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REVIEW

Alginate Lyases: Sources, Mechanism of Activity and Potencial Application

Alginate Liase: Sumber, Mekanisme Aktivitas dan Aplikasi Potensial

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ABSTRACT

Alginate lyases are group of enzymes which catalyze depolymerization of alginate into oligosaccharides. Alginate lyase have been widely used in many applications such as in production of bioactive oligosaccharides, control of polysaccharide rheological properties, and polysaccharide structure analysis. The products of alginate lyase, polysaccharide structure analysis, alginate oligosaccharides (AOS) have many biological activities including act as prebiotics, immune modulator, anticoagulation, antioxidant, anticancer, growth promoting activities, promote production of antibiotics and ethanol. In relation to the importance of alginate lyases, their potential aplications and prospect in development of new bioactive products, we present review of the enzymes, sources, mechanism of activity and potential applications. This paper also discussed some new biological engineering in alginate lyase production.

Keywords: *alginate lyase, alginate oligosaccharides, seaweed, biological activities*

ABSTRAK

Alginat liase adalah kelompok enzim yang mengkatalisis depolimerisasi alginat menjadi oligosakarida. Alginat liase telah banyak digunakan dalam berbagai aplikasi seperti dalam produksi oligosakarida bioaktif, pengaturan sifat rheologi polisakarida dan analisis struktur. Produk dari alginat liase, oligosakarida alginat (AOS) memiliki berbagai aktivitas biologi seperti bertindak sebagai prebiotik, modulasi imun, anti koagulasi, anti oksidan, anti kanker, aktivitas pemacu pertumbuhan, meningkatkan produksi antibiotik dan etanol. Sehubungan dengan pentingnya alginat lyase, potensi aplikasi dan prospek pengebangan produk-produk bioaktif baru, kami menyampaikan review tentang enzim ini, sumber, mekanisme aktivitas dan potensi aplikasinya. Tulisan ini juga membahas beberapa rekayasa biologi dlam produsksi alginat liase.

Kata Kunci: alginat liase, alginat oligosakarida, rumput laut, aktivitas biologi

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Alginate lyase, Specificity and Mechanism of Activity

Alginate lyases catalyze depolymerization of alginate through α-elimination of the 4-O-glycosyl bond accompanied by the formation of a double bond between C-4 and C-5 and the production of 4- deoxy-L-erythro-hex-4-ene pyranosyluronate at nonreducing end of the resulting oligosaccharides (Figure 1) (Gacesa, 1992). Based on their substrate specificity, alginate lyases are devided into two groups. First, alginate lyase which specifically depolymerize polymannuronate, called poly (α-D-mannuronate) lyases (EC4.2.2.3). Some examples of these enzyme are produced from *Pseudomonas aeruginosa* (Boyd et al., 1993), *Azotobacter vinelandii* (Ertesvåg et al., 1998), and *Azotobacter chroococcum* (Haraguchi and Kodama, 1996). Second, alginate lyase which has hydrolitic activity on polyguluronate block, are called poly (α-L-guluronate) lyases (EC 4.2.2.11). Some examples for these enzymes are alginate lyase from *Enterobacter cloacae* (Shimokawa et al., 1997), *Klebsiella pneumoniae* (Baron et al., 1994) and *Vibrio* sp. 510 (Hu et al., 2006). Although lyases are classified based on mannuronate or guluronate specificity, some crude lyase extracts have multiple substrate specificity such as *P. alginovora* strain XO17 and *Sphingomonas* sp. strain A1. It appeares that the organism produce more than one lyases or the enzymes produced have multiple substrate activities (Wong et al., 2000). Boyen et al., (1990) reported that *P. alginovora* strain XO17 produced more than one alginate lyase. It has both poly (M) and poly (G) lyase. Hashimoto et al., (2000) reported

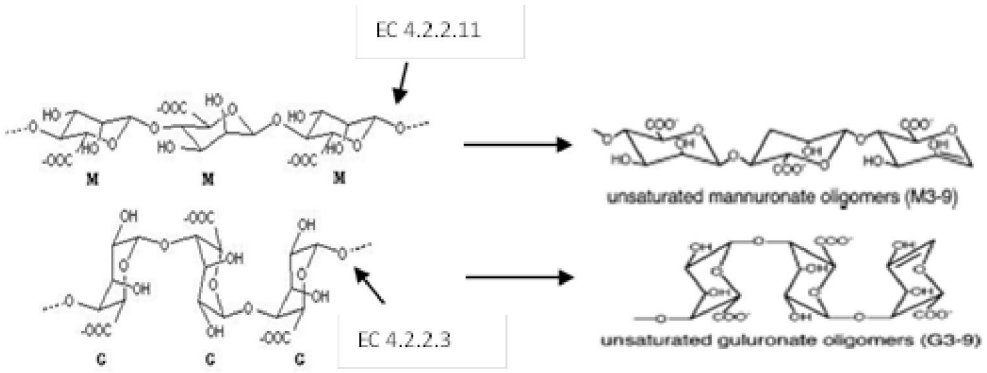

Figure 1. Depolymerization of alginate by alginate lyase producing unsaturated mannuronate and unsatrated guluronate (source: Iwamoto *et al*., 2005; UIO, 2013) M = mannuronate, G = guluronate

a lyase from soil bacteria *Sphingomonas* sp. strain A1 which has activities both on polymannuronate and polyguluronate.

