

ALGINATE LYASE FROM INDONESIAN *Bacillus megaterium* S245 SHOWS ACTIVITIES TOWARD POLYMANNURONATE AND POLYGULURONATE

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Abstract

Screening of alginate lyase producing bacteria associated with seaweed *Sargassum crassifolium* was carried out, and isolate S245, identified as *Bacillus megaterium* S245 was found to produce high alginate lyase activity. This research was conducted to evaluate activity of the alginate lyase enzyme at various pHs, temperatures and substrates. Polymannuronate and polyguluronate were used to evaluate substrate specificities. Alginate lyase activity was assayed by analysis of reducing sugar released using the 3,5 dinitrosalicylic acid (DNS) method. The research showed that the activity of alginate lyase was optimum at pH of 7.0 and temperature of 45 °C. This enzyme was active for both polymannuronate and polyguluronate substrates. The V_{max} and K_m of this enzyme for polymannuronate and polyguluronate were 200 unit/ml/min and 79.8 mg/ml for polymannuronate substrate and 27.78 unit/ml/min and 13.17 mg/ml for polyguluronate substrate. This enzyme showed unique characteristic in working toward the two substrates.

Keywords: alginate lyase, seaweed, *Bacillus*, kinetic activity, *Sargassum* sp.

1. Introduction

Alginate lyase or alginase (EC4.2.2.3 and EC4.2.2.11), an enzyme that catalyze the degradation of alginate by a β -elimination mechanism has been isolated from various sources, such as soil bacteria, marine bacteria, marine mollusks and crustaceae, brown seaweed and fungi (Guan, Wang & Guo, 2011^b; Li et al., 2011^a; Li, Jiang, Matiur, Ling, Akira & Takao, 2012; Subaryono, Peranginangin, Suhartono & Zakaria, 2013). The alginases from alginolytic bacteria, included those of soil and marine bacteria have been isolated from *Pseudomonas* sp. QD03 (Xiao, Han, Yang, Xin-zhi and Wen-gong, 2006), *Pseudoalteromonas atlantica* AR06 (Matsushima et al., 2010), *Pseudoalteromonas* sp. SM0524 (Li et al., 2011^a), *Pseudomonas fluorescens* HZJ216 (Li, Jiang, Guan, Wang & Guo, 2011^b), *Stenotrophomas maltophilia* KJ-2 (Lee, Choi, Lee & Kim, 2012), *Bacillus subtilis* (El Ahwany & Elborai, 2012), and *Flavobacterium* sp. S20 (Lishuxin, Jungang, Qiang,

Hong & Yuguang, 2013). Based on their substrate specificity, alginate lyases are classified into poly (β -D-mannuronate) lyases (EC4.2.2.3) and poly (α -L-guluronate) lyases (EC 4.2.2.11). Examples of alginate lyases with poly (β -D-mannuronate) as substrate are produced from *Azotobacter chroococcum* (Khanafari & Sepahei, 2007), *Azotobacter vinelandii* (Gimmestad et al., 2009), *Littorina* spp (Matiur, Ling, Akira & Takao, 2012), and *Pseudomonas aeruginosa* (Rasamivarak, Labtani, Duez & Jaziri, 2015). On the other hand, alginate lyase from *Klebsiella pneumoniae* (Baron et al., 1994), *Vibrio* sp. 510 (Hu, Jiang & Hwang, 2006) and *Enterobacter cloacae* WD 7 (Prasertsan, Wichienchot, Doelle & Kennedy, 2008), showed specificity toward poly (α -L-guluronate) lyases. Another alginate lyase with the two substrates specificity were produced by *P. alginovora* strain XO17 and *Sphingomonas* sp. strain A1. Hashimoto, Miyake, Momma, Kawai and Murata (2000) also reported alginate lyase from soil bacteria *Sphingomonas* sp.

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strain A1 which has activities both on polymannuronate and polyguluronate.

