup> to Leu<sup>195</sup> which was also known as the discoidin (DS) domain family (Figure 3). Alginate lyase2 domain in aly-IV was from Phe<sup>284</sup> to Lys<sup>545</sup> (Figure 3). It was a member of pfam08787 which have a predominantly beta sheet structure [17]. Analysis of the protein primary structure revealed that the Alginate lyase2 was assigned to polysaccharide lyase family 7 (PL7). Sequence alignment of Alginate lyase2 in aly-IV with other PL7 alginate lyases indicated that the conserved motifs in aly-IV were RTELRL, YFKNGVYNQ and QIH, which were highly conserved motifs in PL7 alginate lyases (Figure 4). The three conserved motifs composed the active center of the alginate lyase and were crucial for substrate recognition and catalysis [18-20].

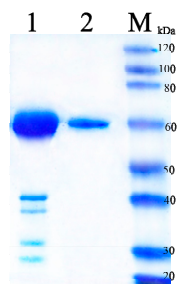


aly-IV	.....FDLTKKLTTF.....FDHDKDGRADDIDEWDLANGFQHSDFYTAD..DGGMVFERSYVK	53
BAV53312.1	KFLCMSVILGCSANAMAQAAPVADKFDMMNKITVDEEDRDKNGKIDIEGVAMF..SYSNPDPYFLDD..DGNMVFQVQNK	78
BAV10560.1	GDGSGEVKEADVSAYGLRTDVPFSENFDLTHWKLTLFADRDNDGKVDIEIEEELQ..GWSDFRFFVYDFATPAGMVFERTAPD	79
ANB32492.1	LILALGTVA..ISAPALANDTP..FAEAFNLDTWKLTLEMDGGDGRVDEIKVADLQ..SYRHADYFLDD..EDHLVVFVTPNK	76
BAJ62034.1	GSSSSSSGSGSGSGGLDPLPFSNFDLAAWYLSVETDDGNGRADSIYEAEINSGYGNNSYFYTG..DGGMVFRCPTA	79
ACM89454.1	C..LRSELPTPALPETPLPGNAPSENFDLISWYLSVETDDGNGRADSIYEAEINSGYGNNSYFYTG..DGGMVFERSYVK	78
BAK40155.1	SSSSTSTSSSSGGFNLNPNAPFSNFNLSCWYLSVETDDGSGTADSIKEGELINSGYENNSYFYTG..DGGMVFRCPTS	79
ADW41663.1	CDLRSKAMPYPT..LPKEPLATNAPGENFDLTHWKLTLTFDHDKDGRAADDIDEWDLANGFQHSDFYTAD..DGGMVFERSYVK	78
BAH79133.1	CNTRTALPVETGLPATPVAGNAPSENFDLTHWYLSQF..FDHDKNGKFDVSEKNNLANGYQHPEIFYTAD..DGGMVFERSYVK	79
ALP75563.1	DSSGNSGGITTP..DVGLDSQAAPSENFDLISAWYLGLE..IDQNNDGKSDSIYEKELTAGFYEPYFHTMDGDDGGMVFLSYVS	79
ALP75562.1	CDTRRSALSAFTGLPATPKAGNKEQNFDLTEWYLSQF..FDHGNGRFLDVSEWNLANGYEHDPVIFYTAD..DGGMVFERTFK	79
↓		
aly-IV	GARTSENKTYAFTELRLMLRAGDKSYSTK...VNPNWVFSAPFVEDQKEAGGVDTLEATIKIDHATTGGSHE...	126
BAV53312.1	AITTSG..SSNARSELRCMLRGTDLTIDVKAP...ANNEFALASHKAK..EYGAVGGKLEATIKVNHVATHANQPNKLP	150
BAV10560.1	G..KTTSGSHYTRSELRLMIRGGDKSIATRVDDGTPNKNWVFSSTAEAAQALAGGVDTMTATLAVNHVTFRTGESGK...	155
ANB32492.1	AFTTPN..SSNARTELRLMLRGTDTKIGTHAP...QNNELASNQHAD..EFAQIGGYLSATIRVHVAFERSKPKDRKS	148
BAJ62034.1	GFKTSTNTSYRTELRLMLRAGDTSISTQG...VNKNWVFSAPFAAREAGGVDTLRLATLAVNHVTTTGDSSQ...	152
ACM89454.1	GVRTSENKTYAFTELRLMLRAGNMSHKTG...VNKNWVFSAPFADLKAAGGIDGVLEATIKIDHATTGAANE...	151
BAK40155.1	GKTSSTGTSYRTELRLMLRAGNNTSIATSG...VNKNWVFSAPSAQAAGGVDTGNMKTALAVNVTTTGDSSQ...	152
ADW41663.1	GARTSENKTYAFTELRLMLRAGDKSYSTK...VNPNWVFSAPFVEDQKEAGGVDTLEATIKIDHATTGGSHE...	151
BAH79133.1	GVRTSENKTYAFTELRLMLRAGDTSISTK...VNKNWVFSAPFADLKAAGGIDGVLEATIKIDHATTGNANE...	152
