主論文

Studies on alginate lyases from an alginolytic bacterium *Hydrogenophaga* sp. strain UMI-18 that produces poly(3-hydroxybutylate)

ポリヒドロキシ酪酸合成アルギン酸資化細菌 *Hydrogenophaga* sp. UMI-18株のアルギン酸リアーゼに関する研究

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Studies on alginate lyases from an alginolytic bacterium

***Hydrogenophaga* sp. strain UMI-18 that produces poly(3-hydroxybutylate)**

Alginate is a major structural polysaccharide from brown seaweeds, which is composed of β-D-mannuronate (M) and α-L-guluronate (G). These uronic acid units build up poly(M) block, poly(G) block and mixed M and G block (poly(MG) block) of the alginate polymer. Alginate has been widely used as a viscosifier and gelling agent in food, pharmaceutical and biotechnological industries and it is recognized as a viable biomass to produce biofuels and biomaterials. Recently, a novel alginolytic bacterium *Hydrogenophaga* sp. strain UMI-18 has been isolated, and it was shown to produce poly(3-hydroxybutylate) (PHB) using alginate as its sole carbon source. While the PHB synthesizing capability of strain UMI-18 have been studied to some extent, there is still a need to understand how this bacterium breakdown the alginate substrate to produce an unsaturated monosaccharide 4-deoxy-L-*erythro*-5-hexoseulose uronic acid (DEH), which is used as a carbon source for PHB synthesis.

In **Chapter 1**, the significance of PHAs as an alternative to petrochemically derived plastics is introduced along with bacteria that can use seaweeds as a fermentable biomass. There are only a few strains described that utilize seaweed carbohydrates in PHA production. To date, strain UMI-18 is the only alginolytic and PHB-producing bacterium reported. The importance of understanding the mechanism of alginate degradation of this strain and its connection to its PHB-synthesizing capability have also been described in this chapter. Furthermore, the alginate lyases of strain UMI-18 can also be used as a tool to produce oligoalginates which have many important applications.

In **Chapter 2**, the characteristics in growth and the PHB production of strain UMI-18 were investigated. This strain produces PHB in the alginate mineral salt (AMS) medium containing 1% (*w/v*) sodium alginate as its sole carbon source. PHB yield by strain UMI-18 was 1.1 ± 0.15 g/L of AMS and 58 ± 4% (*w/w*) of the dried cell pellet. Sugars like glucose, fructose, galactose, mannose, mannitol, and sucrose were also utilized by this strain for PHB production. PHB yield in 1% (*w/v*) carbohydrate media reached 2.03 – 2.24 g/L for glucose and fructose, 0.75 – 1.64 g/L for alginate, galactose, mannitol and sucrose, and ~0.15 g/L for lactose. The PHB produced by this strain had a glass-transition temperature (T_g) at 4°C, melting temperature at 175°C and an average molecular mass of 860 kDa. Draft genome analysis of strain UMI-18 revealed the co-occurrence of alginate-assimilating and PHB-synthesizing gene clusters. Four alginate lyase genes, *HyAly-I* – *HyAly-IV* were annotated in the alginate-assimilating gene cluster.

In **Chapter 3**, primary structures of the alginate lyases encoded by *HyAly-I* – *HyAly-IV* were characterized. Based on the primary structures of HyAly-I, HyAly-II, HyAly-III and HyAly-IV, the four alginate lyases were classified to PL-17, PL-7, PL-15 and PL-5, respectively. HyAly-I and HyAly-III are considered exolytic alginate lyases; whereas, HyAly-II and HyAly-IV are endolytic lyases. The two endolytic alginate lyases contain signal peptides suggesting their

localization in the periplasmic space of the cell, while the two exolytic alginate lyases are thought to be localized in the cytosol. Out of the four alginate lyases, HyAly-I, HyAly-III and HyAly-IV were subjected to the expression in *E. coli*. However, only PL-17 recHyAly-I was successfully produced. The purified recHyAly-I showed a major band of 80 kDa on SDS-PAGE which was well consistent with the molecular mass predicted from the primary structure.

In **Chapter 4**, biochemical properties of the recHyAly-I were investigated. recHyAly-I showed optimal temperature and pH at 40°C and pH 6.0, respectively, while it was unstable at temperature higher than 30°C and pH lower than 6.0 and higher than 7.0. recHyAly-I directly released DEH from polymer substrates and showed the highest activity toward poly(M) and poly(MG) blocks of alginate. The degradation products of recHyAly-I were analyzed by TLC and ESI-MS. Interestingly, recHyAly-I was found to produce dimeric DEH (diDEH), a novel unsaturated disaccharide, along with monomeric DEH. Production of diDEH was prominent in the degradation of trisaccharides. This indicates that diDEH is produced in the final phase of the exolytic degradation of alginate.

In conclusion, *Hydrogenophaga* sp. strain UMI-18 is a promising bacterium that can be used as a tool to explore brown seaweeds as a viable fermentable biomass in bioplastic production. Strain UMI-18 contains alginate-assimilating and PHB-synthesizing genes that can serve as a template to allow researchers to design new metabolic pathways of strains that can utilize seaweed biomass to produce high value materials. recHyAly-I was found to produce a novel dimeric DEH directly from poly(M) and alginate trisaccharides.

Abbreviations

AMS	alginate-mineral salt
AGE	agarose-gel electrophoresis
AOS	alginate oligosaccharides
bp	base pair
BSA	bovine serum albumin
CAZy	Carbohydrate Active enZYmes
CBB	Coomassie Brilliant Blue
DDBJ	DNA Data Bank of Japan
DEAE	diethylaminoethyl
DEH	4-deoxy-L-*erythro*-5-hexoseulose uronate
diDEH	dimeric 4-deoxy-L-*erythro*-5-hexoseulose uronate
DNA	deoxyribonucleic acid
DSC	differential scanning calorimetry
EC	Enzyme Commission
ESI-MS	electrospray ionization mass spectrometry
FPLC	fast performance liquid chromatography
NMR	nuclear magnetic resonance
IPTG	isopropyl 1-thio-β-D-galactoside
kDa	kilodalton
MEGA	molecular evolutionary genetics analysis
MHz	megahertz
MS	mass spectrometry

m/z	mass-to-charge ratio
MWCO	molecular weight cut-off
Ni-NTA	nickel-nitriloacetic acid
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PHA	polyhydroxyalkanoate
PHB	poly(3-hydroxybutylate)
PL	polysaccharide lyase
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SM1	Saturated mannuronate monosaccharide
SM2	Saturated mannuronate disaccharide
SM3	Saturated mannuronate trisaccharide
TBA	thiobarbituric acid
T_g	glass transition temperature
TLC	thin-layer chromatography
T_m	melting temperature
UV	ultraviolet
UG3	unsaturated guluronate trisaccharide
UM3	unsaturated mannuronate trisaccharide

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Chapter I: General Introduction

1.1 The global plastic problem

Plastics are high molecular weight polymers that can easily be prepared and molded into different kinds of products. Since they were invented, plastic production has been heavily reliant on the use of petrochemical materials. Approximately 4% of the oil and gas production which is a non-renewable resource, is dedicated in the production of plastic as a feedstock (Hopewell et al., 2009). Polyethylene terephthalate (PET), high density polyethylene (HDPE), polyvinyl chloride (PVC), low density polyethylene (LDPE) and polypropylene (PP) are the most commonly used plastic derivatives and they account for 90% of the total demand in plastic production (Andrady & Neal, 2009).

There is estimated 8,300 million metric tons of virgin plastics produced and 6,300 million metric tons of plastic have been generated as waste. Unfortunately, only 9% of the waste generated is being recycled; the remaining 12% is incinerated and a staggering 79% is disposed in landfills or in the environment that eventually reach the ocean (Geyer et al., 2017).

The buoyancy and durability of plastics make plastic pollution globally widespread in all the world's oceans. As of 2014, there is 5.25 trillion particles floating in all oceans weighing 268,940 tons (Eriksen et al., 2014). The pollution caused by the accumulation of plastics have brought deleterious effects in the marine environment. Marine animals ingest or are entangled with plastic litter. A large number of marine organisms are harmed and or killed with plastic pollution (Derraik, 2002). For example, there is a 90% plastic ingestion rate in seabirds according to different studies conducted from 1962-2012 (Wilcox et al., 2015). Plastics have entered the food chain of these marine organisms and have also moved into the human food chain. Exposure to plastic pollution

is a potential health risk for humans once the pollution has entered in the marine food chain. For example, exposure to bisphenol A and di-(2-ethylhexyl) phthalate (DEHP) is a serious threat especially in pregnant women because these compounds have endocrine-disrupting properties (Halden, 2010). Accumulation of microplastics has chronic effects in marine organisms. In humans, microplastic accumulation causes alteration in chromosomes which lead to infertility, obesity and even cancer (Sharma & Chatterjee, 2017).

There is an urgent need in the sustainable production and consumption of plastics. While reduced consumption, production and recycling are important strategies to the global plastic problem, other solutions should also be explored. One possible solution is the use of microorganisms and enzymes in the biodegradation of plastics. Another answer is to develop biodegradable plastic polymers as a substitute to the conventional plastics which pollute the environment.

1.2 Polyhydroxyalkanoates (PHAs) as alternative to plastics

The problems caused by plastic pollution and the fluctuating price of crude oil are driving factors to look for alternative materials to plastics. Bioplastics are naturally derived polymers from different biomass sources which are considered to have low impact to the environment and are sustainable compared to conventional plastic production. One example of these bioplastics are the polyhydroxyalkanoates (PHAs) which are considered to be good substitute for plastics and can be used for biomedicine, food packaging, textile and household material applications (Keshavarz & Roy, 2010).

PHAs are produced by different microorganisms grown under nutrient stress conditions. They are accumulated intracellularly and have more than 100 monomer units—producing different

types of polymers that are biodegradable with wide-ranging properties (Reddy et al., 2003). These polymers are grouped into three classes of short chain length (scl), medium chain length (mcl) and long chain length (lcl) based on the number of carbons in their side chains. Scl-PHAs have less than 5 carbon atoms, while mcl-PHAs have 5-14 carbon atoms in the side chains. Lcl-PHAs are rare because they have more than 14 carbon atoms (Raza et al., 2018). In bacteria, carbon sources are converted into scl-PHAs like poly(3-hydroxybutyrate-*co*-3-hydroxyvelerate (P3(HB-*c*o-3HV)) or poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) (P(3HB-*co*-4HB)) and mcl-PHAs like poly(3-hydroxyhexanoate-*co*-3-hydroxyoctanoate) (P(3HHx-*co*-3HO)) (Mozejko-Ciesielska & Kiewisz, 2016).

Despite the prospects of PHAs as an alternative to conventional plastics, there are challenges that are needed to be addressed to support its commercialization and market success. First, there is limited choice of PHA diversity to meet wide range of applications. Second, cultivation of bacteria has high energy demands due to sterilization of materials and intensive aeration. Third, there is low rate of substrate to PHA conversion and unstable PHA structures and irregular molecular weights produced. Most of the strains used in PHA production has slow growth and can be easily affected with contamination. Lastly, downstream processes such as purification of PHAs are expensive (Chen & Jiang, 2017; Zheng et al., 2019).

Novel, robust and high throughput strains must be isolated to increase the quantity and improve the quality of PHAs. These new strains must utilize different types of substrate and synthesize PHAs in larger quantity. The isolation of new strains will also give rise to novel PHAs that can be blended with other materials, thus expanding PHA diversity. The new strains along with other described PHA-producing microorganisms can be used as a genetic template to design streamlined and robust metabolic pathways that are dedicated for PHA synthesis and utilization of

various substrates (Reddy et al., 2003; Singh et al., 2019). Additionally, economically and environmentally sustainable biomass must be selected. There should also be an improvement in fermentation technology, process engineering and the design of bioreactors. All of these enhancements must be done to achieve the principles of producing alternative bioplastics in a sustainable way (Koller, 2007).

1.3 Seaweeds as fermentable raw material for PHA production

Various cheap and sustainable raw materials such as fats, oils, glycerol and wastewater along with terrestrial biomass such as starch, molasses, lipids, whey, and lignocelluloses have been used to produce PHAs (Du et al., 2012). PHA production using terrestrial biomass as a feedstock has been investigated and was observed to synthesize of up to 78% (*w/w*) bioplastic from its total cell biomass. By-products of sugar manufacturing industries have also been used as a fermentable biomass, yet, these materials only produce 30% (*w/w*) PHA in dry cell weight. Waste lipids and industrial wastewaters such as cooking oils, crude glycerol, biodiesel, food processing waste, and municipal wastewater have also been used as a fermentable biomass. However, PHA production was also observed at lower rates (Nor Aslan et al., 2016).

A promising alternative substrate in PHA production are seaweeds. Despite their abundance and great potential, seaweed biomasses remain underutilized as an alternative source for value-added biomaterials like PHA (Cesario, et al., 2018). Since they grow in marine environment, cultivation of these marine plants does not compete with land areas intended for agricultural, industrial and residential purposes. Additionally, seaweeds can be cultivated sustainably without heavy nutrient inputs. Seaweeds contain different kinds of carbohydrates which is likely to be a good substrate for PHA production (Marquez et al., 2014). For example, brown seaweeds contain

specific carbohydrates such as alginate, mannitol, laminarin, galactan, and fucoidan (van Hal et al., 2014), which can be used as carbon sources by different kinds of bacteria (Enquist-Newman et al., 2014; Takeda et al., 2011; Takagi et al., 2017; Wargacki et al., 2012).

1.4 Alginate

Alginate is a complex polysaccharide from brown algae that is composed of (1-4)-linked β-D-mannuronic acid (M moiety) and α-L-guluronic acid (G moiety). It is a major component in the cell walls of brown seaweeds. Its units are arranged in three types of blocks, poly β-D-mannuronate (M-block), poly α-L-guluronate (M-block) and a combination of the two blocks (MG-block) (Gacesa, 1988) (Fig. 1-1). Alginates have varied applications because of its viscous and gel-forming properties. It can be used as an emulsion stabilizer, thickener and binder to different materials used in food, textile, animal feed, cosmetics and pharmaceutical products (Lee & Mooney, 2012). However, the pathogenicity of some bacteria like *Pseudomonas aeruginosa* is attributed to its alginate production in its cell wall (Hassett, 1996).

Oligosaccharides can be produced from the alginate polymer to expand the applications of these polysaccharides. For example, alginates can be depolymerized into oligo-alginates through physical and chemical methods such irradiation and acid hydrolysis. Low molecular weight oligo-alginates can be prepared by acid hydrolysis using hydrochloric acid (Hirst et al., 1964), phosphoric acid (Ikeda et al., 2000) or oxalic acid (Haug & Larsen, 1966). Acid hydrolysis is the method commonly used to produce alginate oligosaccharides (AOS); however, this method is expensive. It generates a lower yield and the AOS must be neutralized. Neutralizing the spent acids also generates salt wastes. AOS can also be prepared through irradiation using γ-rays in sodium alginate (Nagasawa et al., 2000; Lee et al., 2003). Irradiated with γ-rays to alginate solutions

resulted in the reduction of viscosity (Lee et al., 2003). AOS can also be derived using organic synthesis (solution-phase-synthesis) of poly-β-mannuronate hexadecasaccharide (Pan et al., 2019). However, these methods do not produce oligosaccharides with similar properties, uniform chain length and molecular weights. The use of enzymes remains to be more advantageous. Enzymes such as alginate lyases help improve the properties of the alginates and other polysaccharides (Wong et al., 2000).

AOS derived from the degradation of alginate with alginate lyases were found to promote growth on the root elongation of barley seedlings. These functional AOS were able to promote and mediate in key processes of plant development such as growth regulation, signal transduction and even defense response (Tomoda, et al., 1994). AOS were able to increase the resistance of tobacco plants to tobacco mosaic virus infection and was also able to stimulate the growth of marine green microalga *Nannochloropsis oculata* (Gonzalez, Castro, Vera, & Alejandra, 2013). AOS at low concentration stimulate endothelial cell growth and migration. AOS with peripheral guluronic acids has the most stimulatory and migratory activities in human umbilical vein endothelial cells (Kawada et al., 1999). AOS demonstrated antioxidant activities by completely inhibiting lipid peroxidation and showed radical scavenging activity in vitro (Falkeborg et al., 2014). They also demonstrate immunostimulatory effects through cytokine secretion from mouse macrophage cell lines and may be applied as an anti-allergic agent (Yamamoto et al., 2007*)*. AOS also improved nitrogen assimilation, basal metabolism and cell division in plants. It also stimulated the growth of green microalgae and helped in the increased production of fatty acid (González et al., 2013).

1.5 Alginate lyases

Alginate lyases (EC 4.2.2.3, EC 4.2.2.11, EC 4.2.2.26) are enzymes that play an essential role in the saccharification of alginate to AOS. They catalyze the degradation of the alginate polymer by β-elimination mechanism that cleaves the 1→4-*O*-linked glycosidic bonds. This cleavage forms a double bond between the C4 and C5 carbons of the new reducing terminal residues resulting in the formation of an unsaturated oligosaccharides (Gacesa, 1987, 1992; Gacesa & Wusteman, 1990). The β-elimination mechanism has three steps which includes: (1) the carboxyl anion is neutralized by an amino-acid residue (usually Lysine) that acts as a salt bridge; (2) a general base catalyzed abstraction of the proton from C5, where one amino-acid residue may be required as the proton abstractor and another residue as a proton donor, although the proton may be derived from the solvent of the environment and (3) the electrons from the carboxyl group are transferred resulting in the formation of double bonds between C4 and C5 and the β-elimination of 4-*O*-glycosidic linked group (Gacesa, 1992; Wong et al., 2000).

There are two types of alginate lyases based on their pattern of degradation, i.e., endolytic and exolytic alginate lyases. Endolytic alginate lyases are enzymes that cleave the internal glycosidic bonds of the alginate polymer, resulting alginate di-, tri-, tetrasaccharides with unsaturated residues in the non-reducing terminus. Exolytic alginate lyases further act on the terminal region of the oligosaccharide and eventually produce the monosaccharide 4-deoxy-L-*erythro*-hexoseulose uronic acid (DEH). These enzymes come from various organisms like marine bacteria (Sawabe et al., 1997; Sawabe et al., 2000; Sugimura et al., 2000; Sawabe et al., 2001; Inoue et al., 2014; Inoue et al., 2016), seaweeds (Inoue et. al., 2015; Inoue & Ojima, 2019), marine invertebrates (Shimizu et al., 2003; Suzuki et al., 2006; Rahman et al., 2012; Ojima et al., 2019). In bacteria, DEH is reduced to 2-keto-3-deoxygluconate (KDG) and further metabolized through the Entner-Duodoroff pathway (Preiss & Ashwell, 1962; Preiss & Ashwell, 1962). Several bacteria

that assimilate alginate contain more than one alginate lyases. For example, *Flavobacterium* sp. strain UMI-01 contain four different alginate lyases that have distinct roles that facilitate in the complete depolymerization of alginate to DEH (Inoue et al., 2016). FlAlyA, an endolytic alginate lyase would first attack on the polymer and produce AOS. Next, FlAlyB, FlAlyC and FlAlex, which are exolytic alginate lyases, would then cleave the AOS in the terminal region of the AOS resulting in the formation of DEH (Inoue et al., 2016). Alginate lyases have also defined by substrate specificity which is based on their dominant cleaving mechanism on guluronate rich or mannuronate rich alginates. They are either M-specific (EC 4.2.2.3) or G-specific (EC 4.2.2.11) alginate lyases (Gacesa, 1988; Wong et al., 2000; Lee & Mooney, 2012; Kanehisa, 2017). Endolytic alginate lyases split internal glycosidic bonds of the alginate polymer to produce di-, tri-, and tetrasaccharides, whereas exolytic alginate lyases (EC 4.2.2.26) act on the terminal region of the polymer and oligomer alginate, thereby producing di- and monosaccharides.

Enzymes that split uronic acid containing polysaccharides through the β-elimination mechanism are grouped into polysaccharide lyase (PL) families. They are grouped based on amino-acid sequence similarity and biochemical properties (Lombard et al., 2010). Alginate lyases are enrolled in different PL families in the Carbohydrate-Active Enzyme (CAZy) database. These families are: PL-5, PL-6, PL-7, PL-14, PL-15, PL-17, PL-18, PL-32, PL-34, PL-36, and PL-39 (www.cazy.org). Most of the endolytic alginate lyases from bacterial sources are listed in PL-5 and PL-7. Over the last decade, the advancement in genome and metagenome sequencing analyses have led to the identification of more gene or protein sequences. The number of predicted carbohydrate-active enzymes (CAZymes) also needs to be continuously updated (Helbert et al., 2019). Exolytic alginate lyases of bacteria are commonly found in PL-15 and PL-17, whereas those from mollusks are classified in PL-14 (Suzuki et al., 2006). Other interesting alginate lyases, such

as bifunctional alginate lyases which act on both poly(M) and poly(G) residues are found in PL-18 family (Zhu & Yin, 2015).

Alginate lyases show differences in their three-dimensional structure. For example, lyases listed in the PL-7, PL-14, and PL-18 families assume a β-jelly roll type of protein folding while the enzymes grouped in PL-6 typically folded in a triple-strand-β-helix. Enzymes belonging to the PL-5 have an $(\alpha/\alpha)_n$ toroid form. Interestingly, PL-15 and PL-17 enzymes adopt a multidomain protein fold (Garron and Cygler, 2010). The alginate lyases are also characterized based on their molecular masses, e.g., small (25 – 30 kDa), medium (~40 kDa), and large (> 60 kDa) (Zhu et al., 2015).

1.6 The alginolytic PHB-producing bacterium *Hydrogenophaga* sp. strain UMI-18

Recently, a novel alginolytic bacterium *Hydrogenophaga* sp. strain UMI-18 that produces PHB using alginate as its sole carbon source was isolated from a seaweed litter (Yamaguchi et al., 2019). Aside from alginate, this strain also used glucose, fructose, galactose, mannose, mannitol, sucrose and lactose for PHB production. The presence of an alginate-assimilating and PHB-synthesis gene clusters in the genome of the strain further confirms its alginate-degrading and PHB-synthesis capability. Interestingly, all annotated alginate lyase genes of this strain are localized in one single gene cluster. This indicates the possibility that the alginolytic gene cluster have been acquired by strain UMI-18 through horizontal gene transfer. The co-occurrence of an alginate-assimilating gene and PHB-synthesizing gene clusters validates the ability of this strain to assimilate alginate and use it as a carbon source for PHB production.

To date, this is one of the few strains described that can utilize seaweed carbohydrates for PHA production. Moreover, this is the first *Comamonadaceae* described that used alginate as its

sole carbon source in PHB production (Yamaguchi et al., 2019). Various bacteria belonging to this family are known to be PHA producers (Reddy et al., 2017).

The isolation and complete characterization of strain UMI-18 has greater implications in the pursuit to the market success and large-scale PHA production. This strain provides the link of PHB production and seaweed biomass utilization. Strain UMI-18 answers the challenges in high production costs of PHA because it can utilize different types of substrates like alginate from seaweeds.

There is a need to understand how strain UMI-18 use alginate as a carbon source in PHB production. It is important to study the roles of alginate lyases in the complete depolymerization of alginate to DEH and how DEH is used as a precursor in PHB synthesis.

To fully realize this, it is imperative to produce stable and soluble proteins that can be used in structural and functional studies of alginate lyases. Genes coding for these alginate lyases should be subjected to recombinatorial cloning, protein expression, screening and purification. This strategy allows researchers to validate the genomic data (Groisillier et al., 2010). It also explores the potential of the strain's alginate lyases as a possible tool to produce novel oligosaccharides and monosaccharides that may have important biotechnological applications to different industries.

Two approaches can be taken in the development of bacterial strains that produce PHA from cheap and accessible carbon sources like seaweeds. Substrate utilization genes like the alginate-assimilating gene clusters can be introduced to PHA producing bacteria or PHA biosynthetic genes can be introduced to a non-PHA producing strain which can utilize inexpensive fermentable material. Metabolic engineering through heterologous gene expression of PHA-synthesizing genes and alginate-assimilating genes also help in the co-production of oligosaccharides from the seaweed biomass. Oligosaccharides from produced from brown seaweeds can be used in various

ways and may also help reduce the cost in PHA production (Chen et al., 2017; Singh et al., 2019; Zheng et al., 2019).

1.7 Aims of the study

This study aimed to characterize the alginolytic PHB-producing bacterium *Hydrogenophaga* sp. strain UMI-18. Specifically, this study aimed to:

1. Characterize the PHA produced by strain UMI-18;
2. Determine the most suitable carbon source for PHA production;
3. Analyze its genome and identify PHA synthesis and alginate lyase genes;
4. Clone alginate lyase genes;
5. Express recombinant enzyme(s) in *Escherichia coli* and;
6. Characterize recombinant enzyme(s) and its degradation products.