Alginate lyase has been used in many applications such as in producing poly (M) and poly (MG) blocks (Wong, Preston & Shiller, 2000), to degrade alginate in wakame composting process (Tang, Taniguchi, Chu, Zhou & Nagata, 2009), to produce bioactive oligosaccharide (An et al., 2009; Guo et al., 2011; Iwamoto, Araki, Iriyama, Oda, Fukuda & Hayashida, 2005) and to analyze alginate fine structure (Kim, Lee & Lee, 2011). Since many bioactive molecule can be produced using alginate lyase, the future application of this enzyme will continue to increase (Wong, Preston & Shiller, 2000). We had isolated bacteria that produce alginate lyase from decomposing seaweed *S. crassifolium* and found that isolate S245 has the highest lyase activity compared to other isolates. This enzyme has activities on both polymannuronate and polyguluronate substrate (Subaryono, Peranginangin, Suhartono & Zakaria, 2013). Considering the potential application of this enzyme to produce bioactive alginate oligosaccharides, it is important to find identity of this bacteria and the enzyme characteristics. This is done to get the source of alginate lyase-producing bacteria that safe as well as to optimize the application of alginate lyase on the production of alginate oligosaccharides enzymatically. The objective of this study was to identify alginolytic isolat S245, the effect of pH, temperature and substrates on the enzyme activities.

2. Material and Methods

2.1. Material

Bacteria S245 was previously isolated from decomposing seaweed of *S. crassifolium* from Binuangun Water, Province of Banten, Indonesia. The procedure of isolation of this bacteria was reported earlier (Subaryono et al., 2013). The isolate was kept alive in luria bertani agar medium containing 1% of sodium alginate (Sigma Inc.) Other chemicals were purchased form Merck through local distributors.

2.2. Bacteria Identification

Bacteria identification was conducted by morphological, biochemical and molecular analysis. Morphology of bacteria was observed under microscope after Gram staining and spore staining. Bacteria identification using biochemical reaction was conducted to the 24 hours age of isolate using API® bacterial kit identification. Molecular identification of bacteria was conducted with 16S rDNA sequencing.

The 16S ribosomal DNA analysis was carried out with several steps. The bacteria was first incubated for 24 hours, and then DNA extraction was carried out using a Genomic DNA Purification Kit (Fermentas Life Bioscience, EU). The primers used to amplify the 16S rDNA fragment were 63f (5'-CAGGCCT AACAC AGGCAAGTC) and 1387r (5'GGG CGGW GT GTACAAGGC). PCR was carried out by using a GeneAmp PCR System 9700 (Applied Biosystem, Foster City, CA, USA) with the following conditions for amplification : initial denaturation at 95 °C for 5 min, 30 cycles of 1 min of denaturation at 95 °C, 1 min of annealing at 56 °C, and 1.5 min of extension at 72 °C, and final extension at 72 °C for 7 min. The 16S rDNA sequence was blasted and aligned with multiple sequence data in GenBank database by using BLAST algorithm and CLUSTAL W program. The phylogenetic three was constructed by using neighbor-joining algorithm.

2.3. Enzyme Production

The bacteria were cultured in Luria Bertani medium containing alginate (Merck Milipore, 71753) 5 mg/ml (w/v) at 30 °C for 48 hours on a stagnant culture. The culture was then centrifuged at 10,000 x g 4 °C for 20 minutes and supernatant containing alginate lyase was concentrated using amonium sulphate precipitation. To the supernatant, ammonium sulphate was added until concentration of 35% (w/v), stired at cold temperatures for 2 hours and centrifugated at 10,000 x g for 20 minutes. The pellet was dialyzed againts 20 mM sodium phosphate buffer (pH 7.0) at 4 °C for 24 hours to produce crude enzyme.

2.4. Enzyme Activity Asay

Polymannuronate and polyguluronate were prepared from alginate using method of Haug, Larsen & Smidsrød (1967). Briefly, alginate is dispersed in 500 ml 0.3M HCl, and kept in a water bath 100°C for 5 hours. The solution was then cooled and centrifuged at 4500 xg for 10 minutes. The supernatant was removed, and the precipitate containing M and G block was resuspended in aquadest. pH was adjusted to 2.8 using 5 M NaOH solution, and let stand for 24 hours. After centrifuged at 4500 xg for 10 minutes, the supernatant containing M blocks was neutralized, dialyzed and freeze-dried. The insoluble part which is G blocks was also neutralized, dialyzed and freeze-dried (Haug, Larsen & Smidsrød, 1967).