ALP75563.1	GPKTSTNTSYRSELRLMLRAGDTSIKTQG...VNKNWVFSAPFVSDQLSAGGVDTLRLATLAVNHVTTTGDSSQ...	152
ALP75562.1	GVRTSENKTYAFTEMLRAGDTSISTK...VNKNWVFSAPFVEDQKAAAGGVDTLEATIKVDHTTTTGEAGQ...	152
↓		
aly-IV	VGRFIICQIHDKDD.....EPIRLYRRLFDQPTGTAYFAHEKTK....TGTEDYYSLVGDMTGEIGD..	185
BAV53312.1	AYSVVVQIHLANKDKPKLMEANTFGYGNFELKIFKFKWVDHDKGVSFFNYFNPLAKDDPNRIDIAYPVWNTWENKDDPK	230
BAV10560.1	IGRVIICQIHRMDD.....EPIRLYRRLFDQPTGTAYFAHEFVG....GDDLLVNVIGDRGSDIDNP..	214
ANB32492.1	AYSVVVQIHLAGKDEALMDADEGFHGNFELKIFKFKRFDKDTGVSFFWNYFNPLAKEDPKRRTDVSVAWGDWDTGTDPG	228
BAJ62034.1	TGRVIVVQIHRMDD.....EPIRLYRRLFDNSKSGSIYIAHEIKG....GDDTWYEMIGSRSSASNP..	211
ACM89454.1	VGRFIICQIHDQND.....EPIRLYRRLFNQATGAVYFAHESQD....ATKREDFYPLVGLTAEVGE..	210
BAK40155.1	VGRVIICQIHAENK.....EPIRLYRRLFDNSKGGIYIAHEIAD....GGEVWVDMIGSRSSASNP..	210
ADW41663.1	VGRFIICQIHDKDD.....EPIRLYRRLFDQPTGTAYFAHEKTK....TGTEDYYSLVGDMTGEIGD..	211
BAH79133.1	VGRFIICQIHDQND.....EPIRLYRRLFNQPTGAVYFAHESQD....ATKREDFYPLVGLTAEVGE..	211
ALP75563.1	VGRVIICQIHRMDD.....EPIRLYRRLFDNSKSGSIYIAHEFRDG....YGSEQKTYMIGSCSSASNP..	213
ALP75562.1	VGRFIICQIHDQDD.....EPIRLYRRLFDNHNNTGTVYFAHENTL....KGTDAFYDLVGLTAEVGEIGD..	211
↓		
aly-IV	.DGIALGEKFSIILDVKGN.....TMTVTVKRD...GKDDVVQVVDMSDGGYDEGGRYMFKAGVYVNCNMYGNP..	250
BAV53312.1	DAGVAIGEEFSSVVVDVEGNTMHLTFTSRNHRVTEYRIDLKSNLKPDTGVDERDNPRGYAEDSFFKAGAMGQCSVRDDEG	310
BAV10560.1	ADGIALDEVFSYVEIKVTSEEKDGELHPLNLSITRDD...GTVVKAEPYDMFESGYSTDKDFMFKAGAMSCNNSITWP..	290
ANB32492.1	ADGIALGDTFSYKVEVKGDMRLTFNADGHPTRNFENLADNVANGKVDNIDLPAAGYAGDWMYFKAGSYVNCNTRFPS..	306
BAJ62034.1	EDGIALNEIFSYRVEVGN.....LLTVTIFRE...GKDDVIVQVVDMSDGGYDTEEQYMFKAGVYVNCNNSGDD..	277
ACM89454.1	.DGIALGEVFSYRVEVGN.....TMTVTVSRE...GKEDAVQVVDMSDGGYDVGKYMFKAGVYVNCNISGDL..	275
BAK40155.1	SDGIALNEVESYRVEVGN.....MLTVKIYRE...GKSTVTSQYVNVNSGGYDSDWYMFKAGVYVNCNNTGNG..	277
ADW41663.1	.DGIALGEKFSIILDVKGN.....TMTVTVKRD...GKDDVVQVVDMSDGGYDEGGRYMFKAGVYVNCNMYGNP..	275
BAH79133.1	.DGIALGEKFSYRVEVGN.....TMTVSLMRE...GHDDVVQVVDMSDGGYDVGKYMFKAGVYVNCNISGDL..	276
ALP75563.1	SDGIALNEKFSYRKTNGD.....LLTVTIMRD...NKEDIVQVVDMSVNSGVNLGGQYMFKAGVYVNCNNTGDA..	279
ALP75562.1	.DGIALGEKFTYRVEVGN.....LLTVTVKRD...GKEDVQVVDMSDGGYDVGKYMFKAGVYVNCNISGDL..	276
↓		
aly-IV	.....DQYQATFYKIK.....	262
BAV53312.1	FWSPGCGAGTGFATDVRKNGDYNSVTHSKLELTSHKK..	346
BAV10560.1	.....DQFDQVTFYALDVTHGE...	307
ANB32492.1	..SNACEGTGEWAQDEANGDYARIVTKLESSEMQL...	339
BAJ62034.1	.....SDYVQATFYALENSHTEYED	297
ACM89454.1	.....DDYSQATFYKIKATHDSYTE	295
BAK40155.1	.....SDYVQATFYKIKATHD...	293
ADW41663.1	.....DQYQATFYKIKKQSFQKYQG	295
BAH79133.1	.....DDYSQATFYQHDVSHDTYTA	296
ALP75563.1	.....KDYAQATFYHIEHEYGRK.	298
ALP75562.1	.....DDYVQATFYKIKERSHSTYTG	296

