OVER-EXPRESSION AND CHARACTERIZATION OF FOUR ALGINATE LYASES FROM *VIBRIO SPLENDIDUS* 12B01

BY

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THESIS

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ABSTRACT

Alginate is a polysaccharide found within brown seaweeds and has been targeted as a carbon source for biofuel production. Alginate consists of α-L-guluronate (G) and β-D-mannuronate (M) linked in various patterns, which results in either a homo- or heteropolymeric structure. Alginate lyases are enzymes that degrade the linkage between G and M blocks and can have specificity to either polyG, polyM, or polyMG block degradation. The marine bacterium *Vibrio splendidus* 12B01 contains four putative alginate lyases which were investigated in this study. We identified, purified, and characterized the four PL7 alginates lyases. We found that these lyases have optimal activity between pH 7.5-8.5 and 20-25°C, consistent with use in a marine environment. Additionally, Ca^{2+} is necessary for optimal enzyme activity. The binding constant (K_m) of the lyases toward alginate was found to be between 22 and 123 mM alginate and the maximum reaction rate (V_{max}) was found to be between 0.13 and 0.83 μM s^{-1}. The turnover numbers for the lyases was found to be between 0.60 and 7.1 s^{-1}.

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CHAPTER 1: INTRODUCTION

Alginate is a polysaccharide found within the cell wall and intercellular region of brown seaweeds (1), comprising up to 40% by weight (2). Alginate is a polymer consisting of (1-4)-linked α-L-guluronate (G), and β-D-mannuronate (M). Guluronate and mannuronate are epimers. The interlinkage of the M and G block within alginate can take on one of three forms: poly-guluronate (polyG), poly-mannuronate (polyM), and heteropolymeric M/G (polyMG) (3). The heteropolymeric form can take the form of either alternating G or M or alternating short stretches of polyG and polyM.

Alginate is also produced from the bacterial families *Azotobacteriaceae* and *Pseudomonadaceae*. Alginate derived from bacterial sources differs from algae alginates, in that bacterial alginates contain *O*-acetyl groups on the 2 or 3 carbon positions of D-mannuronate (4). An initial investigation of an alginate lyase derived from an *Azotobacter vinelandii* phage suggested that acetylation of the D-mannuronate protects its conversion to L-guluronate via a C-5-mannuronate epimerase (5, 6). This hypothesis was confirmed (4) via epimerization of acetylated and deacteylated alginate from *A. vinelandii*. The acetylated alginate had a minor increase in guluronate fraction, while the deacteylated alginate has a more than 20% increase in L-guluronate fraction, indicating that acetylation of the D-mannuronate protects epimerization. Only 20% of the D-mannuronate residues were acetylated, indicating that acetylation of a D-mannuronate residue has a protective effect on neighboring D-mannuronate residues.

Interestingly, the synthesis of alginate within *A. vinelandii* has been proposed (7) to involve conversion of fructose into mannose-6-phosphate via fuctokinase and phosphoglucose isomerase. Mannose-6-P is then converted into GDP-mannose via the

enzymes phosphomannomutase and GDP-mannose pyrophosphorylase. Finally, GDP-mannose is converted into GDP-mannuronic acid via GDP-mannose dehydrogenase. The GDP-mannuronic acid is linked together to form polymannuronic acid, which is then converted to alginate via the selective epimerization of mannuronic acid residues by C-5-mannuronate epimerase. The acetylation of mannuronate allows for the partial conversion of polymannuronic acid. The formation of alginate in brown seaweeds has been proposed as having a similar pathway (8), however, GDP-guluronic acid has been detected in algal seaweeds, so conversion of the assembled polymer is not needed, and thus acetylation is not present in the brown seaweed derived alginate.

Alginate lyases are a class of enzymes that degrade alginate through β-elimination of the glycosidic bond between polyG (EC 4.2.2.11), polyM (EC 4.2.2.3), or polyMG. Alginate lyases are characterized based on their substrate specificity as polyG-(9, 10), polyM (11), or polyMG-specific (12). Additionally, alginate lyases can have either endo-cleaving or exo-cleaving specificity with the majority of alginate lyases having endo-cleaving preference (3). The mechanism of action for alginate lyase has been proposed as the following three sequential events: 1) removal of the negative charge on the carboxyl anion, possibly through action of a lysine residue, 2) abstraction of the C-5 proton on the sugar ring by an aspartic acid, glutamic acid, histidine, lysine, or cysteine residue, and 3) transfer of electrons from the carbonyl group to form a double bond between C-4 and C-5 (13). The culmination of these steps results in the β-elimination of the glycosidic bond (3, 13-15) , which generates an exposed L-guluronate or a D-mannuronate and a non-reducing end. The non-reducing end contains a 4-deoxy-L-erythro-hex-4-enepyranosyluronate residue that is signified as Δ (16) to

emphasize that it is structurally different from both L-guluronate and D-mannuronate (16). The action of an alginate lyase, along with other polysaccharide lyases (PLs), differs from the action of glycoside hydrolases (GHs). GHs cleave the glycosidic bonds through hydrolysis, wherein the addition of a water molecule across the cleaved bond maintaining the 4-OH group on the new non-reducing end. PHs cleave the glycosidic bond without water, as outlined above for alginate lyases, generating a double-bond on the new non-reducing end termed a hexeneuronic acid moiety.

Some microorganisms can use alginate as a carbon source, and as such, must possess a means of converting alginate to a form metabolized by common metabolic pathways. The L-guluronate and D-mannuronate monomers are non-enzymatically converted into 4-deoxy-L-erythro-hexoseulose uronic acid (DEH) and then the α-keto acid is enzymatically converted into 2-keto-3-deoxy-D-gluconic acid (KDG) via the NADPH-dependent enzyme DEH reductase (17). KDG is then converted into 2-keto-3-deoxy-6-phosphogluconic acid (KDPG) via the enzyme KDG kinase (18). KDPG is then converted to pyruvate and glyceraldehyde-3-phosphate via the enzyme KDG-6-phosphate aldolase (18).

