Acta Biologica Hungarica 67(3), pp. 305–317 (2016)
DOI: 10.1556/018.67.2016.3.8

PURIFICATION AND CHARACTERIZATION OF ALGINATE LYASE FROM LOCALLY ISOLATED MARINE *PSEUDOMONAS STUTZERI* MSEA04

Ehab A. Beltagy,[1] Aliaa El-Borai,[2]* Marina Lewiz[2] and Samy A. ElAssar[2]

[1]Marine Microbiology Department, National Institute of Oceanography and Fisheries (NIOF), Alexandria, Egypt
[2]Botany and Microbiology Department, Faculty of Science, Alexandria University, Alexandria, Egypt

(Received: January 15, 2016; accepted: March 16, 2016)

An alginate lyase with high specific enzyme activity was purified from *Pseudomonas stutzeri* MSEA04, isolated from marine brown algae. The alginate lyase was purified by precipitation with ammonium sulphate, acetone and ethanol individually. 70% ethanol fraction showed maximum specific activity (133.3 U/mg). This fraction was re-purified by anion exchange chromatography DEAE- Cellulose A-52. The loaded protein was separated into 3 peaks. The second protein peak was the major one which contained 48.2% of the total protein recovered and 79.4% of the total recovered activity. The collected fractions of this peak were subjected to further purification by re-chromatography on Sephadex G-100. Alginate lyase activity was fractionated in the Sephadex column into one major peak, and the specific activity of this fraction reached 116 U/mg. The optimal substrate concentration, pH and temperature for alginate lyase activity were 8 mg/ml, pH 7.5 and 37 °C, respectively. While, K_m and V_{max} values were 1.07 mg alginate/ml and 128.2 U/mg protein, respectively. The enzyme was partially stable below 50 °C, and the activity of the enzyme was strongly enhanced by K^+, and strongly inhibited by Ba^{+2}, Cd^{+2}, Fe^{+2} and Zn^{+2}. The purified enzyme yielded a single band on SDS-PAGE with molecular weight (40.0 kDa).

Keywords: Pseudomonas sp. – Alginate lyase – Enzyme purification – Enzyme properties

INTRODUCTION

Alginate is considered as a linear polysaccharide in which β-D-mannuronate (M) and α-L-guluronate (G) are covalently (1-4)-linked in different sequences. α-L-Guluronate is C5 epimer of β-D-mannuronate. The uronic acid monomers are linked to form polymannuronate block (polyM-block), polyguluronate block (polyG-block) and random copolymer (polyMG-block). In the nature, alginates are quite abundant; they are produced as a structural component in brown algae; some bacteria also can synthesize alginates [7, 17].

The mechanism of alginate lyases is by degradation of alginate through β-elimination of the glycosidic bond, yielding various oligosaccharides [1]. These oligosaccharides have an unsaturated uronic acid at the non-reducing terminus and unsaturated uronic acid monomers in the non-reducing end [13], which is an unsatu-

*Corresponding author; e-mail address: aliaa_elborai2007@yahoo.com

rated double bond between C4 and C5, showing strong absorption at 235 nm. However, the enzymatic property and the hydrolyzing site of the alginate vary among different origins [23]. Alginate lyases can be characterized as polyM-, polyG-, and polyMG-specific lyases which depend on the substrate specificity. Also they have either endo- or exo-degradation activity with the corresponding substrate specificity [7]. Moreover, alginate lyases are grouped into three classes, according to their molecular masses: 20–35 kDa class, ~40 kDa class, and ~60 kDa class [16].

Various alginate lyases have been produced from algae, marine invertebrates, marine and some soil microorganisms, more often from brown algae and some bacteria belonging to the genera *Azotobacter* and *Pseudomonas* [18]. Both non-alginate-synthesizing and alginate-synthesizing organisms produce alginate lyases. In the non-alginate-synthesizing organisms, alginate lyases play important roles in assimilation of alginate as a carbon source [20]. In the biosynthesis of alginate, alginate lyase appears to play a certain role in control of alginate polymer length and optimization of polymerization reaction [6].

Alginate lyases have a wide variety of uses. They are used in the production of algal protoplasts and for studying the fine structure of alginate [9]. Alginate lyases are important tools for oligosaccharide preparation, medical treatment, and energy bioconversion [24].