**Figure 4.** Amino acid alignment of aly-IV with other PL7 alginate lyase. BAV53312.1, an alginate lyase from *Vibrio alginovor*; BAV10560.1, an endo-type alginate lyase from *Falsirhodobacter* sp. alg1; ANB32492.1, an alginate lyase from *Halomonas* sp. Victoria JH; BAJ62034.1, an alginate lyase from *Microbulbifer* sp. 6532A; ACM89454.1, an alginate lyase from *Pseudoalteromonas* sp. CY24; BAK40155.1, an alginate lyase from *Saccharophagus* sp. Myt-1; ADW41663.1, an alginate lyase precursor from *Vibrio midae*; BAH79133.1, an alginate lyase from *Vibrio* sp. A9m; ALP75563.1, an alginate lyase from *Vibrio* sp. BZM-1; ALP75562.1, an alginate lyase from *Vibrio litoralis*. Similar amino acid residues among the alginate lyases were shaded in gray. Conserved regions were boxed.

### 2.3. Purification of the recombinant enzyme aly-IV.

To characterize the recombinant enzyme, the gene Aly-IV was cloned into an expression vector pET-22b (+) and expressed in BL21. Tests showed the recombinant enzyme, aly-IV, was expressed in the form of inclusion bodies (IB). For obtain the active enzyme aly-IV, the IB was denatured by Sarkosyl and refolded via dilution. A large amount of renaturation enzyme were obtained and purified by nickel affinity chromatography followed by gel filtration chromatography. The apparent molecular weight was about 62 kDa on the SDS-PAGE (Figure 5). The final activity of the enzyme reached 1256.78 U/mg, which was different from the alginate lyase from other *Vibrio* sp..

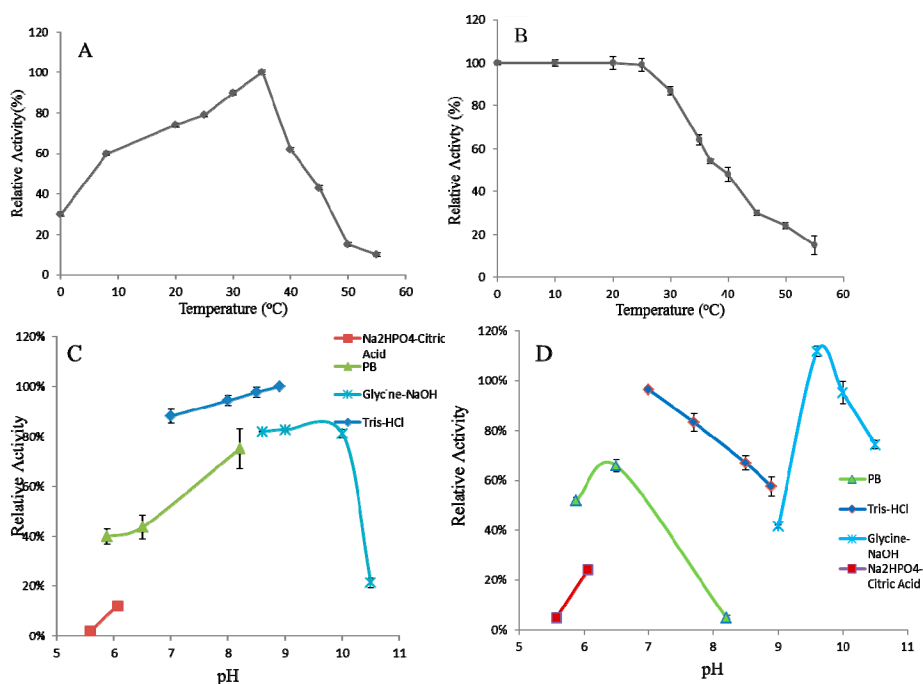


**Figure 5.** SDS-PAGE analysis of the purified enzyme aly-IV. Proteins were electrophoresed on 12.5% acrylamide gel and stained with Coomassie Brilliant Blue G-250. Lane 1: 10  $\mu$ L of the protein (150  $\mu$ g/mL) purified by Ni-NTA affinity chromatography. Lane M: protein mass markers. Lane 2: 20  $\mu$ L of the protein (10  $\mu$ g/mL) purified by Superdex-200 Increase10/300.

