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A novel *Bacillus* sp. isolated from rotten seaweed: Identification and characterization alginate lyase its produced

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Abstract. *Zilda DS, Yulianti Y, Sholihah RF, Subaryono S, Fawzya YN, Irianto HE.2019. A novel* Bacillus *sp. isolated from rotten seaweed: identification and characterization alginate lyase its produced. Biodiversitas 20: 1166-1172.* Alginate lyase has been known as potential biocatalyst not only for industrial but also medicinal application especially for the production of oligosaccharides which have distinct bioactivities. An alginate lyase, AlgT513, has been isolated from rotten seaweed bacterium strain T513 and characterized. The bacterium showed low similarity (95%) with *Bacillus tequilensis* strain 10b based on 16S rDNA sequence indicating that AlgT513 may be a novel Bacillus species. The bacterium forms a clear zone on solid medium with 0.5% sodium alginate addition. The optimum temperature and pH were 50ºC and 8 respectively. AlgT513 maintained stability at board pHs of 4-9 and temperature of 45ºC. Metal ions Mg^{2+}, Ca^{2+} and K^{+} increase the activity of the enzyme while Zn^{2+}, Co^{2+} and Li^{+} strongly inhibit it. NaCl inhibits AlgT513 activity where most of the alginate lyases need it to reach maximum activity. AlgT513 is suggested as a serine metalloenzyme due to inhibition of ethylenediaminetetraacetic acid (EDTA) and phenylmethylsulfonyl fluoride (PMSF).

Keywords: Alginate Lyase, AlgT513, *Bacillus* sp., rotten seaweed

INTRODUCTION

Alginate is an acidic polysaccharide composing of monomeric units β-D-mannuronic acid (M) and α-L-guluronic linked by 1.4 glycosidic bonds comprising M (M-block) or G-consecutive sequence (G-block) and M/G random sequence (MG-block) (Vera et al. 2011). Alginate is widely applied in various industries as stabilizer (Guan et al. 2017; Rescignano et al. 2015; Zhao et al. 2014), viscosifier (Steinert et al. 2003) gelling agent (Ching et al. 2017), biomaterials (Schütz et al. 2017; Bendtsen et al. 2017) and drug delivery device (Agüero et al. 2017; Strand et al. 2017). Alginate is also used in paper and printing factory (Müller et al. 2017).

The enzyme using alginate as a substrate, Alginate lyases (also known as alginases or alginate depolymerases), catalyze the cleavage of the 1-4 glycosidic bond of alginate via the β-elimination mechanism. There are 2 classes of Alginate lyases based on the kind of alginate structure they work on, polyguluronate lyase (EC 4.2.2.1) on G blocks, polymannuronate lyase (EC 4.2.2.3) on M blocks, and alginate lyase degrading both M and G blocks. Alginate lyase degrading G or M block is named monofunctional and those degrading both G and M block named bifunctional alginate lyase. Some organisms were reported generating alginate lyase such as marine algae (Wijesekara et al. 2011), marine mollusca (Boyen et al. 1990: Shimizu et al. 2003), fungi (Perullini et al. 2010; Schaumann et al. 1995), bacteria (Chen et al. 2018; Sun et al. 2019) and viruses (Suda et al. 1999). Alginate lyases have been used to analyze the structure of alginate (Heyraud et al. 1996; Ostgaard 1993; Ostgaard 1992) protoplast seaweed, production of poly G and poly M and to treat the CF sufferers (Mrsny et al**.** 1996). Alginate lyase was also used to depolymerize alginate generating low molecular weight alginate. The degradation product of alginate lyase, oligomeric alginate had been applied as a growth promoter, germination enhancer and elongation shooter in plants (Yonemoto et al. 1993), food sources (Murata et al. 1993). In health and pharmacy field, alginate oligomer has been examined for antitumor (Fujihara et al. 1992), anti-obesities (Nakazono et al. 2016) and anti-bacteria (Pritchard 2017; Oakley 2018)

Enzymes for seaweed polysaccharide degradation are mainly produced by microorganisms (Kim et al. 2012). Screening of those microorganisms from environment which rich of suitable substrate will lead to the discovery of new enzyme as well new application in biotechnology. Seaweed is a promising host for novel microorganism discovery which produces seaweed polysaccharides as revealed by Agusman et al. (2017) that seaweed have high bacteria diversity. *Turbinaria*, which rich of alginate was chosen for alginate lyase-producing microorganisms isolation. Decomposition process was carried out to ensure microorganisms growing on it are alginate lyase-producing ones. In this study, an alginate lyase-producing bacterium, screened from rotten *Turbinaria* and the enzyme its produced, AlgT513, was ammonium sulfate-precipitated and characterized.