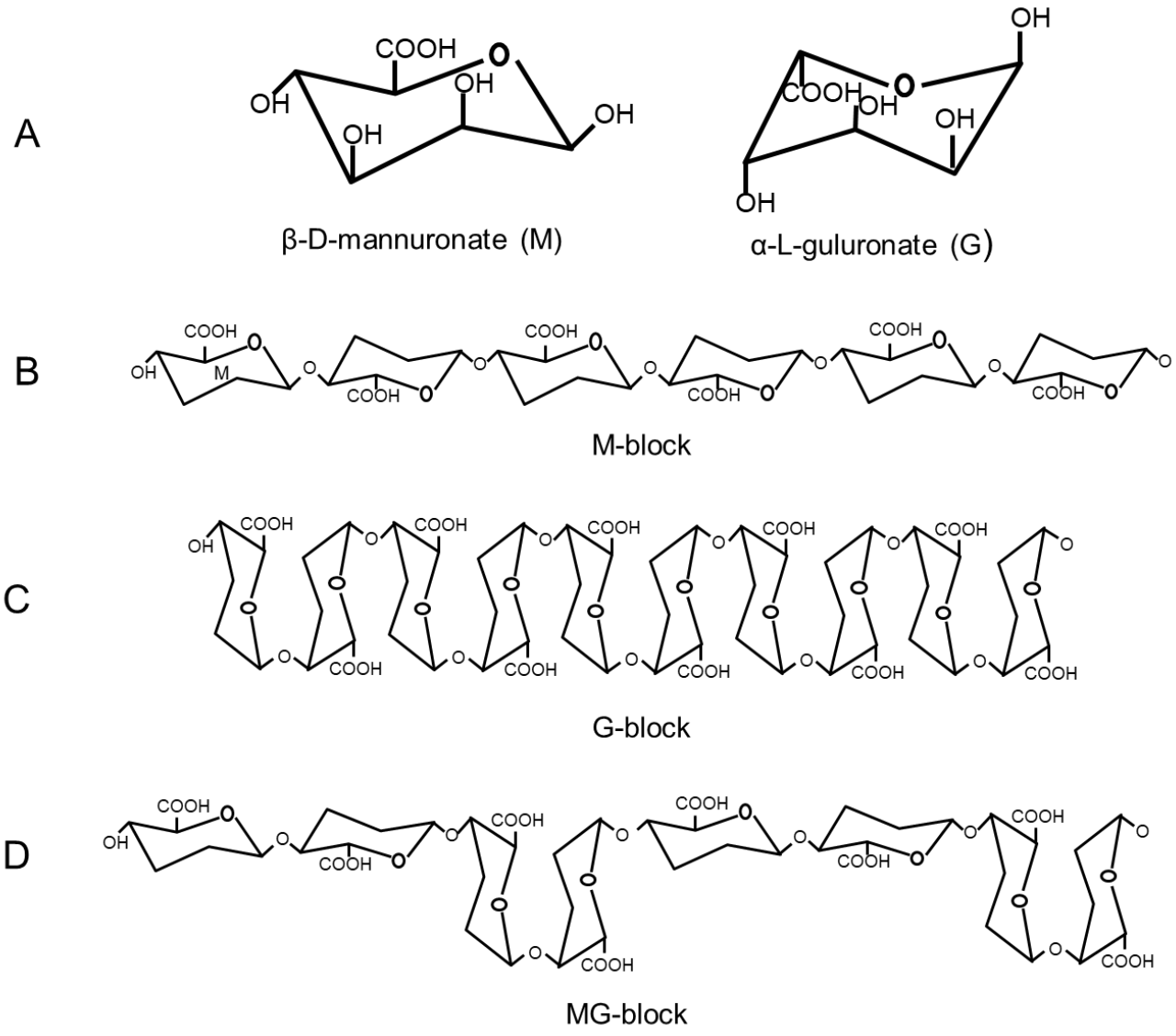


Figure 1-1. Chemical structure of alginate. (A) β-D-mannuronate (M) and α-L-guluronate (G); (B) Covalently linked poly(M); (C) Covalently linked poly(G); (D) Covalently linked poly(MG). Hydroxyl groups in B - D are omitted for simplification.

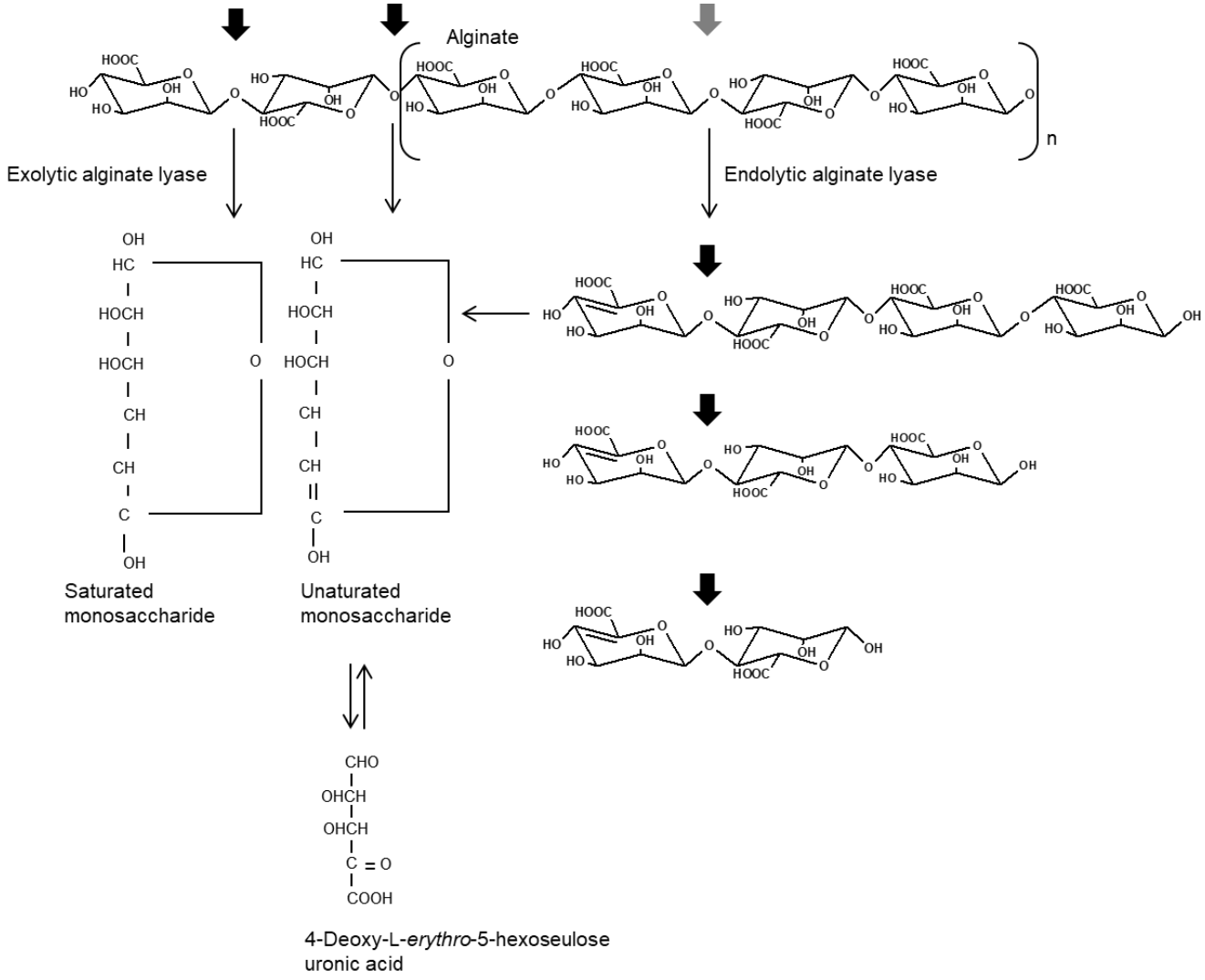


Figure 1-2. Depolymerization of alginate to DEH. Endolytic and exolytic alginate lyases act synergistically to depolymerize alginate to 4-deoxy-L-*erythro*-5-hexoseulose uronic acid (DEH). Black and gray arrows indicated cleavage sites of exolytic and endolytic alginate lyases, respectively. Thin arrows represent the depolymerization pathway (Miyake et al., 2003).

Chapter II: A novel alginolytic bacterium *Hydrogenophaga* sp. strain UMI-18 that uses alginate as a sole carbon source in poly(3-hydroxybutyrate) production

2.1 Introduction

Numerous bacteria produce intracellular carbon- and energy-storage polyesters called polyhydroxyalkanoates (PHA) that are accumulated as granules in the cytoplasm of bacterial cells. PHAs are thermoplastic materials with varying mechanical properties (e.g., brittle, hard, stiff or rubber-like). Thus, these polymers are considered as good substitute for the petroleum derived plastic because of their inherent biodegradability (Lee, 1995).

There are more than 100 monomer units identified in PHA. Poly(3-hydrxoybutytrate) (PHB) is a high molecular mass polymer and the most characterized PHA. Formation of PHB co-polymers such as 3-hydroxyvalerate (3HV) or 4-hydroxybutyrate-co-3-hydroxyvalerate (4HB) monomers are also possible by co-feeding bacteria with different substrates. Combination of 3HV with PHB also results with the formation of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-3HV)] (Reddy et al., 2003).

Despite its great potential as an alternative to petroleum-derived plastics, production of PHAs in an industrial scale has met with limited market success because of expensive carbon source, high production and purification costs (Singh et al., 2019).

Many studies have explored the potential of different bacteria that utilize terrestrial biomass as a carbon source. However, the use of terrestrial biomass in PHB production may give rise to food vs. material conflict as in the case of bioethanol production. On the other hand, seaweed biomass are underutilized carbon sources for PHB production. Brown seaweeds contain approximately 20% alginate and approximately 10% mannitol (*w/w*, dry weight); making them an

attractive carbon source in the production of many biomaterials (Enquist-Newman et al., 2014). Several seaweed-associated bacteria that produce PHB have been isolated such as *Burkholderia* sp. AIU M5M02 and *Massilia* sp. UMI-21 that use mannitol and starch as a sole carbon source in PHB production, respectively (Yamada et al., 2018; Han et al., 2014). Recently, we isolated a PHB-producing marine bacterium *Hydrogenophaga* sp. strain UMI-18 that utilizes alginate as its sole carbon source. It is the first strain reported to have both alginate lyase genes and PHB synthesis genes (Yamaguchi et al., 2019).

This chapter introduces the properties of *Hydrogenophaga* sp. strain UMI-18 and its ability to produce PHB in an alginate mineral salt medium. The yields of the PHB produced from various carbohydrate sources were also determined. PHB-synthesizing genes and alginate-assimilating gene clusters were annotated in the draft genome of strain UMI-18.

2.2 Materials and methods

2.2.1 Isolation of alginolytic PHA-producing bacterium

Decayed brown seaweed material, comprising mostly of *Sargassum* sp. was added to 50 mL of 1% (*w/v*) AMS medium containing 0.36% (*w/v*) Na_2HPO_4, 0.075% (*w/v*) KH_2PO_4, 0.05% (*w/v*) NH_4Cl and 0.02% $MgCl_2$ with 0.2% (*v/v*) trace element solution (0.97% (*w/v*) $FeCl_3$, 0.78% (*w/v*) $CaCl_2$, 0.02% (*w/v*) $CoCl_2.6H_2O$, 0.016% (*w/v*) $CuSO_4.5H_2O$, 0.0125% (*w/v*) $NiCl_3.6H_2O$, and 0.011% (*w/v*) $CrCl_3.6H_2O$ in 0.1 N HCl) and cultivated for a week at 25°C with 150 rpm shaking in a BR-43FL Shaker (TAITEC, Tokyo, Japan). The enriched culture was diluted 10-100 times with MS medium, and each 50 μL of aliquot was spread on a 1.5% (*w/v*) agar plates containing AMS medium and 1 μg/mL Nile-red and cultivated at 25°C for 5 days. The colonies showing red fluorescence upon irradiation at 312 nm were selected as PHA-synthesizing bacteria candidates. Bacterial isolates were identified by the sequence analysis of 16S ribosomal RNA (rRNA) gene

and by the morphological and biochemical analyses at Techno Suruga Laboratory Co. Ltd., (Shizouka, Japan).

2.2.2 Preparation of PHA from *Hydrogenophaga* sp. strain UMI-18

A 25 mL AMS medium preculture was prepared by inoculating strain UMI-18 and incubated at 30°C with shaking at 150 rpm until OD_{600} 1.2. The preculture was then poured into 1 L of AMS medium and further cultivated at 30°C for 72 h. Bacterial pellets were harvested by centrifugation at 4,000x *g* for 20 min and the pellet was added to 1 L of nitrogen-limited AMS medium (NH_4Cl concentration was reduced to 1/10 of the AMS medium) and cultivated at 30°C to induced PHA production. After 72-h cultivation, bacterial cells were collected by centrifugation at 8,000x *g* for 5 min and rinsed with distilled water and then lyophilized. Dried cell pellet was then suspended in 10 mL chloroform and incubated at 60°C for 3 h to extract PHA. The extract was cooled at room temperature and filtered through PTFE membrane. Ten volume of methanol was added to the filtrate to precipitate PHA. Precipitates were then collected by centrifugation at 8,000x g for 5 min and dried in vacuo. The PHA was further purified by repeating methanol precipitation using chloroform solution. The average yield of PHB and its content in the dried cell pellet were estimated from the triplicate experiments and shown with standard deviations.

2.2.3 Characterization of PHA produced by *Hydrogenophaga* sp. strain UMI-18

The crotonic acid method was used to investigate the chain structure of the PHA produced by the strain (Slepecky & Law, 1960). Briefly, 0.01 mL of PHA (~1 mg/ml in chloroform) was added to 10 mL of concentrated sulfuric acid and incubated at 100°C for 10 min. It was then cooled at room temperature for 10 min and the UV absorption spectrum was recorded using a U-3010

spectrophotometer (Hitachi High Tech Science, Tokyo, Japan). Crotonic acid formation from PHB was evaluated by detecting a maximum absorption at 235 nm. Structure of PHA was studied by ^{1}H NMR with ECP-400 NMR spectrometer (400 MHz; JOEL, Tokyo, Japan) at the Global Facility Center of Hokkaido University, Sapporo, Japan. Differential scanning calorimetry (DSC) profile of the PHA was determined using DSC1 calorimeter (Mettler Toledo, Tokyo, Japan). Temperature scanning was carried out at 10°C per min from 50°C to 200°C with a constant nitrogen flow at 50 mL/min. Viscosity of PHA in chloroform was determined using Ostwald viscometer (16 s solvent flow time). Briefly, PHA was dissolved in chloroform at 60°C to make a 1.0 – 4.0 mg/ml and the flow time of the PHA solution was measured at 30°C to determine the intrinsic viscosity [η]. Molecular weight of PHA was estimated using the formula of Mark-Houwink-Sakurada: $[\eta] = KM^{\alpha}$ with K and α values, 1.1 x 10^{-5} ml/mg and 0.78, respectively (Savenkova et al., 2000).

2.2.4 Bacterial PHA production using different carbon sources

Two mL culture of *Hydrogenophaga* sp. strain UMI-18 in AMS medium with an OD_{600} = 1.2 was transferred to a 100 mL of MS medium containing 1% (*w/v*) glucose, fructose, mannose, xylose, mannitol, sucrose, lactose, starch or cellulose. The medium was incubated at 30°C with 150 rpm shaking. For every 12 h, 1 mL of culture was aseptically withdrawn to measure the cell density with OD_{600}. The amount of PHB produced was also evaluated every 24 h by the crotonic acid method. Briefly, 1 mL of culture was centrifuged at 12,000x g for 2 min and the bacterial pellet was rinsed with distilled water and lyophilized. PHB was extracted from the pellet at 60°C for 3 hours with 1 mL of chloroform. The 5 µL extract was then added to 5 mL concentrated sulfuric acid and incubated at 100°C for 10 min. The amount of PHB was determined by measuring the absorbance at 235 nm of the crotonic acid derived from the PHB using a calibration curve

drawn with a known amount of commercial PHB (Sigma-Aldrich). At the end of the cultivation, i.e., at 120-h cultivation, the remaining media were separately centrifuged at 12,000x g for 5 min, and the dried cell weights and PHB yields were determined to estimate PHB contents in the dried cells. The average values of OD_{600} of the media and PHB yields in triplicate experiments were shown with standard deviations.

2.2.5 Genome analysis of *Hydrogenophaga* sp. strain UMI-18

Genomic DNA of the strain was prepared using ISOHAIR DNA extraction kit (Nippon, Gene, Tokyo, Japan) from a single colony of the strain grown in 1% (*w/v*) sodium alginate - 1.5% (*w/v*) agar in MS. Genome sequence was analyzed using an Illumina Hiseq X Sequencer (Illumina, Inc., San Diego, CA, USA) at the Hokkaido System Science Co. Ltd. (Sapporo, Japan).

Twelve FASTA files of raw reverse and forward sequences were generated from Illumina sequencing were uploaded into the Platform for Assembling Nucleotide Sequence (PLATANUS) database (http://platanus.bio.titech.ac.jp/) for genome assembly.

Assembled with 39 contigs in FASTA format was uploaded to the Microbial Genome Annotation Pipeline database (MiGAP) (https://www.migap.org/) for annotation. Alternatively, the genome data files were also subjected to Local Basic Local Alignment Search Tool (BLAST) to search for other alginate lyase genes.

2.3 Results

2.3.1 Characterization of the alginolytic PHA-producing bacterium

Out of the four strains isolated, one strain (strain UMI-18) was the most promising because it showed the highest growth rate in AMS medium. The 16S rRNA (DDBJ accession number

LC435567) of strain UMI-18 indicated that it belongs to the genus *Hydrogenophaga*. It has the highest sequence similarity to that of *Hydrogenophaga taeniospralis* NBRC strain 102512 (GenBank accession number AB681846) (Fig. 2-1). Strain UMI-18 was Gram-negative and rod shaped (0.7-0.8 x 1.2-1.5 μm) and formed smooth, tallow, circular convex and entire edged colony with the size less than 1 mm. The colonies are yellowish under normal visible light and orange under UV light at 312 nm (in Nile-red staining) (Fig. 2-2). It tested positive in catalase and oxidase tests and negative in acid and gas production form glucose and in oxidation and fermentation tests. These properties indicated that strain UMI-18 belongs to the genus *Hydrogenophaga*. Thus, we named this strain *Hydrogenophaga* sp. strain UMI-18.

2.3.2 Properties of the PHA synthesized by strain UMI-18

Dried cell pellet (1.9 ± 0.3 g) of strain UMI-18 was obtained from 1 L of the nitrogen limited AMS culture after 72-h cultivation and 1.1 ± 0.15 g of PHA was extracted from the pellet. The yield of PHA from the dried pellet was 58 ± 4% (*w/v*). The PHA was regarded as polyhydroxybutyrate (PHB) since the crotonic acid-like derivative with a maximal absorbance of 235 nm was produced by sulfuric acid treatment. The UV-absorption spectrum was indistinguishable from the spectrum of the crotonic acid derived from the commercial PHB (Sigma-Aldrich).

Peak signals of the ^{1}H NMR were observed at 1.27 – 1.28 ppm, 2.44 – 2.63 ppm and 5.22 – 5.30 ppm which can be assigned to methyl, methylene and methine groups of PHB, respectively (Fig. 2-3A). The characteristics of the PHB from strain UMI-18 is like the PHB previously reported (Gerngross & Martin, 1995; Yamada et al., 2018). The glass-transition temperature (*Tg*) and melting temperature (T_m) for the PHB were 4°C and 175°C, respectively (Fig. 3B). The average

molecular weight of the PHB was determined as 860 kDa with an average degree of polymerization at 10,000 based on its viscosity measurements.

2.3.3 PHB production using different carbon sources

The strain was cultivated in AMS medium and also MS medium containing 1% (*w/v*) glucose, fructose, galactose, mannose, xylose, mannitol, sucrose, lactose, starch or cellulose and growth rates and PHB production were examined. OD_{600} of the culture reached 6.9 ± 0.1 in AMS medium at 48 h and then slowly decreased to 5.2 ± 0.1 at 120 h. The alkalization of the AMS medium via the consumption of an acidic substrate, such as the alginic acid, in the medium might be the cause of this decrease. Actually, the pH of the AMS medium increased during 48-hour cultivation. This increase in pH was not observed in other media containing neutral carbohydrate substrates. This strain could also grow in the media containing glucose, fructose, galactose, mannose, mannitol, sucrose and lactose. Maximum OD_{600} and appropriate cultivation time for each carbohydrate was as follows: glucose, 15.6 ± 0.5 at 84 h; fructose, 14.1 ± 0.1 at 84 h; galactose, 9.7 ± 0.1 at 96 h; mannose, 6.3 ± 0.1 at 120 h; mannitol, 8.9 ± 0.2 at 120 h; sucrose, 7.5 ± 0.1 at 120 h; lactose, 1.4 ± 0.1 at 120 h (Fig. 2-4A).

No growth was observed in the medium containing starch, cellulose, or xylose. These results indicated that the strain does not possess amylase, cellulase and xylose-assimilating enzymes. Based on its growth rates in various sugars, glucose and fructose are the most preferable substrate for strain UMI-18. It also indicated that alginate is a good substrate for this strain compared with galactose, mannitol and sucrose. While, a long lag phase before a log phase was observed in mannose and lactose.

PHB yield reached 1.25 ± 0.04 g/L in AMS medium after 48-h cultivation and then decreased to 0.75 ± 0.06 at 120 h. The alkalization of the medium lead to the death phase of the cultivated bacteria. Maximal yields of PHB at appropriate cultivation time with different carbon sources were as follows: glucose, 2.30 ± 0.06 g/L at 96 h; fructose, 2.20 ± 0.04 g/L at 96 h; galactose, 1.65 ± 0.08 g/L at 96 h; mannose, 0.95 ± 0.07 g/L at 120 h; mannitol, 1.26 ± 0.05 g/L at 120 h; sucrose, 1.05 ± 0.12 g/L at 120 h; and lactose, 0.15 ± 0.04 g/L. Using glucose and fructose as carbon sources resulted in the highest PHB yield (2.03 – 2.24 g/L) with a high PHB content (71.4 – 71.8% (*w/v*)) in dried cells.

Moderate PHB yields were observed in alginate, galactose, mannitol, mannose and sucrose media (0.75 – 1.64 g/L) and PHB content (43.2 – 68.5% (*w/v*)) (Fig. 2-4B). Lowest PHB yield was observed in lactose with a yield of 0.15 g/L and a content of ~22.0% (*w/v*) in dried cells. Therefore, glucose and fructose are considered to be its preferred substrate for PHB production; while alginate is also regarded as a good carbon source as galactose, mannitol, mannose and sucrose.

2.3.4 Genome analysis of strain UMI-18

The assembled genome of strain UMI-18 was composed of 38 contigs with 1,004 – 653,793 base pairs. Putative alginate lyase genes designated as HyAly-I to HyAly-IV were classified in PL families (www.cazy.org) -17, -7, -15, and -5, respectively. These alginate lyases were included in an alginate-assimilating gene cluster found in contig 8 (453,520 bp). The genes encoding alginate-metabolic enzymes are important in the assimilation of 4-deoxy-L-*erythro*-5-hexoseulose uronic acid (DEH) to pyruvate and glyceraldehyde-3-phosphate (Fig. 2-5A). Genes encoding short chain dehydrogenases/reductases (SDR) (DEH reducatse, HyDehR-I and II), one encoding 2-keto-3-deoxy-D-gluconate kinase (KDG kinase, HyKdgK) and one encoding for 2-keto-3-deoxy-6-

phosphogluconate aldolase (KDPG aldolase, HyKdpgA) were also annotated. Transporter genes such as HyABC transporter-a, -b, -c, and -d; HyMFS transporter, hyporin were also seen in the genome. These transporters are essential in incorporating alginate oligosaccharides and DEH into the cell. The occurrence of these genes in the strain's genome provides genetic basis of the alginate-assimilating capability of strain UMI-18.

Furthermore, the genes encoding three enzymes responsible for PHB synthesis, i.e., β-ketothiolase (HyPhaA), NADPH-dependent acetoacetyl CoA reducatse (HyPhaB) and Class I PHA Synthase (HyPhaC) were found in contig 15 (653,793 bp) (Fig. 2-5B). The presence of the alginate-assimilating genes and the PHB-synthesizing genes in its genome indicated that strain UMI-18 can assimilate alginate and synthesize PHB.

2.4 Discussion

Hydrogenophaga sp. strain UMI-18 is an alginolytic PHB-producing bacterium that was isolated from decayed brown seaweed litter. There have been many species from *Hydrogenophaga* that produce PHA using various carbon sources like sucrose (Tanamool et al., 2011). Strain UMI-18 is closely related to *Hydrogenophaga pseudoflava* produced PHB using glucose, fructose, galactose, xylose, mannose and lactose (Choi et al., 1995; Koller et al., 2008); while *H. palleroni* produced poly(3-hydroxybutyrate-co-hydroxyvarate) P(3HB-co-3HV) utilizing volatile fatty acid as a carbon source. Despite numerous reports of the capability of different *Hydrogenophaga* species to synthesize PHAs, there has been no report yet of a strain that can utilize alginate as a substrate in PHA production.

The amount of PHB produced by strain UMI-18 accounts for 58% ± 4 (*w/v*) of the dried cell weight. This strain utilizes various substrates like glucose, galactose, mannose, mannitol, sucrose

and lactose for PHB production; however, glucose and fructose were the most preferable substrates. Alginate is considered a good substrate for this strain but the alkalization of the alginate medium during cultivation seems to limit the growth of cells. Therefore, it is important to consider maintaining the pH of the medium to obtain a higher yield of PHB. The molecular weight, T_g, and T_m of the PHB produced by this strain is comparable to *Masilia* sp. strain UMI-21 (Han et al., 2014) and *Burkholderia* sp. strain AIU M5M02 (Yamada et al., 2018), *Azotobacter chroococcum* (Savenkova et al., 2000), *Hydrogenophaga* sp. (Tanamool et al., 2011), *Hydrogenophaga pseudoflava* (Koller et al., 2008), *Cupriavidus necator* (Tanadchangsaeng & Yu, 2012) and *Bascillus megaterium* (Pradhan et al., 2018).

Analysis of the draft genome of strain UMI-18 revealed the presence of alginate-assimilating and PHB-synthesizing gene clusters. This strain appears to have similar alginate-assimilating metabolic pathway to *Flavobacterium* sp. strain UMI-01 (Inoue et al., 2016; Nishiyama et al., 2017) and *Sphingomonas* sp. strain A1 (Takeda et al., 2011). Alginate is initially degraded to monosaccharide (DEH) by the synergistic action of endolytic and exolytic alginate lyases in the periplasmic space and the cytosol. Then, DEH is reduced to KDG by DEH reductases in the cytosol and then KDG is phosphorylated to KDPG by KDG kinases. Lastly, KDPG is split to pyruvate and glyceraldehyde-3-phosphate by KDPG aldolase, and then metabolized through the central metabolic pathway. In strain UMI-18, pyruvate derived from the metabolism of alginate is channeled to the PHB synthesis pathway.

Genes encoding the enzymes responsible for PHB synthesis have been identified in the genome of strain UMI-18. The enzymes β-ketothiolase, NADPH-dependent acetoacetyl-CoA synthetase and Class I PHA synthase are designated as HyPhaA, HyPhaB and HyPhaC genes, respectively. Deduced amino-acid sequences for HyPhaA, HyPhaB, HyPhaC showed 93%, 97%

and 84% identities to putative PhaA (GenBank accession number WP_066088257), PhaB (GenBank accession number WP_066088259) and PhaC (GenBank accession number WP_066088259) of *Hydrogenophaga crassostreae*, respectively, while 72%, 73% and 61% identities of PhaA (GenBank accession number WP_010810132), PhaB (GenBank accession number WP_010810131) and PhaC (GenBank accession number WP_011615085) of *C. necator*, respectively.