The reducing sugar produced by enzyme substrate reaction was detected using the 3,5-dinitrosalicylic acid (DNS) method following Tang et al. (2009) with slight modification. 1 ml of sample mixture at (0,8 ml

sodium phosphate buffer + 0.1 mg alginate + 0.1 ml enzyme) was mixed with 1 ml of DNS solution and boiled for 5 min. Absorbance of the reaction solution was observed at 540 nm. Standard curve with mannose at concentration of 0.1, 0.3, 0.5, 0.7 and 0.9 mg/ml followed the same steps. Enzyme activity was expressed as enzyme that released 1 μ g reducing sugar per minute per ml of enzyme under that condition.

2.5. Enzyme Characterization

To study the activity of alginate lyase at different pH, as much as 0.8 ml universal buffer (pH 6-11) was mixed gently with 0.1 ml 1% alginate solution and 0.1 ml of enzyme. The mixture was incubated at 30 °C for 30 minute, and enzyme reaction was stopped by heating 10 min in boiled water. To study the effect of temperature on lyase activity, the same mixture was made and incubated at various temperature: of 25, 35, 45, 65, 75 and 85 °C for 30 minutes. To study the substrate specificities, the alginate solution was replaced by polymannuronate at concentration of 1.0, 7.5, 15.0, 30.0, 60.0 and 120.0 mg/ml and polyguluronate at concentration of 0.5, 1.0, 2.5, 5.0, 7.5, 15.0, and 30.0 mg/ml. The activity of alginate lyase was assayed after the treatment by measuring the released reducing sugar using DNS method. The kinetics curves were drawn by Lineweaver-Burk double reciprocal plot method, and the K_m and V_{max} were determined.

The activity staining assay was performed according to the method Kawamoto et al. (2006) with some modifications. Enzyme was separated using

15% (w/v) SDS-PAGE without merchaptoethanol, and the gel was renaturated three times for 30 minutes incubations in a renaturation buffer (50 mM Tris-HCl buffer [pH 8.0], 10 mg/ml of casein, 2 mM EDTA, 0.01% NaN_3) containing 25% of methanol. The gel was overlayed on a 1% agar gel containing 1 mg/ml of sodium alginate, 10 mg/ml of NaCl, and 100 mM Tris-HCl buffer (pH 7.2), and incubated at 30 °C for 20 hours. Visualization of clear zone was conducted with addition of 10% CaCl_2 for 30 minutes.

3. Results and Disucssion

3.1. Bacterial Identification

Microscopic examination of isolate S245 indicated a Gram-positive bacteria, rod shape and spore producing as seen in Figure 1. Biochemical analysis of the bacteria showed positive result for Gram, calatase, oxidase, gelatin, glucose, saccharose, meleztebiose and amylase. Negative result was shown for arginine dihyrolase (ADH), lysine decarboxylase (LDC), ornithine decarboxylase (ODC), citrate reductase, H_2S reductase, urea, tryptophane deaminase (TDA) and indole. The positive catalase of the isolate showed that this bacteria can survive in aerobic condition, such as in their natural habitate (marine water agitated with sea wave). This bacteria also showed positive oxidase, that mean posessing cytochrome oxidase as a specific characteristic for aerob or facultative anaerob organism (Trivedi, Pandey & Bhadauria, 2010). From the ability to metabolize carbohydrate, it can be seen that these bacteria were able to utilize glucose, saccharose, meltobiose, and