MATERIALS AND METHODS

Screening of alginate lyase producing bacteria

Screening of alginate lyase producing-bacteria was conducted by decomposing 100 g seaweed, *Turbinaria*, naturally in flasks covered by cotton and incubated at 37ºC for 7 days. The rotten *seaweed* was blended and 1 g of the blended was grown in a selective medium containing 0.5% sodium alginate, 0.6% H_3PO_4, 0.3% NH_2SO_4, 0.02%, and $MgCl_2$. The culture was incubated at 37ºC for 3 days in shaking incubator with 100 rpm rotation. 1 mL of culture was spread on selective solid media with a series of dilution. Single colonies with different pattern were collected and spread on new solid media to obtain pure isolates. Iodine solution (10 g I_2 and 5g KI in 1000 mL distilled water) was poured on to the single colony and clear zone around the colony was measured. Alginolitic index was determined as the diameter of the clear zone divided by the diameter of the colony.

Identification of bacterial strain

DNA extraction kit (Guangzhou Dongsheng Biotech Co., Ltd., China) was used to extract genomic DNA of strain T513 following the manufacturers' instruction. Amplification of 16S rRNA gene was performed by PCR using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'- GGCTACCTTGTTACGACTT-3') (Moreno et al. 2002) with the genome of strain T513 as a template. The PCR product was sequenced, and the homology of 16S rRNA sequence was searched using the BLAST program (Altschul et al. 1997) against the GenBank database. The phylogenetic tree was built using MEGA 7.0 software (bootstrap: 1000) (Tamura et al. 2013) with the neighbor-joining method (Saitou and Nei 1987).

Alginate lyase production

Bacterium isolate T513 was refreshed from glycerol stock into 15 mL Luria Bertani Broth and incubated for 24 h. The fresh culture was tricked on to plate agar containing 0.5% sodium alginate, 0.6% H_3PO_4, 0.3% NH_2SO_4, 0.02%, and 0.01 $MgCl_2$ and incubated at 37ºC The isolate of T513 from 24 h-solid culture was inoculated into 50 mL starter media and incubated in a 150 rpm shaker incubator at 37ºC. After 72 h, 5% starter was inoculated into 4x250 mL production in 1 L flasks and incubated at the same condition with the starter culture. The culture was harvested after 72 h and centrifuged for 10 minutes at 10000 rpm 4ºC. The purification process was carried out in 4 steps, ammonium sulfate precipitation, dialysis, ion exchange, and gel filtration. Precipitation step was conducted by addition of 70% saturation ammonium sulfate at low temperature (by putting the enzyme container on ice). After whole night incubation, the ammonium sulfate added supernatant was centrifuged at 10.000 rpm for 30 minutes at 4ºC and the precipitated protein was dissolved in 50mM phosphate buffer pH 7. Remained ammonium sulfate in precipitated protein was removed by dialysis using 10000 Da co cellulose membrane with 25 mM phosphate buffer pH 7 for 12 h. The buffer for dialysis was changed every 3 h.

Alginate lyase activity

Alginate lyase activity was determined by measuring the amount of reducing sugar as the result of sodium alginate hydrolysis using the 3,5 dinitrosalicylic acid (DNS) method (Miller 1959). The mixture of 0.9 mL 0.5% alginate lyase (in 50 mM Tris-Cl buffer pH of 8) and 0.1 mL enzyme was incubated at 45ºC for 30 minutes. The reaction was stopped by heating the mixture in boiled water for 10 minutes before being added by 0.5 L DNS reagent and heated for 10 minutes. The mixture was cooled in room temperature and the absorbance was measured at 540 nm. One unit of alginate lyase activity was defined as the amount of enzyme that releases 1μg the reducing sugar (D glucose equivalent) per minute under assay condition.