In this study, an alginate lyase-producing bacterium was screened from marine rotten kelp. The enzyme produced by this strain was purified and characterized, and its molecular weight was determined.

MATERIALS AND METHODS

Bacterium *and* Culture Medium

Bacterial strains capable of producing alginate lyase enzyme, were isolated from brown algal samples, collected from Aqaba Gulf, Suez Gulf and Red Sea, cut into small pieces and grown in a Mineral salt-medium with the following composition (g/l): sodium alginate, 10; $(NH_4)_2SO_4$, 5; K_2HPO_4, 2; $FeSO_4 \cdot 7H_2O$, 0.01; $MgSO_4 \cdot 7H_2O$, 1; NaCl, 30; agar, 15 and final pH was adjusted to 7.5; plates were incubated at 37 °C for 24–48 hr to form detectable colonies [9]. For alginate lyase production, 50 ml aliquots of the mineral medium was inoculated with 1 ml of overnight bacterial culture in ZoBell medium; then incubated in the shaker at 37 °C and 120 rpm for 24 hr. Production medium was further optimized by statistical ways for maximum alginate lyase production, the optimized medium used for enzyme purification was: 2% sodium alginate, 0.4% yeast extract, 0.2% $K_2HPO_4 \cdot 3H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.1% $MgSO_4 \cdot 7H_2O$, 5% NaCl, and inoculum size: 1 ml.

The target strain was identified by the 16S rRNA gene sequencing analysis by GACT Company-Germany using universal primers; 27F: FAGAGTTTG-ATCMTGGCTCAG and 1492R: CGGTTACCTTGTTACGACTT. NCBI tools including blast search, tree method (Fast Minimum Evolution) and pair wise align-

ment were applied for the providing sequence. PCR amplification program was set according to the following cycling parameters: 5 min of denaturation at 95 °C, followed by 20 cycles of 30 s at 95 °C (denaturing), 30 s at 56 °C (annealing), and 90 s at 72 °C (elongation), with a final extension at 72 °C for 7 min. The nucleotide sequences were analyzed with the BLAST database [10].

Preparation of the crude enzyme, enzyme assay and protein determination

The clear supernatant was considered as the crude enzyme source. Alginate lyase activity was measured depending on the release of reducing sugars from alginate as a substrate according to the 3,5-dinitro salicylic acid (DNS) method as described by El-Katatny et al. [4]. Total soluble protein was also measured as described by Lowry et al. [12].

Purification of Alginate lyase produced by Pseudomonas stutzeri *MSEA04*

Crude culture supernatant obtained from cultures of *Pseudomonas stutzeri* MSEA04 was precipitated using different agents as ammonium sulphate, acetone and ethanol, at different concentrations of each precipitant in a sequential manner [2]. The highest recovered protein which was obtained by precipitation by ethanol was further purified by anion-exchange chromatography column (28 × 1.8 cm) on DEAE-Cellulose, which was used as an anion exchanger as reported by Peterson and Sober [19]. It was equilibrated with 0.05 M Tris buffer (pH 7.5) to be used in chromatography. The fractions were eluted with the same buffer (180 ml) at flow rate 30 ml/h. Four ml fractions were collected and assayed for protein and enzyme activity. The active fractions with the highest specific activity of enzyme were pooled. The pooled fractions of the enzyme were further fractionated by gel filtration through Sephadex G-100 column, eluted with acetate buffer (180 ml) at a flow rate of 30 ml/h. Four ml fractions were collected and assayed for protein and enzyme activity.

Characterization of the alginate lyase produced by Pseudomonas stutzeri *MSEA04*

To determine the optimal substrate concentration, different substrate concentrations ranging from 1–10 mg/ml were used. The reaction was carried at 37 °C for 20 minutes. To determine the optimal pH, the enzymatic reaction was carried out in the range of pH 3.5–8 using 0.05M acetate and phosphate buffer. The optimal enzyme temperature was determined by measuring the activity of the purified alginate lyase toward sodium alginate at pH 7.5 with different temperatures 30, 37, 40, 45, 50 and