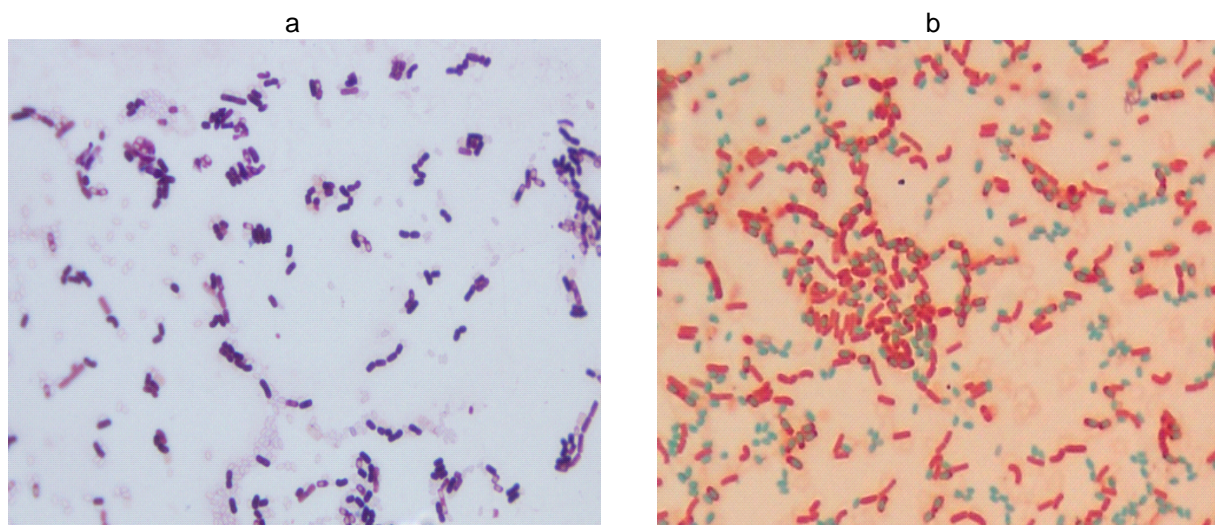


Figure 1. Isolate S245 under microscope after Gram staining (a) and spora staining (b) (magnifications 1000 x).