Effect of temperature and pH

Optimum temperature was determined by incubating substrate with the enzyme at a temperature of 30-65ºC. To obtain optimum pH 0.5% sodium alginate was dissolved in various of pH buffer of acetate (4-6), phosphate (6-7) and Tris-Cl (7-9). The activity was measured as described above. Both optimum temperature and pH were defined as U/mL. To determine thermal stability, the enzyme was incubated at an optimum temperature and 5^o below optimum temperature for 150 minutes. Enzyme activity was measured every 15 minutes. pH stability was determined by incubating enzyme in various pH buffer (4-9) for 12 h at 4ºC. The activity was defined as a relative activity with activity without treatment as 100% activity.

Effect of metal ions

Effect metal ions, Ca^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, Mg^{2+}, Fe^{2+}, Li^{+}, and K^{+}, were determined by incubating the mixture of enzyme, the substrate in optimum pH buffer and metal ion at a final concentration of 1 and 10 mM and the activity was measured as described above. The activity was defined as a relative activity with activity without metal ion addition as 100% activity.

Effect of chemicals and detergent

Effect of chemicals (EDTA, Mercaptoethanol, PMSF) and 0.1-1% detergent (SDS, Tween 80, Triton-100) were determined by incubating the mixture of enzyme, the substrate in optimum pH buffer and the compounds at final concentration mentioned above for 30 minutes at optimum temperature and pH. The activities were measured after treatment and determined as relative activity (%) with enzyme without treatment as 100% activity.

RESULTS AND DISCUSSION

Bacterial strain

The bacterium T513 is one of three isolates forming clear zone from 8 selected isolates (the data was not presented). Clear zone formed by T513 on solid medium with 0.5% sodium alginate as substrate was higher than those shown by other isolates (Index alginolytic = 6.8)

(Figure 1.). In the next discussion, alginate lyase produced by T513 will be mentioned as AlgT513. Identification using 16S rDNA region blasted with the database on NCBI showed that T513 has 95% similarities with *Bacillus tequilensis* strain 10b (Accession Number: NR_104919.1). The 16 rDNA sequence of T513 has been submitted to DDJB GenBank with Accession Number of LC457966. Neighbor-joining phylogenetic tree showing the relationship of T513 with members of *Bacillus* present in GenBank based on 16S rRNA gene was presented in Figure 2.

Some microorganisms were reported producing alginate lyase, i.e. *Wenyingzhuangia fucanilytica* (Pei et al. 2018), *Isoptericola halotolerans* CGMCC 5336 (Chen et al. 2018), *Sphingomonas* sp. (He et al. 2018; Park et al. 2012; Ryu and Lee 2011), *Vibrio furnissii* (Zhu et al. 2018), *Microbulbifer* sp. ALW1 (Zhu et al. 2016; Swift et al. 2014), *Flavobacterium sp.* (Inoue et al. 2014; Huang et al. 2013), *Vibrio* sp. (Wang et al. 2013), *Pseudomonas aeruginosa* (Farrell and Tipton 2012), *Pseudomonas alginovora* (Lundqvist et al. 2012), *Stenotrophommonas maltophilia* (Lee et al. 2012), *Saccharophagus degradans* (Kim et al. 2012), *Pseudomonas fluorescens* (Li et al. 2011), *Agarivorans* sp. (Kobayashi et al. 2009), *Alteromonas* sp. (Iwamoto et al. 2001), *Corynebacterium* sp. (Matsubara et al.1998), *Pseudoalteromonas elyakovii* (Ma et al. 2008), *Pseudomonas syringae* (Preston et al. 2000), and *Streptomyces* sp. (Cao et al. 2007; Kim et al. 2009).