The presence of alginate lyase genes was investigated in some members of the family *Comamonadaceae* using BLAST search which included *Hydrogenophaga* sp. and *Makilia* sp. However, no species possessing alginate-assimilating genes was found. This makes strain UMI-18 the first documented alginolytic species of the genus *Hydrogenophaga*. It can be considered that the alginate-assimilating genes were acquired by this strain through horizontal gene transfer from another alginolytic bacteria. BLAST search of the deduced amino-acid sequences of HyAly-I (PL-17), HyAly-II (PL-7), HyAly-III (PL-15) and HyAly-IV (PL-5) of strain UMI-18 shows their sequence identity to other alginate lyases. HyAly-IV and HyAly-III has 50% and 57% sequence identity to A1-III (PL-5 alginate lyase) (GenBank accession number BAB03312.1) and A1-IV (PL-15 alginate lyase) (GenBank accession number BAB03319.1) of *Sphingomonas* sp. strain A1, respectively. HyAly-I and HyAly-II shows 25.1 – 54.2% sequence identity to other PL-17 and PL-7 alginate lyases, respectively.

HyAly-II and HyAly-IV were regarded as endolytic alginate lyases which are secreted in the periplasmic space of the cell (Yamaguchi et al., 2019). While, HyAly-I and HyAly-III were regarded as exolytic alginate lyases which are localized in the cytosol. Alginate polymer is assumed to be initially degraded to AOS by HyAly-II and HyAly-IV in the periplasmic space. The AOS are then transported into the cytoplasm via the ATP binding cassette transporter. This is

followed by the degradation of the oligosaccharides into the monosaccharide DEH by exolytic alginate lyases HyAly-I and HyAly-III.

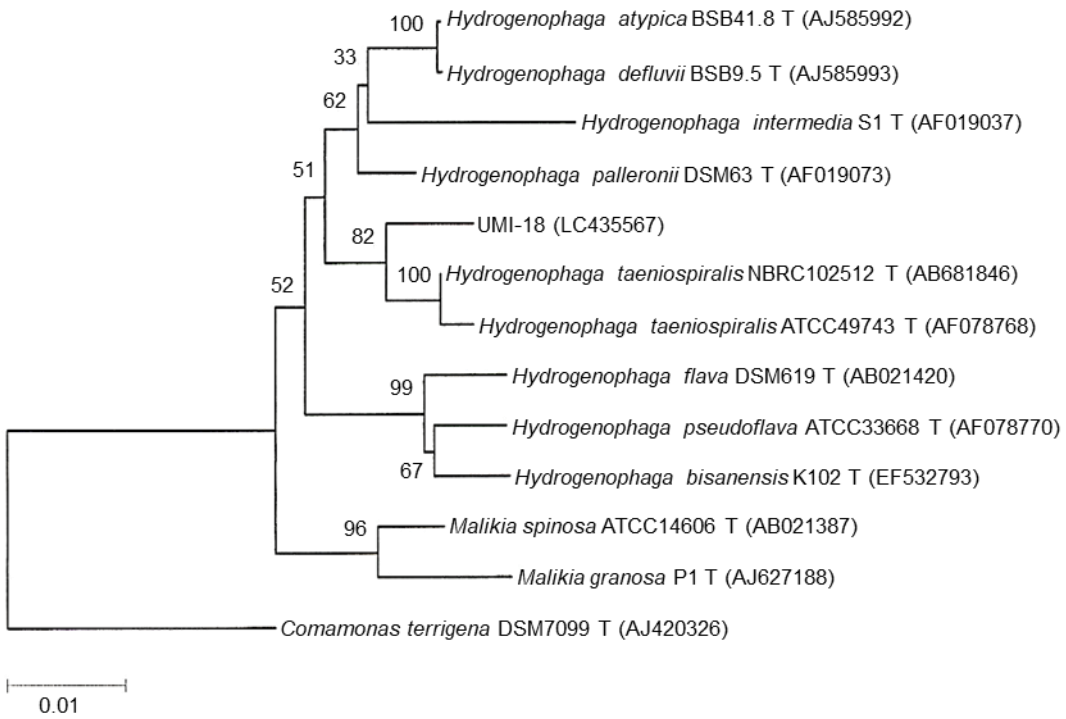


Figure 2-1. Phylogenetic tree of the 16S rRNA gene sequences of *Hydrogenophaga* sp. strain UMI-18 and other associated bacteria. The tree was generated using neighbor-joining method. *Comamnas terrigena* DSM7099T was used as an outgroup. Numbers at the tree branches indicate the bootstrap values using 1,000 replicates.

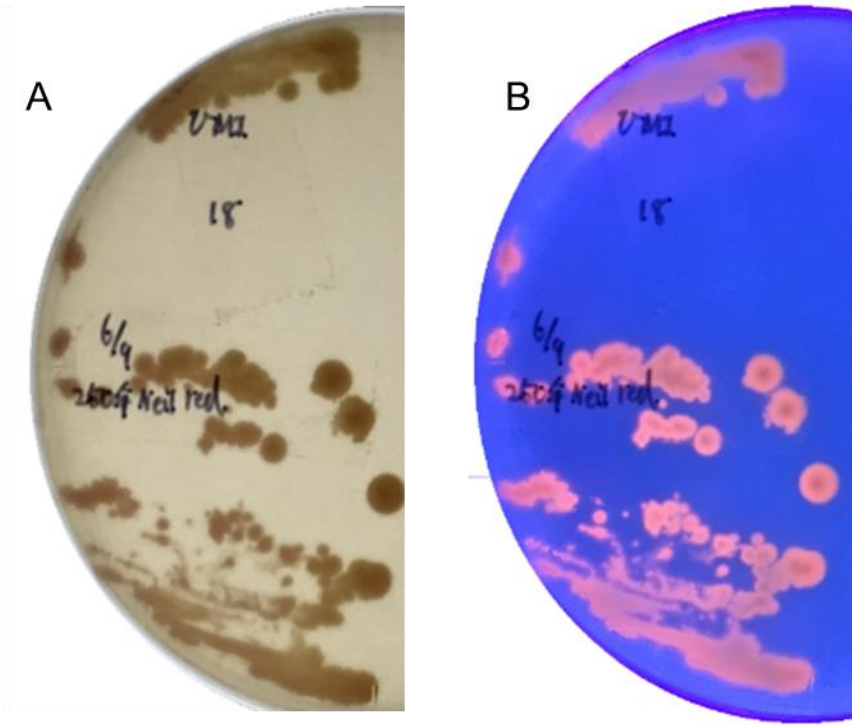


Figure 2-2. Growth of *Hydrogenophaga* sp. strain UMI-18 in 1% alginate in MS-agar plate with Nile red. (A) Agar plate viewed under visible light. (B) Agar plate viewed under UV light at 312 nm.

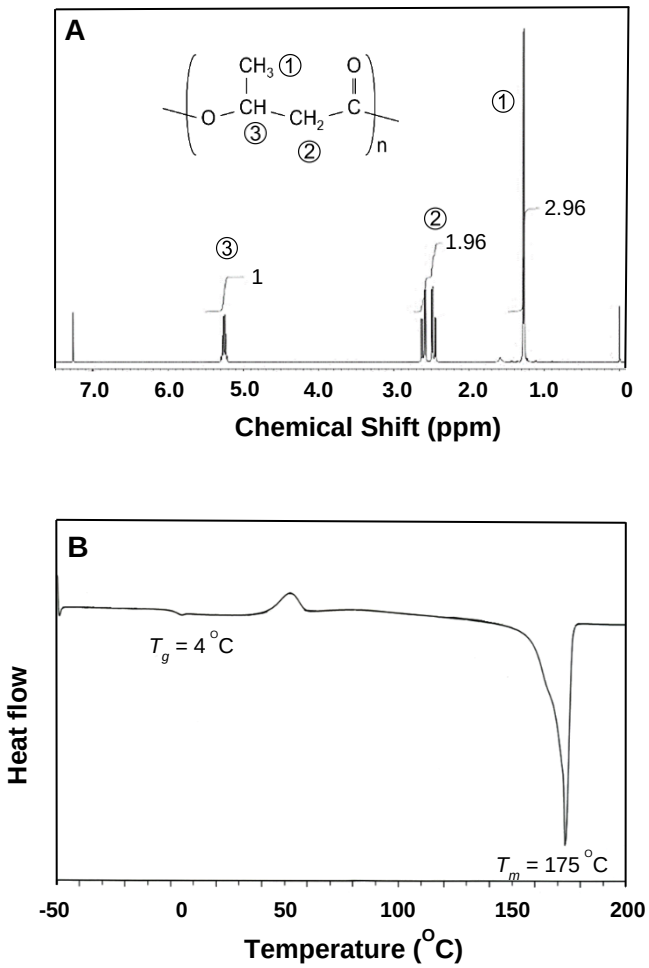


Figure 2-3. NMR and DSC analyses of PHA produced by *Hydrogenophaga* sp. strain UMI-18. (A) ^{1}H NMR spectrum of PHA from strain UMI-18. PHA from strain UMI-18 was dissolved in deuterated chloroform to make 5 mg/mL and the ^{1}H NMR was measured at 27°C using an EPC - 400 NMR spectrometer (400 MHz, JOEL). Numbers in circles indicate the respective proton atoms and their corresponding signals, respectively. Integral values for the respective signals are also shown. (B) DSC thermogram of PHB from strain UMI-18. DHC profile of PHB was recorded under nitrogen gas flow of 50 mL/min and temperature increasing rate of 10°C/min using DSC1 calorimeter (Metler Toledo). T_g, glass transition, T_m melting/temperature.

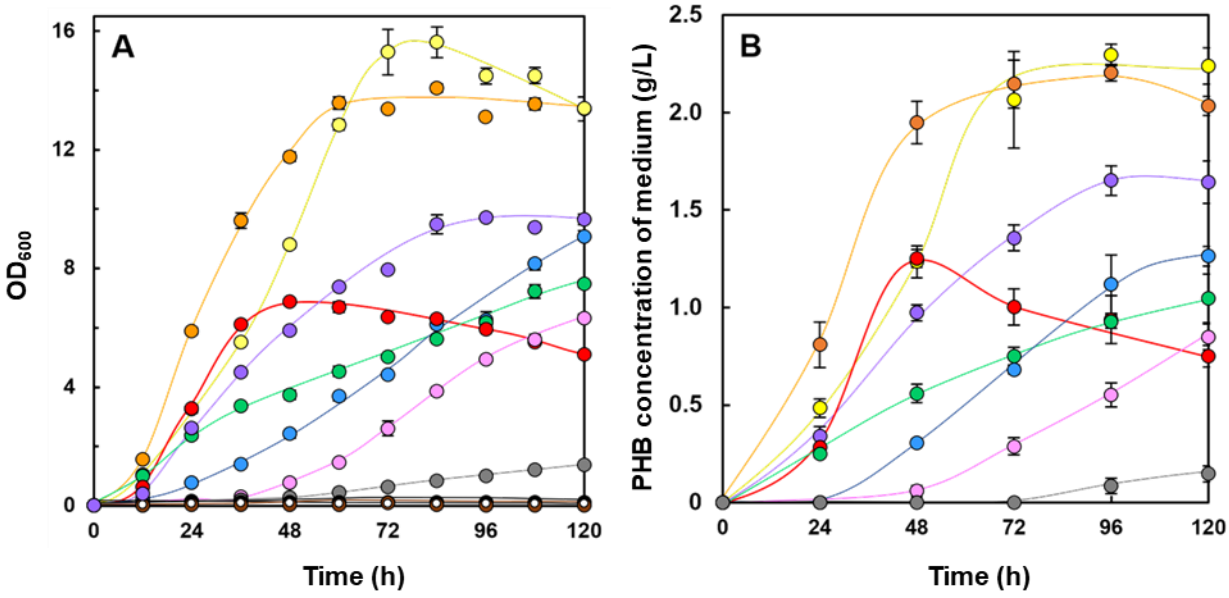


Figure 2-4. Growth of strain UMI-18 and production of PHB in MS media containing different carbon sources. (A**)** Growth curves of strain UMI-18. Strain UMI-18 was grown at 30°C in MS media containing 1% different carbohydrates, i.e., alginate (AMS medium, red), glucose (yellow), fructose (orange), galactose (purple), mannose (pink), mannitol (blue), xylose (white), sucrose (green), lactose (gray), starch (brown), and cellulose (black). (B) Production of PHB by strain UMI-18. One millimeter of each culture medium was withdrawn and centrifuged at appropriate time intervals and PHB was extracted from the cell pellet after lyophilization. The amount of PHB extracted was determined by crotonic acid method. Color and symbols are the same as in A.

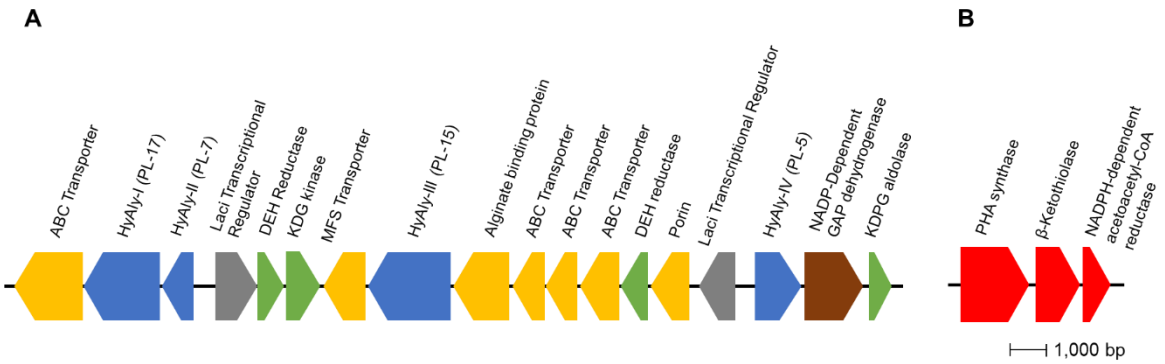


Figure 2-5. Schematic representation of alginate-degrading gene cluster and PHB-synthesizing gene cluster of *Hydrogenophaga* sp. strain UMI-18. (A) four alginate lyase genes (HyAly-I, HyAly-II, HyAly-III and HyAly-IV) are all found in one single gene cluster along with other carbohydrate transporter genes. Alginate lyase genes (blue), DEH-metabolic enzyme genes (green), transporter genes (yellow), transporter regulator gene (gray) (B) PHB synthesis genes.

TABLE 1. Dried cell weights, PHB yields and PHB contents of strain UMI-18

Carbon sources (1% (*w*/*v*))	Dried cell weight (g/L)	PHB yield (g/L)	PHB content (% (*w*/*w*))
alginate	1.73±0.24	0.75±0.06	43.2±3.21
glucose	3.13±0.15	2.24±0.09	71.4±2.99
fructose	2.83±0.15	2.03±0.05	71.8±1.74
galactose	2.40±0.25	1.64±0.11	68.5±4.54
mannitol	2.27±0.18	1.26±0.05	55.7±2.27
mannose	1.73±0.15	0.85±0.07	49.0±3.85
sucrose	1.97±0.10	1.05±0.12	53.2±6.27
lactose	0.67±0.12	0.15±0.04	22.0±6.15

TABLE 2. Properties of PHB produced by *Hydrogenophaga* sp. strain UMI-18 and other PHB-producing bacteria.

Strain	Carbon sources	T_g[a] (°C)	T_m[b] (°C)	Mw[c] (KDa)	References
Hydrogenophaga sp. strain UMI-18	Alginate	4	175	860[d]	Present study
Azotobacter chroococcum	Sucrose	2.6-4.1	177.6-179.8	1740 – 2378[d]	Savenkova et al. (2000)
Hydrogenophaga pseudoflava	Lactose	N.D.[f]	178.9	827[e]	Koller et al. (2008)
Hydrogenophaga sp.	Sucrose	2.32	173.31	N.D.[f]	Tanamool et al. (2008)
Cupriavidus necator	Glucose	3.8	175.1	890[e]	Tanadchangsaeng & Yu (2012)
Massilia sp. strain UMI-21	Starch	N.D.[f]	N.D.[f]	760[e]	Han et al. (2014)
Burkholderia sp. strain AIU M5M02	Mannitol	N.D.[f]	N.D.[f]	1860[e]	Yamada et al. (2018)
Bacillus megaterium	Fructose	6	176	N.D.[f]	Pradhan et al. (2019)

[a]T_g, glass transition temperature.

[b]T_m, melting temperature.

[c]*Mw*, molecular weight; [d]calculated by viscosimetric measurements; [e]measured by size exclusion chromatography.

[f]N.D., not determined.

Chapter III: Gene cloning and recombinant protein expression of alginate lyases from *Hydrogenophaga* sp. strain UMI-18

3.1 Introduction

Hydrogenophaga sp. strain UMI-18 is an alginolytic bacterium that produces poly(3-hydroxybutylate) (PHB) using alginate as its sole carbon source. Genome analysis of strain UMI-18 revealed that it has both PHB-synthesizing and alginate-assimilating gene clusters. Four alginate lyase genes, *HyAly-I – HyAly-IV*, were annotated in the alginate-assimilating gene cluster. The translational products of these genes, i.e., HyAly-I, HyAly-II, HyAly-III and HyAly-IV, were predicted as members of the polysaccharide lyase (PL) family-17, -7, -15 and -5, respectively. These enzymes synergistically depolymerize alginate to the monosaccharide, which is then enzymatically and non-enzymatically converted to DEH. After, DEH is reduced to 2-keto-3-deoxy-D-gluconate (KDG) and then metabolized through the Entner-Doudoroff pathway to pyruvate and glyceraldehyde-3-phosphate. These are eventually used by the strain in PHB synthesis.

This chapter focuses on the molecular cloning and expression of alginate lyase genes of strain UMI-18. First, the primary structure of HyAly-I – HyAly-IV were studied through phylogenetic analysis and comparison with other characterized enzymes from their respective PL families. Then, the recombinant alginate lyases were expressed with the *Escherichia coli* expression system comprising pCold-I or pET-21b (+) expression vector and *E. coli* BL21 (DE3). Some basic properties of the recombinant enzymes were also investigated.

3.2 Materials and methods

3.2.1 Materials

*Bam*HI and *Hind*III, Premix Taq polymerase, and In-Fusion HD cloning kit were purchased from TaKaRa Bio (Tokyo, Japan). The plasmids pET-21(b+) and pCold-I, *Escherichia coli* DH5α and *Escherichia coli* BL21(DE3) were from Novagen-Merck KgaA (Dermstadt, Germany). ISOHAIR DNA extraction kit was from Nippon Gene Co., Ltd. (Tokyo, Japan). TOYOPEARL DEAE 650-M was from Tosoh (Tokyo, Japan). Other reagents used were purchased from FUJIFILM Wako Pure Chemical Industry Ltd. (Tokyo, Japan).

3.2.2 Amino acid sequence analyses of alginate lyases

Amino acid sequences of alginate lyases were retrieved from the DNA Data Bank Japan (DDBJ) (https://www.ddbj.nig.ac.jp/index-e.html), GenBank (https://www.ncbi.nlm.nih.gov/genbank/), and the CAZy databases. Phylogenetic tree was generated using the NJ model through MEGA X software (Kumar et al., 2018). Signal peptide of the alginate lyases was predicted by SignalP 5.0 (http://www.cbs.dtu.dk/services/SignalP/). Molecular weight was calculated from the amino-acid sequences of the alginate lyases using ExPASy Bioinformatic Resource Portal (https://web.expasy.org/compute_pi/).

3.2.3 DNA purification

Genomic DNA of *Hydrogenophaga* sp. strain UMI-18 was prepared using ISOHAIR DNA extraction kit from a single colony of the strain grown in an MS agar plate containing 1% (*w/v*) sodium alginate.

3.2.4 Agarose gel electrophoresis

DNA samples were analyzed by 1% (*w/v*) agarose gel electrophoresis using 1x TAE as a running buffer. The electrophoresis was conducted at 100 V for 30 - 35 minutes, and the DNA band was detected by the transilluminator (E-Graph, ATTO) after staining with 0.5 μg/ml ethidium bromide.

3.2.5 PCR

Coding regions of *HyAly-I*, *HyAly-II*, *HyAly-III* and *HyAly-IV* were amplified by the PCR using specific forward and reverse primers (Table 1) and the genomic DNA template. Briefly, 1 μl of forward and reverse primers, 1 μl of genomic DNA, 9.5 μl distilled water, and 12.5 μl of Quick Taq HS Dye Mix (TOYOBO, Japan) were added in a PCR tube, then PCR was carried out with the Thermal Cycler Dice Mini (TaKaRa, Tokyo Japan). After the initial denaturation (95°C, 60 s), the reaction cycle comprising denaturation (95°C, 30 s), annealing (60°C, 30 s; 58°C, 30 s) and extension (72°C, 2:30 min) was repeated 30 times. Amplified DNA were subjected to AGE and then target DNA bands were purified using Macherey-Nagel PCR clean-up gel extraction kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany).

3.2.6 Plasmid construction

The pET-21b(+) plasmid was linearized by the digestion with *Bam*HI and *Hind*III and ligated with the PCR products using In-Fusion HD cloning kit according to the manufacturer's protocol. The recombinant plasmids were introduced to *E. coli* strain DH5α and transformants were grown on LB-agar plates containing ampicillin at a final concentration of 100 μg/ml. Individual colonies

from the agar plate were picked and inoculated to a 2 mL Amp-LB broth (100 μg/mL ampicillin, 1% (*w/v*) tryptone, 1% (*w/v*) sodium chloride, 0.5% (*w/v*) yeast extract) and cultured at 37°C for 12 h with 150 rpm shaking. Recombinant plasmids were purified from the cell pellets using Macherey-Nagel plasmid DNA purification kit (Macherey-Nagel, Durin, Germany).

3.2.7 Transformation of *E. coli* BL21 (DE3)

Chosen plasmids were used in the transformation of *E. coli* strain BL21 (DE3). Briefly, recombinant plasmids were added to 50 μl of *E. coli* BL21 (DE3) competent cells then the cells were heat-shocked at 45°C for 90 seconds. Transformants grown on the Amp-LB-agar plates were selected then used for recombinant protein expression.

3.2.8 Recombinant protein expression

A single colony was picked and transferred aseptically to an Amp-LB broth pre-culture. The culture was incubated overnight at 37°C with 150 rpm shaking. Then, 5 mL of the culture was transferred to a 250 ml 2X YT media (1.6% (*w/v*) tryptone, 1% (*w/v*) yeast extract, 0.5% (*w/v*) sodium chloride) and grown until OD_{600} reached 0.5 - 0.6. Protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to make final concentration 1.0 mM. The culture was further incubated at 15°C for 18 - 20 with 150 rpm shaking.

3.2.9 Purification of recombinant enzyme

Culture media were centrifuged at 8,000 x *g* for 10 min at 4°C. Bacterial pellets were dissolved in a lysis buffer containing 10 mM potassium phosphate (pH 7.0), 10 mM imidazole, 1% (*v/v*) Triton-x 100 and then placed in a -80°C freezer for an hour and then thawed at 4°C. The

suspension was then sonicated ten times (each for 10 s) using an Ultrasonic Homogenizer (TAITEC VP 050, Tokyo, Japan) on ice. Cell lysate was collected by centrifugation of the homogenate at 8,000 x *g* 4°C for 10 min.

Recombinant enzymes recHyAly-I, recHyAly-III and recHyAly-IV were successfully expressed after IPTG induction; however, the concentration of recHyAly-I in the soluble fraction was most promising for further purification. Thus, recHyAly-I was further purified (see Fig. 3-6 – Fig. 3-8). Although the recombinant alginate lyases were expressed in an N-terminal His-x6-tagged form, these did not adsorb to the Ni-NTA resin. Therefore, recombinant enzymes were purified by ammonium sulfate fractionation followed by conventional column chromatography. Namely, proteins in the soluble fraction were precipitated in 80% saturation of ammonium sulfate and then dissolved in and dialyzed against 10 mM sodium phosphate buffer (pH 7.0). The proteins were then applied to TOYOPEARL DEAE 650-M column (2.5 x 19 cm) preequilibrated with the same buffer and eluted with a linear gradient of 0 to 0.3 M NaCl in sodium phosphate buffer (pH 7.0). Fractions containing target proteins were pooled and dialyzed against 10 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl. The pooled fractions were then concentrated to 0.5 mL by ultrafiltration using Vivaspin 20/10 kDa MWCO (GE Healthcare, Uppsala, Sweden) and further purified by AKTA FPLC equipped with a Superdex 200 10/300 (GE Healthcare, Uppsala, Sweden).

3.2.10 SDS-PAGE

SDS-PAGE was conducted by the method of Porzio and Pearson (1977) using a 10% polyacrylamide slab gel. After the electrophoresis, the gel was stained with 0.15% (*w/v*) Coomassie Brilliant Blue R-250 in 50% (*v/v*) methanol- 10% (*v/v*) acetic acid and destained with

7% (*v/v*) acetic acid - 5% (*v/v*) methanol. Protein Molecular Weight Marker (Broad) (TaKara, Tokyo, Japan) was used as a molecular weight standard.

3.2.11 Assay for alginate lyase activity

Alginate lyase activity of each fraction was determined using a standard reaction mixture containing 10 mM sodium phosphate buffer (pH 7.0), 0.1% (*w/v*) sodium alginate and 50 μl enzyme. Progress of the reaction was monitored by measuring the absorbance at 235 nm using U-3010 spectrophotometer (HITACHI, Tokyo, Japan) with a thermal controlling unit SP12R (TAITEC). One unit (U) of alginate lyase activity was defined as the amount of enzyme that increases absorbance at 235 nm to 0.01 for 1 min.

3.2.12 Thin-layer chromatography for reaction products

A 100 μL reaction mixture containing 0.5 – 1% sodium alginate, 50 mM sodium phosphate buffer (pH 7.0), and 5 – 10 μL recombinant enzyme was incubated at 30°C for 12 h. Aliquots were collected at 0 h, 1 h, 3h, 6 h and 12 h. Ten μg of the reaction products were applied to the TLC-60 plate (Merck KGaA, Darmstadt, Germany) and developed with a solvent of 1-butanol – acetic acid – water (2:1:1, *v:v:v*). Sugars on the plate were stained by 10% sulfuric acid in ethanol followed by heating at 130°C for 15 min.

3.3 Results

3.3.1 Primary structure of HyAly-I – HyAly-IV

HyAly-I, HyAly-II, HyAly-III and *HyAly-IV* genes are comprised of 2,139 bp, 891 bp, 2,304 bp and 1,293 bp, respectively. The predicted molecular masses of translational produces, HyAly-I, HyAly-II, HyAly-III and HyAly-IV were 78.44 kDa, 31.79 kDa, 86.84 kDa, and 46.78 kDa, respectively. Presence of signal peptide was predicted in the N-terminal regions of HyAly-II and HyAly-IV. Thus, these were considered to be secreted enzymes. According to the phylogenetic analysis, HyAly-I, HyAly-II, HyAly-III and HyAly-IV were regarded as members of PL-17, PL-7, PL-15 and PL-5, respectively (Fig. 3-1).