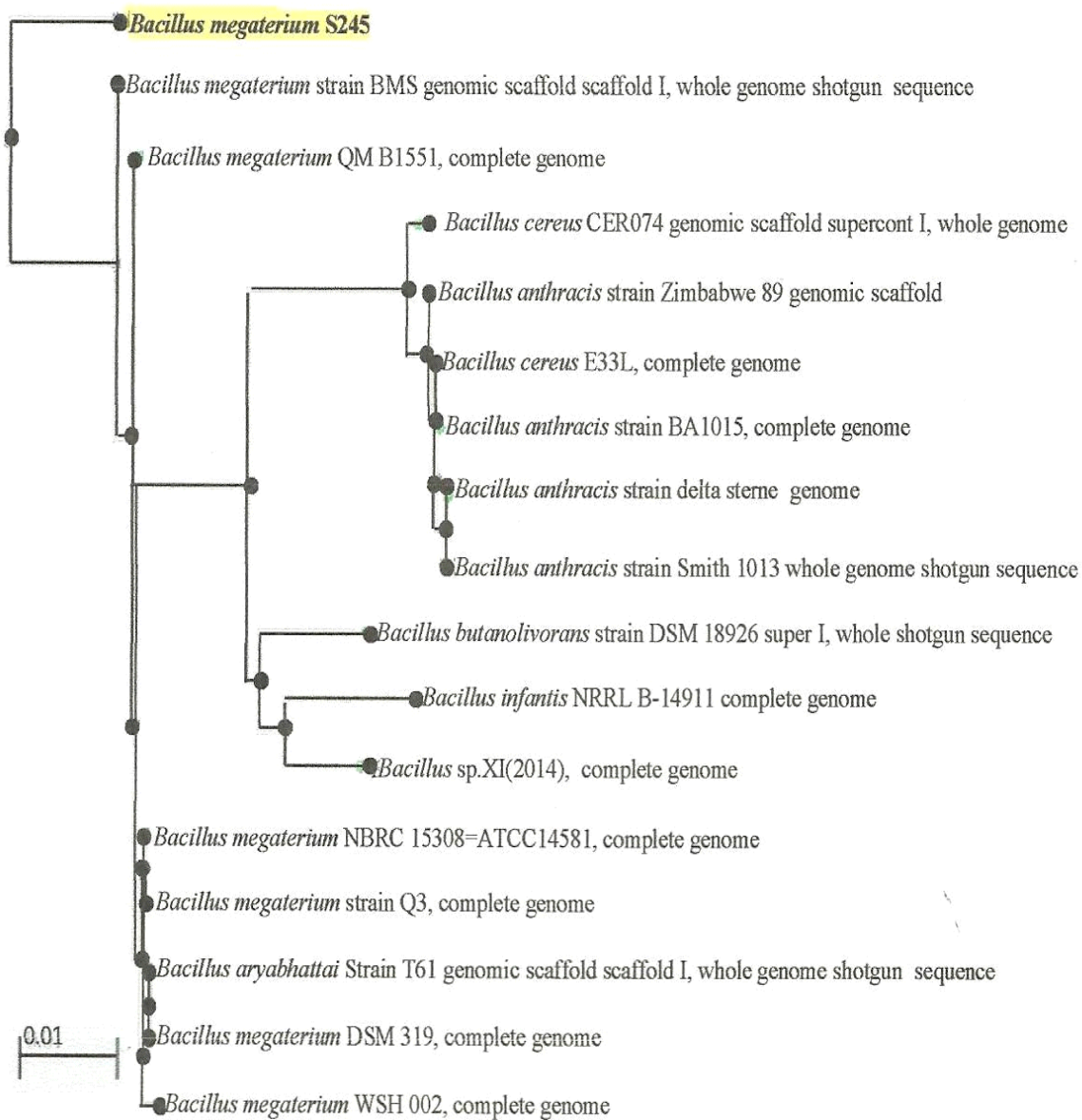


Figure 2. Phylogenetic tree based on 16S rRNA of *B. megaterium* S245 and other related species.

amylum. It can be concluded that alginate was not the only carbon source for this bacteria. From biochemical analysis result (using API kit bacteria identification) the S245 isolate was closely related to *B. megaterium* (ID 85%).

Partial 16S rDNA analysis suggested that S245 was most closely related to *B. megaterium* DSM 319 (96% similarity), *B. megaterium* QM B1551 (96% similarity), *B. megaterium* WSH 002 (96% similarity), *Bacillus* sp.1NLA3E (94% similarity), *B. infantis* NRRL B-14911 (94% similarity) and relatively close to other species of *Bacillus*. Therefore, S245 is a strain of genus *Bacillus* and we identified this as *B. megaterium* S245. *Bacillus* species such as *B.*

megaterium was commonly found in soil, sea water, sediment, or decomposing material (Scholle, White, Kunialaiyaan & Vary, 2003). The phylogenetic tree of this bacteria was shown in Figure 2.

Previous studies reported that marine bacteria producing alginate lyase were *Alteromonas* sp. strain H-4 (Sawabe, Otsuka & Ezura, 1997), *Vibrio* sp. O2 (Kawamoto et al., 2006), *Pseudoalteromonas agarovans* (Choi, Piao, Shin & Cho, 2009), *Pseudoalteromonas atlantica* AR06 (Matsushima et al., 2010), and *Pseudomonas fluorescens* HZJ216 (Li et al., 2011^b). On the other hand, alginate lyase reported from soil bacteria were *Corynebacterium* sp (Matsubara, Iwasaki & Muramatsu, 1998),