Another classification of alginate lyase was published by Henrisat et al. (2005). Based on their primary structures, alginate lyases are grouped into three families, PL-5, -7, and -14. Most of the PL-5 and -7 alginate lyases family specifically depolymerize poly(M) and poly(G), although family PL-14 contains enzymes specific for poly(M) or poly(G). Similar to this classification, Matsushima et al. (2010) categorized alginate lyases into three groups on the basis of their substrate specificities. Two groups are PM lyases (EC 4.2.2.3) and PG lyases (EC 4.2.2.11) that depolymerize only PM and PG, respectively. The enzymes belonging to the last group, often designated as bifunctional alginate lyases, and able to depolymerize PM, PG, and the heteropolymeric form.

Kawamoto et al. (2006) isolated two alginate lyase from marine bacterium *Vibrio* sp. O2 as aly VOA and aly VOB. He estimated the sizes of these alginate lyases as 28.4 and 25.2 kDa. These enzymes are consisted of 285 and 275 amino acid residues. The modelling structure of alginate lyase from *Vibrio splendidus* A3UR44 and *Vibrio splendidus* A3UT33 are shown in Figure 2.

Alginate as substrate for lyase activity is a polymer consisted of the monomeric units of α-L-guluronate (G) and α-D-mannuronate (M). Based on the arangement of the monomeric units, there are three type of alginate: alginate with polymannuronate (poly M), that with polyguluronate (poly G), and alginate with random M & G (poly MG). Alginate may have the three block on single polimer molecule, but some alginate predominantly consisted of one type of block (Draget et al., 2005).

Many research have been conducted to find out the mechanisme of alginate lyase activity in depolymerizing alginate. The lattest theory, proposed that mechanism of activity of alginate lyase included three steps of reaction. First, alginate lyase removed the negative charge on the carbonyl anion. This enzyme neutralized the charge of anion by a salt bridge, with lysine as a proposed residue responsible

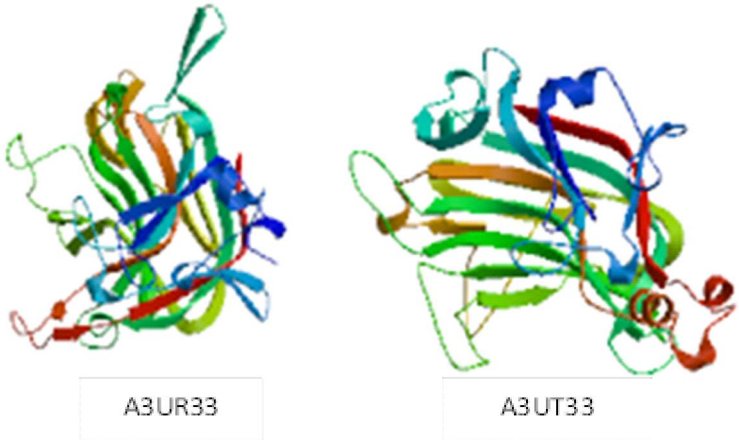

Figure 2. Modelling structure protein of alginate lyase isolated from *Vibrio splendidus* A3UR44 and *Vibrio splendidus* A3UT33. Source: Anon. (2013)

for this action. Secondly, an abstraction of the proton on C5 catalized by a general base (this role was predicted taken by aspartic acid, glutamic acid, histidyne, lysine and cysteine), where one residue act as proton donor and another as proton abstractor. In addition, the proton may be derrived from the solvent environment. Thirdly, forming a double bond between C4 and C5 by transfered electrons from the carbonyl group resulting in the α-elimination of the 4-O-glycosidic bond (Gacessa et al., 1992).

The former theory for alginate lyase activity in depolymerize alginate proposed by Haugh et al., (1967) stated that depolimerization alginate was a â-elimination mechanism, targeting the glycosidic 1 à 4 O-linkage between monomers. A double bond is formed between the C4 and C5 carbons of the six-membered ring from which the 4-O-glycosidic bond is eliminated, resulting in depolymerization of alginate and simultaneously yielding a product containing 4-deoxy-L-erythro-hex-4-enopyranosyluranic acid as the nonreducing terminal moiety (Wong et al., 2000).

Organism Producing Alginate Lyase

Alginate lyase is produced by brown seaweed, marine molluscs, fungi and microorganism including bacteria and macrophage (Wong et al., 2000). Microorganisms such as soil and marine bacteria were the most important sources for alginate lyase production because they are easy to culture, grow fast and uncomplicated to improve their productivity through genetical engineering (Baron et al*.,* 1994; Xiao et al., 2006). Alginate can serve as a carbon and energy sources for many microorganism (Wong et al., 2000). Most of bacteria producing alginate lyase used this polymer as a carbon and energy source, and depolymerize alginate into mannuronate or guluronate to increase the absorption of the moleculles. Organism producing alginate lyase and the specificity of the enzyme are shown in Table1.