HyAly-I (713 amino-acid residues) formed a cluster with well characterized PL-17 alginate lyases such as Alg17C from *Saccharophagus degradans* 2-40 (Kim et al., 2012) (Fig. 3-1A). The sequence alignment of HyAly-I showed a relatively low amino-acid identity to other PL-17 enzymes (i.e., 33.6% to 50.1%). HyAly-I consists of two conserved domains like other enzymes in the PL-17 family (Figure 2). It has an AlgLyase superfamily domain (NCBI conserved domain pfam05426) (residue numbers 73 - 270) and a Heparinase II/III-like protein domain (NCBI conserved domain pfam07940) (residue numbers 359-621). HyAly-I has conserved catalytic residues (His176 and Tyr233), substrate-binding residues (Gln120, Asn123, Asn175, Tyr232, Arg235, His392, Arg417 and Glu640), and a metal-binding residue (His394), which had been identified in Alg17C. These alginate lyases catalyze the degradation of alginates via β-elimination, generating a molecule containing 4-Deoxy-L-*erythro*-hex-4-enepyranosyluronate at the non-reducing end. This family of alginate lyases assumes an all alpha fold (Ertesvåg et al., 1999; Yoon et al., 1999).

HyAly-II (297 amino acid residues) grouped with PL-7 enzymes like AlyA from *Klebsiella pneumoniae* subsp. *aerogenes* (Baron et al., 1994) and A1-II' from *Sphingomonas* sp. A1 (Miyake et al., 2004) (Fig. 3-1B). HyAly-II also showed relatively low amino-acid identity (24.1% - 33.1%)

to other PL-7 enzymes (Fig. 3-3). It contains signal peptide (amino-acid residue 0 – 36) in its N-terminal region and has an Alginate_lyase2 superfamily domain (NCBI conserved domain pfam0878) (amino acid residue 48-259). This family forms an all beta fold and is different from all alpha fold of conserved domain pfam05426 (Marchler-Bauer et al., 2017).

HyAly-III (768 amino acid residues) is a unique PL-15 enzyme which has 54% to 57% amino-acid identity to other oligoalginate lyases such as A1-IV from *Sphingomonas* sp. A1 and Atu3025 from *Agrobacterium fabrum* C58 (Fig. 3-4). Like other PL-15 enzymes, HyAly-III contains two catalytic domains: DUF4962 (NCBI conserved domain pfam16332) and Hepar_II_III (NCBI conserved domain pfam07940). HyAly-III has conserved catalytic residues His302, Tyr356 and Tyr542. Conserved substrate-binding residues Trp189, Tyr190, Tyr295, Trp458 and His522 were also found in HyAly-III.

HyAly-IV (431 amino acid residues) also exhibited low sequence identity to other PL-5 alginate lyases like A1-I from *Sphingomonas* sp. A1 (50.0%) and AlgL from *Pseudomonas aeruginosa* PAO1 (46.14%) (Fig. 3-5). It has an AlgLyase superfamily conserved domain (NCBI conserved domain pfam05426) like HyAly-I.

3.3.2 Preparation of recombinant enzymes

Recombinant enzyme recHyAly-I was successfully expressed by pET-21(b+) and *E. coli* BL21 (DE3) (Fig. 3-6), and substantial amount of recHyAly-I could be obtained. recHyAly-III and recHyAly-IV were also successfully expressed by pCold-I and *E. coli* BL21 (DE3) (Fig. 3-6 – 3-9); however, majority of the recHyAlyIII and recHyAly-IV were produced as insoluble proteins. While, recHyAly-II protein could not be produced due to unknown reasons. These results indicate that the expression of recombinant enzymes is not so easy except for HyAly-I.

3.3.3 Initial screening of alginate lyase activity of recombinant enzymes

The *E. coli* BL21(DE3) harboring pET-21b(+) – *HyAly-I – IV* and pCold-I – *HyAly III – IV* were cultured for protein expression. First, alginate lyase activity of bacterial cell lysates was determined with a standard reaction mixture by measuring absorbance at 235 nm. Then, aliquots of the reaction mixture obtained at reaction time 1 h, 3 h, 6 h and 12 h were subjected to TLC to analyze degradation products. Among the three recombinant enzymes, i.e., recHyAly-I, recHyAly-III and recHyAly-IV, only recHyAly-I exhibited distinct and promising activity. The activity was greatly improved by the purification with TOYOPEARL DEAE-650M column (Fig. 3-10). Although no activity was detected in recHyAly-II – recHyAly-IV by measuring the absorbance at 235 nm, degradation products of recHyAly-IV were detected by TLC upon reaction with poly(M) substrate overnight. This indicates that small amount of active recHyAly-IV was released in the soluble fraction of cell lysate and degraded poly(M) block. TLC analyses also indicated that the recHyAly-I has a substrate specificity towards poly(M) (Fig. 3-11).

3.4 Discussion

The primary structure of HyAly-I was found to consist of two domains: one is the alginate lyase (AlgLyase) superfamily domain and another is the heparinase II/III-like protein domain (Hepar_II_III domain) (Fig. 3-2.). AlgLyase domain is common among various bacterial alginate lyases (Ertesvåg et al., 1998). The Hepar_II_III domain is commonly seen in PL-15 and PL-17 alginate lyases, e.g., OalS17 from *Shewanella* sp. Kz7 (Wang et al., 2014), AlgL from *Sphingomonas* sp. MJ-3 (Park et al., 2012), alyII from *Pseudomonas* sp. OS-ALG9 (Kraiwattanapong et al., 1999; Kraiwattanapong et al., 2009), Alg17C from *Saccharophagus*

degradans 2-40 (Kim et al., 2012), OalB and OalC from *Vibrio splendidus* (Jagtap et al., 2014), OAL from *Strenotophomas maltophilia* KJ-2 (In Lee et al., 2012), OalC17 from *Cellulophaga* sp. SY116 (Li et al., 2018), TcAlg1 from *Thalassotalea crasostreae* (Wang et al., 2018) and FlAlyB from *Flavobacterium* sp. UMI-01 (Inoue et al., 2016). However, heparinase activity was not observed in AlgL, OAL, and BP-2 (Park et al., 2012; In Lee et al., 2012; Huang et al., 2019). It is suggested that the Hepar_II_III domain is involved in alginate binding (Wang et al., 2018). Therefore, this domain may have an auxiliary function to these alginate lyases. Its definite function is currently unknown.

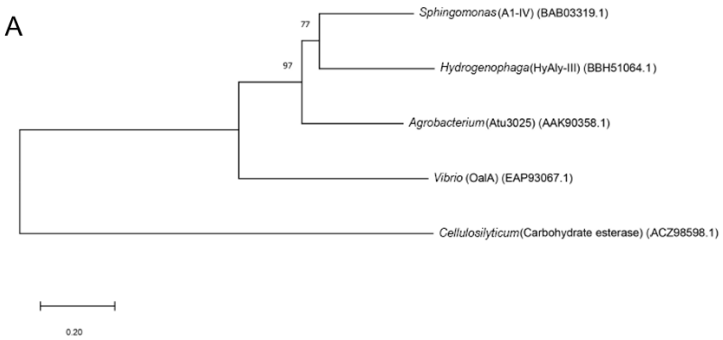
HyAly-II is regarded as a PL-7 alginate lyase based on its sequence similarity with other PL-7 alginate lyases. However, it showed relatively low amino-acid identity to other PL-7 alginate lyases. While, it showed the highest sequence identity to an uncharacterized alginate lyase from *Cellivibrio* sp. OA-2007. HyAly-II is predicted to be an endolytic enzyme based on other alginate lyases from the PL-7; however, expression of recHyAly-II was unsuccessful.

HyAly-III is PL-15 exolytic alginate lyase which also has two domains: DUF4962 alginate lyase domain (amino-acid 16 – 445) and Hepar_II_III domain (amino-acid 483 – 656). Alginate lyases Atu3025 from *Agrobacterium fabrum* str. C58, A1-IV from *Sphingomonas* sp. A1, and OalA from *Vibrio splendidus* 12B01 are well-characterized members of this PL family. To date there are 144 enzymes that belong to PL-15. All these enzymes are from prokaryotic bacteria. recHyAly-III protein could be expressed by the *E. coli* expression system; however, no soluble and active enzyme was obtained.

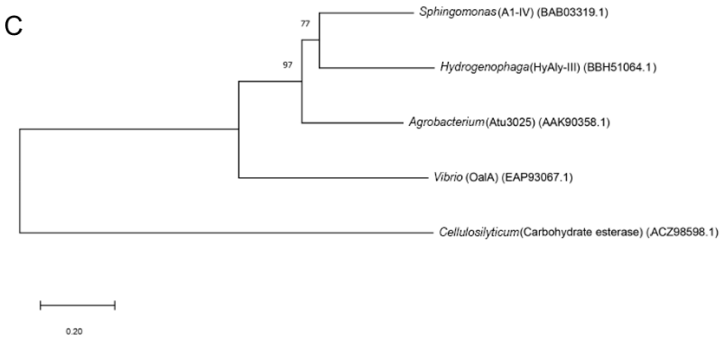
HyAly-IV is regarded as a member of PL-5 family. It contains an alginate lyase domain Alg_Lyase (NCBI conserved domain pfam05426) (amino acid residue 120 – 355). HyAly-IV showed 51.26% amino-acid identity to the alginate lyase A1-III from *Sphingomonas* sp. A1

(Protein Databank: 1HV6), which was characterized as poly(M)-specific alginate lyase. Thus, based on its sequence identity, HyAly-IV is also considered as a poly(β-D-mannuronate) lyase. The TLC analysis indicated that recHyAly-IV preferably degraded poly(M).

Based on the above initial screening of alginate lyase activity, recHyAly-I and recHyAly-IV were considered to be the promising alginate lyases of strain UMI-18 that can further be characterized using recombinant enzymes. But the amount of recHyAly-I contained in the cell lysate was much higher than that of recHyAlyIV. This allows us to further purify recHyAly-I and characterize its enzymatic properties. It is noteworthy that purification of recHyAly-I by Ni-NTA affinity chromatography was unsuccessful. Namely, recHyAly-I was expressed as the N-terminal His-6x-tagged protein; however, Ni-NTA affinity chromatography was not applicable to the purification of recHyAly-I because of the weak binding of recHyAly-I to the resin. This may be partly due to the steric hindrance of the His-6x-tag by the large catalytic domain of recHyAly-I, which may interfere the binding of His-6x-tag of the Ni-NTA resin. Hence, in the present study recHyAly-I was purified by conventional column chromatography.


A
77
97
Sphingomonas (A1-IV) (BAB03319.1)
Hydrogenophaga (HyAly-III) (BBH51064.1)
Agrobacterium (Atu3025) (AAK90358.1)
Vibrio (OalA) (EAP93067.1)
Cellulosilyticum (Carbohydrate esterase) (ACZ98598.1)
0.20


B
49
49
48
53
Corynebacterium (alyPG) (BAA83339)
Hydrogenophaga (HyAly-II) (BBH51063.1)
Flavobacterium (FlAlyA) (BAP05660.1)
Sphingomonas (A1-II) (BAD16656.1)
Pseudomonas (PA1167) (AAG04556.1)
Klebsiella (AlyA) (AAA25049.1)
Haloterrigena (PL-40 Pectate lyase) (ADB63558.1)
0.20


C
77
97
Sphingomonas (A1-IV) (BAB03319.1)
Hydrogenophaga (HyAly-III) (BBH51064.1)
Agrobacterium (Atu3025) (AAK90358.1)
Vibrio (OalA) (EAP93067.1)
Cellulosilyticum (Carbohydrate esterase) (ACZ98598.1)
0.20

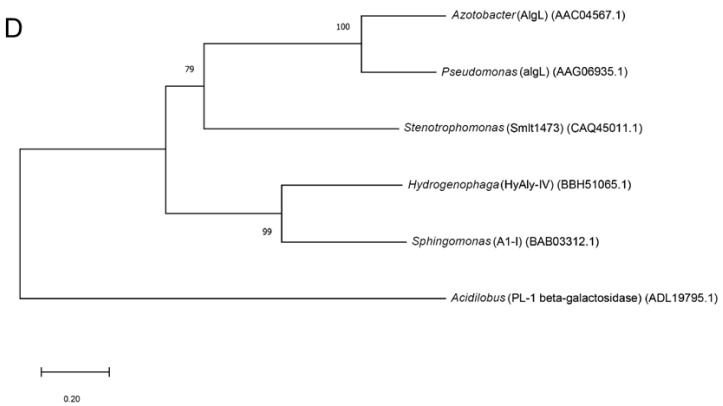

D
100
79
99
Azotobacter (AlgL) (AAC04567.1)
Pseudomonas (algL) (AAG06935.1)
Stenotrophomonas (Smlt1473) (CAQ45011.1)
Hydrogenophaga (HyAly-IV) (BBH51065.1)
Sphingomonas (A1-I) (BAB03312.1)
Acidilobus (PL-1 beta-galactosidase) (ADL19795.1)
0.20

Figure 3-1. Molecular phylogenetic trees based on amino-acid sequences of alginate lyases. Phylogenetic tree was generated using Neighbor-Joining model with 1,000 bootstrap replicates through MEGA X software using alginate lyases from different PL families. Labels in the tree branches indicate genus names, enzyme names and the GenBank accession numbers. (A) Phylogenetic tree of HyAly-I and other PL-17 alginate lyases. Carbohydrate esterase from *Arthrobotys oligospora* ATCC29427 is the outgroup. (B) Phylogenetic tree of HyAly-II and other PL-7 alginate lyases. PL-1 beta-galactosidase from *Acidolobus saccharovans* is the outgroup. (C) Phylogenetic tree of HyAly-III and other PL-15 alginate lyases. Carbohydrate esterase from *Cellulosilyticum ruminucola* is the outgroup. (D) Phylogenetic tree of HyAly-IV and other PL-5 alginate lyases. PL-1 beta-galactosidase from *Acidolobus saccharovorans* is the outgroup.

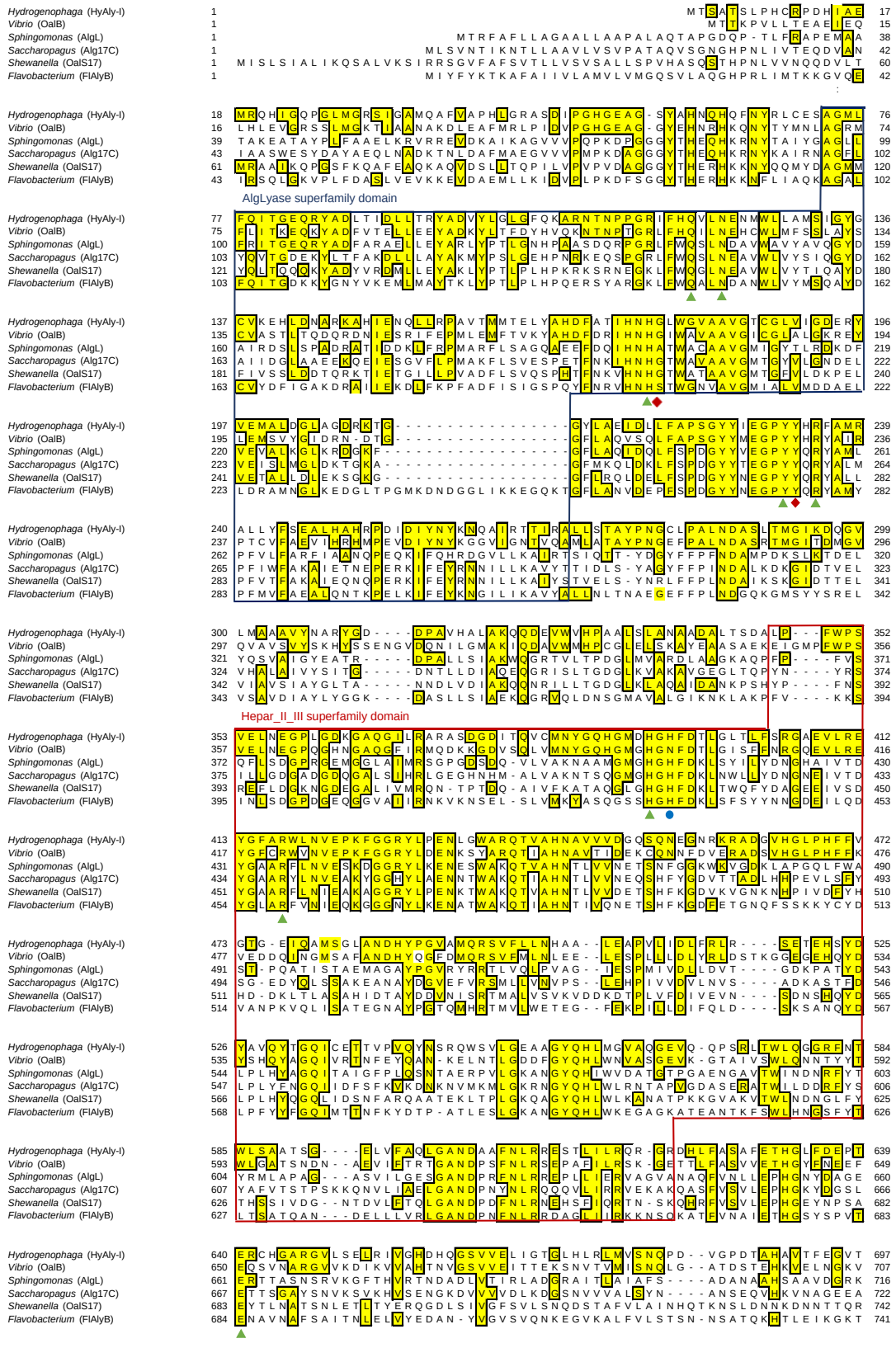

Hydrogenophaga (HyAly-I)
Vibrio (OalB)
Sphingomonas (AlgL)
Saccharopagus (Alg17C)
Shewanella (OalS17)
Flavobacterium (FlAlyB)
AlgLyase superfamily domain
Hepar_II_III superfamily domain
Identity
713 Identity
708 50.1%
717 37.5%
723 34.1%
743 33.6%
742 31.3%

Figure 3-2. Alignment of amino-acid sequences of HyAly-I and other PL-17 enzymes. The alignment was carried out using the sequences of HyAly-I (GenBank Accession number: BBH51062.1), OalB from *Vibrio splendidus* 12B01 (GenBank accession number: EAP93062.1), AlgL from *Sphingomonas* sp. MJ-3 (GenBank accession number: AEM45874.1), Alg17C from *Saccarophagus degradans* 2-40 (GenBank accession number: ABD82539.1), OalS17 from *Shewanella* sp. Kz7 (GenBank accession number: AHW45238) and FlAlyB from *Flavobacterium* sp. strain UMI-01 (GenBank accession number: BAQ25533.1) Blue and red boxes indicate the AlgLyase superfamily domain (NCBI conserved domain database: pfam05426) and the Hepar_II_III domain (NCBI conserved domain database: pfam07940), respectively. Green triangles indicate substrate binding site. Blue circles are metal binding sites and the red diamond are the catalytic sites.

Figure 3-3. Alignment of amino-acid sequences of HyAly-II and other PL-7 enzymes. The alignment was carried out by using the sequences of HyAly-II (GenBank accession number: BBH51063.1), AlyA from *Klebsiella pneumoniae aerogenes* (GenBank accession number: AAA25049.1), AlyPG from *Cornybacterium* sp. PAO1 (GenBank accession number: BAA83339.1, PA1167 from *Pseudomonas* sp. strain A1 (GenBank accession number: AAG04556.1), A1-II from *Sphingomonas* sp. A1 (GenBank accession number: BAD16656.1), FlAlyA from *Flavobacterium* sp. strain UMI-01 (GenBank accession number: BAP05660.1)

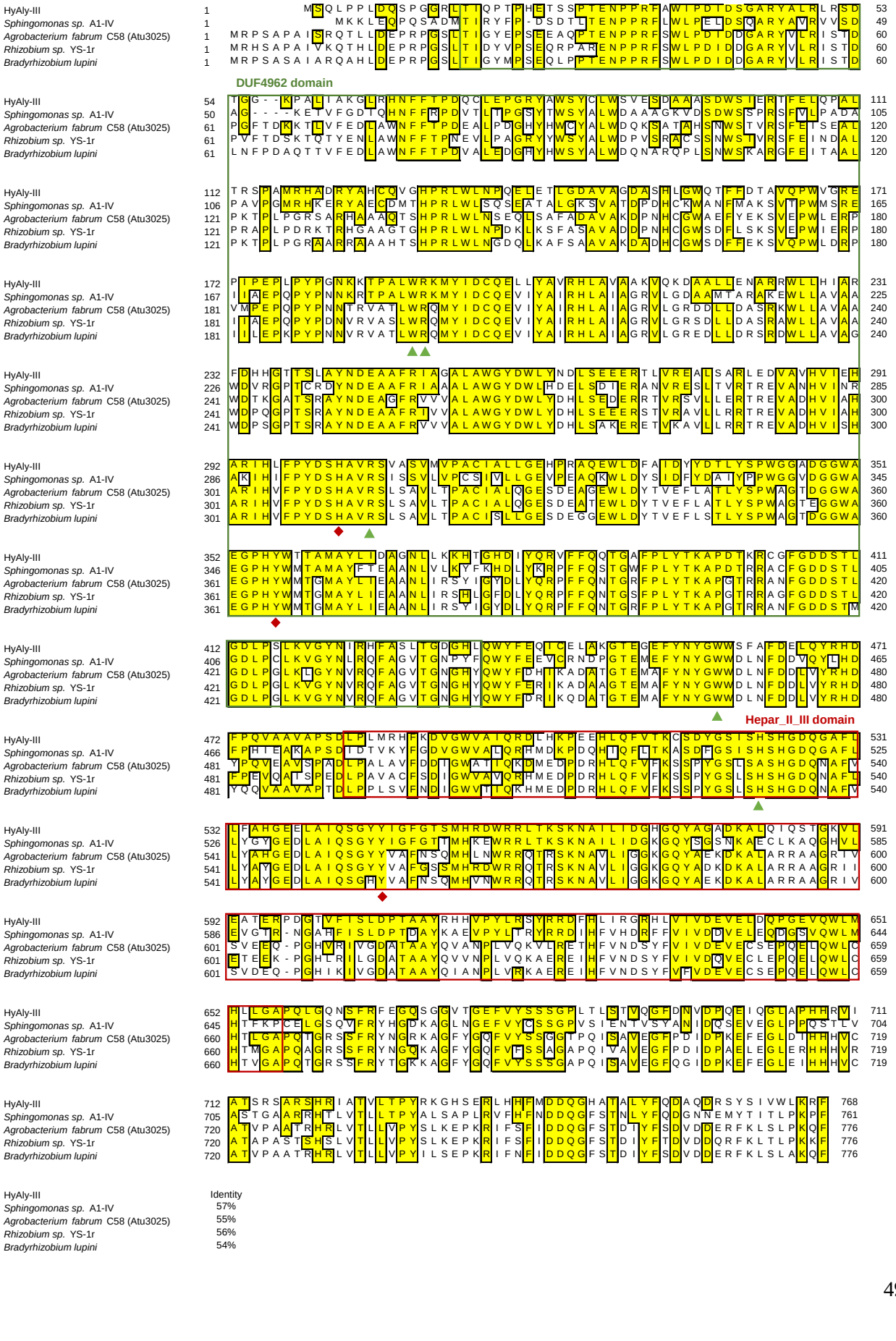

HyAly-III
Sphingomonas sp. A1-IV
Agrobacterium fabrum C58 (Atu3025)
Rhizobium sp. YS-1r
Bradyrhizobium lupini
DUF4962 domain
Hepar_II_III domain
Identity
57%
55%
56%
54%

Figure 3-4. Alignment of amino-acid sequences of HyAly-III and other PL-15 enzymes. The alignment was carried out using the sequences of HyAly-III (GenBank Accession number: BBH51064.1), A1-IV from *Sphingomonas* sp. A1 (GenBank accession number: BAB0339.1), Atu3025 from *Agrobacterium fabrum* C58 (GenBank accession number: AAK90358.1), DUF4692 domain containing protein from *Rhizobium* sp. YS-1r (GenBank accession number: WP_037153080.1), and DUF4692 domain containing protein from *Bradyrhzobium lupini* (GenBank accession number: WP_006697908.1). Green and red boxes indicate the DUF4962 domain (NCBI conserved domain database: pfam16332) and the Hepar_II_III domain (NCBI conserved domain database: pfam07940), respectively. Red diamonds indicate catalytic active sites while green triangles indicate substrate binding sites.

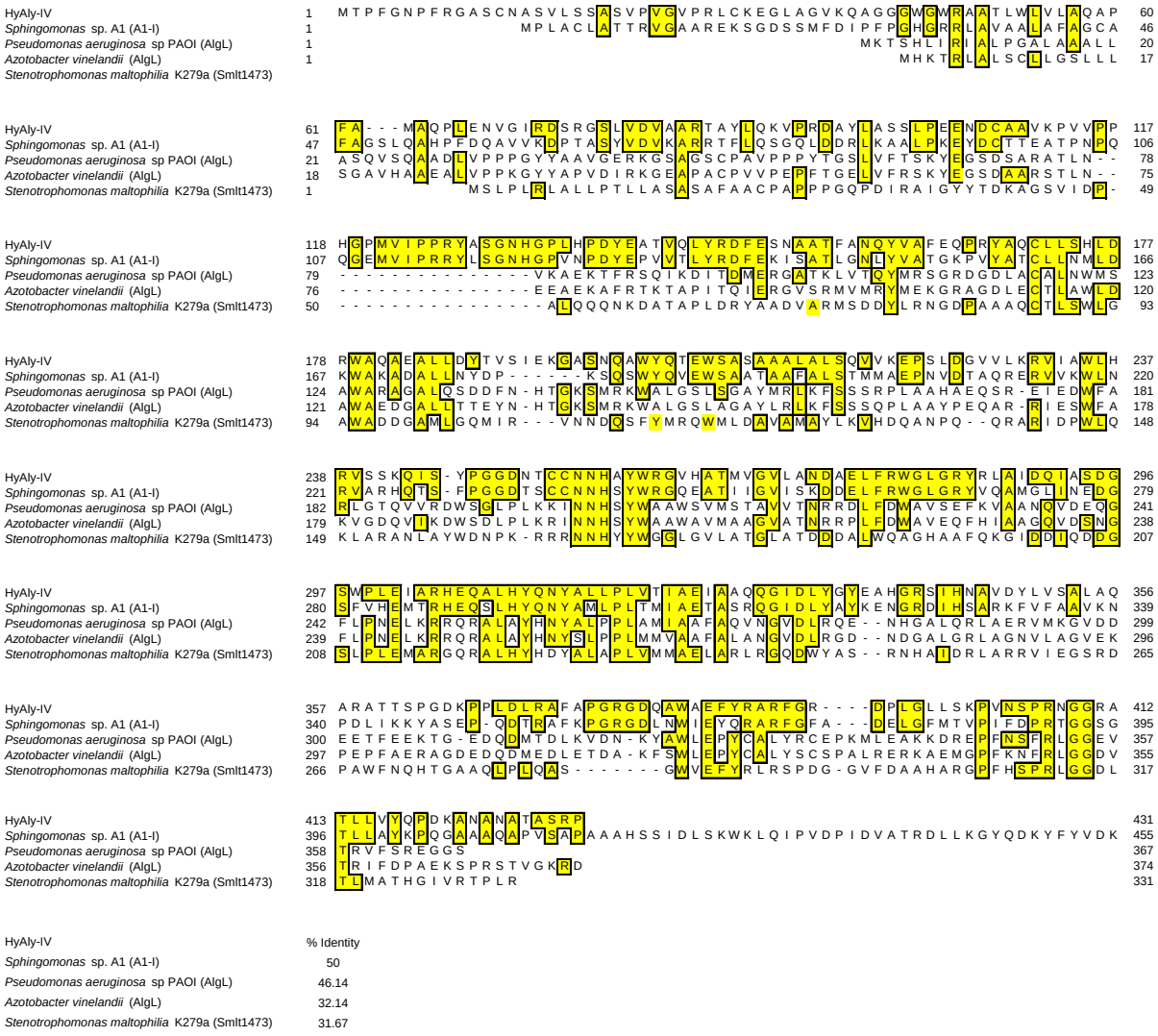


Figure 3-5. Alignment of amino acid sequences of HyAly-IV with other PL-5 enzymes. Amino acid sequences of HyAly-IV from *Hydrogenophaga* sp. strain UMI-18 (GenBank Accession number: BBH51046.1), A1-I from *Sphingomonas* sp. A1 (GenBank accession number: BAB03312.1), AlgL from *Pseudomonas aeruginosa* sp. PAOI (Genbank accession number: AAG06935.1), AlgL from *Azobacter vinelandii* (GenBank accession number: AAC04567.1), Smlt1473 from *Strentophomas maltophilia* (GenBank accession number: CAQ45011.1) were aligned.