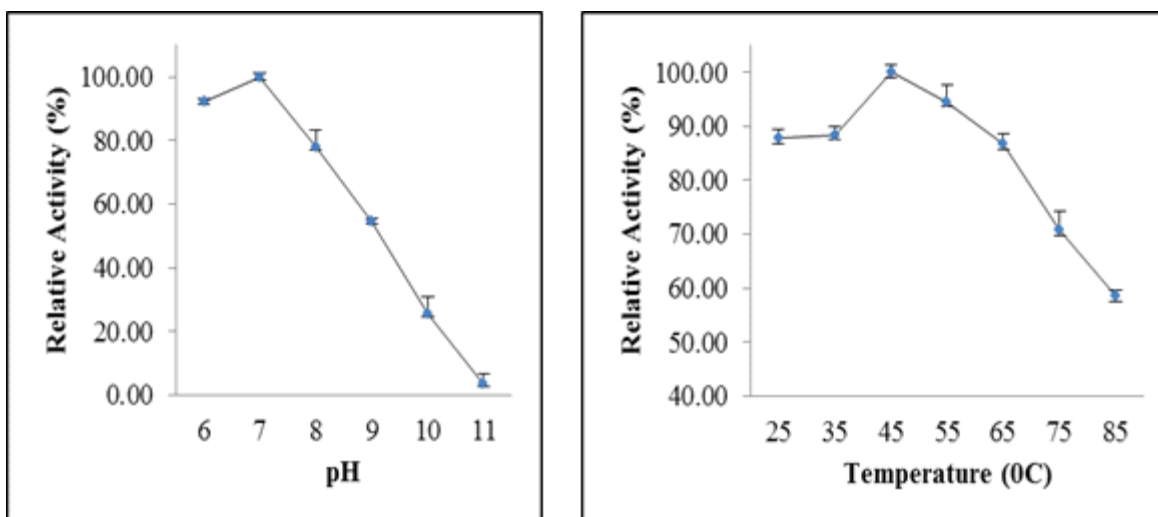


Figure 3. Activity of alginate lyase from *Bacillus megaterium* at various pHs (a) and at different temperature (b). The relative activity was compared to its maximum activity.

Sphingomonas sp. strain A1 (Hashimoto et al., 2000), *Pseudomonas* sp. QD03 (Xiao, Han, Yang, Xin-zhi & Wen-gong, 2006), *Azotobacter vinelandii* (Gimmestad et al., 2009), and *Corynebacterium* sp (Kim et al., 2011). This research was the first finding of a new alginate lyase producing *Bacillus megaterium* S245. Another work that reported bacteria producing alginate lyase from genus *Bacillus* were *Bacillus* sp ATB-1015 (Nakagawa, Ozaki, Chubachi, Hosoyama & Okubo, 1998) and *Bacillus subtilis* (El Ahwani & Elborai, 2012).

3.2. Characterization of Enzyme

Figure 3. showed activity of alginate lyase from *B. megaterium* S245 at various pHs and temperatures. The activity was slightly increased at pH of 7 then gradually decreased to pH of 11. At pH, of the 11 activity of enzyme was only 3,38% compared to the highest activity at pH of 7. The optimum pH for this enzyme was 7 with maximum activity as 8.72 U/ml/min. Other reserchers reported that optimum pH of alginate lyase were 7 from *Vibrio* sp. YKW-34 and *Alteromonas* sp. strain H-4 (Fu, Hong & Kim S., 2007; Sawabe et al., 1997), 7.5 from *Vibrio* sp YKW-34 (Xiao et al., 2007), 7.5-8 from *Alteromonas* sp. strain no 272 (Iwamoto et al., 2001), and 8.5 from *Pseudoalteromonas* sp. SM0524 (Li et al., 2011^a).

The activity of alginase from *B. megaterium* S245 at different temperatures indicated an increase from 25 °C and reach maximum at 45 °C. After that, the activity of alginat lyase was slightly down at temperature 65 °C, and dropped at 85 °C. At this temperature, the activity of aginate lyase remained 58.62% compared to the highest activity. The minimum activity at temperature >65 °C showed that this enzyme