Soil bacteria producing alginate lyase reported by Kaneko et al. (2000) include species from *Flavobacterium* sp., *Alkaligenes* sp., and *Bacillus* sp. The alginate lyase activity in these strains is low when they were grown separately, but it is high when these strains were cultured together in the same medium indicating that there are mutual simbiotic activities between these bacteria (Kaneko et al., 2000). A Soil bacteria *Sphingomonas* sp. strain A1 was also reported having alginate lyase activity. There were three kinds of cytoplasmic alginate lyases: A1-I (66 kDa), A1-II (25 kDa), and A1-III (40 kDa) from this bacteria (Hashimoto et al., 2000). These enzymes not only specific for mannuronate but also for guluronate. Kinoshita et al. (1991) isolated alginate lyase from *Pseudomonas* sp. and reported that the bacterium produced alginate-degrading enzymes both intra- and extracellularly. He found that the growth and alginate-degrading enzyme production were optimum at pH 7.0 and 30 °C. An alginate lyase which has specificity for mannuronate substrate had been found in *Pseudomonas aeruginosa* (Boyd et al., 1993). The optimum pH for optimal activity was 7.0 and the apparent isoelectric point (pI) was 9.0. Another *Pseudomonas* such as *Pseudomonas* sp. QD03 and *Pseudomonas aeruginosa* FRD1 were two types of soil bacteria which produce lyases that are effective on acetylated alginate (Xiao et al., 2006). Preston et al. (2000) characterized alginate lyase from *Pseudomonas syringae* pv syringae, and found that maximum activity for lyase was at pH 7.0 and 42 ^{0}C.

An mannuronate specific alginate lyase reported from *Azotobacter vinelandii* which has pI value of 5,1. The optimum enzyme activity were found between pH 8.1 and 8.4 and with the presence of 0.35 M NaCl (Ertesvåg et al., 1998). Another mannuronate specific alginate lyase was also found in *Azotobacter chroococcum* which has pI value of 5,1 (Haraguchi and Kodama, 1996).Two guluronate specific alginate lyases from soil bacteria were isolated from *Enterobacter Cloacae*. These enzymes were both extracellular and intracellular with pI of 8,9 (Nibu et al., 1995; Shimokawa et al., 1997).

Gram-positive soil bacteria such as *Clostridium* sp. and *Bacillus* sp. also produced alginate lyase. Kaiser et al. (1968) reported a lyase activity from *Clostridium alginolyticum*. Soil bacterium *Bacillus* sp. ATB-1015 was also reported to produce an extracellular alginate lyase (Nakagawa et al., 1998). *Corynebacterium* sp. produced an endo-poly (G) lyase that cleaves G-G bond (Matsubara et al., 1998).

Another important source of alginate lyase is marine bacteria including bacteria or microorganism associated with marine algae and mollusks. Li et al., (2011) found three alginate lyases from marine bacterium *Pseudomonas fluorescens* HZJ216. Molecular masses of the three enzymes were 60.25, 36, and 23 kDa with isoelectric points of 4, 4.36, and 4.59, respectively. The highest activity of these enzyme were obtained at pH 7.0 and 35 °C. Alginate lyases from marine *Alteromonas* sp. had been characterized. *Alteromonas* sp. strain H-4 produced more than five different alginate lyases (Sawabe et al., 1997). Besides the extracellular lyase, *Alteromonas* sp. strain H-4 also produced at least four intracellular alginate lyase. Hu et al. (2006) characterized alginate lyase from marine bacterium *Vibrio* sp. 510 which was found as extracellular enzyme which specific for poly (G) block.

Matsushima et al. (2010), characterized an extracellular alginate lyase from marine bacteria

Table 1. Organism producing alginate lyase and the specificity of its enzyme

Organism	Substrate Specifity	Reference
Soil bacteria:		
Sphingomonas sp. strain A1	Poly (M)	Hashimoto et al., 2000
Pseudomonas aeruginosa	Poly (M)	Boyd et al., 1993
Pseudomonas sp. QD03	Poly (M)	Xiao et al., 2006
Azotobacter vinelandii	Poly (M)	Ertesvåg et al., 1998
Azotobacter chroococcum	Poly (M)	Haraguchi and Kodama 1996
Enterobacter Cloacae	Poly (G)	Nibu et al., 1995; Shimokawa et al., 1997
Bacillus sp. ATB-1015	-	Nakagawa et al., 1998
Corynebacterium sp	Poly (G)	Matsubara et al., 1998
Marine bacteria:		
Pseudomonas fluorescens HZJ216	-	Li et al., 2011
Alteromonas sp. strain H-4	Poly (M), Poly (G) and poly (MG)	Sawabe et al., 1997
Vibrio sp. 510	Poly (G)	Hu et al., 2006
Pseudoalteromonas atlantica AR06	-	Matsushima et al., 2010
Vibrio sp. O2	Poly (M)	Kawamoto et al., 2006
Vibrio sp. QY 101	Poly (M), Poly (G)	Han et al., 2004
Pseudoalteromonas agarovans	-	Choi et al., 2009
Streptomyces sp ALG-5	Poly (G)	Kim et al., 2009
Pseudoalteromonas sp. SM0524	Poly (M), Poly (G)	Li et al., 2011
Mollusks and Crustaceae		
Littorina spp.	Poly (M)	Matiur et al., 2012
Littorina brevicula	Poly (M), Poly (G)	Wang et al., 2012
Haliotis spp	Poly (M)	Wong et al., 2000
Haliotis tuberculata	Poly (M), Poly (G)	Heyraud et al., 1996
Haliotis discus	Poly (M), Poly (G)	Mami et al., 2009
Haliotis iris	Poly (M), Poly (G)	Mami et al., 2009
Orchonectes rusticus	Poly (M), Poly (G)	Mami et al., 2009