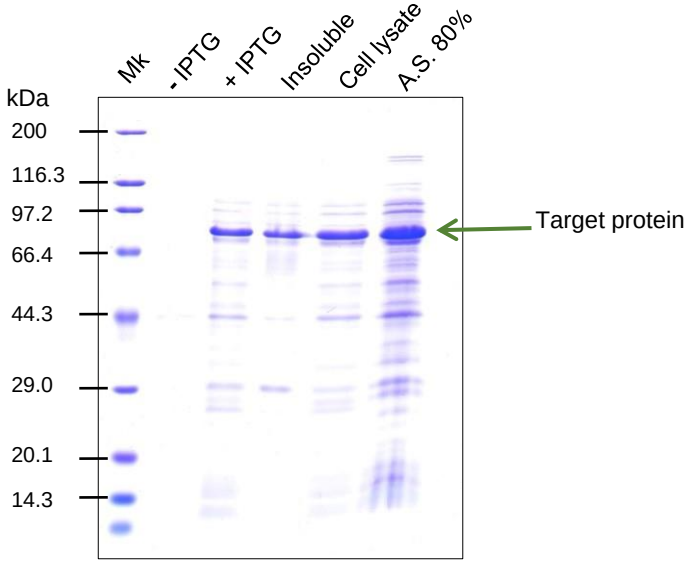


Figure 3-6. Expression of recHyAly-I. Samples obtained in various purification steps are subjected to SDS-PAGE. Mk, molecular weight marker (broad range); -IPTG, before IPTG induction; +IPTG, after IPTG induction; Insoluble, insoluble proteins after sonication of bacterial pellets; Cell lysate, supernatant of cell lysate; A.S. 80%, proteins precipitated by 80% saturation of ammonium sulfate.

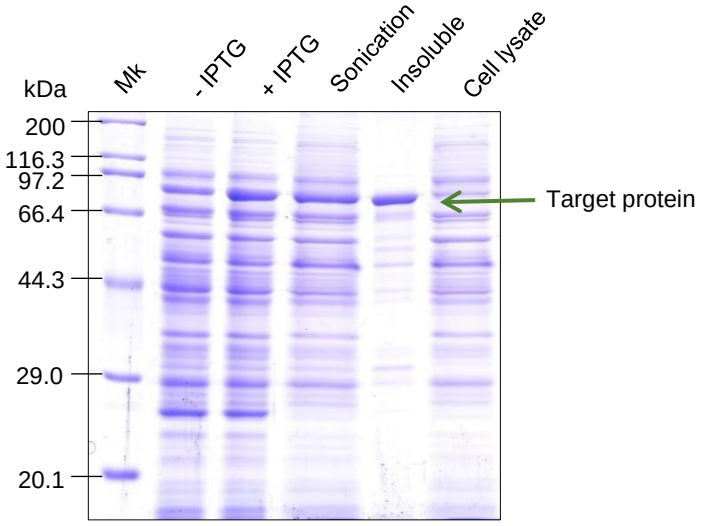


Figure 3-7. Protein expression of recHyAly-III. Different fractions obtained from recHyAly-III purification were subjected to SDS-PAGE. Captions used are the same as in Figure 3-6.

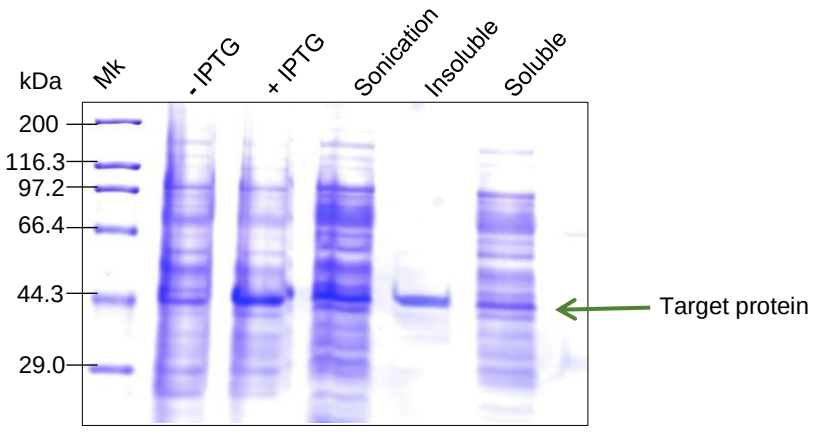


Figure 3-8. Protein expression of recHyAly-IV. Different fractions obtained from recHyAly-III purification were subjected to SDS-PAGE. Captions used are the same as in Figure 3-6.

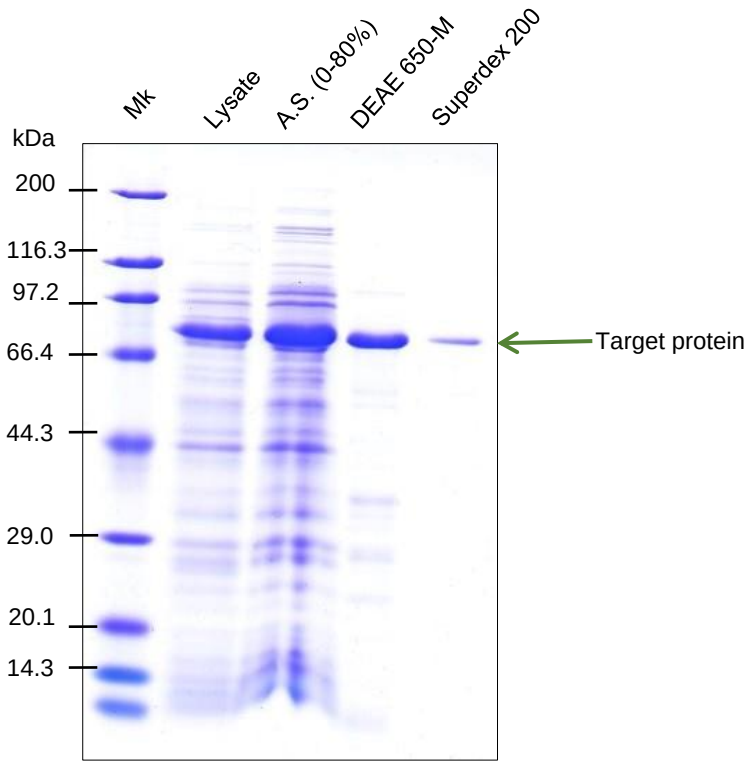


Figure 3-9. Summary of purification of recHyAly-I. Proteins in various purification steps were subjected to SDS-PAGE. Mk, molecular weight marker (broad range); Lysate, cell lysate from *E. coli* BL21 (DE3) expressing recHyAly-I, AS (0-80%), 80% ammonium sulfate fraction of the cell lysate; DEAE 650-M, pooled fractions after anion exchange chromatography; Superdex 200, fraction after size exclusion chromatography Superdex 200 3.2/300.

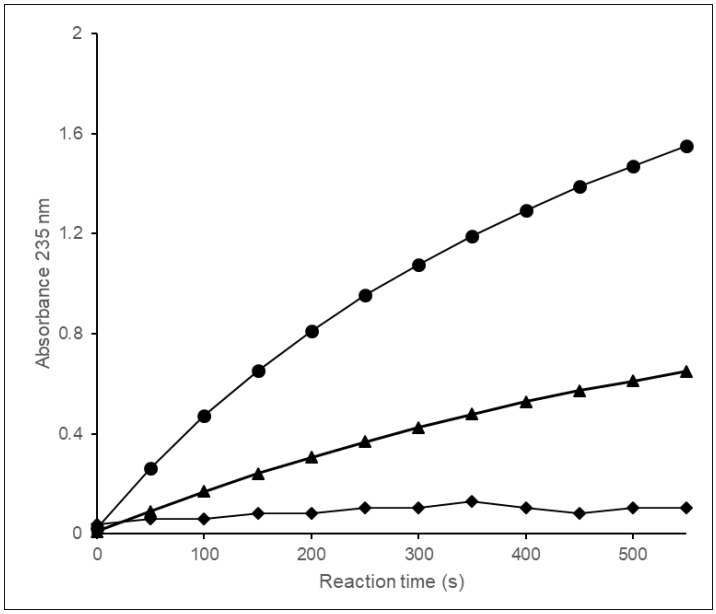


Figure 3-10. Alginate lyase activity of recHyAly-I. Enzyme assay was conducted in a standard reaction mixture containing 0.1 mg/ml enzyme and the progress of reaction was recorded by measuring absorbance at 235 nm. Diamond symbols indicate the reaction with crude cell lysate; triangle symbols indicate the reaction with 80% ammonium cell fraction and circle symbols indicate the reaction with the fraction showing highest activity in TOYOPEARL DEAE-650M chromatography.

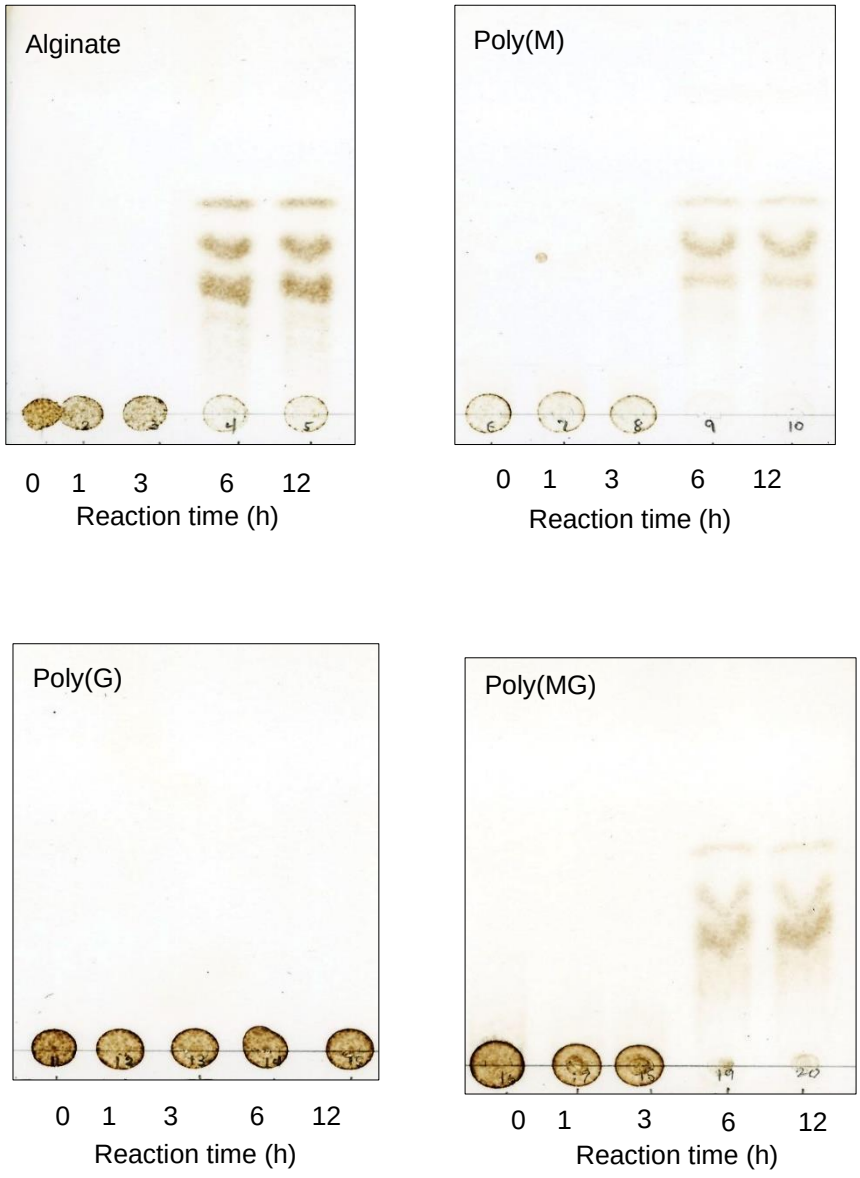


Figure 3-11. TLC analysis for the degradation products of polymer substrates by recHyAly-IV. Staining intensity of the original spots decreased in 6 h – 12 h reaction with alginate, poly(M) and poly(MG) substrates, while no degradation products were detected in the reaction with poly(G). Reaction conditions: recHyAly-IV (0.1 mg/mL) was added in a reaction containing 0.1% (*w/v*) substrate, 10 mM sodium phosphate buffer (pH 7.0) and incubated at 30 °C for 12 h.

Table 3-1. Primers used for the cloning of alginate lyases from strain UMI-18 genome. Underlines indicate the restriction sites.

Gene	Plasmid		Primer	Restriction enzyme site
HyAly-III	pCold-I	F	AGGTAATACACCATGTCCCAGCTCCCCCCTCTTGA	*NcoI*
		R	CACCTCCACCGGATCCGAACCGCTTGAGCCAGACGA	*BamHI*
HyAly-IV	pCold-I	F	AGGTAATACACCATGGCGCAGCCCCTGGAGAACGT	*NcoI*
		R	CACCTCCACCGGATCCTGGACGTGAGGCGGTTGCGT	*BamHI*
HyAly-I	pET-21(b+)	F	ATGGGTCGGGATCCGATGACATCCGCCACTTCCTT	*BamHI*
		R	TGCGGCCGCAAGCTTCGGCGCGCCCGACCAGATCCT	*HindIII*
HyAly-II	pET-21(b+)	F	ATGGGTCGGGATCCGGCTGGCTGTGCGGCCCCCAC	*BamHI*
		R	TGCGGCCGCAAGCTTCGTCGAGCGACAGTGCCTGGA	*HindIII*
HyAly-III	pET-21(b+)	F	ATGGGTCGGGATCCGATGTCCCAGCTCCCCCCTCT	*BamHI*
		R	TGCGGCCGCAAGCTTCGAACCGCTTGAGCCAGACGA	*HindIII*
HyAly-IV	pET-21(b+)	F	ATGGGTCGGGATCCGCAGGCGGGCGGCGGCTGGGG	*BamHI*
		R	TGCGGCCGCAAGCTTCTGGACGTGAGGCGGTTGCGT	*HindIII*

Chapter IV: Characterization of the PL-17 exolytic alginate lyase HyAly-I and the analysis of its degradation products

4.1 Introduction

Alginate has been widely used in different applications in food, pharmaceutical and other biotechnological industries (Augst et al., 2006; Wong et al., 2009). It is also recognized as a promising alternative biomass for biofuel and high-value materials production (Takagi et al., 2017; Lee & Mooney, 2006). The range of possible application of alginates can be improved if its solubility in water is increased and its high viscosity can be adjusted (Liu et al., 2019). There are many approaches to adjust the properties of alginate and produce alginate oligosaccharides (AOS) such as the use of weak acids (Holtan et al., 2006), irradiation of gamma rays (Mohdy, 2017), chemical synthesis (Pan et al., 2019), and enzymatic degradation (Gacesa, 1987). Enzymatic techniques in AOS production is more advantageous versus physical, chemical and organic synthesis approaches because they are safe and precise. Enzymatic degradation of alginate to AOS can be done in mild conditions, require less energy or material and can also result to a higher yield (Liu et al., 2019). Alginate monosaccharide (AMS) is also useful as a material for the production of biofuels and biomaterials. AMS is usually produced by exolytic alginate lyases as unsaturated monouronate, i.e., 4-deoxy-L-*erythro*-5-hexoseulose uronic acid (DEH).

Exolytic alginate lyases (EC 4.2.2.26) have been classified to PL-6, PL-7, PL-15 and PL-17 families in the CAZy database (http://www.cazy.org). They act on the non-reducing terminus of the substrates and produce DEH. In the previous chapter, HyAly-I was regarded as a PL-17 alginate lyase and successfully expressed as a soluble recombinant enzyme. Thus, recHyAly-I appears to be applicable for the detailed characterization of PL-17 exolytic alginate lyase, whose enzymatic properties have not been thoroughly investigated. Therefore, in this chapter, the general

properties of HyAly-I from strain UMI-18 was characterized using recHyAly-I, focusing on substrate preference, optimum temperature, thermal stability, optimum pH and pH stability. Additionally, effects of sodium chloride and other salts, and different reagents were also examined. Finally, the the degradation products of various polymer and oligomer substrates produced by recHyAly-I were characterized by TLC and electrospray ionization mass spectrometry (ESI-MS).

4.2 Materials and methods

4.2.1 Preparation of block substrates, saturated oligosaccharides and unsaturated oligosaccharides

Poly(M), poly(G) and poly(MG) blocks were prepared by the limited hydrolysis of alginate (*Macrocystis pyrifera* origin, 300-400 cp, FUJIFILM Wako Pure Chemical Industry Ltd.) with 0.3 M HCl (Gacesa & Wusteman, 1990). Saturated oligo(M) and oligo(G) substrates were prepared from the poly(M) and poly(G) blocks by the limited sulfuric-acid hydrolysis. Briefly, 0.25 ml of 70% of sulfuric acid was added to the 30 mg dried powder of poly(M) and poly(G) and incubated at room temperature for 20 min. Then, the solution was diluted ten times with distilled water and further hydrolyzed at 100°C for 1 h. The hydrolysate was neutralized with barium carbonate powder and the clear hydrolysate was obtained by centrifugation at 12,000 x *g* for 20 min. The hydrolysate was then concentrated by rotary evaporation and applied to a column of Bio-Gel P2 (BioRad, CA, USA) (2.2 x 90 cm) preequilibrated with 50 mM ammonium acetate. Elution position of the oligosaccharides was detected by phenol-sulfuric acid reaction (Saha and Brewer, 1994) and thin-layer chromatography using TLC-silica gel 60 plate (Merck KgaA, Darmstadt, Germany). The separated oligosaccharides were lyophilizyed and stored at -20°C until use.

Unsaturated oligosaccharides were prepared by the degradation of poly(M) and poly(G) blocks with recFlAlyA (Inoue et al., 2014). After the digestion at 30°C for 12 h, solutions were centrifuged at 12,000 x *g* for 20 min to remove insoluble materials formed during reaction. Then, the supernatant was concentrated by rotary evaporation, and applied to Bio-Gel P2 column (2.2 x 90 cm). Elution positions of the di-, tri-, and tetrasaccharides were determined by TLC and measurement of UV absorption at 235 nm. Each oligosaccharide fractions were separately lyophilized and stored at -20°C.

4.2.2 Assay for exolytic alginate lyase activity

Alginate lyase activity was assayed in a 500 μL of a standard reaction medium containing 10 mM sodium phosphate buffer (pH 7.0), 0.1 mg/mL recHyAly-I, 0.1% (*w/v*) substrate and 0.1 mg/mL bovine serum albumin (BSA) as a stabilizer. Aliquots (100 μL) of the reaction mixture were withdrawn at reaction time 0, 2, 4, 6, and 8 min and the unsaturated sugars produced were determined by the thiobarbituric acid (TBA) method (Inoue, 2018). One unit of alginate lyase activity was defined as the amount of enzyme that releases 1 μmol of β-formyl-pyruvic acid per min. Optimal temperature of recHyAly-I was determined at 0 to 60°C. Thermal stability of recHyAly-I was assessed by measuring the activity remained after the 30-min incubation at 20 to 70°C. The effects of pH on the activity was determined by incubating the enzyme in different buffers: 20 mM sodium phosphate (pH 4.5 to 8.1), 20 mM Tris-HCl (pH 7.3 to 9.1) and 20 mM glycine-NaOH (pH 8.5 to 10). The pH stability was determined by measuring the residual activity of the enzyme after 3 h incubation with sodium phosphate buffer (pH 5 to 8.5) on ice. The effects of salts and other reagents on recHyAly-I were determined in the standard reaction medium

supplemented by 1 mM or 100 mM each reagent. All assays were repeated three times and the activity was expressed as a mean value with a standard deviation.

4.2.3 Determination of substrate preference

Substrate preference of recHyAly-I was determined with a reaction mixture containing 0.1% (w/v) sodium alginate, poly(M), poly(MG) or poly(G) block substrates, and 0.1 mg/mL enzyme. The reaction was carried out at 30°C for 8 min, and alginate lyase activity was determined by the TBA method.

4.2.4 Degradation of alginate polymer and alginate oligosaccharide substrates

Alginate, poly(M), poly(G), poly(MG), saturated trisaccharide (SM3), unsaturated trisaccharide (UM3), saturated disaccharide (SM2) and unsaturated disaccharide (UM2) were dissolved in 10 mM sodium phosphate buffer (pH 7.0) to make 6 mg/ml. They were degraded with 0.3 mg/ml recHyAly-I for 15 - 180 min in the standard conditions. Reaction was terminated by the addition of chloroform. Approximately 10 μg of reaction products were applied to a TLC plate and developed with 1-butanol – acetic acid – water (2:1:1, *v:v:v*). Sugars developed on the plate were stained by spraying 10% sulfuric acid in ethanol or 4.5% TBA, followed by heating at 130 ˚C for 15 min. Reaction products were also subjected to ESI-MS using an LTQ Orbitrap Discovery Mass Spectrometer (Thermo Fischer Scientific K. K., Tokyo, Japan) at the Global Facility Center of Creative Research Institution, Hokkaido University (Sapporo, Japan). Approximately 10 ng of materials dissolved in 5 μL of 80% methanol was subjected to the spectrometer and analyzed in negative ion mode.

4.3 Results

4.3.1 General Properties of recHyAly-I

The optimal temperature of recHyAly-I was observed at around 35°C (Fig. 4-1A), while the activity of recHyAly-I decreased to 65% to 8% of the original activity by the incubation for 30-min at 35°C and 40°C, respectively (Fig. 4-1B). No residual activity was detected after the incubation at temperature above 50°C.

recHyAly-I showed an optimum pH at around 6.0 (Fig. 4-1C). The highest activity was observed in 20 mM Tris-HCl buffer (pH 7.2), suggesting that Tris buffer stabilizes and or activates recHyAly-I. The enhancement of activity by Tris buffer was also reported in an exolytic alginate lyase OAL from *Strenotophomas maltophilia* KJ-2 (In Lee et al., 2012). The highest stability of recHyAly-I was shown at pH 6.5 (Fig. 4-1D) and the activity decreased to 20% by the incubation at pH 8.5 and pH 5.0.

recHyAly-I was activated 1.5 and 2 times by the addition of 100 mM NaCl and KCl, respectively (Table 4-1). The addition of 1 mM Co^{2+}, Mg^{2+}, EDTA and DTT slightly decreased the activity, while the addition of Ni^{2+} and Fe^{3+} strongly inhibited the enzyme. Cu^{2+} and SDS almost completely inactivated the enzyme.

4.3.2 Substrate preference

To investigate the substrate preference of recHyAly-I, the activity was determined using alginate, poly(M), poly(G) and poly(MG) block as substrates. As shown in Fig. 4-2, the highest activity was observed in poly(M) block. Poly(MG) block was also a preferable substrate; however, poly(G) block gave about a half activity compared with poly(M) and poly(MG) block substrates.

Thus, poly(M) and poly(MG) blocks were regarded as the most preferable substrate of recHyAly-I. Alginate polymer exhibited the lowest activity. This may be attributed to the lesser action points in the polymer substrate than oligomer substrate for the exolytic enzyme. In other words, shorter substrates can provide higher concentration of substrate for exolytic recHyAly-I than longer substrate if the weight concentrations of shorter and longer substrates are equal in the reaction medium.