In order to transport the extracellularly degraded alginate derived L-guluronate and D-mannuronate oligomers into the cell, a means of transport is required on both the outer-membrane and inner-membrane. The marine bacterium *Vibrio splendidus* contains both transport systems that allow transport of oligomers into the cytoplasm (18). The outer-membrane porin KdgMN is used to transport longer chain oligoalginate polymers into the periplasm. The longer chain oligoalginate polymers are degraded into chains of length 2-to-4 within the periplasm, which are then transported into the

cytoplasm via the symporter ToaABC. The short chain oligomers are then degraded and metabolized. An alternative means to transport alginate can be found in the bacterium *Sphingomonas sp*. strain A1 which contains a periplasmic alginate binding protein-dependent ATP binding cassette (ABC) transporter (19). The transporter contains three components: a pit on the cell surface which allows the alginate polymer into the periplasm, alginate-binding proteins within the periplasm, and an ABC transporter in the inner-membrane. Alginate lyases are then contained within the cytoplasm that degrades alginate. The two transport mechanisms outlined above demonstrate that alginate must be carefully transported into the cell either following initial degradation or via chaperone-like transport continuously.

In analyzing the known alginate lyases, a number of different lytic motif structures have appeared, termed PL families. PL families show wide variety in fold types, including β-helices and α/α barrels. Since, PLs are characterized by the catalytic function rather than their structures, PLs can have the same catalytic function, but vastly different structures, indicating that PLs have been invented many times. Alginate lyases belong to seven PL families (20, 21): PL5, PL6, PL7, PL14, PL15, PL17, and PL18. PL5 and PL15 contain an $(\alpha/\alpha)_6$ barrel structure, which consists of α-helices interlinked. PL7, PL14, and PL18 contain a β-jelly roll that consists of β-sheets in an antiparallel, adjacent barrel forming a cleft. PL6 contains a parallel β-helix forming a long series of β-strands. PL17 has recently been determined to contain a structure similar to an $(\alpha/\alpha)_6$ barrel structure along with layers of antiparallel β-strands (22). PLs belonging to the same family are expected to have a similar evolutionary origin. Thus, analysis of PLs must take into account the PL families, in order to make an equal comparison.

Alginate lyases are a prevalent class of enzyme within prokaryotes and eukaryotes. Alginate itself is produced in a number of organisms, both terrestrial and aquatic environments. Alginate lyases have been discovered that are produced by micro-organisms that co-inhabit these environments. A survey of bacterial isolates associated with brown algae *Fucus distichus* found dramatically variable alginate lyase activity and M and G specificity (23). Interestingly, while alginate is produced by many organisms, an alginate lyase from the soil bacterium *Azotobacter vinelandii* has been found to degrade alginate produced from aquatic algae and the bacterium *Pseudomonas aeruginosa*, despite having different chemical properties (24).

An alginate lyase Aly from *Pseudomonas alginovora* has been over-expressed and classified based on its substrate specificity (25). Aly was found to have a polyM specificity based on its high enzymatic activity with a polyM substrate, intermediate to low activity with an alginate substrate, and an almost 14-fold decrease under a polyG substrate in comparison to the polyM substrate. Additionally, Aly from *P. alginovora* was found to have 50% lower activity in a 75 mM sodium phosphate (pH 7.5)/450 mM NaCl buffer in comparison to a 225 mM Tris/HCl (pH 7.5). Additionally, a preliminary classification of Aly from *P. alginovora* assigned the gene with the corresponding Aly gene from *K. pneumoniae*. Further studies of Aly using NMR analysis confirmed the substrate specificity of polyM substrates (26). The NMR analysis found Aly to act only on the M-M diads. No cleavage occurred between M-MG, G-MM, or G-MG. Following expectations, the only oligosaccharides found were ΔM, ΔMM, ΔMMM, and ΔMMMM. The NMR analysis confirms that Aly from *P. alginovora* has indeed polyM specificity.

The alginate lyase, AlgL, from the bacterium *Azotobacter chroococcum* was cloned and over-expressed (27). This lyase has high sequence similarity to another *Azotobacter* species lyase (90% identity), while has low homology (15 to 20% identity) to other alginate lyases, so AlgL can serve as a representative from a distinct PL5 lyase. It was found that this lyase had optimal activity at 30°C and pH 7.5. Additionally, by combinatorially adding potassium, sodium, and magnesium ions, the authors found that maximal activity was found at 100 mM K^+/Na^+/Mg^+ in a 10 mM Tris-HCl (pH 7.5) buffer. The addition of Ca^{2+}, Co^{2+}, Mn^{2+}, and Zn^{2+} had no effect on the activity of AlgL.

An alginate lyase from the soil bacterium *Azotobacter chroococcum* was isolated from the culture supernatant during growth on 0.3% sodium alginate (28). The lyase was found to have optimal activity at pH 5.5 to 6.0 and 60°C. Additionally, the lyase was found to be stable for 24 h exposure at pH 6.0 to 10.0 and 30 to 60°C. The metal ion effect was also investigated; 1mM Ca^{2+} was found to increase enzymatic activity 50%, while 1 mM Hg^{2+} was found to abolish activity. Other metal ions, 1 mM Ba^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, and Mn^{2+}, were found to have moderate decrease to no effect on enzymatic activity. Using polyG and polyM as a substrate, the alginate lyase was found have activity only with polyM substrate.

The alginate lyase $AlxM_B$ from the marine bacterium ATCC 433367 (29) has been over-expressed and characterized (30). $AlxM_B$ was found to have two-fold increase in activity on alginate derived from *P. aeruginosa* in comparison to the brown seaweed *L. hyperborean*. Increasing ionic strength was found to increase enzymatic activity; above 1M ionic strength enzymatic activity plateaus, while enzymatic activity is linear below 1M ionic strength. The maximal enzymatic activity of $AlxM_B$ under high

ionic strengths is expected, and should follow for other alginate lyases derived from marine bacteria.

The vertebrate sea hare *Aplysia kurodai* contains two alginate lyases AkAly28 and AkAly33 (31). The lyases were isolated from the gastric lumen and then dialyzed and centrifuged in order to isolate the lyases. The crude enzymes were then purified to yield ~28 kDa and ~33 kDa products. Both lyases had an identical optimal pH of 6.7 and optimal temperature of 40°C. The NaCl concentration effect on enzymatic activity differed between AkAly28 and AkAly33: AkAly28 showed no activity in the absence of NaCl, while AkAly33 showed 20% of maximal activity without NaCl. Both AkAly28 and AkAly33 showed optimal activity at approximately 0.2 M NaCl. In analyzing the substrate specificity, the authors found that both AkAly28 and AkAly33 did not have activity with a polyG-rich substrate, while activity was found with polyM- and polyMG-rich substrates, indicating that AkAly28 and AkAly33 have preference to polyM substrates.