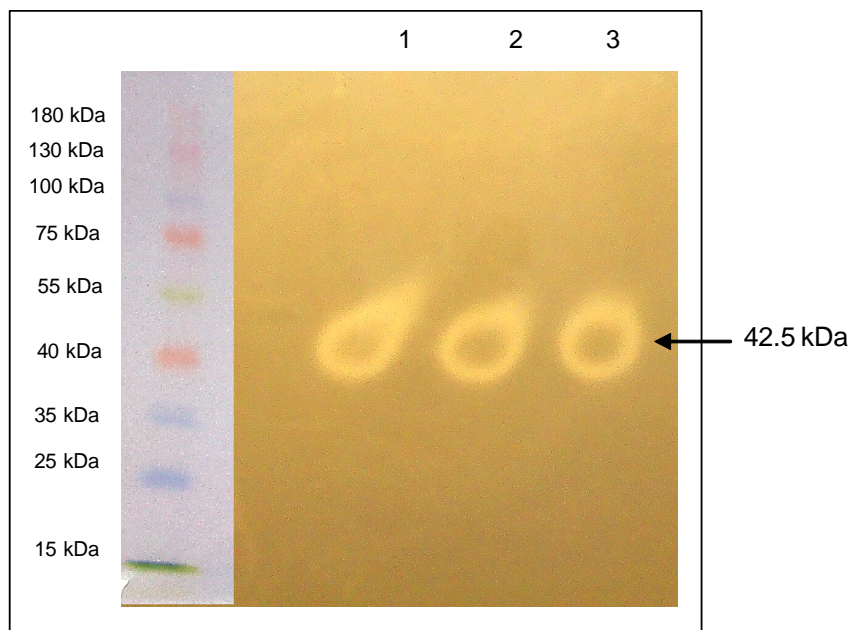
was heat unstable. The optimum activity was obtained at 45 °C reaching 9.00 unit/ml/min and the minimum was at 85 °C reaching 5.28 unit/ml/min. The optimum temperature for alginate lyase from *Pseudomonas aeruginosa* FRD1, *Vibrio* sp YKW-34, *Pseudomonas syringae* pv. *Syringae*, *Pseudoalteromonas* sp. SM0524 were respectively 37, 40, 42, 50°C (Fu et al., 2007; Li et al., 2011^a; Preston, Wong, Bender & Schiller, 2000; Xiao et al., 2006).

The zymogram insitu enzyme activity of this alginate lyase were shown in Figure 4. It showed that molecular mass of this enzyme was around 42.5 kDa. The molecular mass of this enzyme was close to alginate lyase from *Pseudomonas* sp. QD03 cloned into *Pseudomonas aeruginosa* FRD1 as 42,8 kDa (Xiao et al., 2006). Other researches reported that molecular mass of lyase from *Pseudoalteromonas* sp. SM0524 as 32 kDa (Li et al., 2011^a), *Vibrio* sp. YKW-34 as 60 kDa (Fu et al., 2007), and *Vibrio* sp. mutant Strain 510-64 as 34.6 kDa (Hu et al., 2006). The lowest molecular mass of this enzyme was reported as 23 kDa from *Pseudomonas fluorescens* HZJ216 (Li et al., 2011^b) and from *Alteromonas* sp. strain 272 (Iwamoto et al., 2001).

Table 1 represent the activity of alginate lyase at different polymannuronate and polyguluronate concentrations. It showed that the enzyme activity was increased sharply with the increasing of polymannuronate concentration. The maximum enzyme activity was found at polymannuronate concentration 120 mg/ml 131.44 unit/ml/min. Using Lineweaver-Burk double reciprocal plot method, the kinetics of this lyase activity follows the equation of $Y = 0.339X + 0.005$ ($R^2=0.999$) (Figure 5). From this

Table 1. Activities of alginate lyase at different polymannuronate and polyguluronate concentrations as substrate

Polymannuronate Substrate		Polyguluronate Substrate	
Substrate Concentration (mg/ml)	Enzyme Activity (U/ml/min)	Substrate Concentration (mg/ml)	Enzyme Activity (U/ml/min)
1	2,47	0,5	0,99
7,5	19,99	1	2,32
15	28,76	2,5	3,66
30	43,44	5	6,07
60	84,30	7,5	8,60
120	131,44	15	24,18
		30	27,97