Pseudoalteromonas atlantica AR06 and found that there were three protein bands (molecular weight) that had alginate lyase activity. From this research he found a gene that responsible for alginate lyase activity called alyA. Kawamoto et al. (2006) found an alginate lyase from marine bacterium *Vibrio* sp. O2, This lyase was specific to polymannuronate and did not degrade polyguluronate. Choi et al. (2009) isolated an lyase from marine bacterium *Pseudoalteromonas agarovans* and it was used for production of alginate oligosaccarides. The optimum concentration of sugar in this production was obtained at 30 rpm and 29^{0}C at the addition of 30 g/Lof NaCl. Li et al. (2011) isolated alginate lyase from bacterial strain *Pseudoalteromonas* sp. SM0524 that associated with rotten kelp, and found that this lyase has bifunctional activities. This lyase depolymerized not only polymannuronate but also polyguluronate, and named aly-SJ02. The optimal temperature and pH of aly-SJ02 toward sodium alginate was 50 °C and 8.5, respectively.

Other sources of alginate lyase are molluscs and algae. Matiur et al. (2012) isolated alginate lyase from the digestive tracts of herbivorous marine snails like *Littorina* spp. The *Littorina* enzymes have been identified as endolytic polymannuronate (poly(M))

lyases (EC 4.2.2.3). A marine abalone, *Haliotis* spp produced two different lyases, most were endo-poly (M) lyases (Wong et al., 2000). On the other hand, another mollusks *Turbo cornutus* produced two lyase isozymes. While lyase from *T. cornutus* has preference to cleave M-M linkage, lyase from *H. tuberculata* pefered on M-M and G-M linkages (Heyraud et al., 1996). Another abalone, *Haliotis gigantea* produced alginate lyase, and a supplemented diet with probiotics *Pediococcus* sp. Ab1 led to the improvement of environment gut abalone and increased the alginate lyase activity (Shunpei et al., 2010). Some algae, such as brown seaweed also produced alginate lyase (Wong et al., 2000).

Mami et al. (2009) analyzed alginate lyases from three Archeogastropoda, i.e., *Haliotis discus hannai*, *H. iris*, and *Omphalius rusticus*, and one Mesogastropoda, i.e., *Littorina brevicula*. These enzymes were identified as poly(M) lyase (EC 4.2.2.3) since they preferably degraded poly(M)-rich substrate. *H. discus, H. iris*, and *O. rusticus* enzymes were classified as polysaccharide-lyase family-14 by analysis of partial amino acid sequences.

A heat stable alginate lyase isozyme LbAly35 from a marine snail *Littorina brevicula* had been studied (Wang et al., 2012). From this study, it is known that although the N-terminal region of LbAly35 was significantly deviated from those of other molluscan alginate lyases, the catalytic domain of LbAly35 showed ~45 % identity to other molluscan enzymes which had been classified under polysaccharide-lyase-family-14 (PL-14). In addition, the amino-acid residues crucially important for the catalytic actions of PL-14 enzymes were also conserved in LbAly35. Accordingly, LbAly35 was regarded as a member of PL-14 as other molluscan alginate lyases despite the significant deviation of its N-terminal region (Figure 4).

Biological Engineering in Alginate Lyase Production

Some organism producing alginate lyase were difficult to cultivate and others have low productivity. The efforts to increase productivity and easiness in production of alginate lyase have been conducted. Biological engineering was the most powerfull tool to gain new microorganism that had ability to produce alginate lyase by cloned gen encoding this enzyme from another organism.

In order to increase the productivity of alginate lyase, Hu et al. (2006) used marine *Vibrio* sp. 510 as a parent strain for screening high producers of alginate lyase using the complex mutagenesis of Ethyl Methanesulphonate and UV radiation treatments. The mutant strain *Vibrio* sp. 510-64 was selected and its alginate lyase activity was increased by 3.87-fold (reaching 46.12 EU/mg) over that of the parent strain. The substrate specificity analysis demonstrated that the alginate lyase had specificity for poly G block. Expression of gene encoding alginate lyase (algL) and the catalytic subunit of the alginat polymerase complex (alg8) on *Azotobacter vinelandii* ATCC 9046, followed by optimation of inlet sucrose concentrations has increased alginate production up to 2.4 g/l and specific alginate production rate to 0.1 g/g/h at an inlet sucrose concentrations (ISC) of 15 g/l on a continuous culture (Alvaro et al., 2012). Carbon recovery of about 100% was obtained at an ISC of 10 g l, whereas it was close to 50% at higher ISCs, suggesting that cells growing at lower sucrose feed rates utilize the carbon source more efficiently. In each of the steady states evaluated,

An increase in algL gene expression was not related to a decrease in alginate molecular weight, whereas an increase in the molecular weight of alginate was linked to higher alg8 gene expression, demonstrating a relationship between the alg8 gene and alginate polymerization in *A. vinelandii* for the first time. The results obtained provide a possible explanation for changes observed in the molecular weight of alginate synthesized and this knowledge can be used to build a recombinant strain able to overexpress alg8 in order to produce alginates with higher molecular weights (Alvaro et al., 2012).