4.3.3 Analysis of degradation products of recHyAly-I

Poly(M) substrate was degraded with recHyAly-I for 180 min and the degradation products were analyzed by TLC and ESI-MS (Fig. 4-3A - B). The major product released by recHyAly-I was identified as DEH as in the cases of other exolytic enzymes (Li et al., 2018; Wang et al., 2018). Aside from DEH, three bands (a – c) were detected in the TLC plate stained with 10% sulfuric acid (Fig. 4-3A). Band "a" showed similar mobility as the SM1 marker and was not stained by TBA (Fig. 4-3B). Thus, this band was regarded as a saturated monosaccharide (SM1) derived from the non-reducing terminus of poly(M). Band "b" was stained by both sulfuric acid and TBA and was regarded as an unsaturated disaccharide (UM2). This band was slowly disappeared along with the extension of the reaction time to 180 min indicating the slow degradation of UM2 by recHyAly-I. Contrary, band "c" was produced with the decrease of band "b" (UM2). This strongly suggested that the band "c" was a compound derived from UM2 and was not further degraded by recHyAly-I. Band "c" or compound **C** was positive in TBA staining. Thus, **C** was considered to possess a methylene carbon as did DEH and UM2 (Fig. 4-3B). However, **C** could not be assigned to DEH or UM2 because its mobility on the TLC plate is distinctly different from band "a" (DEH) and band "b" (UM2). To identify **C**, the silica-gel containing **C** was scraped from an unstained TLC

plate which was prepared in parallel with the stained TLC plate and the **C** extracted from the silica-gel debris with distilled water was subjected to ESI-MS (Fig. 4-3C). **C** showed a prominent divalent molecular ion peak at *m/z* 175.0252 (*z* = 2) while a monovalent molecular ion peak was also detected at *m/z* 351.0565 (*z*=1) (data not shown). These results indicated that the molecular mass of **C** is 352 Da and it can be deduced that it possesses two carboxyl groups. Consequently, the peak at *m/z* 175.0252 (*z*=2) was identified as $[\mathbf{C} - 2H]^{2-}$, while the peak at *m/z* 351.0565 [*z*=1] was identified as $[\mathbf{C} - H]^{-}$. It should be noted that unsaturated disaccharide (UM2, 352 Da) also provides same peaks, i.e., m/z 175.0252 (*z*=2) and *m/z* 351.0565 (*z*=1) in ESI-MS. However, **C** was distinguishable from UM2 by TLC (Fig. 2A and B), indicating that **C** has a different structure from UM2. The molecular ion peak *m/z* 175.0252 (*z*=2) of C was then subjected to MS/MS. As shown in Fig. 3B, three major peaks appeared at *m/z* 157.0139, *m/z* 131.0347 and *m/z* 113.0241. The fragment showing *m/z* 157.0139 was identical to the fragment derived from the open chain form of DEH (Fig. 4-4). Based on these TLC and ESI-MS analyses, it was considered that **C** is a dimeric form of DEH (diDEH) which is composed of a linear-chain-form DEH and a ring-form DEH (Fig. 4-3C inset). A possible structure and element composition of diDEH are shown in the inset of Fig. 4-3A; while the putative structures of the fragment ions from diDEH and their element compositions are shown in the inset of Fig. 3B.

Production of diDEH by recHyAly-I was more clearly detected in the degradation of saturated trisaccharide (SM3) and unsaturated trisaccharide (UM3) (Fig. 4-5A-C). Namely, three major bands corresponding to bands a – c in the degradation products of poly(M) (Fig. 4-3A) were detected in the degradation products of SM3 (Fig. 4-5A). They were regarded as saturated monosaccharide (SM1), unsaturated disaccharide (UM2) and diDEH based in the order of their mobility. Whereas, the three unsaturated sugars, DEH, UM2 and diDEH were detected in the

degradation products of both SM3 and UM3 (Fig. 4-5B-C). The prominent production of diDEH from trisaccharide substrates may also indicate that the diDEH is a product in the final phase of the exolytic degradation of alginate.

4.4 Discussion

Specific activity of recHyAly-I was not as high compared to OalS17 from *Shewanella* sp. Kz7, OalC17 from *Cellulophaga* sp. SY 11 and TcAlg1 from *T. crasostreae* but comparable with Alg17C from *S. degradans* 2-40 and alyII from *Pseudomonas* sp. OS-ALG9. Activity of exolytic alginate lyases are typically affected by reaction conditions such as size of substrate and the buffer used in the reaction medium. Polymer substrates tend to give lower enzyme activity compared to oligomer substrates. Buffers such as Tris and carbonate buffers can enhance enzyme activity (In Lee et al., 2012). The varied specific activity among these exolytic enzymes may be due to such difference in the assay conditions used in the enzyme.

recHyAly-I was activated to 1.5 – 2.0 times by NaCl and KCl (Table1) and retained 20% of the optimal activity even in the presence of 0.4 – 0.6 M NaCl (data not shown). Similar halotolerance was reported with OAL from *S. malpophilia* KJ-2 (In Lee et al., 2012) and Alg17C from *S. degradans* 2-40 (Hirayama et al., 2016). Salt-activated alginate lyases have also been isolated from marine bacteria (In Lee et al., 2012; Wang et al., 2018; Ryu & Lee, 2011) and eukaryotic organisms (Rahman et al., 2010; Inoue & Ojima, 2019). Halotolerant properties of enzymes appear to be advantageous for industrial applications especially in the utilization of marine seaweed biomasses which are usually contaminated by sea salt.

recHyAly-I showed the highest activity toward poly(M) and poly(MG) blocks. Relative activities toward alginate, poly(M), poly(MG), poly(G) were 100%, 648%, 632% and 278%,

respectively. This enzyme exhibited similar substrate preference to alyII from *Pseudomonas* sp. OS-ALG9 (Kraiwattanapong et al., 2009), OalC17 from *Cellulophaga* sp. SY116 (Li et al., 2018), TcAlg1 from *Thalassotalea crasostreae* (Wang et al., 2019) and OalC from *Vibrio splendidus* (Jagtap et al., 2014).

In this study, PL-17 exolytic alginate lyase HyAly-I was characterized and was found to produce a novel unsaturated disaccharide diDEH. This is the first report for the production of diDEH by exolytic alginate lyase; however, it is currently uncertain if diDEH was produced by the catalytic activity of recHyAly-I itself or by non-enzymatic reaction. It is hypothesized that diDEH is produced by the nucleophilic addition reaction between the carbonyl group of one DEH molecule and the hydroxyl group of another DEH. It was confirmed in a different experiment that incubating recHyAly-I with DEH alone in a standard reaction condition does not produce diDEH. This indicated that recHyAly-I is essential in the production of diDEH. There is a need to study how recHyAly-I produces diDEH from polymer and oligomer alginate substrates. Additional experiments such as further purification and structure elucidation are also needed to fully characterize diDEH.

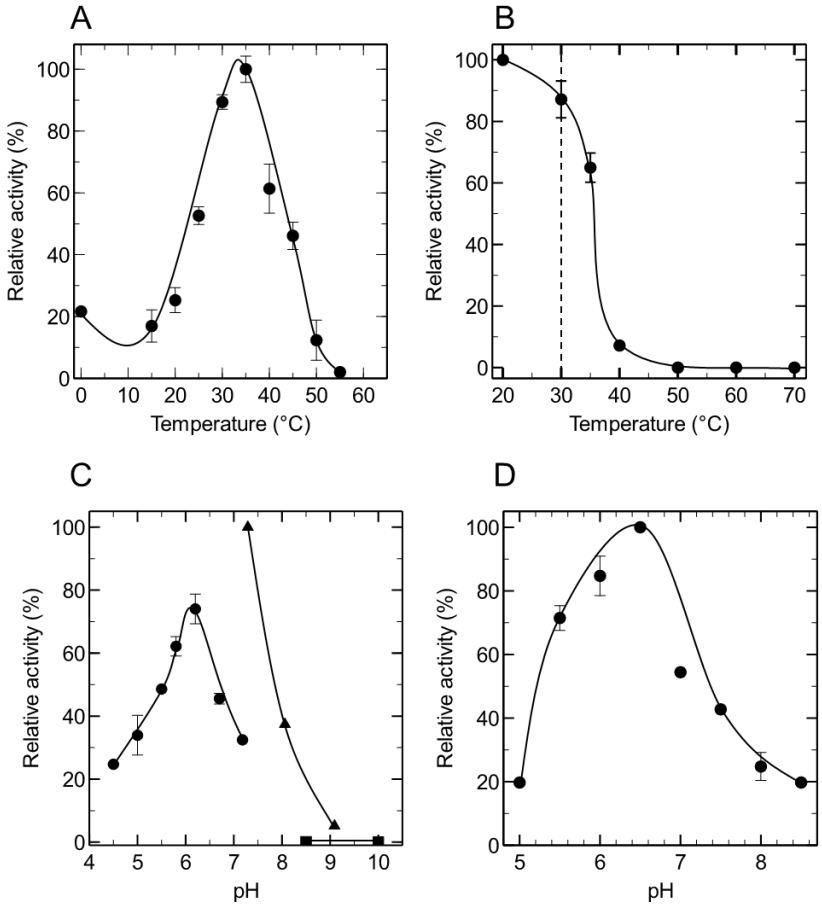


Figure 4-1. General properties of recHyAly-I. (A) Temperature dependence of recHyAly-I. Highest alginate lyase activity was observed at around 40°C. (B) Thermal stability of recHyAly-I. Residual activity after the incubation at 20 – 70°C was determined using a standard condition. 100% relative activity was observed at 20°C. (C) pH dependence of recHyAly-I. Activity was measured at 30°C in reaction mixtures containing 20 mM sodium phosphate buffer (pH 4.5 to 8.1, closed circles), 20 mM Tris-HCl buffer (pH 7.3 to 9.1, closed triangles) and 20 mM glycine-NaOH (pH 8.5 to 10, closed squares). Highest activity was observed at 20 mM Tris-HCl (pH 7.3). (D) pH stability of recHyAly-I. 20 mM sodium phosphate buffer with different pH was measured in a

standard condition. Residual activity was highest at pH 6.5. Relative activity 100% was 0.04 – 0.07 U/ml in the above assays.

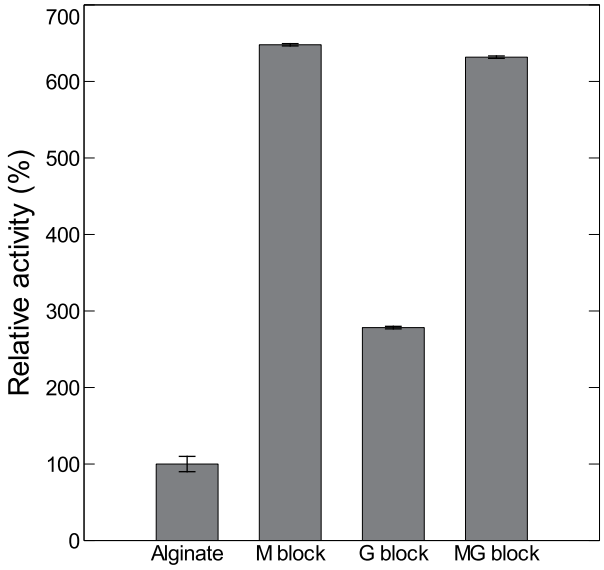


Figure 4-2. Substrate preference of recHyAly-I. Activity was assayed at 30°C using a standard reaction mixture containing 1 mg/mL alginate, poly(M), poly(G) or poly(MG) blocks and 0.1 mg/mL recHyAly-I. The highest specific activity was shown in poly(M) block was 0.44 U/mg.

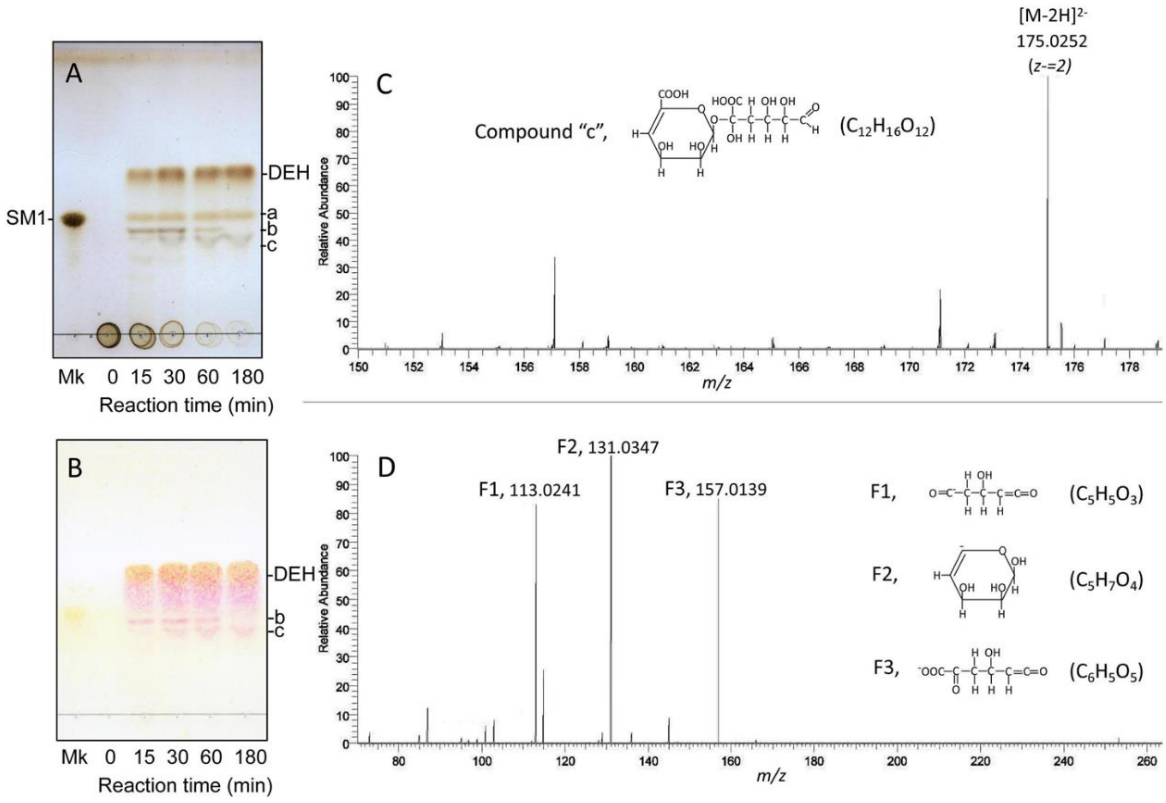


Figure 4-3. Analysis for the degradation products of poly(M) produced by recHyAly-I. (A) TLC analysis for degradation products of poly(M) produced by recHyAly-I. Poly(M) (6 mg/ml) was degraded with 0.25 mg/ml of recHyAly-I at 30°C for 15 – 180 min and the reaction was terminated by mixing with an equal volume of chloroform. Two μL of degradation products in the aqueous layer was subjected to TLC-60 plate. Sugars developed on the plate were stained with 10% sulfuric acid. Mk, marker monosaccharide (β-D-mannuronate, SM1). DEH, 4-deoxy-L-*erythro*-5-hexoseulose uronic acid. (B) TLC analysis for the degradation products of poly (M). Sugars were stained with 4.4% TBA. (C) ESI-MS spectrum of compound "c". The divalent molecular ion peak $[M-2H]^{2-}$ of m/z 175.0252 (z=2) indicates the molecular mass of compound "c" is 352 Da. A possible structure and the element composition of compound "c" are shown in the inset. Ring-form and open-chain-form moieties of DEH are also indicated. (D) ESI-MS/MS spectrum of compound

"c". The putative structures of fragment ions F1 – F3 and their element compositions are shown in the inset.

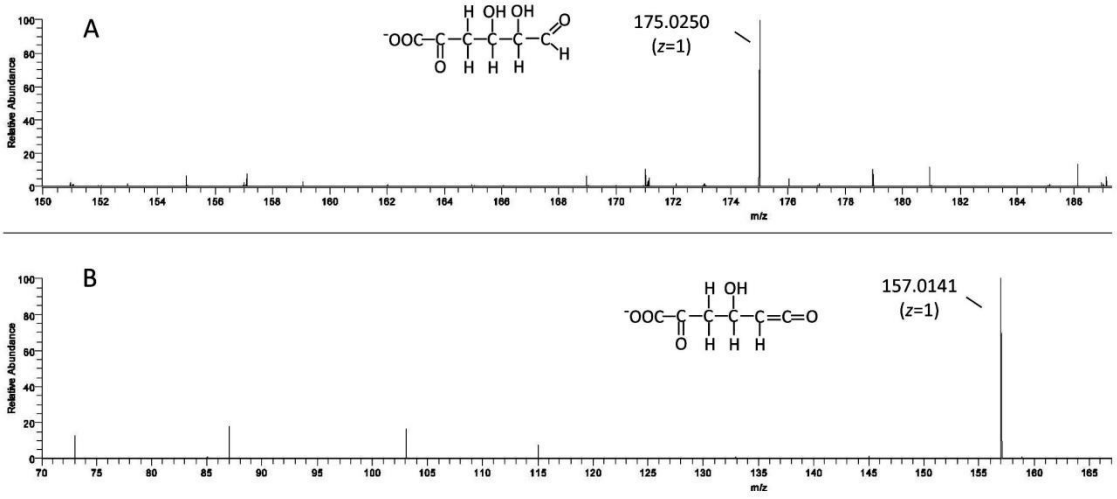


Figure 4-4. ESI-MS analysis for DEH. (A) ESI-MS spectrum of DEH. The peak at *m/z* 175.0250 is monovalent (*z*=1), which differs from the peak of compound "c" (*z*=2). A linear-chain-form DEH is shown in the inset. (B) MS/MS spectrum of the peak at *m/z* 175.0250 in A. A peak at *m/z* 157.0141 appeared as a major fragment. A possible structure for this fragment is shown in the inset.

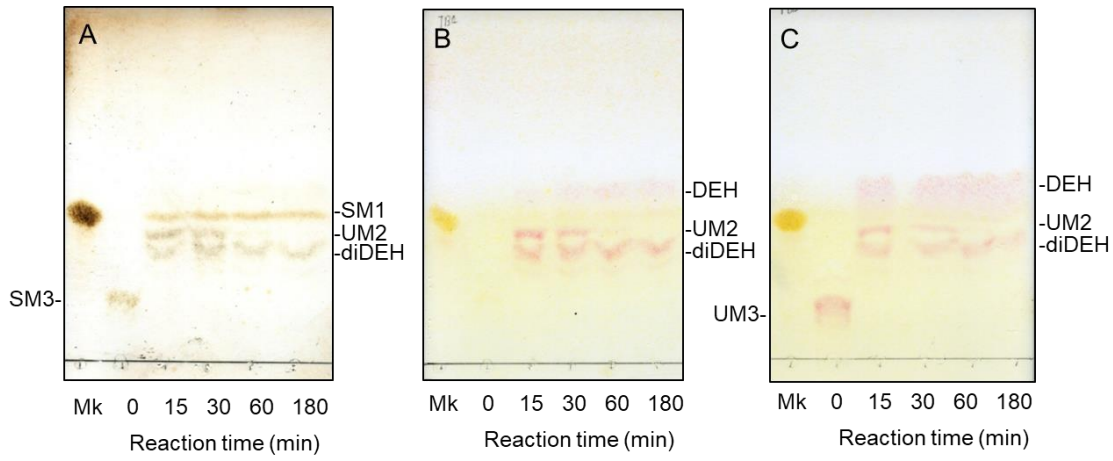


Figure 4-5. TLC analysis for degradation products of trisaccharides produced by recHyAly-I. Saturated trisaccharide (SM3, 6 mg/ml) and unsaturated trisaccharide (UM3, 6 mg/ml) were degraded by recHyAly-I for 15 – 180 min and analyzed by TLC. (A) Degradation products of SM3 stained by 10% sulfuric acid. (B) Degradation products of SM3 stained by 4.5% TBA. (C) Degradation products of UM3 stained by 4.5% TBA. Mk, monosaccharide SM1.

Table 4-1. Effects of various salts and compounds on recHyAly-I

Chemicals	Concentration (mM)	Relative activity (%)		
None		100	±	3.8
NaCl	100	148.2	±	28.5
KCl	100	198.8	±	20.6
$CoCl_2$	1	71.8	±	2.6
$MgCl_2$	1	80.7	±	1.6
$CaCl_2$	1	62.7	±	3.8
$MnCl_2$	1	71.5	±	2.4
$NiCl_2$	1	4.5	±	4.6
$CuCl_2$	1	1	±	0.2
$FeCl_3$	1	40.2	±	7.6
SDS	1	0.5	±	0
EDTA	1	58.3	±	4
Dithiothreitol	1	65.1	±	1.9

Chapter V: General discussion

5.1 Seaweed as carbon source for PHB production

Bioplastics such as poly(3-hydroxybutylate) (PHB) and other polyhydroxyalkanoates (PHAs) are known as biodegradable and environmentally friendly polyesters that are recognized as a material to replace petrochemically derived plastics (Keshvarz and Roy, 2010). However, one of the major disadvantages of industrial scale PHA production is the expensive fermentable biomass and the complicated extraction process (Nielsen et al., 2010). PHAs are generally produced by using terrestrial biomass such as starch, molasses and lipids; however, this may potentially cause food vs. bioplastic conflict. PHA production can be made more sustainable and inexpensive using cheap non-food fermentable materials (Han et al., 2014). There have been many studies on PHA production from terrestrial biomass; however, only few studies have explored the feasibility of seaweeds as a fermentable biomass (Yamada et al., 2018; Azizi et al., 2017; Bera et al., 2015; Ghosh et al., 2019; Yamaguchi et al., 2019). Despite the abundance and great potential of seaweed biomass, they remain underutilized as an alternative source for high-value biomaterials including PHA (Cesario et al., 2018). Seaweeds contain fermentable carbohydrates and other complex polysaccharides such as alginate, mannitol, laminarin, galactan, and fucoidan (van Hal et al., 2014; Enquist-Newman et al., 2014; Takeda et al., 2011; Wargacki et al., 2012). However, only a few bioplastic-producing and algal polysaccharide-degrading bacteria have been described (Yamada et al., 2018; Han et al., 2014). *Hydrogenophaga* sp. strain UMI-18 is the first reported strain the utilize alginate as a sole carbon source in PHB production. Therefore, this strain will serve as a tool in exploring the viability of using seaweed biomass in PHB production.

Many strains of *Hydrogenophaga* have been reported to produce PHA using different carbon sources. For example, *H. pseudoflava*, which is closely related to strain UMI-18, produced PHA using glucose, fructose, galactose, xylose, mannose and lactose (Choi et al., 1995; Koller et al., 2008). Volatile fatty acids were also used as a carbon source by *H. palleron*i to produce poly(3-hydroxybutyrate-co-hydroxyvarate) P(3HB-co-3HV).

5.2 Alginolytic and PHB-producing *Hydrogenophaga* sp. strain UMI-18

Strain UMI-18 produced 58% ± 4% (*w/w*) of the total dried cell weight when it was grown in 1 L of AMS medium. Aside from alginate, glucose and fructose were its most preferable substrate. The general properties of its PHB was characterized and its molecular weight, T_g and T_m were comparable to other reported PHB producers like *Massilia* sp. strain UMI-21 ((Han et al., 2018), *Burkholderia* sp. strain AIU M5M02 (Yamada et al., 2018), *Azotobacter chroococcum* (Savenkova et al., 2000), *Hydrogenophaga* sp. (Tanamool et al., 2011), *H. pseudoflava* (Koller et al., 2008), *Cupriavidus necator* (Tanadchangsaeng et al., 2012) and *Bacillus megaterium* (Pradhan et al., 2018).

The draft genome analysis of strain UMI-18 revealed a deduced biochemical pathway in alginate depolymerization and PHB synthesis. Alginate-assimilating gene cluster and PHB-synthesis gene cluster were identified in its genome. The alginate-assimilating gene cluster is comparable to those of *Flavobacterium* sp. strain UMI-01 (Inoue et al., 2016; Nishiyama et al., 2017). In this pathway, alginate metabolism starts when the alginate polymer is transported inside the cell membrane via the major facilitator superfamily transport protein. Then, alginate is degraded to monosaccharide (DEH) with the synergistic actions of the exolytic and endolytic alginate lyases in the periplasm and cytosol. DEH reductases reduce DEH to KDG and then

phosphorylated to KDPG by KDG kinase. KDPG is finally split to pyruvate and glyceraldehyde-3-phosphate by KDPG aldolase and then metabolized through the cell's central metabolic pathway. In strain UMI-18, the alginate-derived pyruvate is then channeled to the PHB synthesis pathway. The enzymes responsible for PHB synthesis from pyruvate such as β-ketothiolase, NADPH-dependent acetoacetyl-CoA synthetase and Class I PHA synthase were annotated in the genome of strain UMI-18. PHB-synthesis gene cluster is common to many members of the *Comamonadaceae* such as *Hydrogenophaga* sp. and *Makilia* sp.; however, the presence of alginate lyase genes in this group of bacteria is unreported. Therefore, UMI-18 is considered a new alginolytic species of the *Hydrogenophaga* genus. It is necessary to characterize these alginate lyases to understand its role alginate in alginate depolymerization and its importance in PHB production.

5.3 The alginate lyases of strain UMI-18

Translational products of the alginate lyase genes, i.e. HyAly-I, HyAly-II, HyAly-III and HyAly-IV are listed to the PL-17, PL-7, PL-15 and PL-5 (EC 4.2.2.3, EC 4.2.2.11 and EC 4.2.2.26). Interestingly, BLAST search suggested that HyAly-I to HyAly-IV have unique primary structures with up to ~57% identity with other alginate lyases. HyAly-I and HyAly-III are exolytic alginate lyases with two catalytic domains; whereas HyAly-II and HyAly-III are endolytic alginate lyases that have signal peptides in their amino acid sequences. HyAly-II and HyAly-IV are believed to be localized in the periplasmic space, while HyAly-I and HyAly-III are concentrated in the cytosol.

The primary structures of HyAly-I (PL-17) and HyAly-III (PL-15) consist of two catalytic domains. HyAly-I has an heparinase II/III protein-like domain and an alginate lyase domain,

whereas HyAly-III consist of DUF4962, a domain with an unknown function and heparinase II/III protein-like domain. Alginate lyases listed in PL-17 and PL-15 have similar primary structures to HyAly-I and HyAly-III, respectively. In this research, recHyAly-I exhibited alginate lyase activity; however, recHyAly-III did not have alginate lyase activity in both the insoluble and soluble fractions of recombinant *E. coli*. Taking account the primary structures and the alginate lyase activity of the recombinant enzymes, it is believed that HyAly-I is the main exolytic alginate lyase of *Hydrogenophaga* sp. strain UMI-18 mainly responsible in the complete depolymerization of alginate to DEH. Thus, HyAly-I plays an essential role in the strain's PHB production.

To understand the precise biological functions of the alginate lyases of strain UMI-18, it is essential to characterize these enzymes biochemically through overexpression of the target genes to expression platforms in order to produce sufficient, stable and soluble recombinant protein. This is an essential step before structural and functional studies of the enzymes in interest (Groisillier et al., 2010). In the case of strain UMI-18, four alginate lyase genes were cloned and ligated to pET-21(b+) and pCold-I vectors and then introduced to *E. coli* BL21 (DE3). Out of the four, HyAly-I, HyAly-III and HyAly-IV were successfully overexpressed in *E. coli*, as observed in the SDS-PAGE after induction with 1 mM IPTG. However, recHyAly-I was the only promising recombinant protein produced. Majority of the proteins of recHyAly-III and recHyAly-IV were localized in the insoluble fraction of the cell. Therefore, it was difficult to further purify recHyAly-III and recHyAly-IV. recHyAly-I was also expressed with an N-terminal His-x6 tag; however, it cannot be purified using the Ni-NTA resin. This enzyme was purified by ammonium sulfate fractionation followed by TOYOPEARL DEAE-650M column chromatography and Superdex 200 10/300 size exclusion chromatography.