In this study, we characterized the alginate lyases from *Vibrio splendidus* 12B01. The marine bacterium *Vibrio splendidus* (32) is a member of both planktonic and animal associated microbial communities and is the dominant *Vibrio* species in the temperate oceans (33). Alginate has been shown as a viable feedstock for biofuel production (34) by expressing *V. splendidus* 12B01 alginate lyases and other metabolic enzymes and an alginate lyase from Pseudoalteromonas sp. SM0524 to produce ethanol in *E. coli* (18). Use of alginate for biofuel production mitigates the drawbacks of terrestrial biofuel sources, since brown seaweeds require no arable land, fertilizer, or fresh water sources (35). We identified, purified, and characterized four PL7 alginates lyases in *V.*

splendidus 12B01. We found that these lyases have optimal activity between pH 7.5-8.5 and 20-25°C, consistent with use in a marine environment (36). Additionally, Ca^{2+} is necessary for optimal enzyme activity. Finally, the four tested alginate lyases kinetic parameters were determined.

CHAPTER 2: MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *Vibrio splendidus* 12B01 was obtained from Martin Polz (Massachusetts Institute of Technology). Cloning and protein expression were performed in *Escherichia coli* strains DH5α and BL21(DE3), respectively. *V. splendidus* was grown 20°C in M9 minimal salt medium (per liter tap water: 11.28 g M9 minimal salts (Sigma-Aldrich), 2 g casamino acids, and 18 g NaCl) supplemented with 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and either 0.2% glucose, 0.1% alginate, or 1% alginate (37). *E. coli* was grown at 37°C in Luria-Bertani (LB) medium (15 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl). Kanamycin was used at a concentration of 40 µg/mL.

Homology modeling. The structure of AlyD was modeled using the SWISS-MODEL software package (http://swissmodel.expasy.org/) (38) based on the structure of the alginate lyase AlyA5 from *Zobellia galactanivorans* (PDB entry 4be3, chain B) (26). Visualization was accomplished using the VMD software package (http://www.ks.uiuc.edu/Research/vmd/) (39).

Plasmid construction. AlyA, AlyB, AlyD, and AlyE were expressed from a T7 promoter using the plasmid pET28a. The lyases were first analyzed for the presence of signal peptides using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) (40). Signal peptides were identified for AlyB, AlyD, and AlyE. These peptides were removed prior to cloning. The over-expression vectors were constructed first by amplifying the alginate lyase genes *alyA* (region 4103 to 5845), *alyB* (region 6319 to

7836), and *alyD* (region 11675 to 12649) and then cloning the fragments into pET28A using the restriction enzymes NdeI and XhoI. The alginate lyase gene *alyE* (region 99593 to 100570) was cloned into pET28A using the restriction enzymes NheI and XhoI. The two domains of AlyA (domain 1, region 4259 to 4960; domain 2, region 4973 to 5845) were amplified to include (5'-CTT TCC AGC-3') upstream and (5'-ACC ACC ACA-3') downstream of domain 1 and (5'-TCA AAC GAT-3') upstream and (5'-AAC TGA-3') downstream of domain 2. The additional nucleotides upstream and downstream were included to add the hydrophobic amino acid residues flanking each domain. AylA domains then cloned into pET28A using restriction sites NdeI and XhoI. Two additional stop codons were added to all genes. **Table 1** lists the primers used in this study.

Protein purification. Cells were grown overnight in LB medium supplement with 1% glucose. They were then subcultured 1:33.3 into fresh medium and gown to an OD_{600} of 0.6. Protein expression was then induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Cells where then grown an additional four hours and harvested by centrifugation at 5,000 x *g* for 15 minutes. The pellets were lysed by resuspending in Buffer B (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 8.0) and inverting the tube for 1 hour at 20°C. Supernatants were clarified by two serial centrifugations (7,000 x *g*, 10 minutes; 40,000 x *g*, 60 minutes) and then passed through a 0.45 µm filter. The lysates for each protein were individually loaded onto three 5 mL HiTrap Chelating HP columns (charged with 0.1 mM $NiSO_4$) installed on an AKTA prime FPLC system (GE Healthcare). The columns were then washed with

5 column volumes Buffer B, followed by 5 column volumes Buffer C (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 6.3). Elution was then preformed with 5 column volumes Buffer E (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 4.5). Fractions containing the purified proteins were identified with SDS-PAGE. The fractions containing the purified protein were pooled and refolded by dialyzing three times against TKMD-G (50 mM Tris-Cl pH 8.0, 5 mM MgCl, 50 mM KCl, 0.1 mM dithiothreitol, 10 % glycerol, pH 8.0) at 4°C for 2 hours followed by an overnight charge at 4 °C (41). Gel filtration was employed to further purify the refolded lyases using a 16 mm diameter HiPrep Sephacryl S-200 HR (GE Healthcare) gel filtration column charged with TKMD-G. Fractions were collected using TKMD-G at 0.5 mL per minute to elute the purified protein.

Native size was determined by size exclusion high-performance liquid chromatography using a Shimadzu high-performance liquid chromatography system containing an SPD-10A UV-VIS detector set at 280 nm. A Bio-Sil SEC-250 column (300 by 7.8 mm) was used to determine protein size using a mobile phase of 0.05 M Na_2HPO_4, 0.05 M NaH_2PO4, and 0.15 M NaCl, pH 6.8.

Alginate lyase activity assay. Enzymatic activity was determined using the thiobarbituric assay (42-44). Enzymatic reactions were performed in 100 µL of APT buffer (20 mM sodium acetate, 20 mM monosodium phosphate, and 20 mM Tris base) containing 0.33 µg of protein and 0.05% sodium alginate, unless noted otherwise. The reactions were incubated for 20 minutes at the designated temperature, pH, and NaCl concentrations. Reactions were placed on ice following incubation if activity was not

immediately determined. Activity was determined by adding 0.125 mL of 0.025 N H_2IO_6 in 0.125 N H_2SO_4 to the reaction mixture and then incubating for 20 minutes at 20°C. Next, 0.25 mL of 2% sodium arsenite in 0.5 N HCl was added and the mixture was incubated for two minutes. Last, 1 mL thiobarbituric acid (0.3%, pH 2) was added and the mixture was heated at 100°C for 10 minutes. The increase in absorbance at 548 nm was measured using a Shimadzu BioSpec-1601 spectrophotometer. 2-Deoxy-D-glucose was used as a standard because it reacts with thiobarbituric acid (45). Activities were reported as 2-Deoxy-D-glucose equivalent concentrations.

Kinetic parameters were determined by fitting the alginate dose response data to the Michaelis-Menten kinetic model. The MATLAB R2013b (MathWorks) function fit was employed to determine the K_m and V_{max} parameters. Using the determined parameters, a turnover number was determined and is defined as the number of monomer molecules created per alginate lyase molecule per second. In order to determine the K_m of alginate, an average chain length of 70 monomers was used to evaluate the concentration in solution.