Liu et al. (2009) amplified alginate lyase structural gene (AlyVI gene) from plasmid pET24-ALYVI carrying the alginate lyase gene from the marine bacterium *Vibrio* sp. QY101 which is a pathogen of *Laminaria* sp. This gene was succesfully expressed in cell of *Yarrowia lipolytica*, forming a clear zone on the plate containing alginate indicated they had high alginate lyase activity. Futhermore, the cell displaying alginate lyase can be used to hydrolize poly-â-D-mannuronate (M), poly-á-L-guluronate (G) and sodium alginate to produce various length of oligosaccharides from alginate. Similar to that research, alginate lyase gen of *Streptomyces* sp. ALG5 was succesfully cloned and expressed on *E. Coli* BL21(DE3) using pColdI expression vector (Kim et al., 2009). This gene was succesfully expressed, and produce alginate lyase with higher specifity on poly (G) blocks than poly (M) block.

A new alginate lyase was isolated from *Flavobacterium* sp. S20, and the alginate lyase Alg 2A gene was constructed and overexpressed on *E. Coli*. Substrate specificity assays indicated Alg2A preferred poly-α-l-guluronate as a substrate over poly-

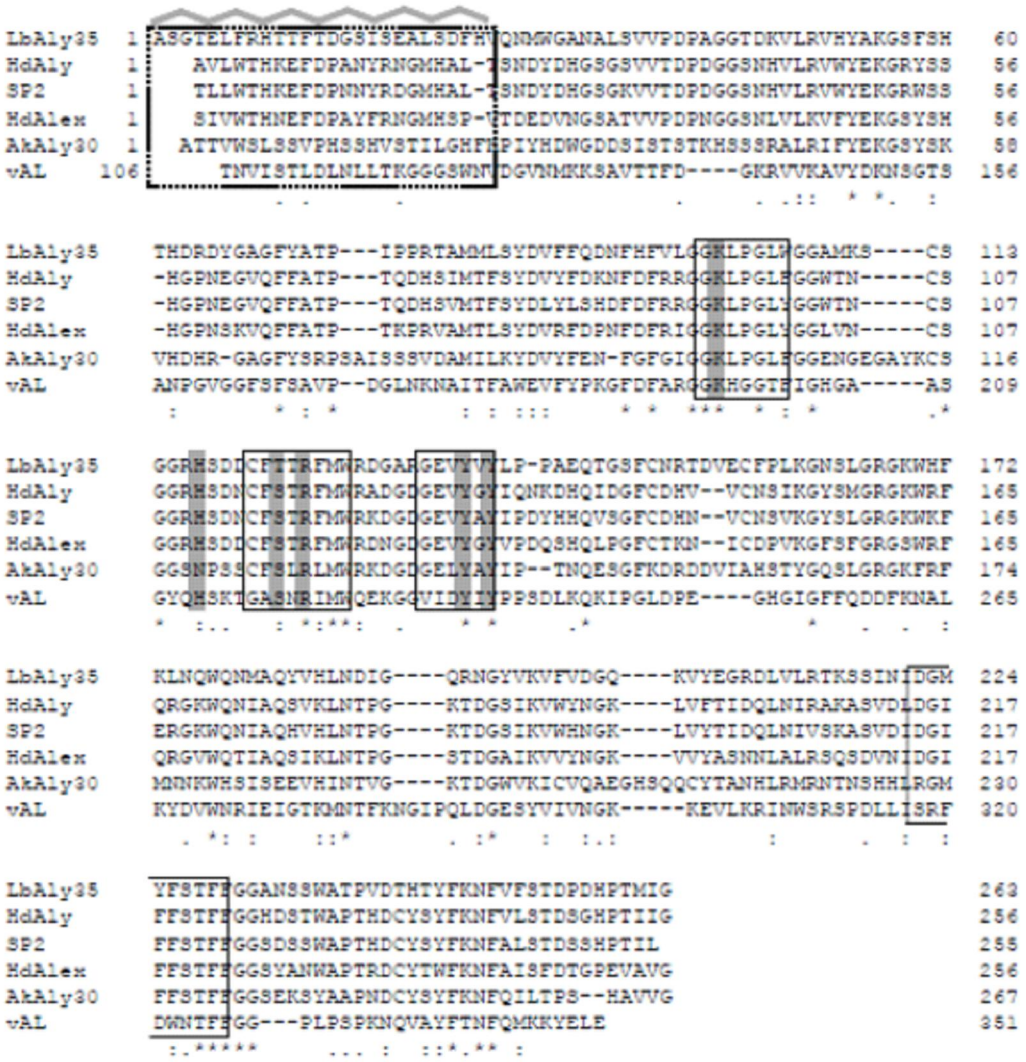

Figure 4. Alignment of amino acid sequences of LbAly35 with those of other PL-14 enzymes. The amino-acid sequence of LbAly35 was aligned with those of abalone HdAly, HdAlex, turbanshell SP2, sea hare AkAly30 and *Chlorella* virus vAL-1. The amino acid residues conserved among PL-14 enzymes are boxed and the catalytically important amino-acid residues for PL-14 enzymes are shaded. Highly deviated N-terminal region among molluscan enzymes at their order level are indicated by a dotted box. Source: Wang et al., 2012.