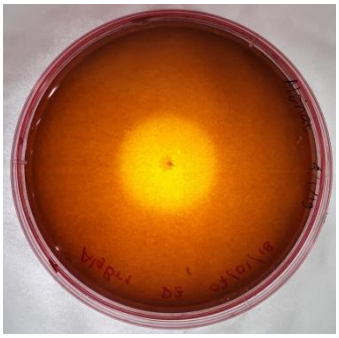

Figure 1. Clear area around T513's colony grown on plate agar containing 0.5% sodium alginate. Incubation was carried out at a temperature of 37^{o}C for 2 days.

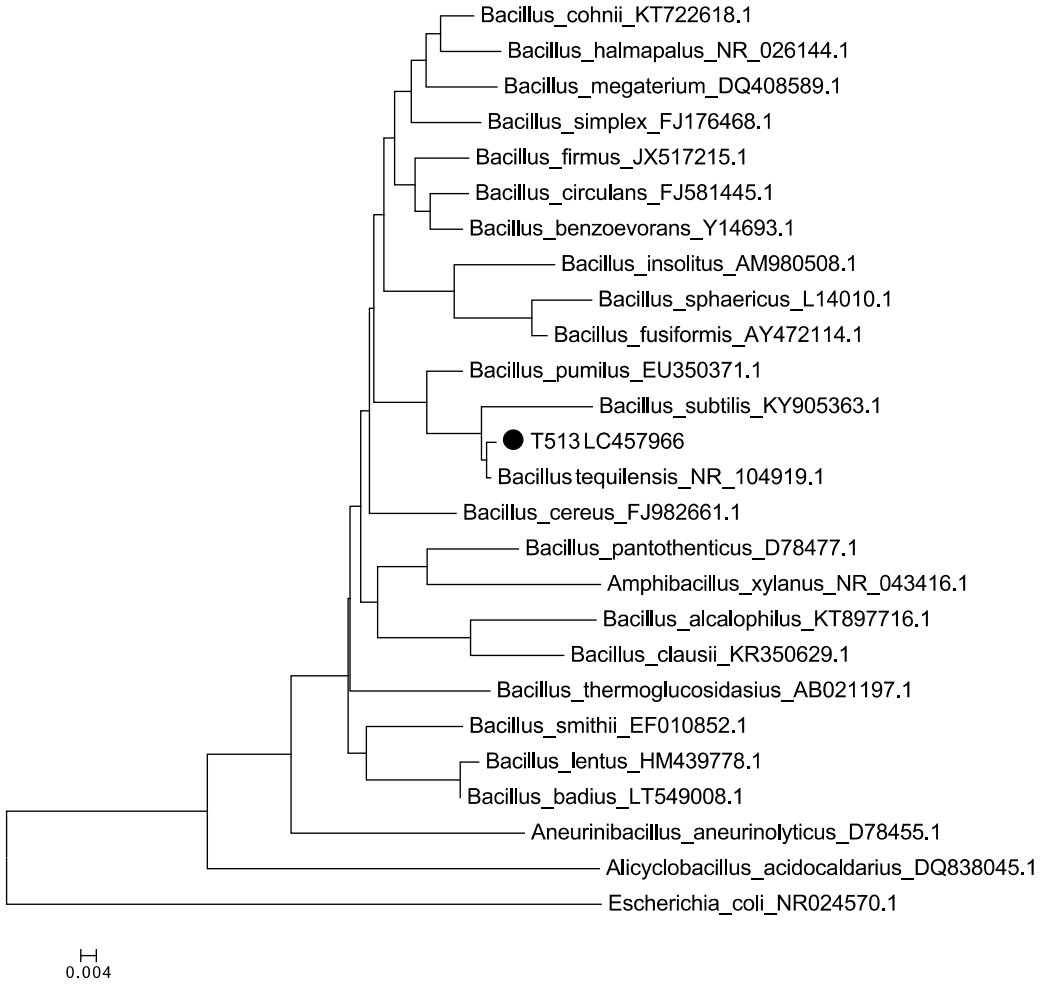

Figure 2. Neighbor-joining phylogenetic tree (bootstrap = 1000) constructed by MEGA7 showing the relationship of T513 with members of *Bacillus* present in GenBank based on 16S rRNA gene analysis

Although *Bacillus* is known as industrial genus of bacteria due to their potential to produce various important enzyme, but rare of this genus reported as alginate lyase producer. When this report is written, there was no report about *Bacillus tequilensis* as Alginate lyase producer. The low similarity of T513's 16s rRNA gene sequence with those of in database identified as Bacillus species (95%) indicating that Alg07 may be a novel Bacillus species. It showed possibility for T513 to be proposed as a new species as revealed by Drancourt et al. 2000 that the similarity level of 16S rRNA gene for new taxa at genus and species level are below of 97% and 99%, respectively.