#### 2.4. Characteristics of the recombinant enzyme aly-IV

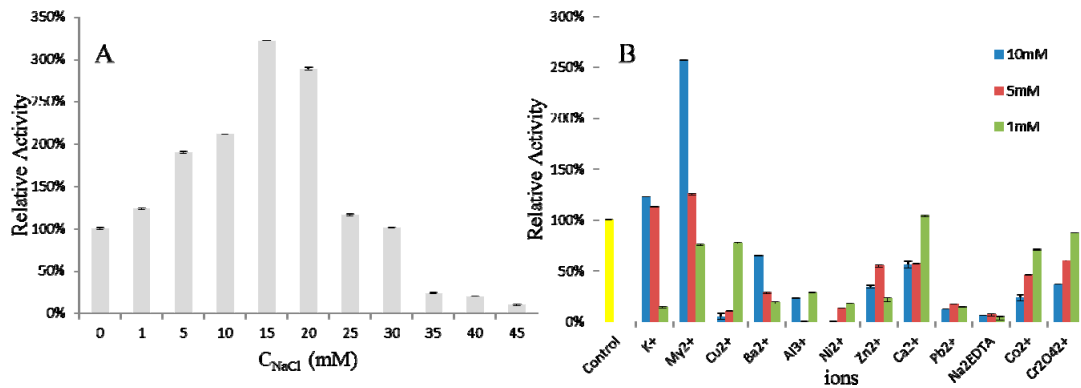
The optimal temperature of the enzyme aly-IV was 35  $^{\circ}$ C (Figure 6A) and remained stable when incubated in the temperature below 30  $^{\circ}$ C for 30 min (Figure 6B). The optimal pH of the enzyme activity was 8.9 (Figure 6C). The enzyme activity was more than 80% when the reaction pH ranges from pH 7.0 to pH 10.0. The residual activity of the enzyme showed more than 70% after being incubated in the Glycine-NaOH buffer (pH9.6-10.5) for 3 h on the ice (Figure 6D).

The pH stability of the enzyme was related to the type of the buffer. In the  $\text{Na}_2\text{HPO}_4$ -Citric acid buffer (pH 5.5-6.0), the residual activity was maintained less than 25%. In the PB buffer (pH 5.8-8.0), the residual activity was lower than 70% of the initial activity. In the Tris-HCl buffer (pH 7.0-8.9), the activity was more than 50%. Notably, in the Tris-HCl buffer pH 7.0, the enzyme activity was almost unaffected. When the glycine-NaOH buffer was used the trend of the effects on the activity was a parabolic curve. The highest residual activity was in the buffer of pH 9.6; the lowest residual activity was in the buffer of pH 9 with 42% against the initial activity. The enzyme aly-IV showed different activity in the different buffers at the same pH, suggesting that the buffer ions affect the enzyme activity.



**Figure 6.** Biochemical characterization of the recombinant alginate lyase aly-IV. (A) The optimal temperature of aly-IV. (B) Thermal stability of aly-IV. (C) The optimal pH of aly-IV. (D) pH stability of aly-IV was determined by measuring the residual activity after the enzyme incubated in the pH range of 5-10.5 with the above buffer for 3 h in ice-bath.

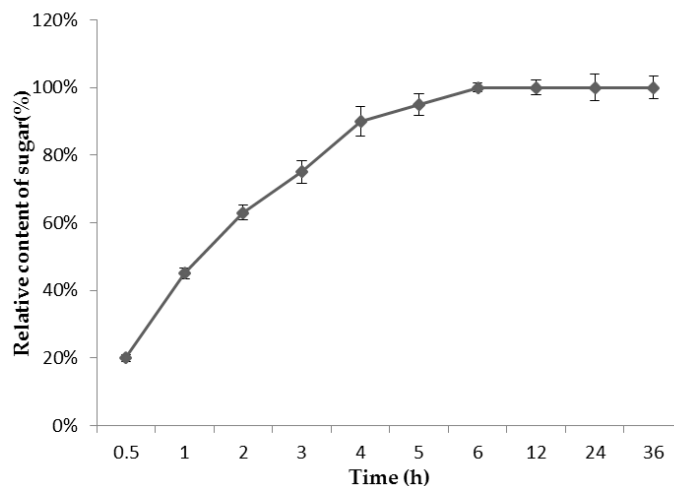
The effects of the NaCl on the activity of the enzyme aly-IV showed in Figure 7A. The enzyme activity was inhibited when the concentration was more than 20 mM. Moreover, the activity of aly-IV was strongly inhibited by 1 mM, Ba<sup>2+</sup>, Al<sup>3+</sup>, Ni<sup>2+</sup>, Zn<sup>2+</sup>, Pb<sup>2+</sup> and EDTA<sup>2-</sup>, but promoted by 1 mM Ca<sup>2+</sup> (Figure 7B), increased with K<sup>+</sup> and Mg<sup>2+</sup> at the concentration of 5 mM and 10 mM.



**Figure 7.** The effects of NaCl (A) and metal ions (B) on the enzymatic activity of aly-IV.

### 2.5 Enzymic Hydrolysis of sodium alginate by aly-IV

To study degradation characteristics of aly-IV, the amount of degradation product was determined by the time length. As shown in Figure S8, when the reaction time increased from 0.5 h to 6 h, the amount of product was increasing considerably. After 6 hours, the increase of the product was not significant due to the loss of the enzyme activity after incubation at 35 °C for a long time.



**Figure S8.** Enzymic Hydrolysis of sodium alginate by aly-IV. The highest OD<sub>520</sub> was set to be 100%.

### 2.6. Substrate Specificity and Km Values of the Recombined Enzyme

To research into the substrate specificity, sodium alginate, polyG, polyM and polyGM were used as the substrates of the recombinant enzyme aly-IV. The data revealed that aly-IV preferred sodium alginate and polyG, no activity toward polyM and polyGM (Table 1)

The K<sub>m</sub> values of the enzyme towards sodium alginate and polyG were 0.2223 g/ml, and 0.3274 g/ml, respectively (Table 1). The lowest K<sub>m</sub> values of the enzyme with sodium alginate suggested that the enzyme had the higher affinity to sodium alginate than polyG.