α-d-mannuronate. In the saccharification process of a high content (10 %, w/v) of sodium alginate, the recombinant alginate lyase Alg2A yielded 152 of mM the reducing sugars after 69 h of reaction, and the amounts of oligosaccharides with different degree of polymerization (DP) generated by Alg2A gradually accumulated without significant variation in the distribution of oligosaccharide compositions (Lishuxin et al., 2013). Su In et al. (2012) cloned, purified, and characterized a novel poly MG-specific alginate lyase in *Stenotrophomas maltophilia* KJ-2. The recombinant alginate lyase consisting of 475 amino acids exhibited the highest activity at pH 8 and 40 °C. Interestingly, the recombinant alginate lyase was expected to have a similar catalytic active site of chondroitin B lyase but did not show chondroitin lyase activity. In the test of substrate specificity, the recombinant alginate lyase preferentially degraded the glycosidic bond of polyMG-block than polyM-block and polyG-block.

Jung et al. (2012) characterized alginate lyase gene using a metagenomic library constructed from the gut microflora of abalone. The library gave an alginate lyase positive clone (AlyDW) harboring a 31.7-kbp insert. The AlyDW insert consisted of 22 open reading frames (ORFs). The deduced amino acid sequences of ORFs 11-13 were similar to those of known alginate lyase genes, which are found adjacent in the genome of *Klebsiella pneumoniae* subsp.

aerogenes, *Vibrio splendidus*, and *Vibrio* sp. belonging to the phylum Gammaproteobacteria. Among the three recombinant proteins expressed from the three ORFs, alginate lyase activity was only observed in the recombinant protein (AlyDW11) coded by ORF 11. The expressed protein (AlyDW11) had the highest alginate lyase activity at pH 7.0 and 45°C in the presence of 1 mM AgNO. The alginate lyase activity of ORF 11 was confirmed to be endolytic by thin-layer chromatography. AlyDW11 preferred poly(â- d-mannuronate) as a substrate over poly(á- l-guluronate). AlyDW11 contained three highly conserved regions, RSEL, QIH, and YFKAGVYNQ, which may act to stabilize the three-dimensional conformation and function of the alginate lyase.

Potential Applications of Alginate Lyase and the Oligo Alginate

Alginate lyase have been widely described for their applications such as in production of bioactive oligosaccharides, control of polysaccharide rheological properties, and polysaccharide structure analysis. The products of alginate lyase, alginate oligosaccharides (AOS) have many biological activities: prebiotics, immune regulation, anticoagulation, antioxidant, anticancer, growth promoting activities, promoting production of antibiotics, promoting yield in ethanol production, etc (Iwamoto et al., 2005; Gibson & Roberfroid, 1995; Spizzirri et al., 2010; Guo et al., 2011; Khan et al., 2011).

Alginate lyases have been used to analyze alginate fine structure to understand how chemical composition influences the physical properties of this important industrial polysaccharide. Alginate lyases also have been used in making protoplast from seaweed. Alginate lyases from both marine and bacterial sources have been applied succesfully in the extraction of protoplasts for food research and regeneration of a variety of algal species (Wong et al., 2000). Another application of alginate lyase includes use of mannuronate specific lyase to produce poly (M) and poly (MG) blocks that could then be used to investigate the substrate specificity for other lyases. Similarly, a guluronate specific lyase which degrade poly (G) and poly (M/G), has been applied in the preparation of poly (M) blocks from sodium alginate for substrate specificity study (Wong et al., 2000).

Hee et al. (2012) used Alg17C, an exooligoalginate lyase to depolimeryze alginate into a monomeric sugar acid. Based on the results of this study, Hee declared that Alg17C could be used as the key enzyme to produce alginate monomers in the process of utilizing alginate for biofuels and chemicals production. Sung-Mook and Jae-Hua (2011) used alginate lyase to help the saccharification and fermentation of a brown seaweed *Laminaria japonica*, to produce *nuruk* (fermentation starter from Korea), which then would be used for production of traditional Korean alcohol.

As uronic acid containing polysaccharides interact with various cations, alginate lyases are used to obtain depolymerized alginates, proposed as food additives to supply metal ions. Another important aplication of alginate lyase was related with the fruit juice industry. Pectin and rhamno-galacturonate lyase are used for clarification of fresh fruit extracts (Michaud et al., 2003). Tang et al. (2009) used bacteria producing alginate lyase strain A7 (*Gracilibacillus* sp.) to degrade alginate in *wakame* composting process. In a laboratory-scale composting experiment, the alginate content in *wakame* compost decreased from an initial value of 36 to 14.3% after 72 h of composting, indicating the effectiveness of the alginate decomposition.