Determination of cation sensitivity. Divalent cations were removed by adding 5 mg of Chelex 100 resin (Bio-Rad) to 100 µL of the lyase in TKMD-G. The mixture was then inverted for 1 h at 20 °C and then spun for 5 minutes at 16,000 x *g* to remove the resin.

Preparation enriched polyG and polyM fractions. Enriched polyG and polyM were produced using the method of Chhatbar and coworkers (46). Briefly, 4 g of sodium

alginate dissolved in 0.25 M H_2SO_4 were microwaved for 5 minutes. The polyG and polyM fractions were then separated based on their pH solubility, lyophilized, and then dissolved in APT buffer.

Gene expression. *V. splendidus* 12B01 was grown overnight in M9 minimal salt medium and then subcultured 1:50 in fresh medium. Samples were harvested at an OD600 of 1.0. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) using the manufacturer's suggested protocol. cDNA was generated using the QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's protocol. Quantitative PCR (qPCR) was performed in triplicate using the primers listed in **Table 1**, which were designed using Primer3Plus software *(*www.bioinformatics.nl/primer3plus) (47). Reactions were carried out using HotStart-IT SYBR Green qPCR Master Mix with UDG (Affymetrix) and a BioRad MiniOpticon Real-Time PCR System**.** To quantify the amount of mRNA, standards were constructed using serial diluted *V. splendidus* 12B01 genomic DNA. The *V. splendidus* 12B01 gene *rpoA* was used to control for differences in total mRNA quantities (48).

CHAPTER 3: RESULTS

Identification of Four Alginate Lyases within *V. splendidus* 12B01. *V. splendidus* 12B01 possesses four putative alginate lyases: AlyA (V12B01_24254), AlyB (V12B01_24259), AlyD (V12B01_24274), and AlyE (V12B01_09446). Another alginate lyase within *V. splendidus* 12B01, AlyC (V12B01_24264), was found, but was not active (data not shown). AlyB, AlyD, and AlyE contain one PL7 alginate lyase domain whereas AlyA contains two PL7 domains (20, 21). Catalytic sites were then identified through comparison to other PL7 domains. (**Figure 1A and B**). Arginine has been proposed as neutralizing the negative charge on the carboxyl group. A tyrosine or glutamine is then used to remove the C5 proton, while histidine is used to stabilize the carboxylate dianion. Finally, a double bond is formed via donation of the proton to form a double bond. These residues all lie within the anti-parallel β-sheets of the PL7 domain cleft (**Figure 1c and d**). AlyB also contains a carbohydrate-binding module family 32 domain (CBM32) in addition to the PL7 alginate lyase domain. The role of this CBM32 domain has not yet been determined.

Expression of alginate lyases. To determine whether the four putative lyases were expressed during growth on alginate, we measured gene expression using quantitative PCR. Briefly, the cells were grown in M9 minimal salt medium supplement with either alginate or glucose. As shown in **Figure 2**, the expression of all four lyases was strongly induced when grown on alginate as compared to glucose. Moreover, expression increased with the alginate concentration. The effect was most pronounced for *alyA, alyD*, and *alyE*, where expression increased 2-fold when the alginate concentration was

increase from 0.1% (wt) to 1% (wt). However, expression of *alyB* increased only moderately as it was already strongly expressed at low alginate concentrations. These results demonstrate the alginate lyases are not constitutively expressed but rather conditionally expressed in response to alginate. In addition, these results suggest that AlyB may be employed at low alginate concentration whereas AlyA, AlyD, and AlyE at high concentrations.

Purification of alginate lyases. AlyA, AlyB, AlyD, and AlyE were cloned with an N-terminal 6xHis-tag and expressed in *E. coli* from a T7 promoters. The lyases were then purified under denaturing conditions and re-folded to yield a functional enzyme **(Figure 3)**. The native molecular weights were determined, and each protein was found exist as a monomer (**Table 2**).

Determination of optimal enzymatic conditions. To determine optimal conditions for each lyase, a universal buffer was formulated that allows a wide range of pH concentrations to be tested. The optimal pH, temperature, and NaCl concentrations are listed in **Table 3** and associated data in **Figure 4A,-C**. The optimal pH for all tested enzymes was found to lie within pH 7.5-8.5, while the optimal temperature was found to lie between 20 and 25 °C. AlyB, AlyD, and AlyE were found to have optimal activity at 400 mM NaCl, while AlyA had optimal activity at 1000 mM NaCl.

To determine whether divalent cations are necessary for enzymatic function, each protein was stripped of divalent cations using Chelex 100 resin and then individual divalent cations were then added to the reaction mixture at a concentration of 1 mM

(**Figure 4d)**. $CaCl_2$ was found to moderately increase enzymatic activity for all four lyase. All other cations tested were found to either decrease or not change enzymatic activity.

Activity of AlyA domains 1 and 2. AlyA possesses two PL7 domains. To determine if these domains both have enzymatic activity, both domains were over-expressed independently and tested for enzymatic activity (**Figure 5**). AlyA domain 1 was found to have no enzymatic activity whereas AlyA domain 2 was found to possess approximately 1.4-fold greater activity than the full enzyme. When accounting for the gram loading of each protein, AlyA domain 2 has almost 3-fold greater activity per µg of protein than the full enzyme.

Determination of enzymatic kinetics. We next determined the enzymatic parameters for each enzyme by determining the initial velocities at different substrate concentration (**Figure 6)**. This was accomplished by dosing alginate and measuring enzymatic activity at the optimal environmental conditions listed in **Table 3** and 1 mM $CaCl_2$. All four enzymes exhibited Michaelis-Menten type kinetics. The associated K_m, V_{max}, and turnover number are found in **Table 3**. All four enzymes had micromolar affinity for alginate. AlyB had the highest apparent affinity, with a K_m equal to 20 µM. This result is consistent with our gene expression results, where *alyB* was found to be strongly expressed at low alginate concentrations. However, the apparent affinities for the other three enzymes were not significantly lower, with K_m's ranging from 35 to 123 µM.

Tables

Table 1. Oligonucleotides used in this study.