Optimum temperature and thermal stability

Temperature range of 40-70ºC was used to determine AlgT513 optimum temperature. The activity had been detected at a temperature of 40ºC, increasing at 45ºC and although showing the highest activity at 50ºC, the activity was still detected up to the temperature of 70ºC (Figure 3). The activity of ALT513 retained about 50% after 105 minutes incubation at 50ºC and still showed 100 % activity after 180 minutes at 45ºC. Alginate lyase produced by *Microbulbifer* sp. 6532A also showed the highest activity at 50ºC (Swift et al. 2014) as well as this produced by *Pseudoalteromonas* sp. SM0524 (Li et al. 2011). Some alginate lyases showed different optimum temperature such as those produced by *Microbulbifer* sp. ALW1 (Zhu et al. 2016) which had an optimum temperature of 45ºC. Others showed the optimum temperature at 40ºC such as produced by marine Vibrio sp. NJ-04 (Zhu et al. 2018) and Marine Bacterium *Bacillus* sp. Alg07 (Chen et al. 2018). The higher optimum temperature of 55ºC was showed by alginate lyase produced by *Flavobacterium* sp. Strain UMI01 (Inoue et al. 2014). *Sphingomonas sp*. ZH0 produced four alginate lyases, ZH0-I, ZH0-II, ZH0-III and ZH0-with a different optimum temperature of 42, 47, 52 and 37ºC respectively (He et al. 2018).

Optimum pH and its stability

AlgT513 showed the highest activity in 0.05 M Tris-Cl at pH of 8 among tested pH range of 5-9 (Figure 3) and tended to be more active at higher pH that showed by high activity at pH of 9 compared to pH of 4-6. AlgT513 also showed stability in all of tested pH (4-9) (Figure 4). Most of the alginate lyases were stable at a broad of pH range such as produced by Marine *Vibrio* sp. NJ-04 which exhibited maximum activity at pH 7.0 and showed stability at broad pH range of pH 4.0 to 10.0 (Zhu et al.2018). For instance, alginate lyase produced by Marine bacteria *Wenyingzhuangia fucanilytica* was active the most at pH 8.5 and stable at pH range from 5.5 to 9 (Pei et al. 2018). *Microbulbifer* sp. ALW1 has an optimum pH of 7.0 and stable over a broad pH range of 5.0-9.0 (Zhu et al. 2016). AlySJ-02 from *Pseudoalteromonas* sp. SM0524 exhibited its maximal activity at pH 8.0 and retained its stability between pH 7.0-9.0 (Lee et al. 2011). The AlyIH from *Isoptericola halotolerans* CGMCC5336 showed the highest activity at pH 7.0 and was stable at pH 7.0-8.0 (Dou et al. 2013).

Effect of metal ion

Addition of 5 mM $CaCl_2$, $MgCl_2$ and KCl enhanced the activity of AlgT513 by 45, 26 dan 48%. While $CoCl_2$, $MnCl_2$ and $ZnCl_2$ repressed activity by 20, 94 and 92% (Figure 5). Some Alginate Lyases showed the same reaction in the presence of $MgCl_2$, $CaCl_2$, and KCl such as those produced by *Pseudoalteromonas* sp. SM0524 (Li et al.2011), *Microbulbifer* sp. ALW1 (Zhu et al. 2016) and FlAlyA from *Flavobacterium* sp. Strain UMI-01 (Inoue et al.2014). *Sphingomonas* sp. ZH0 produced three alginate lyases where their activity strongly inhibited by $ZnCl_2$ (He et al. 2014) as well those produced by *Microbulbifer* sp. ALW1 (Zhu et al. 2016) *Microbulbifer* sp. 6532A (Swift et al. 2014) and *Vibrio* sp. NJ-04 (Zhu et al. 2018).