5.4 General properties of recHyAly-I

recHyAly-I is exolytic and poly(M) specific like other PL-17 alginate lyases (Table 1). It also has similar optimum temperature to OAL from *Strenotophomas maltophilia* KJ-2 (In Lee et al., 2012), Alg17C from Saccharophagus degradans 2-40 (Kim et al., 2012), TcAlg1 from *Thalassotalea crasostreae* (Wang et al., 2019). recHyAly-I is also thermally unstable like OalS17 from *Shewanella* sp. Kz7 (Wang et al., 2015) and TcAlg1. Its optimum pH is like alyII from *Pseudomonas* sp. OS-ALG9 (Kraiwattanapong et al., 1999; Kraiwattanapong et al., 2009), TcAlg1 and OalB from *Vibrio splendidus* (Jagtap et al., 2014). The pH stability of recHyAly-I is similar to OalS17. It also exhibited improved alginate lyase activity with Tris-HCl buffer than with sodium phosphate buffer. Similarly, OAL preferred Tris-HCl buffer compared to the phosphate buffer but exhibited the highest preference toward the carbonate buffer. An opposite buffer preference was observed in AlgL from *Sphingomonas* sp. MJ-3 (Park et al., 2012) favoring sodium phosphate buffer than Tris-HCl. recHyAly-I is slightly activated by NaCl like alyII and OalC17. Salt-activated alginate lyases are usually isolated from marine bacteria (Inoue et al., 2014; Zhu et al., 2019) and some alginate lyase from eukaryotic organisms (Inoue & Ojima, 2019; Rahman et al., 2010). At 0.4 M NaCl, recHyAly-I retained 19.25% of its activity, thus exhibiting halotolerance towards NaCl. Halotolerant enzymes are advantageous for industrial applications especially in the utilization of marine algal biomass which contains considerable amount of salt (Hirayama et al., 2016).

5.5 Production of diDEH by recHyAly-I

Of all the studies published related to PL-17 alginate lyases, this is the first study to use different types of substrate e.g., saturated disaccharide, saturated trisaccharide, unsaturated

trisaccharide, and unsaturated disaccharide. Furthermore, the reaction products produced by recHyAly-I from polymer and oligomer substrates were unique compared to other PL-17 alginate lyases. Unlike other PL-17 enzymes, recHyAly-I released not only substantial amount of DEH but also saturated monosaccharide (SM1), unsaturated disaccharide (UM2) and diDEH. diDEH is a putative novel dimeric DEH which consists of a linear-chain-form DEH and a ring-form DEH. It is believed that diDEH is comes from alginate trisaccharides in the final phase of the exolytic degradation of substrates. Additional studies are needed to confirm the structure of diDEH and to investigate the possible applications of diDEH.

Expensive substrates, high production costs, and the limited choice of PHA have resulted to the limited market success of PHA production on an industrial scale. Advances in metabolic engineering of microbes have allowed researchers to design new metabolic pathways of host strains to utilize inexpensive carbon substrates and to expand PHA diversity with varying functional groups (Zheng et al., 2019). Alginate-assimilating and PHB-synthesis genes of *Hydrogenophaga* sp. strain UMI-18 can be used as a template for this kind of strategy. The first approach involves the introduction of alginate lyase genes from strain UMI-18 into high PHB-producing bacteria and the second approach is to introduce the PHB synthesis genes to non-PHA-producing bacteria that can degrade seaweeds (Reddy et al., 2003). The co-production of oligoalginates and other high-value algal oligosaccharides from the use of seaweeds in PHB production is another strategy to reduce PHA production costs. PHAs are accumulated intracellularly, whereas extracellular by-products from the substrate can still be obtained from the culture media (Singh et al., 2019).

5.6 Future prospects

Out of the four alginate lyases annotated in the genome of *Hydrogenophaga* sp. strain UMI-18, only HyAly-I, HyAly-III and HyAly-IV could be produced in an *E. coli* expression system; however, recHyAly-III was not recovered in the soluble fraction. Production of recHyAly-I was the most promising because of the high concentration of recombinant proteins in the soluble fraction of the cell. Thus, it was purified and characterized. The second promising enzyme is HyAly-IV which was expressed with the pCold-I plasmid. Optimizing growth conditions for the recombinant *E. coli* and other purification methods are needed to extract sufficient amount soluble proteins to further purify and characterize the recombinant enzyme. HyAly-II and HyAly-III can be ligated using other plasmids and overexpressed in a different expression system.

recHyAly-I produced a novel dimeric DEH. Its structure was proposed in Chapter IV, but additional studies are required to fully elucidate the chemical structure of this new compound. First, diDEH should be purified using high performance liquid chromatography (HPLC) and then subjected to nuclear magnetic resonance (NMR) spectroscopy. Other compounds released by the reaction of recHyAly-I from various substrates should also be studied. Furthermore, the application of the oligoalginates, DEH and diDEH produced by recHyAly-I in different fields can also be investigated in future studies.

The genes coding for the PHB-synthesis enzymes of strain UMI-18 have been annotated in Chapter II. Future studies should investigate the feasibility of the heterologous expression of these genes for PHB production. The co-occurrence of alginate-assimilating and PHB-synthesizing gene clusters in strain UMI-18 can be utilized as a template to design new metabolic pathways that would introduce capabilities of host strains to use inexpensive substrates like seaweeds in PHB production. This would also allow the expansion of PHA diversity with varying functional groups. Alginate-assimilating genes can be introduced to high PHA producers or PHB-synthesizing genes

can be introduced to hosts that use seaweed polysaccharides as a carbon source. Since PHA is synthesized and stored intracellularly by the cells, co-production of oligo-alginates can be taken advantage to decrease the price of PHA production.

5.6 Conclusion

Hydrogenophaga sp. strain UMI-18 is the first PHB-producing bacterium that can use alginate as its sole carbon source. Its genes allow it to synthesize PHB and utilize algal polyaaccharides like alginate as a carbon source.

In this research, the exolytic alginate lyase, HyAly-I, a PL-17 enzyme was characterized using a recombinant enzyme. The primary structure and the biochemical properties of recHyAly-I were fully characterized. Based on the enzyme's properties, HyAly-I indeed plays an important role in the strain's PHB production since it degrades the alginate substrate to produce monomeric DEH — a precursor in PHB synthesis. Aside from DEH, a saturated monosaccharide and a novel dimeric DEH compound were discovered from the reaction of the poly(M) and trisaccharide substrates.

Along with the improvement of the downstream process in PHA production, the discovery of novel high throughput strains like *Hydrogenophaga* sp. strain UMI-18 which can utilize algal polysaccharide particularly alginate as a carbon source helps in reducing the costs of PHA production. The information presented in this study provides a link to the viability of using seaweed as an inexpensive raw fermentable material in the production of bioplastics and other high-value materials.

Table 5-1. Comparison of other PL-17 enzymes

Source/ Enzyme	kDa	Substrate preference	Specific Activity (U/mg)	Optimum temperature	Thermal stability	Optimum pH	pH stability	NaCl dependence (M)	Hepar II/III domain	Reference
Hydrogenophaga sp. UMI-18 (HyAly-I)	78.4	Poly M/ MG/	0.4	40°C	20°C	6.0	6.5	0.1	+	Present study
Sphingomonas sp. MJ-3 (AlgL)	79.9	N.R.	N.R.	50°C	N.R.	6.5	N.R.	N.R.	+	Park et al. 2012
Shewanella sp. Kz7 (oalS17)	85.6	Alginate	32	50°C	≤ 30°C	6.2	6.5	N.R.	+	Wang et al. 2014
Strenotophomas maltophilla KJ-2 (OAL)	47.8	Poly MG	N.R.	40°C	N.R.	8.0	N.R.	0.1	+	In Lee et al. 2012
Pseudomonas sp. OS-ALG9 (alyII)	79.8	Poly M	0.07	30°C	N.R.	7.0	N.R.	N.R.	+	Kraiwattanapong et al. 1999 Kraiwattanapong et al. 2009
Saccharophagus degradans 2-40 (Alg17C)	81.6	N.R.	2.3	40°C	N.R.	6.0	N.R.	0.1	+	Kim et al. 2012 Hirayama et al. 2016
Cellulophaga sp. SY116 (OalC17)	85.7	Poly M	67.9	45°C	0°C	7.8	6	0.402	+	Li et al. 2018
Thalassotalea crasostreae (TcAlg1)	82.8	Poly M	20.9	40°C	≤ 40°C	7.0	N.R.	N.R.	+	Wang et al. 2018
Vibrio splendidus (OalB)	82.8	Poly MG	79	30°C	N.R.	7.0	N.R.	N.R.	+	Jagtap et al. 2014
Vibrio splendidus (OalC)	80.5	Poly M	76	35°C	N.R.	7.5	N.R.	N.R.	+	

N.R. not reported

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Appendices

Appendix 1

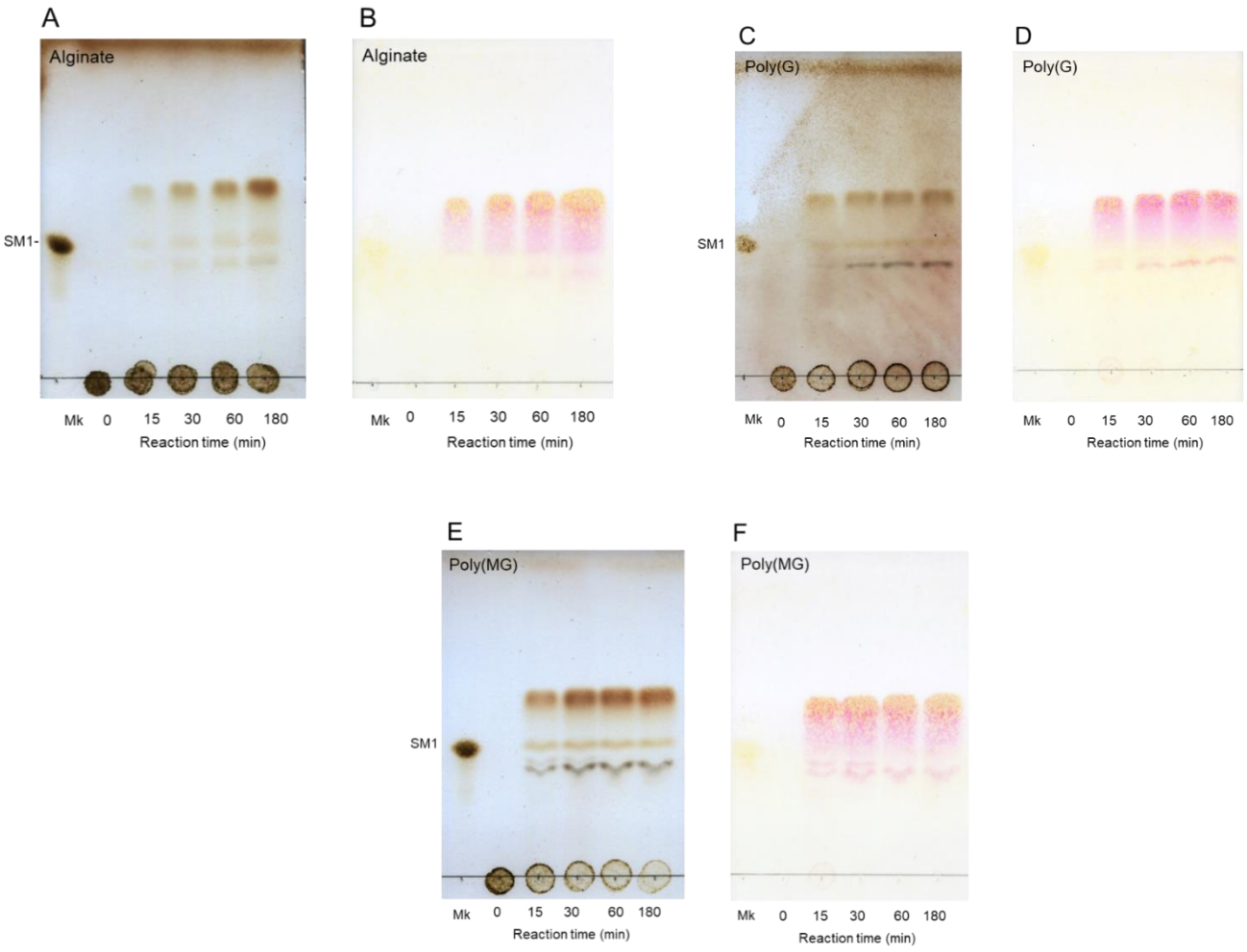

Figure A1. TLC analysis of the degradation products of alginate, poly(G) and poly(MG). Substrates were degraded by recHyAly-I in standard conditions for 15 - 180 min. (A) alginate substrate stained with 10% H_2SO_4 in ethanol; (B) alginate substrate stained with TBA; (C) poly(G) stained with 10% H_2SO_4 in ethanol; (D) poly(G) stained with TBA; (E) poly(MG) stained with 10% H_2SO_4 in ethanol; (F) poly(MG) stained with TBA. SM1- Marker.

Appendix 2

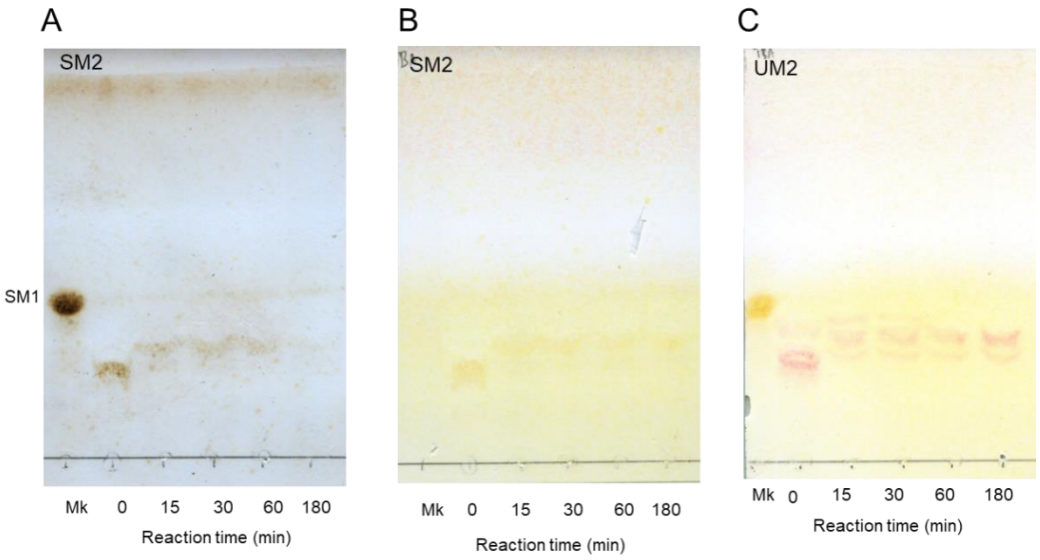

Figure A2. TLC analysis of SM2 and UM2. Substrates were degraded by recHyAly-I in standard conditions for 15 - 180 min. (A) SM2 stained with 10% H_2SO_4 in ethanol; (B) SM2 stained with TBA; (C) UM2 stained with TBA. SM1- Marker

Appendix 3

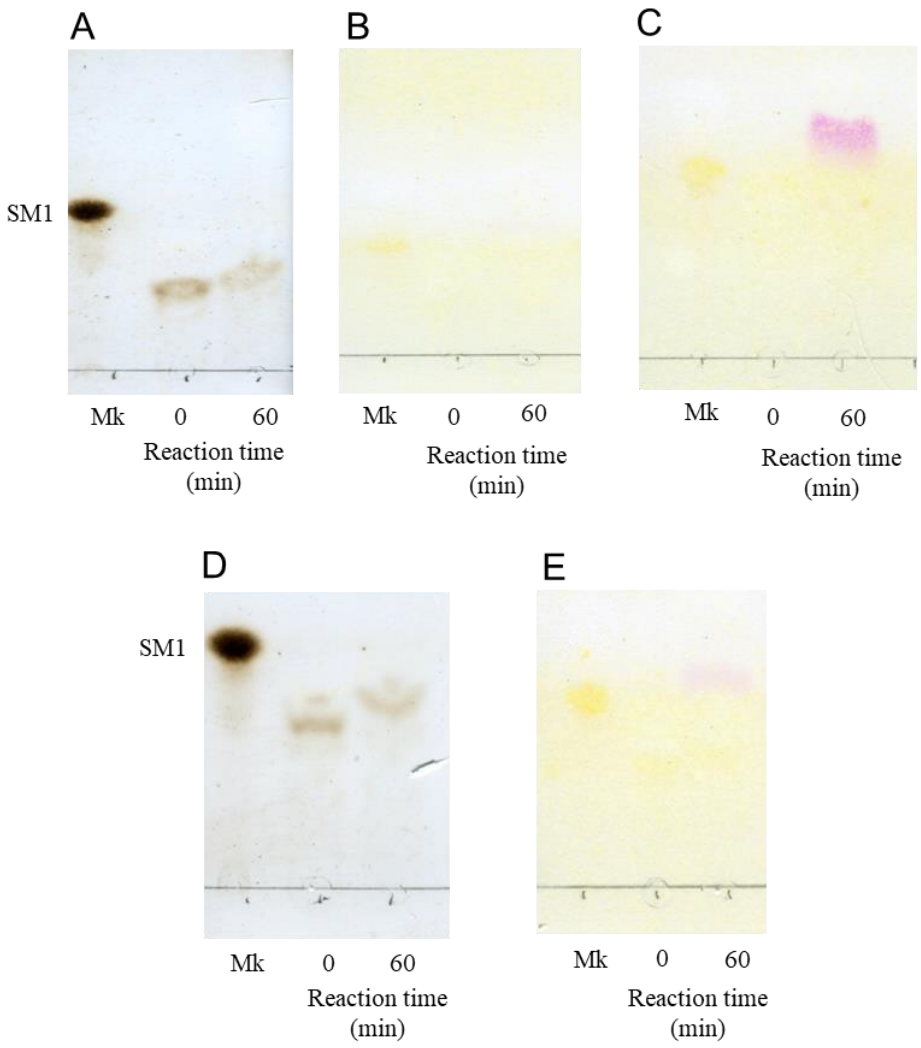

Figure A3. TLC Analysis of the degradation products of SM3, SM2 and poly(G) using FlAlex. FlAlex (PL-6) was used as positive control enzyme. (A) SM3 as substrate stained with 10% H_2SO_4 in ethanol; (B) SM3 as substrate stained with TBA; (C) poly(G)as substrate stained with TBA; (D) SM2 as substrate stained with 10% H_2SO_4 in ethanol; B, SM2 as substrate stained with TBA. SM1- Marker

Appendix 4

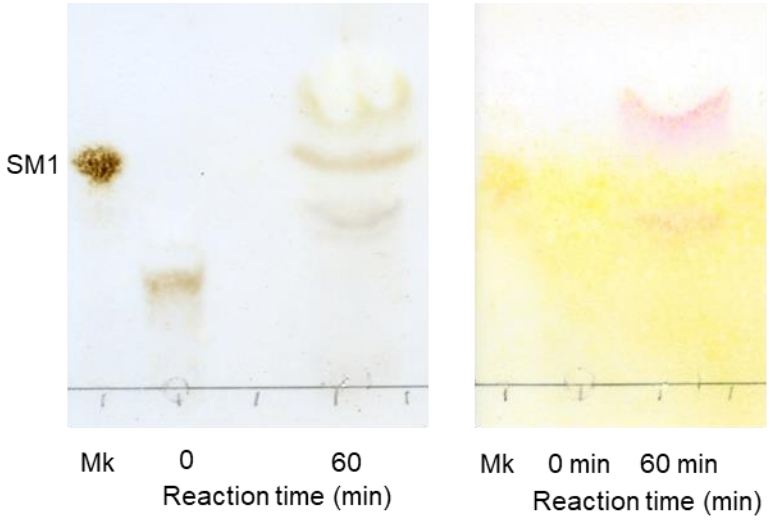

Figure A4. Degradation of SM3 by recHyAly-I and FlAlex. SM3 was used as a substrate in a standard condition with final concentration 0.3 mg/ml recHyAly-I and 0.3 mg/ml FlAlex incubated for 60 min. (A) reaction products stained with 10% H_2SO_4 in ethanol; (B) reaction products stained with TBA. SM1- Marker

Appendix 5

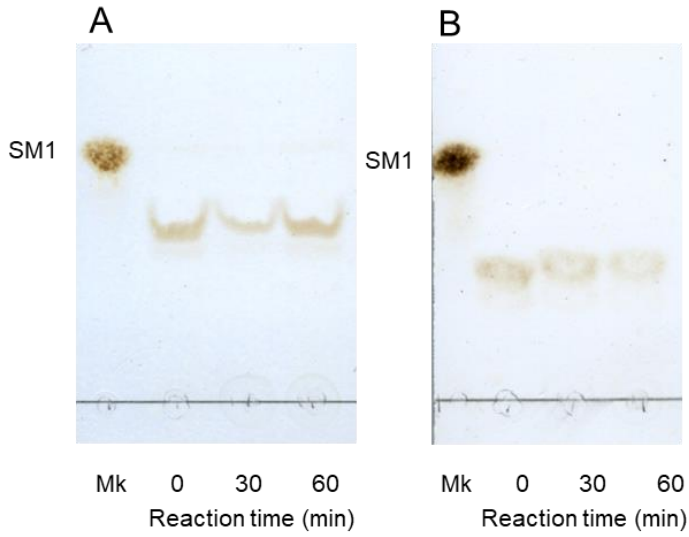

Figure A5. Degradation of SM2 and SM3 by *E. coli* cell lysates. Cell lysates from SM2 and SM3 were incubated with cell lysates from *E. coli* in a standard condition for 60 min. (A) SM2 used as substrate and stained with 10% H_2SO_4 in ethanol; (B) SM3 used as substrate and stained with TBA. SM1- Marker

Appendix 6

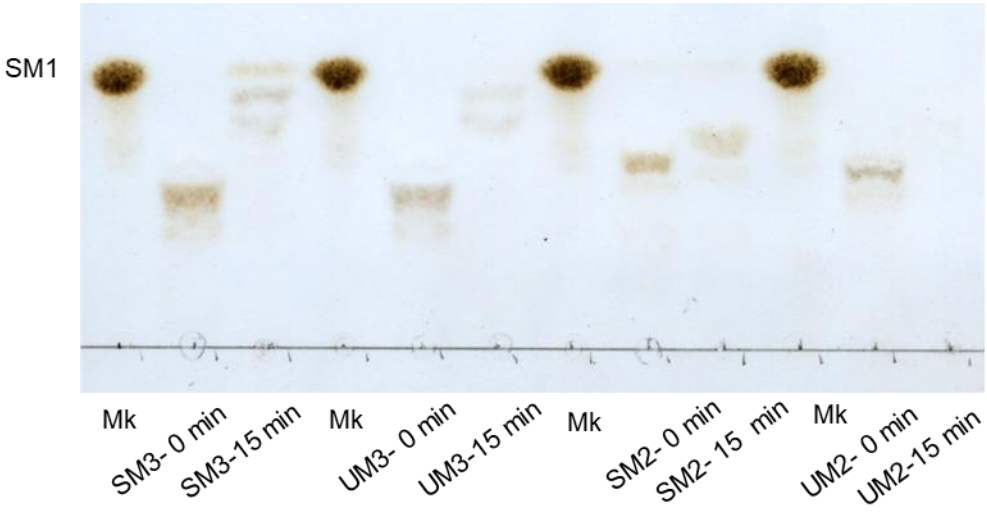

Figure A6. TLC analysis of the degradation products of SM3, UM3, SM2, and UM2 by recHyAly-I. Oligosaccharides were degraded with recHyAly-I for 15 min in standard reaction conditions. SM1- marker

Appendix 7

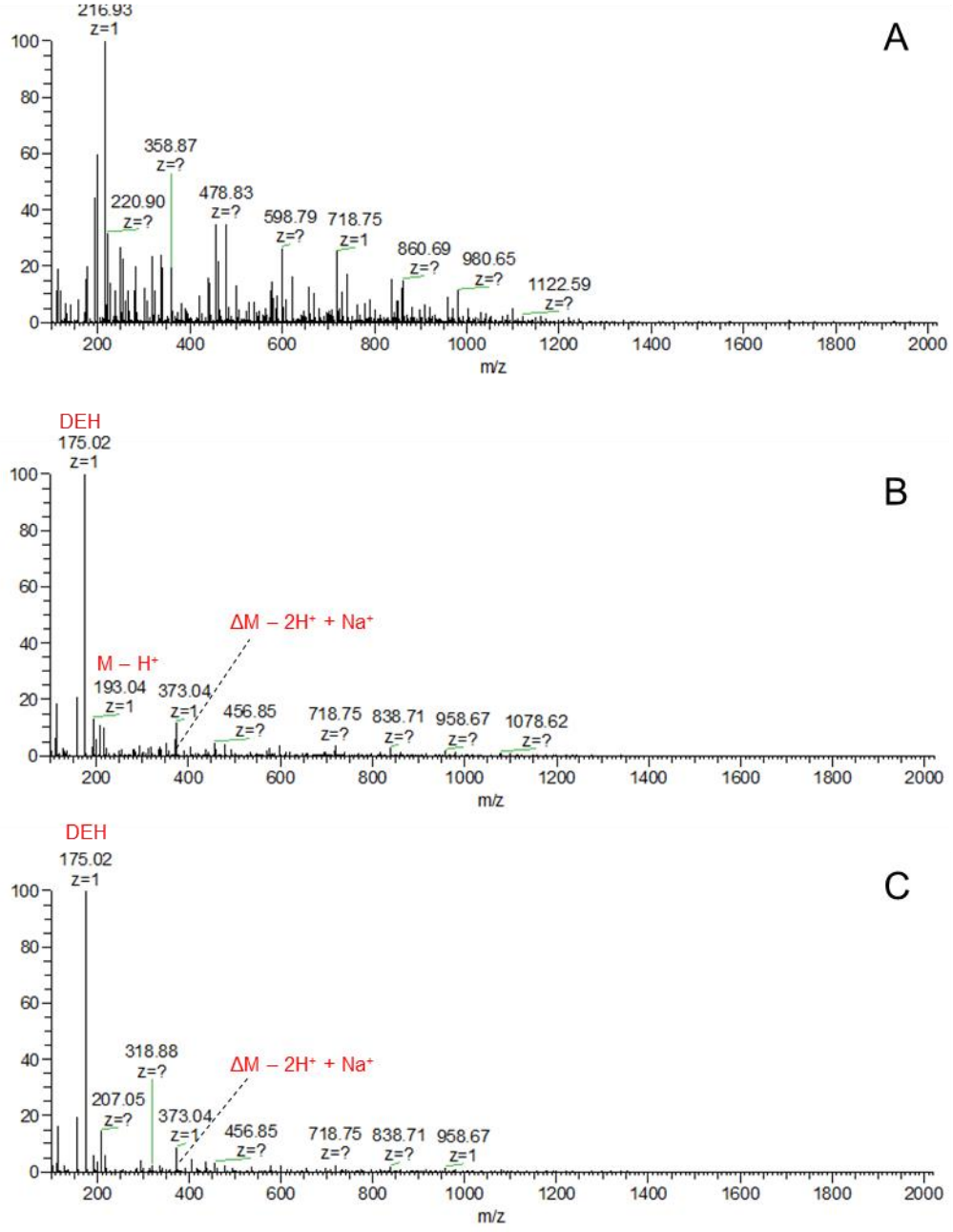

Figure A7. ESI-MS analyses of poly(M) degradation products by recHyAly-I. (A) 0 min; (B) 15 min; (C) 180 min.