Primer	Sequence	Characteristic
AB241F	5’-ACG TCA TAT GAA TAA GCC AAT CTT TGT CGT CGT ACT CG-3’	AlyA, NdeI
AB241R	5’-ACG TCT CGA GTT ATT ATC AGT TAT GCT CTG CTC TTA GAG AAC TAA AG-3’	AlyA, XhoI
AB305F	5’-ACG TCA TAT GCT TTC CAG CTC AGA TCT TCA GGT G-3’	AlyA domain 1, NdeI
AB305R	5’-ACG TCT CGA GTT ATT ATG TGG TGG TGT ATC GCA GTG C-3’	AlyA domain 1, XhoI
AB306F	5’-ACG TCA TAT GTC AAA CGA TTG GGA TAT TAA TGA TTG GAA GTT G-3’	AlyA domain 2, NdeI
AB306R	5’-ACG TCT CGA GTT ATT ATC AGT TAT GCT CTG CTC TTA GAG AAC-3’	AlyA domain 2, XhoI
AB272F	5’-ACG TCA TAT GGT TGG TTG TGC GAG CAC GAG-3’	AlyB, NdeI
AB238R	5’-ACG TCT CGA GTT ATT ATT ACT TTT TGT ATT GAT CGT GCG ATA CAT CTA GC-3’	AlyB, XhoI
AB273F	5’-ACG TCA TAT GAA TAA CGG TGT TTC TTA CCC CGT ACC-3’	AlyD, NdeI
AB239R	5’-ACG TCT CGA GTT ATT ACT ATT TAC CGT TTA ACT TAA GCG CAG AGA AAG TC-3’	AlyD, XhoI
AB274F	5’-ACG TGC TAG CTC TAA TCA CGA TAT TGG TCA ACA GTT CAA TC-3’	AlyE, NheI
AB240R	5’-ACG TCT CGA GTT ATT ATT ATT GAT TAA GAA CTA ACT GGT AGA AGC TTG CTT G-3’	AlyE, XhoI
AB324F	5’-CGA ATC GAG CAA AGA CCT TC-3’	AlyA, RT-PCR
AB324R	5’-CTT CAT CGC TGG TGC TAC AA-3’	AlyA, RT-PCR
AB321F	5’-TAA CTC GCC AAG CGA AAA CT-3’	AlyB, RT-PCR
AB321R	5’-GCC GTC ATC AGC TGT GTA GA-3’	AlyB, RT-PCR
AB322F	5’-TGG CGT TAT TAG CGA CTG TG-3’	AlyD, RT-PCR

AB322R	5’-AAT CGC TTG GTT CTG CAC TT-3’	AlyD, RT-PCR
AB323F	5’-CCA TGG GTC GGA TAA TGA AC-3’	AlyE, RT-PCR
AB323R	5’-GGC GAA TGT CTT TAC GAA GC-3’	AlyE, RT-PCR
AB326F	5’-CCG GTT GAT AAA ATC GCC TA-3’	RpoA, RT-PCR
AB326R	5’-CGC ATC CAG TTG TTC AGC TA-3’	RpoA, RT-PCR

Table 2. Native molecular weight of alginate lyases.

	AlyA	AlyB	AlyD	AlyE
Elution Time (min)	7.58	7.70	8.10	8.13
Predicted Size (kDa)	67.4	57.5	38.3	38.6
Measured Size (kDa)	68.2	59.0	36.5	35.2

Table 3. Optimal environmental conditions and enzyme kinetics of alginate lyases.

	AlyA	AlyB	AlyD	AlyE
pH	8.5	7.5	8.0	7.5
Temperature (°C)	25	20-25	20	25
NaCl (mM)	1000	400	400	400
K_m (μM Alginate)	36 ± 7	22 ± 5	60 ± 2	123 ± 6
V_{max} (μM s^{-1})	0.13 ± 0.01	0.66 ± 0.06	0.52 ± 0.06	0.83 ± 0.02
Turnover (s^{-1})	0.60 ± 0.02	3.7 ± 0.3	4.5 ± 0.5	7.1 ± 0.2

Figures

Figure 1. a) Structure of alginate and the β-elimination of alginate to form α-L-guluronate and Δβ-D-mannuronate. b) Domain structure of alginate lyases AlyA, AlyB, AlyD, and AlyE, drawn to scale. The indicated amino acid residues are the hypothesized catalytic sites. PL7 is the PL7 alginate lyase domain. CBM32 is the carbohydrate-binding module family 32 domain. Signal peptides are indicated with a flag. c) Homology model of AlyD PL7 domain. The depicted amino acid residues are the catalytic residues indicated in the PL7 domain structure. d) AlyD PL7 domain cleft. The catalytic residues lie within antiparallel, adjacent β-sheets within the cleft.