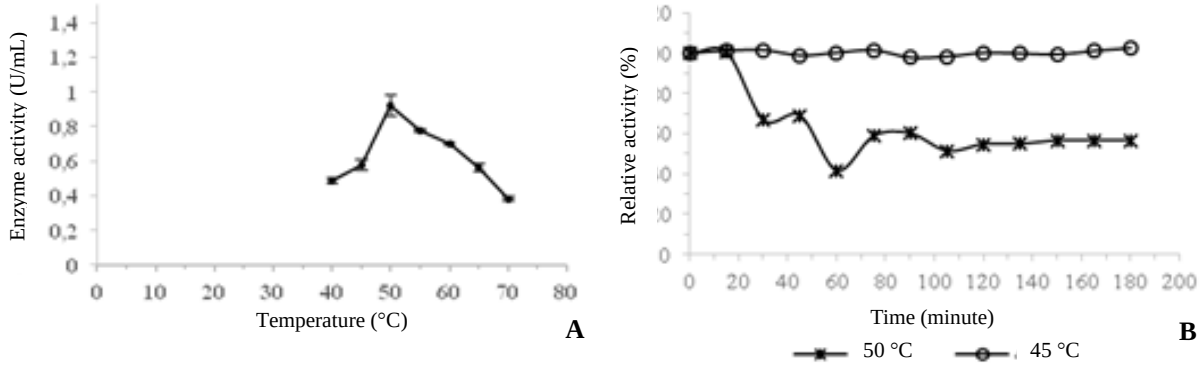

Figure 3. The activity of AlgT513 at various temperature (A) and its thermal stability (B)

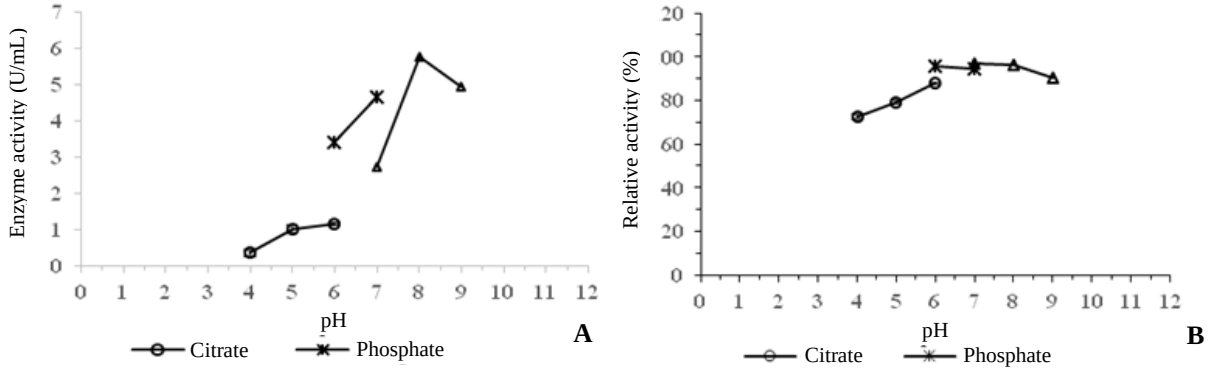

Figure 4. The activity of AlgT513 in various pH buffer (A) and its stability after 12 h incubation (B)