Appendix 8

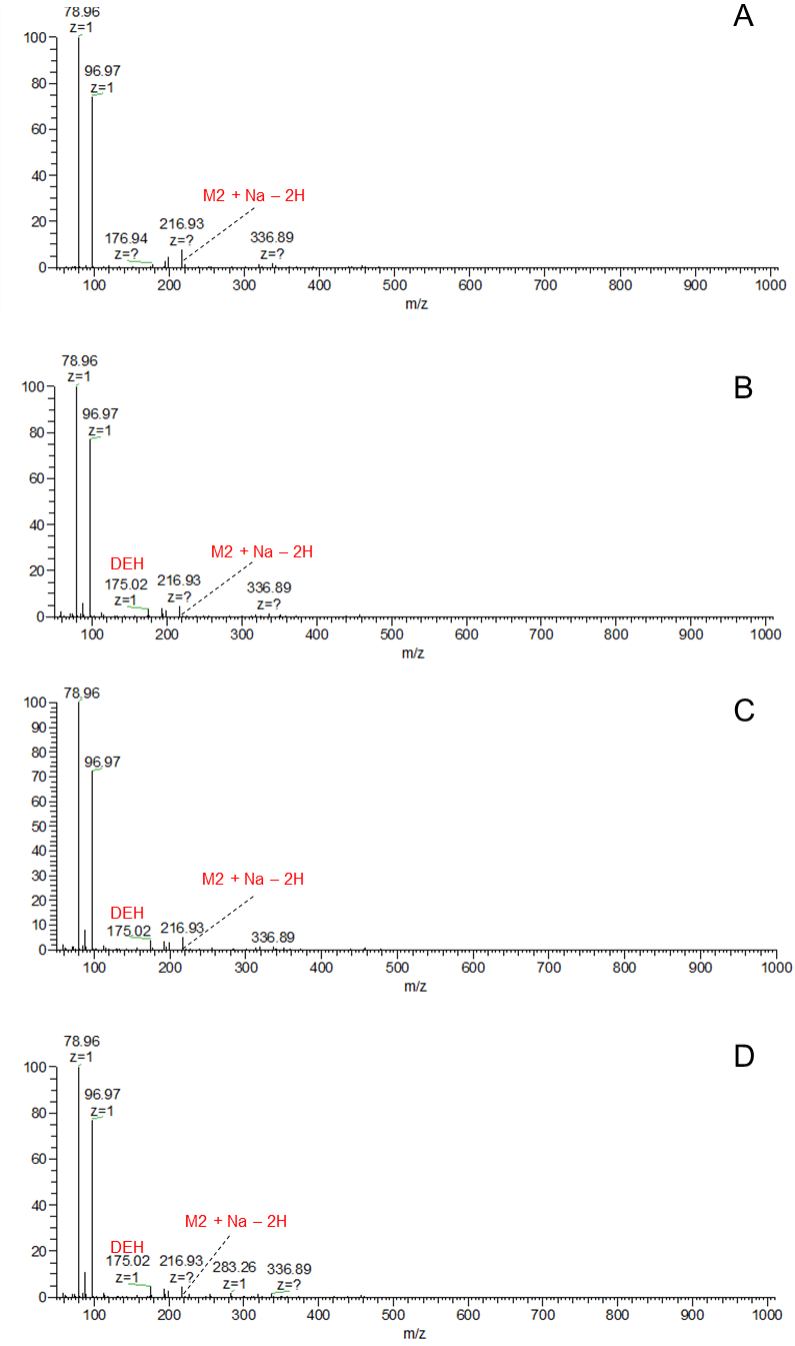

Figure A8. ESI-MS analyses of SM3 degradation products by recHyAly-I. (A) 0 min; (B) 15 min; (C) 30 min; (D) 180 min.

Appendix 9

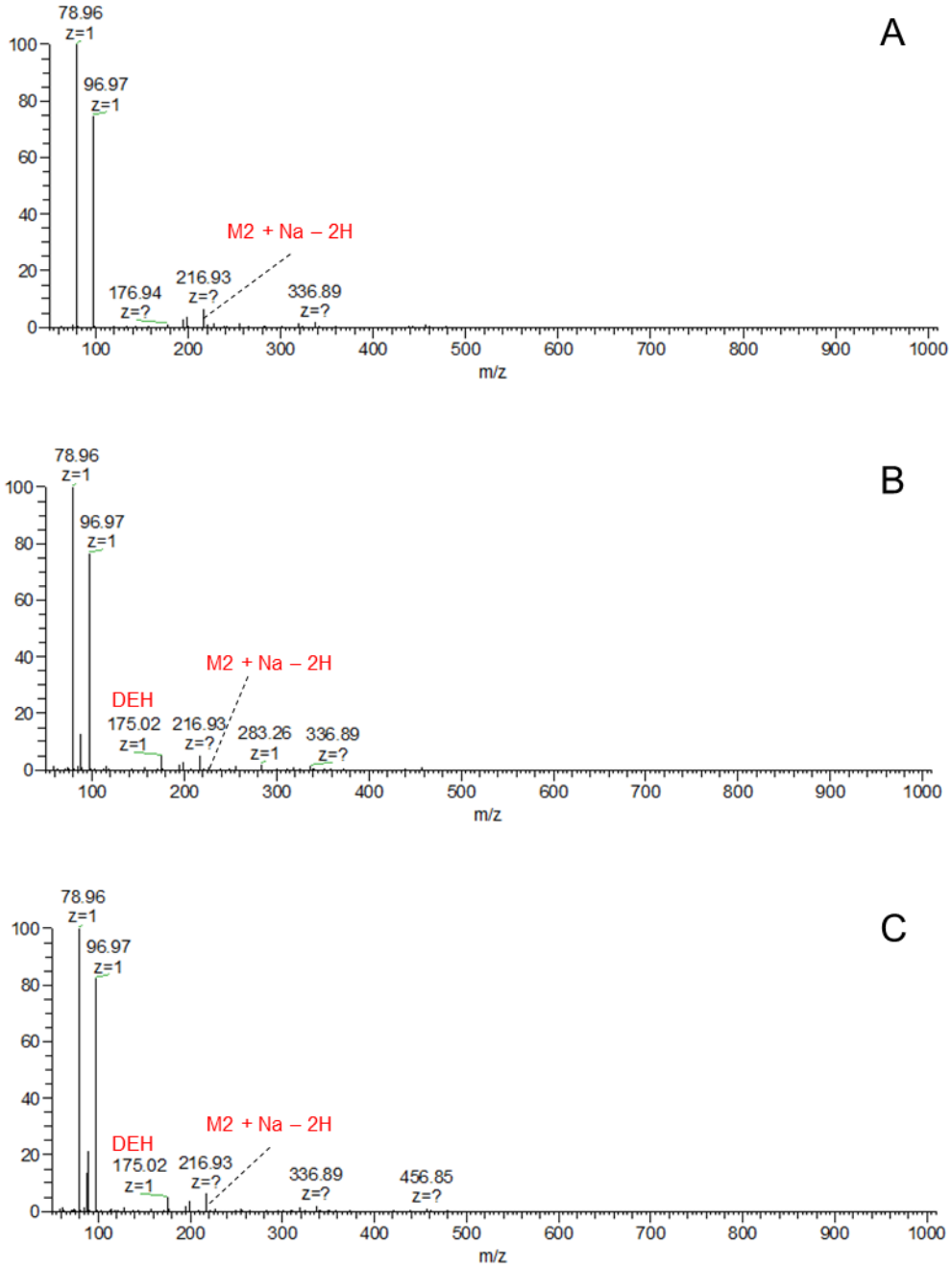

Figure A9. ESI-MS of UM3 degradation products by recHyAly-I. (A) 0 min; (B) 30 min; *C*, 180 min.

Appendix 10

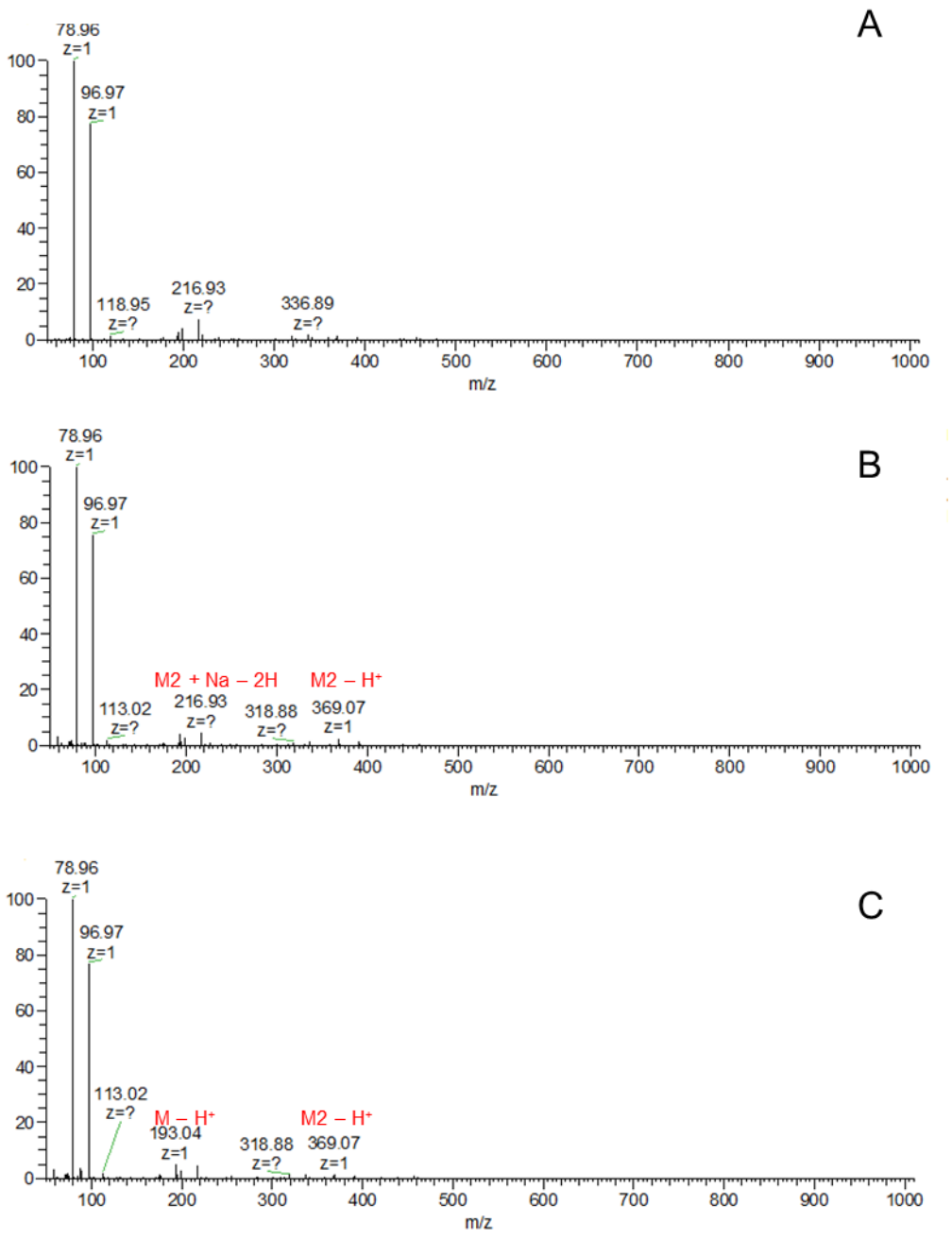

Figure A10. ESI-MS of SM2 degradation products by recHyAly-I. (A) 0 min; (B) 30 min; (C) 180 min.

Appendix 11

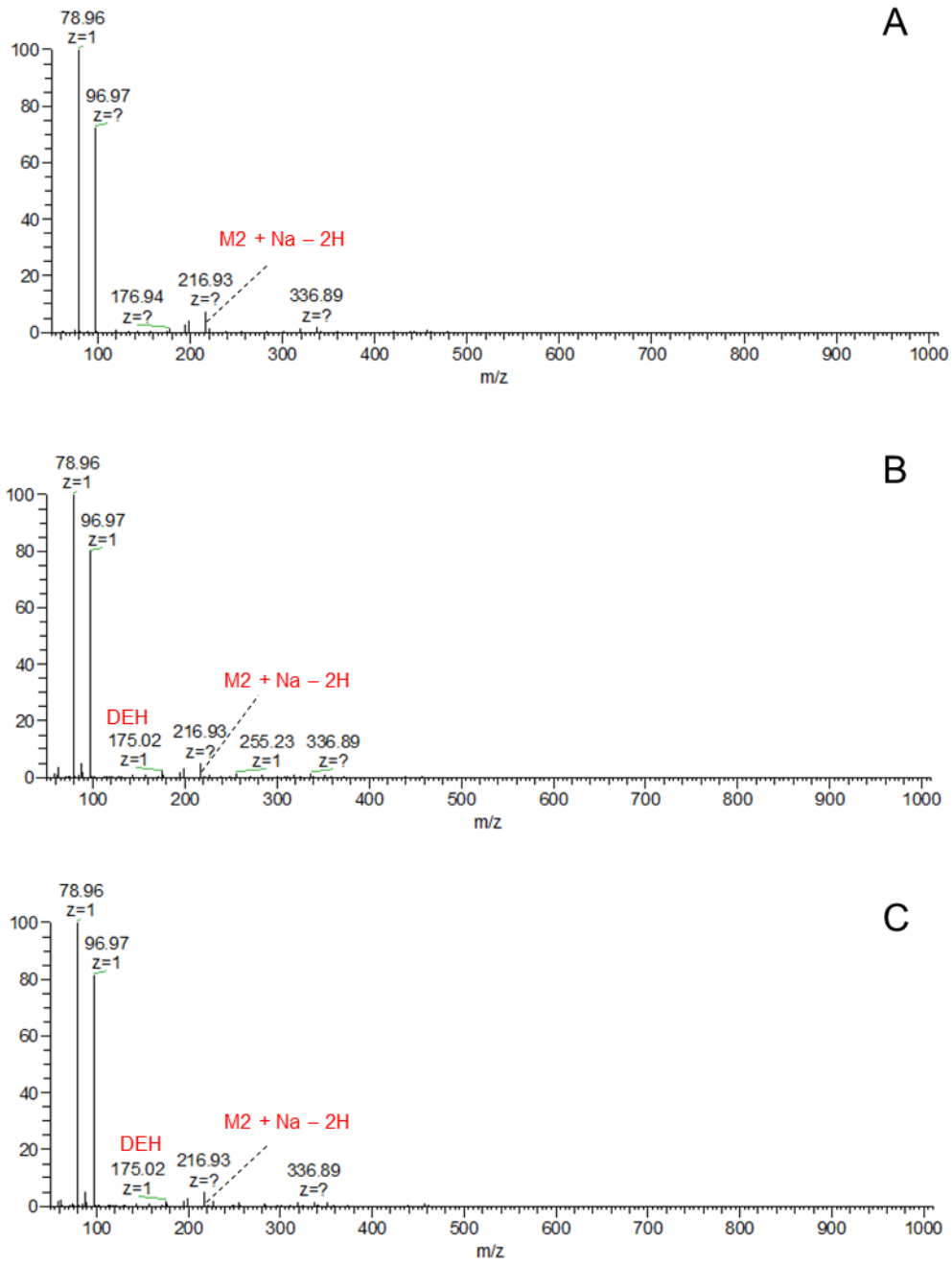

Figure A11. ESI-MS of UG3 degradation products by recHyAly-I. (A) 0 min; (B) 30 min; (C) 180 min.

Appendix 12

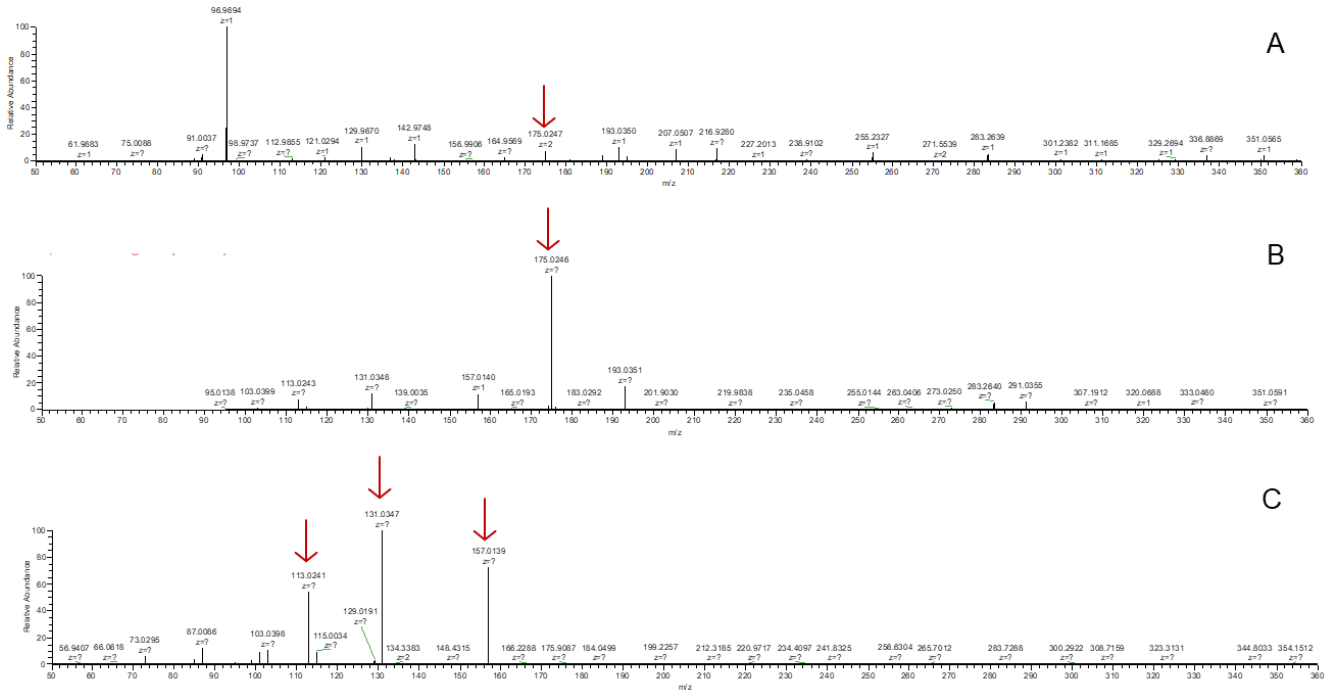

Figure A12. ESI-MS (m/z351) of reaction products of poly(M) degraded with recHyAly-I. Red arrows indicated significant major peaks or ion fragments. (A) MS at z/351, 175.0247 *m/z* (*z*=2) indicated a dimeric precursor ion. (B) MS/MS at z/351, 175.0247 m/z (z=?) indicated molecular mass of fragment ion is similar to monomeric DEH. (C) MS/MS/MS at z/351, have a major fragment of 113.0241 *m/z* (F1), 131.0347 *m/z* (F2) and 157.0139 *m/z* (F3).

Appendix 13

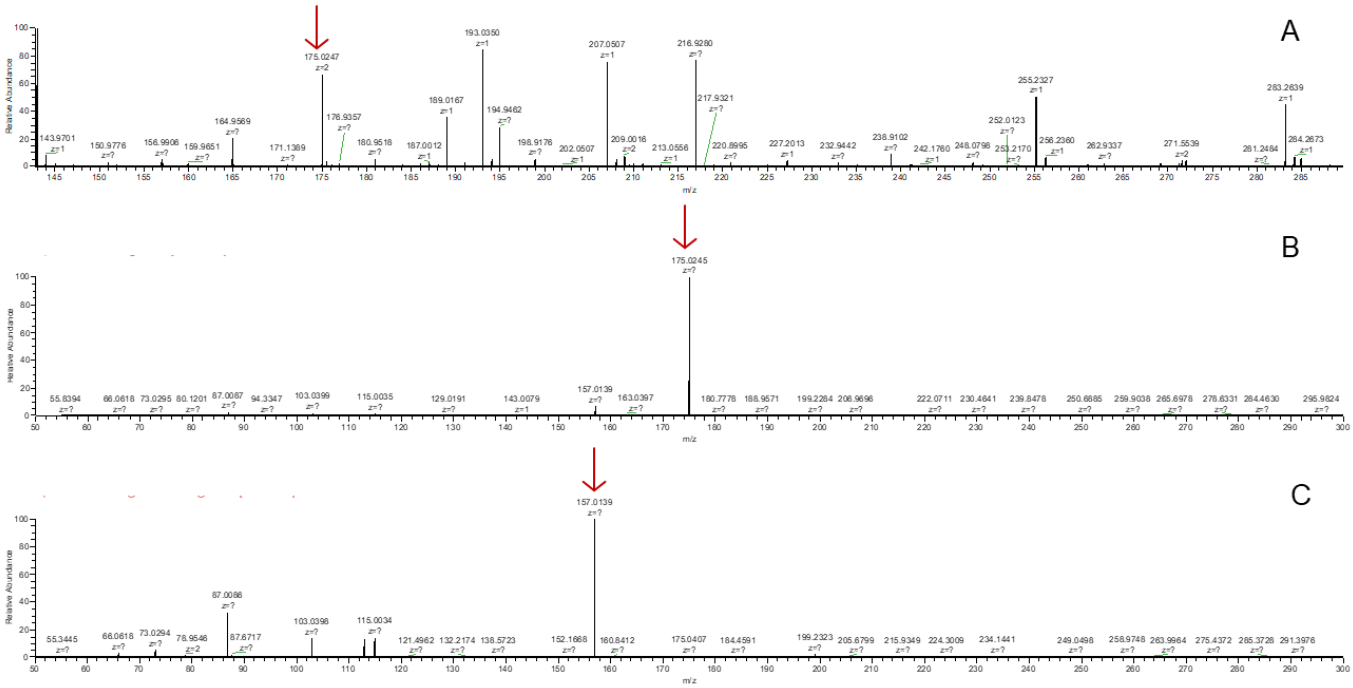

Figure A13. ESI-MS (m/z207) of reaction products of poly(M) degraded with recHyAly-I. Red arrows indicated significant major peaks or ion fragments. (A) MS at z/207, 175.0247 *m/z* (*z*=2) indicated a dimeric precursor ione. (B) MS/MS at z/207, 175.0247 *m/z* (*z*=?) indicated molecular mass of monomeric DEH. (C) MS/MS/MS at *m/z* 207 have a major fragment of 157.0139 *m/z* (F3).

Appendix 14

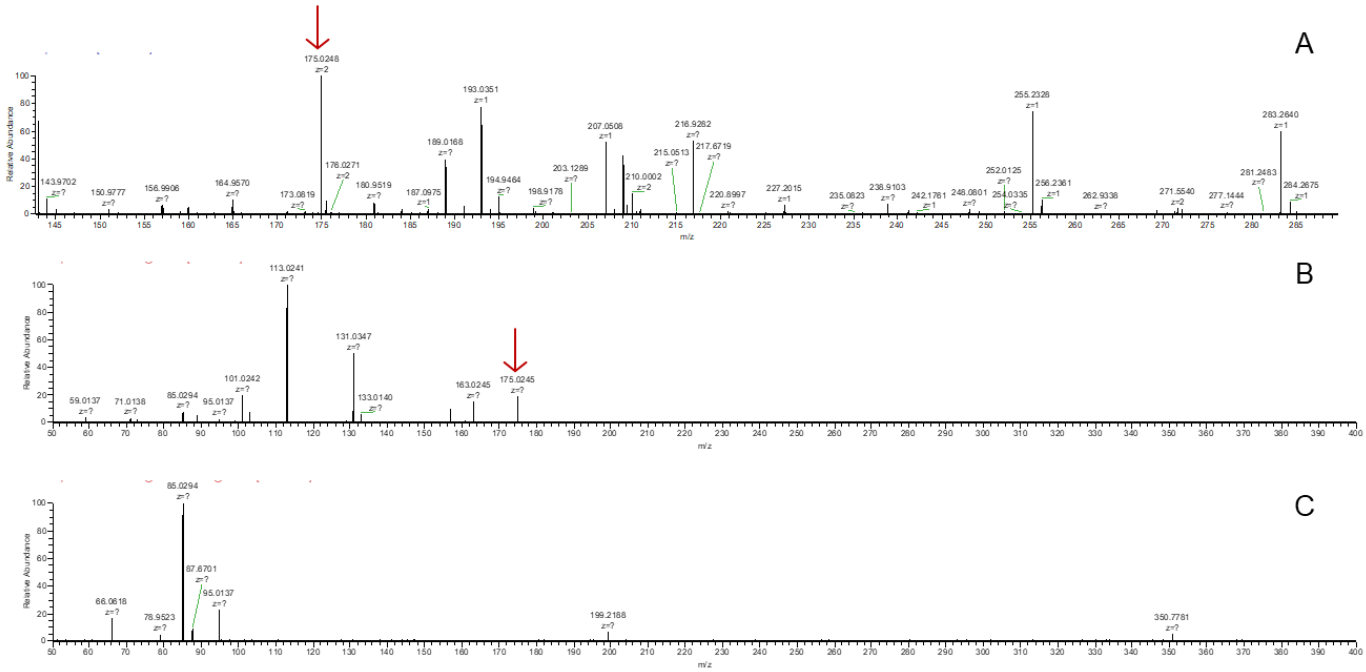

Figure A14. ESI-MS (m/z193) of reaction products of poly(M) degraded with recHyAly-I. Red arrows indicated significant major peaks or ion fragments. (A) MS at *m/z* 193, 175.0247 *m/z* (*z*=2) indicated a dimeric precursor ione. (B) MS/MS at *m/z* 193, 175.0247 *m/z* (z=?) indicated molecular mass of monomeric DEH. (C) MS/MS/MS at *m/z* 193.

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