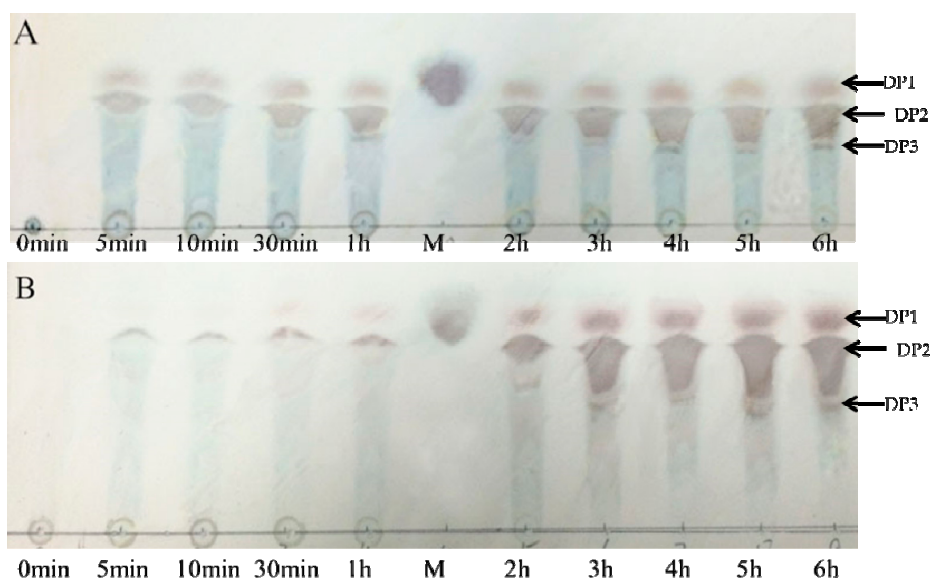
**Table 1.** Specific activity and kinetic parameters of the recombinant enzyme aly-IV towards sodium alginate and polyG.

Substrate	Km(g/ml)	Vmax(OD235/h)	Relative Activity
sodium alginate	0.2223	3.6	100%
polyG	0.3274	2.8321	68%

The datas were the means of three experimental repeats with the  $SD < 0.05$ . The initial reaction velocity was defined as the increasing absorbance at 235 nm of the products produced by recombinant enzyme aly-IV at the first five minutes.

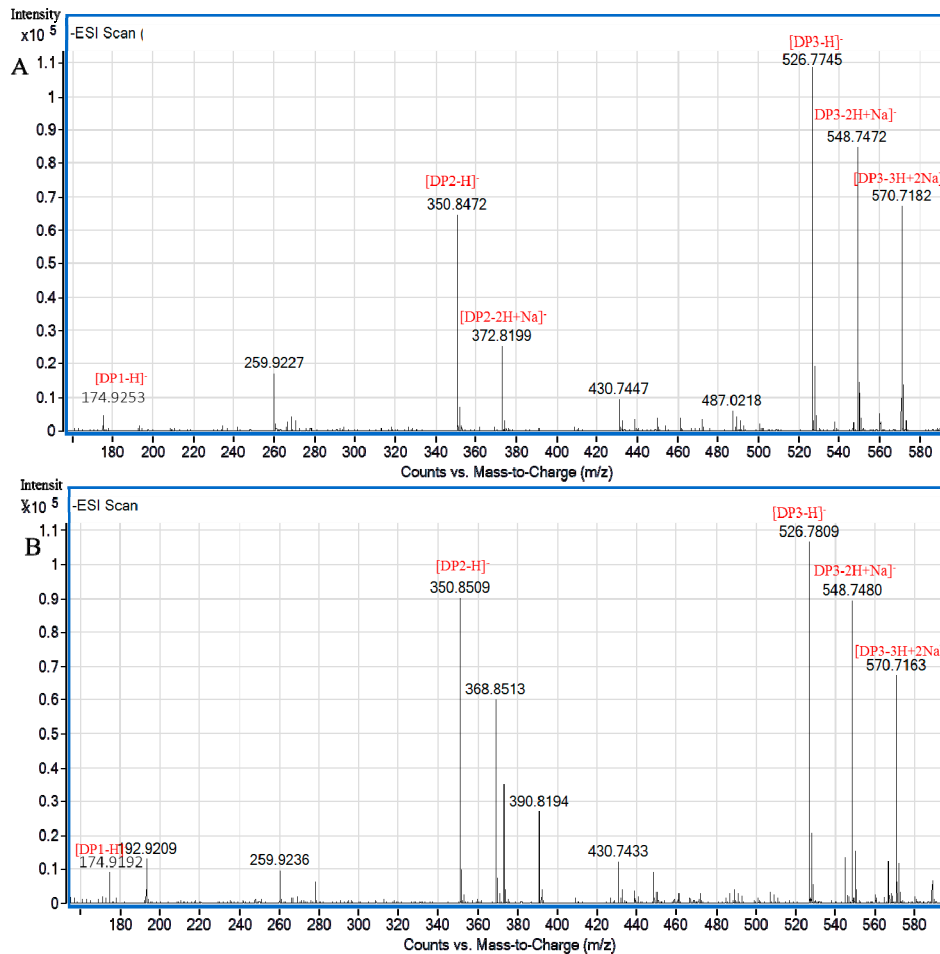
### 2.7. Reaction products and modes of action