Alginate oligosaccharides are potential as imunomodulator since it can induces production of cytokine in human monocyte (Otteriei et al., 1991). Iwamoto et al. (2005) also confirmed that alginate oligomer has capability to induce production of cytokine in mouse macrofage cell (RAW264.7 cells). In addition, enzymatically depolymerized unsaturated alginate oligomers induced tumor necrosis factor (TNF)-α secretion in RAW264.7 cells in a structure-depending manner, while the activities of saturated alginate oligomers prepared by acid hydrolysis were fairly low or only detected at trace levels. These results suggest that unsaturated end-structure of alginate oligomers is important for the TNF-α-inducing activity. Among the unsaturated guluronate (G3–G9) and mannuronate (M3–M9) oligomers, G8 and M7 showed the most potent activities. Bio-Plex assay revealed that interleukin (IL)-1a, IL-1b, and IL-6 secretion from RAW264.7 cells were also induced by unsaturated alginate oligomers with similar structure–activity relationship profiles as seen in TNF-α, and the most potent activities were observed with G8 and M7. These results suggest that G8 and M7 may have the most suitable molecular size or entire structural conformation as stimulant for cytokine secretion. Since antibodies to Toll-like receptor (TLR)2 and TLR4 effectively inhibited the G8- and M7-induced production of TNF-α, these alginate oligomers may stimulate innate immunity through the pattern recognition receptors on macrophages similar to microbial products.

Many researchers reported the ability of alginate oligomer in promoting the growth of good intestine

microflora or probiotics. Alginate oligosaccharides increased the growth of intestinal *Bifidobacteria* (Gibson & Roberfroid, 1995). Similarly, Akiyama et al. (1992) found that enzymatically alginate oligosaccharide promote the growth of *Bifidobacteria*, while alginate polymer (before degradation) have no effect on the growth of these bacteria. Alginate oligosaccharide prepared through enzymatic hydrolysis of alginate polymer increased the numbers of bifidobacteria or lactobacilli (Wang et al., 2006). In addition, alginate oligosaccharides stimulated the growths of *Bifidobacterium bifidum* ATCC 29521 and *Bifidobacterium longum* SMU 27001 more significantly in comparison with that by fructo-oligosaccharides (FOS). Although *Bifidobacterium* cannot utilize laminaran from brown seaweed, it can utilize the degraded products of laminaran produced by *Clostridium ramosum*, a general human intestinal bacterium strains (Kuda et al., 2005).

An et al. (2009) examined the potential uses of alginate oligosaccharide in protection again pathogens. According to this research, alginate oligosaccharides showed elicitor activity stimulating the accumulation of phytoalexin and inducing phenylalanine ammonia lyase in soybean cotyledon, and antimicrobial activity on *Pseudomonas aeruginosa*. It can be concluded that oligosaccharide degraded from alginate by partially purified alginase has maximal bioactivity at average degree of polymerization (DP) 6-8. It was the first report that alginate oligosaccharides showed elicitor and antibacterial activities and potential to be used as a biological agent for protection against plant or human disease. Similarly to that activities, *Pseudomonas mendocina* NK-01 can synthesize medium-chain-length polyhydroxyalkanoate (PHAMCL) and alginate oligosaccharides (AOS) simultaneously from glucose under conditions of limited nitrogen. It is potential to be used as biological agent for protection against colon cancer and pathogen bacteria (Guo et al., 2011).

Alginate oligosaccharides had been used to increase production of crysogenin, a yellow pigment produced by *Penicillium crysogenum* (Asilonu et al., 2000). He found that additions of 50 to 100 µg of acid-hydrolysed alginate oligosaccharides ml-1 and enzyme-hydrolysed pectin oligosaccharides from 24 to 48 h cultures of *Penicillium chrysogenum*, ATCC 9480, led to enhance production of chrysogenin by over 30 to 40% in shaken flasks and bioreactors. Similar to this research, alginate oligosaccharide also increased growth, yield and production of alkaloid from opium poppy (*Papaver somniferum* L.). These studies have revealed that application of alginate oligosaccharides (AOS), obtained from sodium alginate irradiated by Co-60 gamma rays, significantly enhanced certain physiological/biochemical parameters as well as the overall growth of opium poppy (Khan et al., 2011).

The recently use of alginate lyase was to control alginate fouling. Since alginate was one of typical polysaccharide secreted by microorganisms in waste water that may cause significant membrane fouling, the use of alginate lyase can degrade this polymer and significantly reduced membrane fouling and made the fouling easy to clean (Dong & Michael, 2011). Oligoalginate also had been used as grafting material for producing antioxidant for food application (Spizzirri et al., 2010). The use of catechin-alginate and catechin-inulin showed good antioxidant activities, and this kind of systems could be very useful in the optimization of food preservation and to help manufacturers in elaboration of new food products and packaging. Alginate lyase also recently used in degumming ramie fibre (Basu et al., 2009) and help to produce well-defined sizes of microcapsule (Shinji et al., 2009).