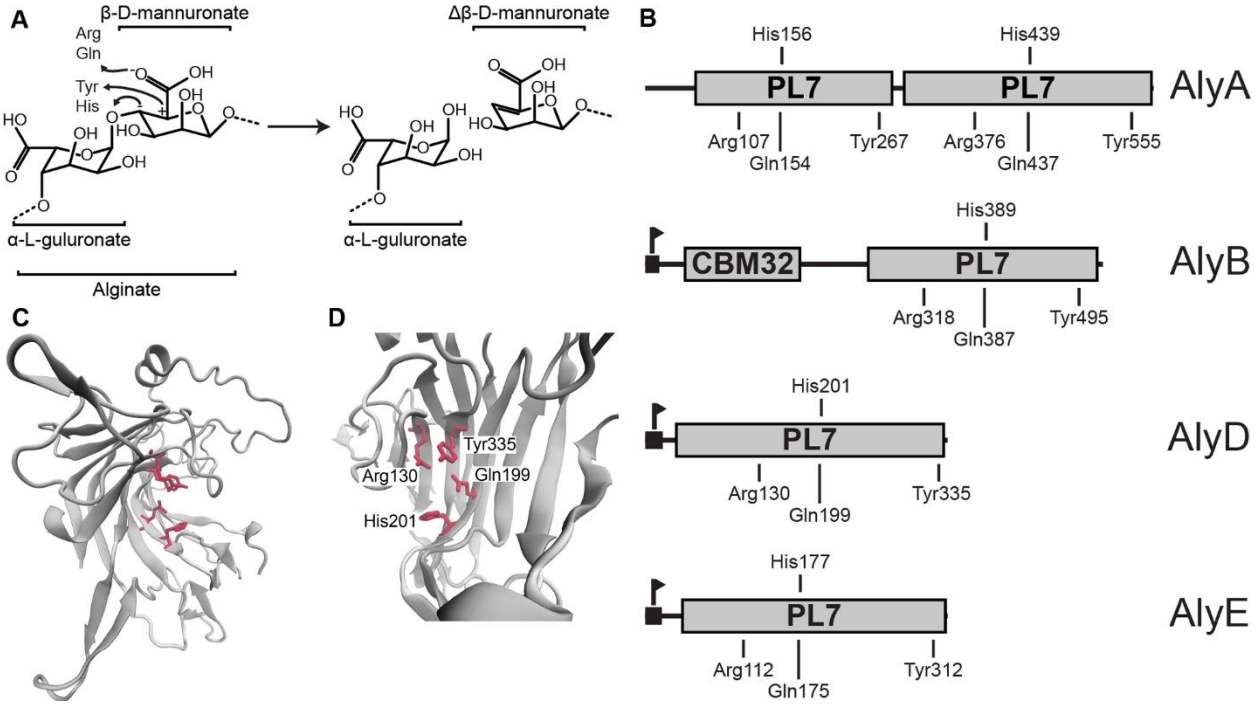


Figure 2. RT-PCR of AlyA, AlyB, AlyD, and AlyE. The mRNA levels of *alyA*, *alyB*, *alyD*, and *alyE* in *V. splendidus* 12B01 were determined. The housekeeping gene *rpoA* was used as an internal control.

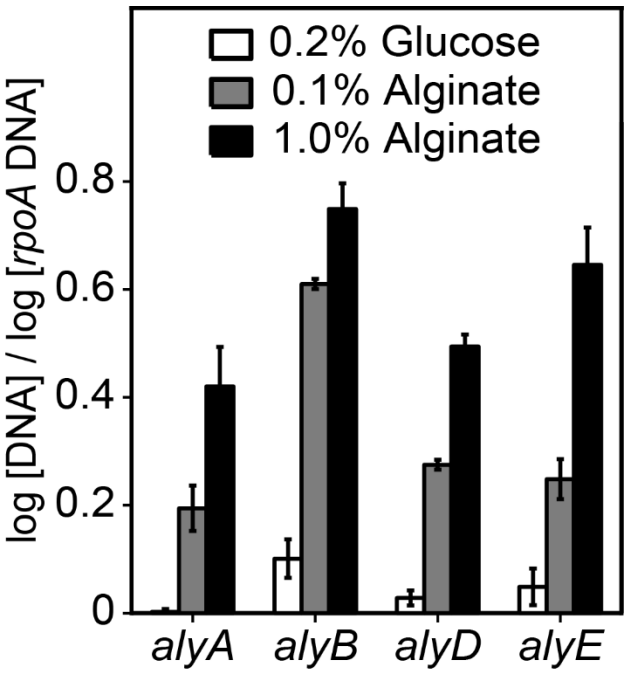


Figure 3. SDS-PAGE of over-expressed and purified AlyA, AlyB, AlyD, and AlyE.

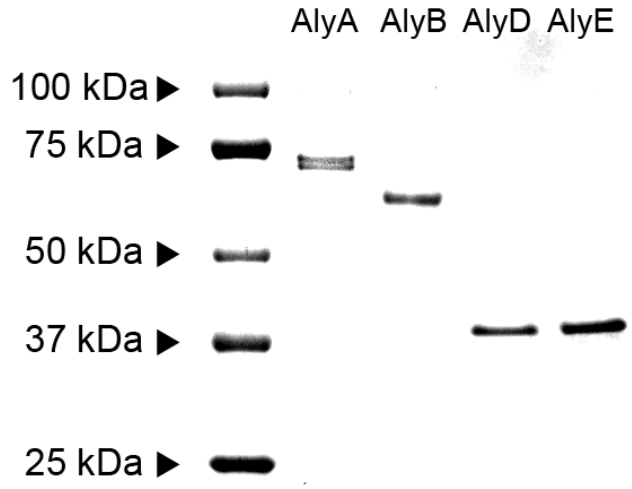


Figure 4. Determination of optima and required cations for enzymatic activity. a) The optimal pH for enzymatic activity was determined in 0.05% alginate, 20 mM APT at indicated pH, and 250 mM NaCl incubated at 20 °C. Activity was normalized to the maximal pH. b) The optimal temperature for enzymatic activity was determined in 0.05% alginate, 20 mM APT at pH 7.5, and 250 mM NaCl incubated at indicated temperatures for 20 min. Activity was normalized to the optimal temperature. c) The optimal NaCl concentration for enzymatic activity was determined in 0.05% alginate, 20 mM APT at pH 7.5, and indicated NaCl concentration incubated at 20 °C for 20 min. Activity was normalized to the optimal NaCl concentration. d) The effect of divalent cations was determined in 0.05% alginate, 20 mM APT at pH 7.5, and 250 mM NaCl incubated at 20 °C for 20 min. 400 mM NaCl was used for AlyE. Divalent cations and EDTA were added at 1 mM. Activity was normalized to the no addition experiment.