To analyze the degradation products of the recombinant enzyme aly-IV, 5  $\mu$ L samples taken at different time were subjected to TLC assay. The distributions of the products from the substrates of sodium alginate and polyG were similar (Figure 8). The reaction products were further identified by negative ESI-TOF-MS. As reported in Figure 9, the degradation products of sodium alginate and polyG mainly consist of DP1, DP2, and DP3. The enzyme had no activity to polyM and polyGM (The dates not listed).



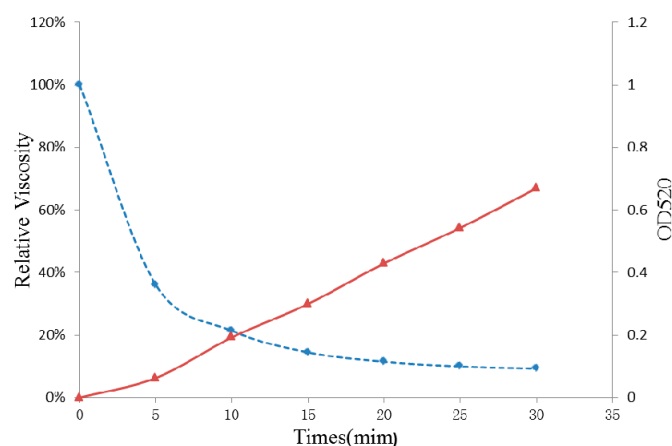
**Figure 8.** TLC analysis of the degradation products of the recombinant enzyme towards sodium alginate and polyG. Samples were taken out at intervals. Line M: Galacturonic acid; (A) the TLC analyses for degradation products of sodium alginate. (B) the TLC plate analyses for degradation products of polyG. The solvent system was 1-butanol/ ethanol/ water 2:1:1 and products were visualized by heated the plate at 85 °C for 10 min after spray with a diphenylamine/ aniline/ phosphate reagent.





**Figure 9.** ESI-TOF-MS analyses of the end products of aly-IV. The samples applied to ESI-TOF-MS was reacted for 6 h (A) The degradation products of sodium alginate. (B) The degradation products of polyG.

The behavior of the recombined enzyme aly-IV was monitored in the viscometric assay. The viscosity of the alginate solution rapidly decreased in the first 5 minutes, whereas the values of  $OD_{520}$  nm increased very slowly (Figure 10). the distribution of the hydrolysate on the TLC plate suggested that the enzyme aly-IV acts in an endo-lytic mode. The endo-lytic mode actions of enzyme Alm from *Agarivorans* sp. JAM-A1m [22] and enzyme AlySY08 from *Vibrio* sp. SY08 [23] were confirmed in the same method.



**Figure 10.** Viscosity reduction during enzymatic degradation of alginate. The initial viscosity of the reaction mixture was taken as 100%. squares filled with dotted line rate of viscosity reduction; triangles filled with solid line absorbance at 520 nm.

### 3. Experimental Section

#### 3.1. Isolation of alginate-degrading bacteria

The rotten kelp samples collected from the seashore of Qingdao, China were suspended in sterile seawater and then spread on the alginate medium (pH 7.2) plates containing (w/v) 0.5 % sodium alginate, 0.5% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.2% K<sub>2</sub>HPO<sub>4</sub>, 0.1% MgSO<sub>4</sub>, 0.001% FeSO<sub>4</sub>·7H<sub>2</sub>O, 2 % agar power. The plates were incubated at 28 °C for about 72 h. Then the colonies showing hollow zone (Figure 1A) were picked up and purified on the same medium several times.

To determine the phylogenetic position of the strain QD-5, the 16S rRNA gene sequence was amplified by polymerase chain reaction (PCR), and aligned with its closely related sequences retrieved from Genbank. The phylogenetic tree was constructed through neighbor-joining algorithm using MAGA 6.0.

#### 3.2. Genome sequencing and assembly

Extracted from *Vibrio* sp. QD-5 using CTAB method, Genome DNA was sequenced using an Illumina HiSeq sequencing system accordingly. The acquired sequences were assembled using Velvet (2013) and submitted to Genbank (accession number of PRJNA382465). Contigs obtained from the HiSeq sequencing run were integrated with formerly sequenced reads and reassembled using paired-end.

#### 3.3. Construction, cloning and sequencing of the alginate lyase gene Aly-IV

*Vibrio* sp. QD-5 was sequenced and the data of the genome were annotated (BerryGenomics, Beijing, China). A cluster of alginate lyase genes was found (Figure 2B), based on which Primers for cloning Aly-IV were designed. The forward primer was designed as 5'-TAGGTGGGATCCGATCATGAAGC-3' and the reverse primer was designed as 5'-AGAACTCGAGTTGGTACTTACC-3'. The DNA fragments with 1683 bp (Figure 3) were amplified by PCR, and ligated into the vector pET-22b (+) in succession. The resultant plasmids were transformed into *E.coli* BL21 (DE3) cell. The positive clones were picked up, and the nucleotide sequences inserted into the plasmids were confirmed by DNA sequencing (GenScript, Nanjing, China).