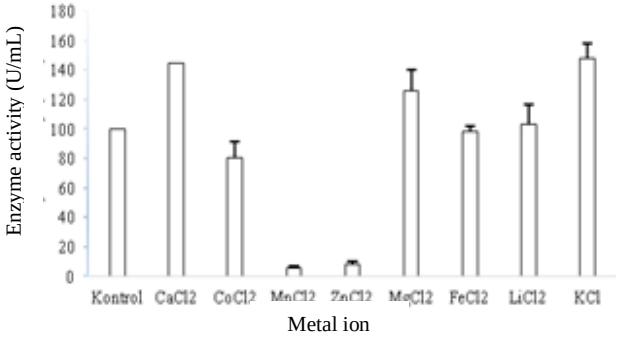

Figure 5. The activity of AlgT513 in the presence of 5 mM metal ion

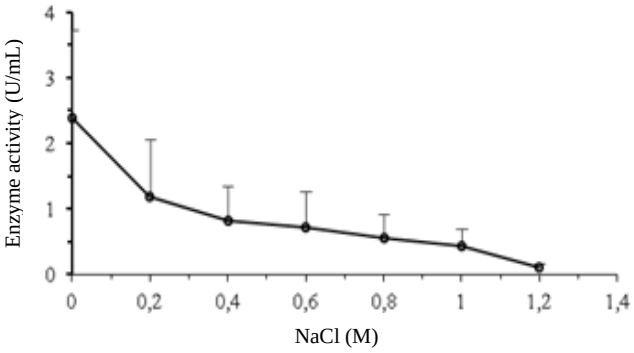

Figure 6. The activity of AlgT513 in the presence of NaCl at various concentration

Table 1. The activity of AlgT513 in the presence of metal ion and detergent

Chemical/ detergent	Concentration	Relative activity (%)		
None		100.00	±	13.58
EDTA	1 mM	10.62	±	1.66
	10 mM	8.64	±	2.54
PMSF	1 mM	106.35	±	0.89
	10 mM	30.83	±	10.18
ME	1 mM	95.36	±	6.18
	10 mM	103.88	±	10.78
Triton X100	1%	120.53	±	2.81
	0.1%	92.46	±	11.39
SDS	1%	31.35	±	2.26
	0.1%	90.51	±	13.75
Tween 80	1%	74.98	±	4.68
	0.1%	3.86	±	0.23

Effect of chemicals and detergent

As showed in Table 1. the present of 1 and 10 mM EDTA strongly inhibited AlgT513 activity as well as 10 mM PMSF. It showed that AlgT513 probably metalloserine enzyme. The activity of AlgT513 was not affected by the presence of ME and Triton-100. However, the addition of SDS and Tween 80 at 1 and 0.1 % concentration respectively strongly inhibited AlgT513 activity (Table 1). As a comparison, Alginate lyase produced by *Microbulbifer* sp. ALW1 inhibited by the presence of EDTA but not by the presence of PMSF and ME (Zhu et al. 2014). Alginate lyase produced by *Pseudoalteromonas* sp. SM0524 also suggested as metalloalginate lyase which is showed by the inhibition of EDTA on its activity (Li et al. 2011).

NaCl showed inhibition effect on AlgT513 activity. Its activity got decreasing by the higher of NaCl concentration and showed no activity at the present 1.2 M NaCl (Figure 6). The presence of NaCl has been reported promoting the activity of most alginate lyases such as produced by *Pseudoalteromonas* sp. SM0524 (Li et al.2011), *Microbulbifer* sp. ALW1 (Zhu et al. 2016) *Flavobacterium* sp. Strain UMI-01 (Inoue et al. 2014), *Bacillus* sp. Alg07 (Chen et al.2018), *S. degradans* (Kim et al. 2012), *Microbulbifer* sp. 6532A (Swift et al. 2014), *Vibrio* sp. NJ-04 (Zhu et al. 2018) and three alginate lyases produced by *Sphingomonas* sp. ZH0 (He et al. 2014). The concentration needed by each enzyme to reach maximum activity was different ranging of 0.2-1.2 M.

In conclusion, bacteria are known as source of valuable enzymes beneficial as degradation agent (Lee et al. 2012; Ethica and Sabdono 2017). In this study, Alginate lyase, an important biofilm degradation enzyme had been isolated from a bacterial novel strain T513 and successfully

characterized. AlgT513 is suggested as a serine metalloenzyme due to inhibition of ethylenediaminetetraacetic acid (EDTA) and phenylmethylsulfonyl fluoride (PMSF). Inhibition of NaCl on AlgT513 makes it unique due to most of Alginate lyase activity produced by microorganisms enhancing by the presence of NaCl. Further study to explore the application of ALgT513 on alginate degradation is needed to obtain the pattern of oligomers its produced and their bioactivity.

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