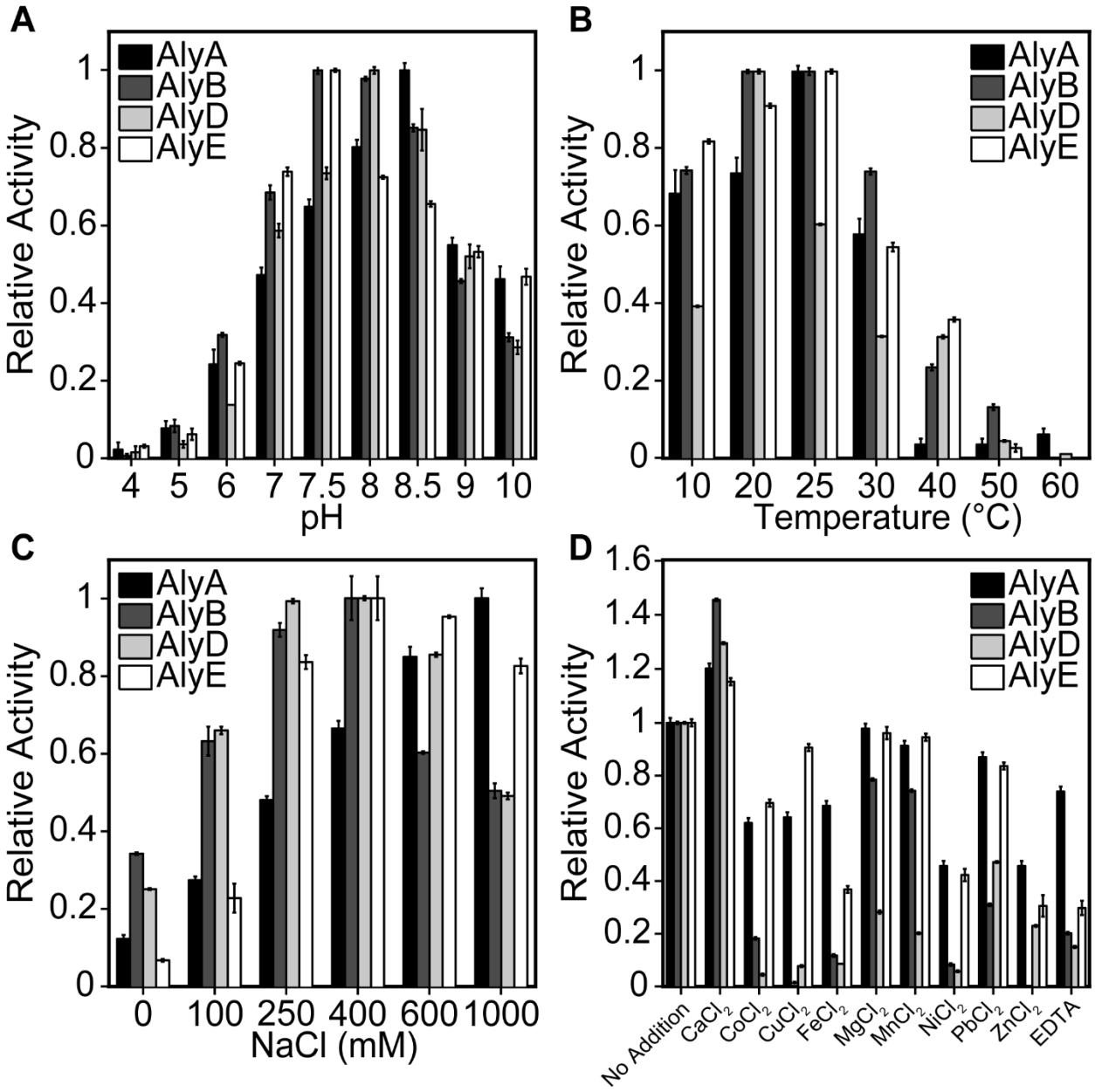


Figure 5. Activity of full AlyA alginate lyase and AlyA domains 1 and 2. The activity is defined as the production of 2-Deoxy-D-glucose equivalent concentration per second per µM protein. The enzymatic activity of the full AlyA enzyme and each of the alginate lyase domains independently over-expressed was evaluated in 0.05% of alginate dissolved in 20 mM APT at pH 8.5 with 250 mM NaCl and incubated for 20 minutes at 20 °C. When considering the gram loading, domain 2 was found to have an almost 3-fold greater activity per µg enzyme than the full form.

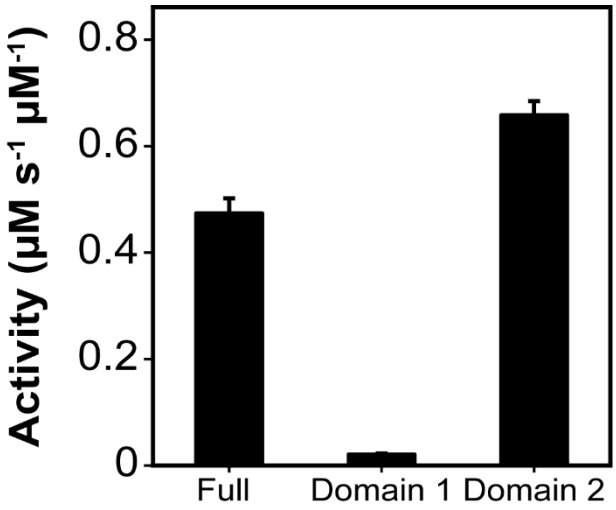


Figure 6. Alginate dose curve of AlyA, AlyB, AlyD, and AlyE. Optimal pH, temperature and NaCl concentration from **Table 3** and 1 mM $CaCl_2$ were used to find the V_{max}, K_m, and turnover number for each lyase. The mixture was incubated for 20 min. a) AlyA, b) AlyB, c) AlyD, d) AlyE.

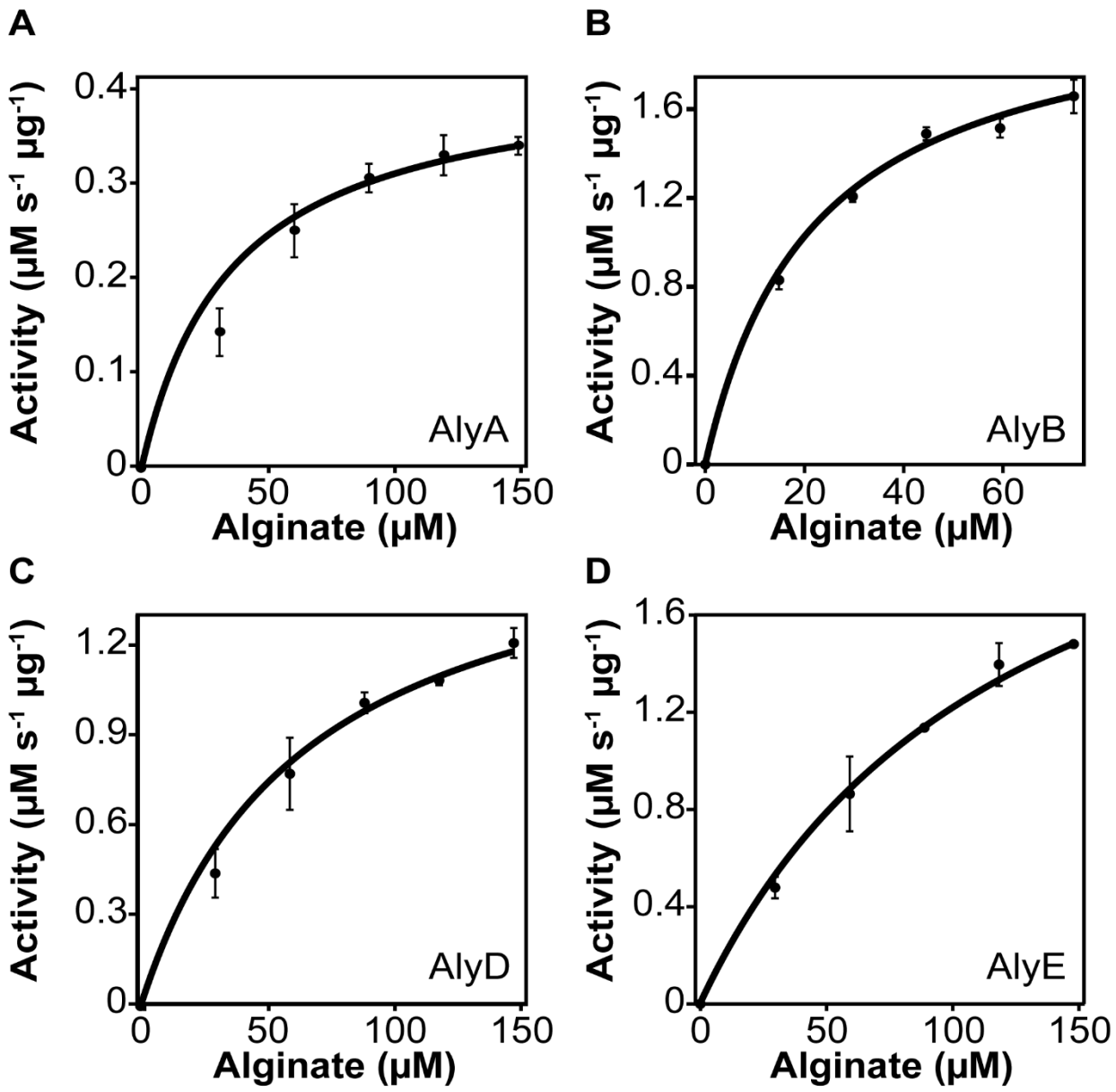
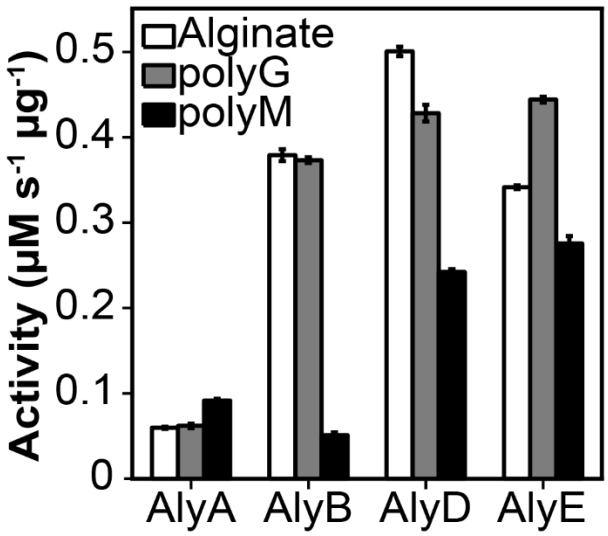