#### 3.4. Sequence analyses of gene and proteins

The ORFs (open reading frames) of the DNA sequence were translated into corresponding amino acid sequences using the translate tool on the ExpASy sever of SIB Bioinformatics Resource

Portal (<http://www.expasy.org/>). Various physical and chemical parameters of the predicted protein were calculated using ProtParam (<http://web.expasy.org/protparam/>) tool in Swiss-Prot. The signal peptide in the alginate lyase was predicted using the SignalP 4.1 server (<http://www.cbs.dtu.dk/services/SignalP/>). Functional information and the similarity sequences of the putative protein were performed on the National Center for Biotechnology Information server (NCBI) (<https://www.ncbi.nlm.nih.gov/>). Protein domains were identified using the NCBI and the Carbohydrate-Active Enzyme (CAZy) database (<http://www.cazy.org>). Phylogenetic tree of the predicted protein was constructed using MEGA 6.0.

### 3.5. Production of recombinant aly-IV

The recombined *E. coli* BL21 (DE3) cells with the constructed plasmid pET-22b (+)-Aly-IV were cultured in LB broth supplemented with 100 µg/ml ampicillin for about 3 h at 37 °C and 200 rpm in a shaking incubator. When cells grown up to an OD<sub>600</sub> of 0.6–0.8, the culture was supplemented with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM to induce the expression of aly-IV. The cells were cultured for another 6 h at 37 °C, 200 rpm and harvested by centrifugation. For majorization expression, a series of parameters including IPTG concentration, postinduction temperature and time were varied.

### 3.6. Preparation of inclusion body (IB) and purification of aly-IV

The cells were harvested by centrifuging and disrupted by the high pressure cell cracker. After centrifuging at 10,000×g for 10 min, the reserved pellets were washed twice using the wash buffer (50 mM Tris-HCl, 2 % TritonX-100) and then resuspended in the lysis buffer (50 mM Tris-HCl, 10 % (w/v) sarkosyl, 10 mM β-mercaptoethanol) for overnight [24]. The solubilized IB was harvest by centrifuging at 10,000×g for 15 min. Before affinity purification, the 10 % (w/v) sarkosyl-solublized IB solution was diluted with the lysis buffer to yield a 1% sarkosyl solution. For the efficiently purification, 2 % TritonX-100 and 20 mM CHAPS in the 1% sarkosyl solution were necessary. The prepared solution was subjected to Ni-NTA Sepharose column (GE Healthcare, Uppsala, Sweden) pre-equilibrated with binding buffer (20 mM Na<sub>3</sub>PO<sub>4</sub>·12H<sub>2</sub>O, 0.5 M NaCl, 10 mM imidazole). The fractions containing most of the target enzyme was eluted using elution buffer (20 mM Na<sub>3</sub>PO<sub>4</sub>·12H<sub>2</sub>O, 0.5 M NaCl, 200 mM imidazole) and pooled for the further purified using Superdex-200 Increase10/300 (GE Healthcare, Uppsala, Sweden). The enzyme was eluted with Tris-HCl buffer (pH 8.6) at a flow rate of 0.15 mL/min. Fractions with alginate lyase activity were gathered and stode at -20 °C. The purity of the fractions and the molecular weight of the enzyme were determined by SDS-PAGE (Figure 5). The concentration of the protein was measured by Coomassie Brilliant Blue G-250 with bovine serum albumin (BSA) as standard.

### 3.7. Assay of the Recombined Alginate Lyase Activity

The activity of the alginate lyase aly-IV was measured in a mixture solution containing 0.3% (w/v) sodium alginate solubilized in 50 mM Tris-HCl pH 8.9 (0.9 mL) and a suitably enzyme solution (0.1 mL) at 35 °C for 10 min. The reaction was terminated in boiling water for 10 min. The enzyme activity was determined as the increase in the absorbance 235 nm. One unit (U) was defined as the amount of enzyme required to increase by 0.1 the absorbance at 235 nm per minute.

### 3.8 Enzymatic degradation of sodium alginate

0.1 mL 0.5% sodium alginate, 0.05 mL of enzyme and 0.15 mL 50 mM Tris-HCl were mixed in 1.5 mL EP tube, and then the mixture was incubated at 35 °C . After incubated for 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 12 h 24 h, and 36 h, the samples were taken to determine the absorbance at 520 nm.

### 3.9. Substrate Specificity and Kinetic parameters of the Recombined Enzyme

50  $\mu$ L purified enzyme was added to the reaction mixture containing 0.5% of sodium alginate and polyG, respectively, for the substrate specificity. Enzyme activity was measured as described previously.

Kinetic parameters of the enzyme towards sodium alginate and polyG were obtained by measuring the enzyme activity with substrate of different concentrations (0.1-1 g/mL) (Table 1). The initial velocities were measured in the first five minute in the standard assay mixture. The  $K_m$  and  $V_{max}$  values were analyzed using the Eadie-Hofstee method.