Present Status of Alginate Lyase Research and its Application in Indonesia

As one of megadiversity countries, Indonesia has thousands biota, including bacteria that is potential as source of new alginate lyase. Exploration of bacteria producing alginate lyase including marine and soil bacteria are not yet intensified. Indonesian coastal areas have many brown seaweeds such as *Sargassum* sp and *Turbinaria* sp. that are producing low MG ratio alginate (alginate rich in poliguluronate), so that the probability to obtain new poliguluronat specific alginate lyase (EC.4.2.2.11) is high. Nevertheless, research on alginate lyase and its application is still very limited in Indonesia.Our screening on bacteria from local brown seaweed showed that there were many bacteria isolates that have high lyase activity index. Seaweed *Sargassum* sp. from Binuangeun Waters Province of Banten Indonesia was decomposed in beaker glass at ambient temperature for 5 days before the isolation. Bacteria were plated on media Luria Bertani agar containing 3 mg/ml of sodium alginate, and incubated for 3 days at 30 ^{0}C. Colonies bacteria showed different characteristic were isolated separately in another same medium. Solution of 10% cetyl pyridinium cloride were poured into the colony, let stand for 30 minutes at ambient temperature, and the clearing zone appears around the bacterial colony showed the alginate lyase activity.

From this screening, ten isolates were obtained, eight of them were positive with alginate lyase activity.

Table 2. Alginate lyase activity index of isolates collected from brown seaweed *Sargassum* sp. harvested in Binuangeun waters, Province of Banten Indonesia

Isolate	Alginate lyase activity index (average + Sdev)
S113	0.14 ± 0.03
S123	0.18 ± 0.05
S133	0.29 ± 0.07
S155	2.09 ± 0.37
S215	1.85 ± 0.19
S225	0.28 ± 0.05
S235	2.42 ± 0.43
S245	2.78 ± 0.09

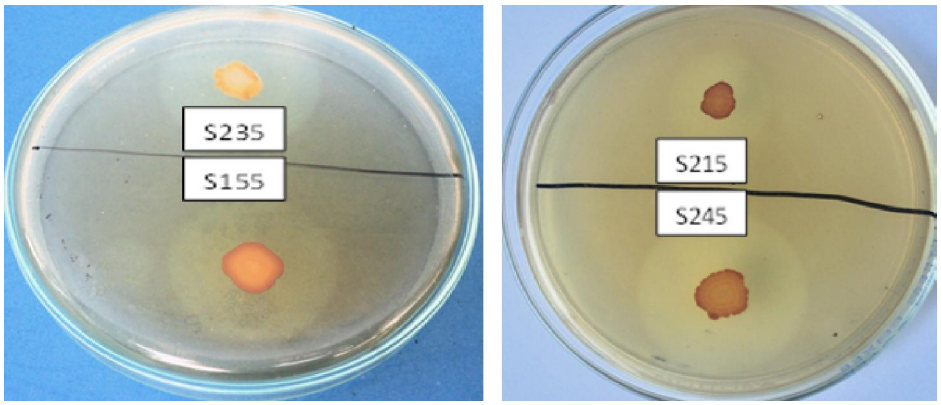

Figure 3. Isolate bacteria from Indonesian seaweed *sargassum* sp. showing high alginate lyase activity.

The alginate lyase activity of these positive isolates were calculated as alginate lyase activity index, which is a ratio of diameter of the clearing zone devided by diameter of bacterial colony. Four isolates showed high alginate lyase activity index, while four other have low activity. The alginate lyase activity index of these isolates were shown in Table 2. Four isolates with high alginate lyase activity were shown in Figure 3. The molecular identification of these bacteria is still on progress.

Alginate lyase isolated from local microorganism is potential to be used in depolimerized low MG ratio alginate from tropical waters into its oligomer or monomer. Since monomer alginate can be used in biofuel production, potential application of this enzyme in biofuel industry in the future is high, because of rising in fuel consumption and declining fosil fuel production in Indonesian. This enzyme also potential to be used in a composting process of brown seaweed to produce biofertilizer. As an agicultural country with a great number of farmer and a major producer of *agricultural* products, future demand of biofertilizer in Indonesia was so high.

Besides the potential use of alginate lyase in the production of biofuel and biofertilizer, the most potential application of this enzime is in production of alginate oligosachharides (AOS) as an immunomodulator substance. The reason for this estimation is the prediction that demand of immunomodulator products will significantly increase in the future, especially for naturally immunomodulator substance. Immune system was significantly affected by social stress and polution that repeatedly encounter human population in many big cities in the world. Traffic jam and air polution from industry and motor vehicle in Indonesian city had lead to increase social stress and decrease immune system. Therefore, demand for immunomodulator products will increase, especially for those which derived from natural resources, because the increase in public awareness of negative effect of chemically substance. On the other hand, the prodution of naturally immunomodulator substance in Indonesian was so small. So far, development of organically immunomodulator in Indonesian were derived from terestrial herbal plant such as *Phyllanthus niruri*, *Curcuma* sp. and *Echinacea*. Unfortunatelly, the development of terestrial herbal plat as source of

immunomodulator susbtance was competed with the demand of land for other purpose such as food agriculture, property, *etc*. Therefore, marine plant and organism such as brown seaweed offer an alternativesources of raw materials for immunomodulator substance. In the future, utilization of marine plant and organism as source of immunomodulator material are expected considerably increased.

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