Figure 7. Substrate specificity of AlyA, AlyB, AlyD, and AlyE. 0.05% of alginate, polyG-enriched, and polyM-enriched was dissolved in 20 mM APT at pH 7.5 with 250 mM NaCl, incubated for 20 minutes at 20 °C, and the enzymatic activity was determined.



CHAPTER 4: DISCUSSION

A number of studies have recently explored alginate metabolism in *V. splendidus* 12B01 (48). These studies are motivated by the potential of producing fuels from alginate (18, 35). In the present study, we investigated the alginate lyases from *V. splendidus* 12B01. Four enzymes – AlyA, AlyB, AlyD, and AlyE – were purified, and the enzymatic properties were characterized. An additional gene, *alyC*, is annotated as a putative alginate lyase, however, we did not detect any alginate lyase activity (data not shown). Unlike the other lyases, AlyC contains a PL6 domain whereas the other enzymes contain PL7 domains.

The PL7 domain contained within AlyA, AlyB, AlyD, and AlyE contains three adjacent β-strands within a structurally rigid cleft. Through comparison to other PL7 family alginate lyases, we identified the catalytic residues within the cleft **(Figure 1b)**. AlyB also contains a CBM32 domain; this domain has been demonstrated to bind galactose, N-acetylglucosamine, and the disaccharide N-acetyl-D-lactosamine (49). The presence of this domain within AlyB indicates the possibility of AlyB binding other carbohydrates. The CBM32 domain appears in a PL7 alginate lyase *from Zobellia* galactanivorans (50), in addition to a characterized homolog of AlyB found in *Pseduoalteromonas* sp. CY24 (51). The role of CBM32 within these alginate lyases remains to be determined.

The investigated alginate lyases were found to have an optimal pH for enzymatic activity between 7.5 and 8.5, an optimal temperature between 20 and 25 °C, and an optimal NaCl concentration of either 400 mM or 1000 mM NaCl **(Table 3 and Figure 4A-C)**; these conditions coincide with marine environments (36). The conditions for

optimal enzymatic activity were investigated to further understand how *V. splendidus* 12B01 metabolizes alginate. Previous work sought to exploit these lyases (18), so an understanding of the activity of these lyases in different environmental situations is critical for their commercial use. The presence of signal peptides in AlyB, AlyD, and AlyE **(Figure 1B)** indicates possible secretion. The investigation of divalent cations found that only Ca^{2+} increased enzymatic activity, while all other cations either decreased activity or had no effect **(Figure 4D)**. The stimulatory effect of $CaCl_2$ has been shown in an alginate lyase from a *Littorina sp* sea mollusc. (52), along with a lyase from the bacterium *Azotobacter chroococcum* (28).

The K_m values of the other investigated alginate lyases from marine bacteria range from 6.8 µM to 6.18 mM (11, 22, 48, 50, 53, 54), however, the majority of these enzymes have K_m values less than 238 µM, in accordance with the 22 to 123 µM affinity of AlyA, AlyB, AlyD, and AlyE. AlyA, AlyB, AlyD, and AlyE were found to have turnover numbers less that those reported (22, 48, 50, 53, 54); the reported turnover numbers ranged from 0.052 to 164 s^{-1} with the majority of lyases having turnover numbers two-fold to more than 23-fold greater than those investigated in this study.

The oligoalginate lyases OalA, OalB, and OalC (lyases which degrade alginate exolytically) of *V. splendidus* 12B01 have been recently characterized (48). The enzyme kinetics of OalA, OalB, and OalC were found to be remarkably different than the lyases in this study. The K_m values were of similar magnitude, however turnover numbers were all greater for OalA, OalB, and OalC, indicating that these lyases process alginate at greater rates. The differing kinetic rates indicate that exolytic and endolytic activity have different rates of degrading alginate in *V. splendidus* 12B01. This presents a picture of

metabolism of alginate within *V. splendidus* 12B01 wherein AlyA, AlyB, AlyD, and AlyE slowly degrade alginate into longer chains of oligomers. The oligoalginate lyases are then employed to quickly degrade these smaller oligomers into the monomers needed for metabolism.

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