Figure 4. Zymogram of alginate lyase from *B. megaterium* S245. Enzyme was separated in three line (1, 2, 3 (three replication)) of 15% (m/v) SDS-PAGE without mercaptoethanol. M = marker (15-180 kDa).

equation, K_m and V_{max} for polymannuronate substrate can be calculated as 79.8 mg/ml and 200 U/ml/min respectively. For polyguluronate as enzyme substrate, the maximum enzyme activity was only 27.97 unit/ml/min at concentration substrate 30 mg/ml, significantly lower compared to that of polymannuronate substrate. The kinetics of lyase activity on polyguluronate substrate follows the equation of $Y = 0.475X + 0.037$ ($R^2=0.984$). Therefore, K_m and V_{max} for polyguluronate substrate can be

calculated as 13.17 g/L and 27.78 unit/ml/minutes respectively. Xiao, Hong and Sang, (2007) reported the K_m alginate lyase from *Alteromonas* sp was 0.27 mg/ml (6.8×10^6 M). Li et al (2011^a) reported that K_m of *Pseudoalteromonas* sp. SM0524 was 1.086 mg/ml, 0.465 mg/ml and 2.751 mg/ml on sodium alginate, polyguluronate and polymannuronate. Two types of alginate lyase from *Vibrio* sp had K_m value of 5.1×10^{-5} and 5.4×10^{-5} M (Iwamoto et al., 2001). The K_m of this enzyme was higher than previous research

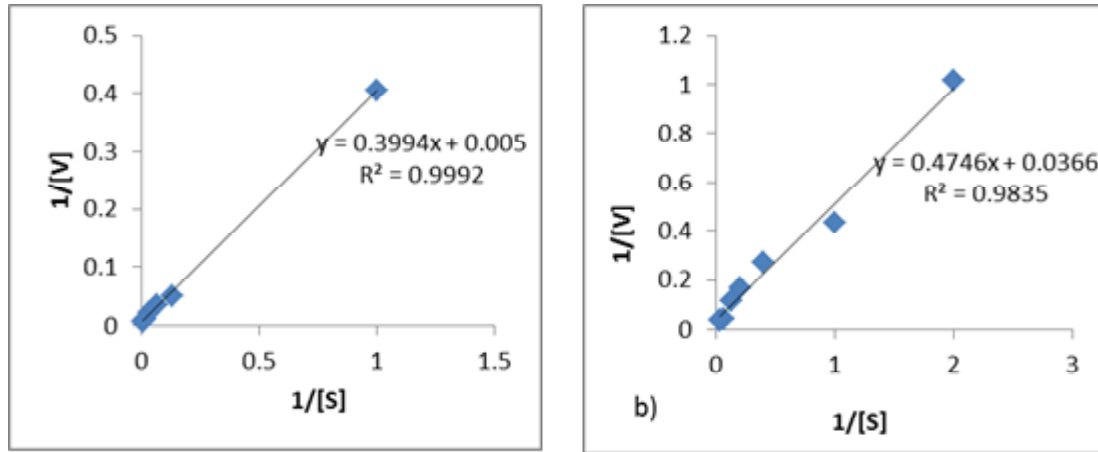


Figure 5. Lineweaver–Burke plots for the degradation of polymannuronate (a) and polyguluronate (b) by the alginate lyase produced by *B. megaterium* S245.

because the enzyme used in this research was not purified yet.

Presently the enzyme is being applied at its optimum activity to produce bioactive oligomannuronate and oligoguluronate.

4. Conclusion

Based on sequence of 16S rDNA, bacteria S245 producing alginate lyase was identified closed to *B. megaterium* (ID 96%). Activity of this alginate lyase was optimum at pH of 7.0 and temperature of 45 °C. This enzyme was active toward polymannuronate substrate as well as polyguluronate substrates, and the V_{max} and K_m of this enzyme were 200 unit/ml/min and 79.8 g/L for mannuronate substrate and 27.78 unit/ml/min and 13.17 g/L for guluronate substrate respectively. The apparent molecular mass of this enzyme was 42.5 kDa.

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