### 3.10. Characterization of the Recombined Alginate Lyase

The temperature effects on the enzyme towards sodium alginate were performed at pH 8.9 in different temperature of 0-55  $^{\circ}$ C (Figure 6A). The thermal stability of the enzyme towards sodium alginate was determined by measuring the enzyme activity after incubated at 0-55  $^{\circ}$ C for 30 min (Figure 6B). The pH effects on the enzyme towards sodium alginate were performed at 35  $^{\circ}$ C in different pH of 5-10.5 (Figure 6C). The pH stability of the enzyme towards sodium alginate was determined by measuring the residual enzyme activity after incubated at different pH for 3 h in ice-bath(Figure 6D).

The influences of NaCl on the enzyme were performed by adding different concentration of NaCl (1-45 mM) to the reaction system as mentioned before. The enzyme activity without NaCl served as control (Figure 7A). The effects of metal ions on the enzyme were carried out by measuring the enzyme activity in the present of metal ions. The reaction mixture without any metal ion was taken as control (Figure 7B).

### 3.11. Analysis of reaction mode and products

The degraded products of the recombined enzyme aly-IV were analyzed using thin-layered chromatography (TLC) (TLC silica gel 60 F254, Merck KGaA, Darmstadt, Germany). The digested samples were periodically withdrawn for TLC analysis. The products on the TLC plates were developed using a solvent system of 1-butanol/ ethanol/ water 2:1:1 and then visualized by heating the plates at 85  $^{\circ}$ C for 10 min after spraying with a diphenylamine/ aniline/ phosphate reagent (Figure 8). To further determine the products of the enzymatic action, the ESI-TOF-MS was conducted. 5  $\mu$ L samples with 6 h reaction time were loop-injected to a G1969A time of flight mass spectrometer (TOF-MS) (Agilent Technologies Inc., California, USA) after filtration with 0.22  $\mu$ m filter membrane (Figure 9). The products were detected in a negative-ion mode using the following settings: pressure of the atomizer ( $N_2$ ), 40 psi; flow rate of the dryer ( $N_2$ ), 11 L/min; temperature of the dryer ( $N_2$ ), 350  $^{\circ}$ C; capillary voltage, 4000 V; scanning the mass range, 100-1000 m/z.

To determine the reaction mode of the recombined enzyme aly-IV, the viscometric parameters were measured with a rotary viscosimeter (SNB-1; Sunny Hengping Scientific instrument co., LTD, shanghai, China). The reaction mixtures contained 3 mL enzyme aly-IV and 27 mL 1.5% sodium alginate dissolved in 50 mM Tris-HCl, pH 8.9. The viscosity was measured at 35  $^{\circ}$ C at timed interval and the timed samples (0.3 mL) were taken out for the measurement of the absorbance at 520 nm (Figure 10).

## 4. Conclusions

In this study, an alginate lyase gene, Aly-IV, was cloned and expressed in *E.coil* BL21. The purification and characterization of this enzyme was reported for the first time. Its optimal temperature and pH was 35  $^{\circ}$ C and 8.9, respectively. Like other alginate lyases, such as AL-128 from *Vibrio harveyi* [25], rA9mT from *Vibrio* sp. JAM-A9m [26], AIYSY08 from *Vibrio* sp. SY08 [23], aly-IV was also a salt-activated enzyme, which its activity was increased 3 times at 15 mM NaCl. Aly-IV may be activated by  $K^+$ ,  $Mg^{2+}$  and  $Ca^{2+}$ , that was similar to other polyG-specific alginate lyase.

As shown in Figure 4, PL7 alginate lyases contain three highly conserved amino acid sequences, (S/T/N)EL, Q(I/V)H, and YFKAG(V/I)YNQ, which were thought to play an important role in

catalytic activity or the folding of the structure [27]. Researches revealed that enzymes containing QIH or QVH in the conserved region had substrate specificity of G-block and M-block, respectively [28]. However, aly-IV seems to prefer poly-G, like ALY-1 from *Corynebacterium* sp. ALY-1 [29], A1-I from *Sphingomonas* sp. A1 [30] and A1m from *Agarivorans* sp. JAM-A1m [31], whose of them preferred polyG as the substrate.

Alginate extracted from seaweed has had a wide range of applications. A lot of organisms are also able to exploit M and G units from depolymerized alginates as carbon and energy sources. Most of them can secrete the alginate lyase to degrade alginate. So far, hundreds of alginate lyases were found from a variety of organisms, such as marine algae, marine mollusks (*Littorina* spp., *Haliotis* spp., *Turbo cornutus*.), and a wide range of marine and terrestrial bacteria. Alginate lyases belong to a member of the polysaccharide lyases. But the question concerned is why so many organisms produce alginate lyase, including even the bacteria from deep-sea [32]? Isn't the alginate everywhere? Why are the gene sequences of alginate lyases from the different sources so diverse [33]? But so far, these questions remained unanswered. We speculate that alginate lyase can not only degrade alginate, may also degrade other polysaccharides, for example, alginate lyase in organisms has the multiple substrate specificities. Although no report has been seen yet, we will attempt to confirm this speculation in future.

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