60 °C. The thermal stability of the enzyme was determined by preheating identical portions of each preparation separately in absence of the substrate for a period of 15, 30 and 60 min. at different temperatures (40, 50 and 60 °C). In each case, a control was carried out using inactivated enzyme solution. To assay the effects of metal ions and EDTA on the activity of the enzyme, 100 mM of each metal ion (or EDTA) was incubated with the enzyme at 37 °C for 1 hr, and then the activity of the enzyme toward sodium alginate was measured. The reaction mixture without any metal ion or inhibitor was taken as control. The K_m and V_{max} values for alginate lyase produced acting on sodium alginate at different concentrations of 1–10 mg/ml were determined by linear regression analysis of Lineweaver–Burke [11], double reciprocal, plots of initial velocity data obtained under the condition described above. To determine the molecular weight of the enzyme, purified alginate lyase was electrophoresed into SDS-PAGE gel, according to the method of Laemmli [8]. Gel electrophoresis was performed in Delta Scientific Services Company, Alexandria, Egypt.

RESULTS

Strain identification

The partial sequence of 16S rRNA (1111 bps) of the isolate was provided by GACT Company-Germany. According to the blast search of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the sequence similarity was found to be 99% similar to *Pseudomonas stutzeri* A1501 and *Pseudomonas stutzeri* TS44 contig0058. Consequently, the strain

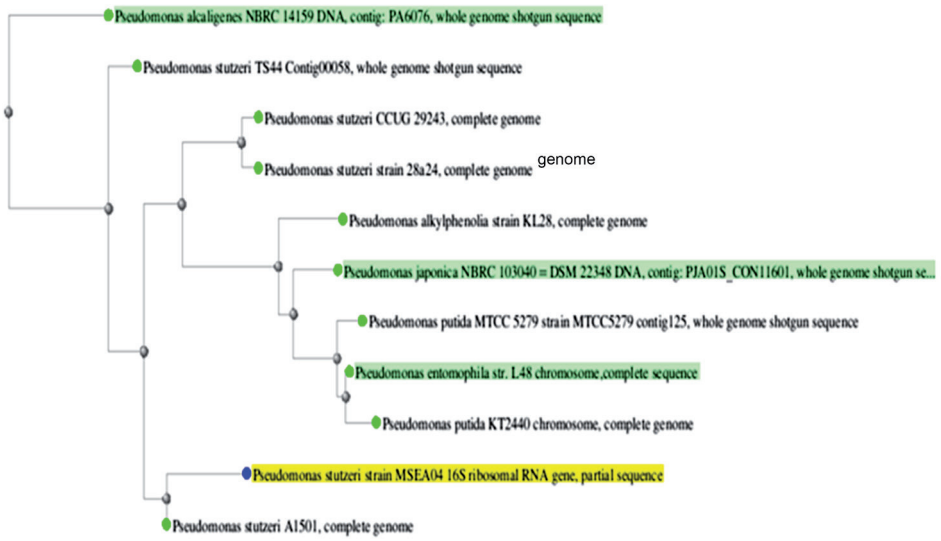


Fig. 1. The phylogeny tree of the identified isolate *Pseudomonas stutzeri* MSEA04 according to the partial sequence of 16S rRNA; showing the relationships between the strain and other related strains

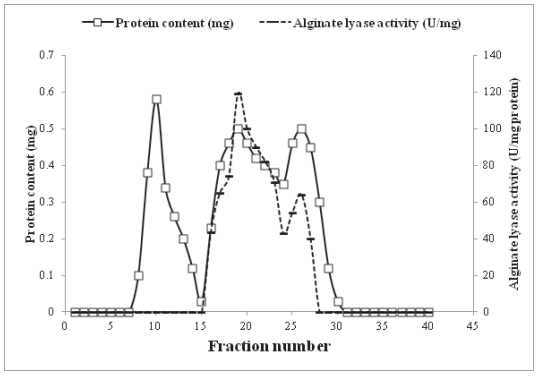


Fig. 2. Purification of semi-purified alginate lyase from *Pseudomonas stutzeri* MSEA04 using ion-exchange chromatography on DEAE Cellulose A-52; (1–3 are protein peaks; alginate lyase is located in two peaks)

was identified as *Pseudomonas stutzeri* strain MSEA04 with accession number GenBank KT867679. In addition, the neighborhood relationship to other strains is illustrated in Figure 1 showing the phylogeny tree of the identified strain. According to the molecular identification of the identified isolate *Pseudomonas stutzeri* MSEA04, belongs to Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; *Pseudomonas stutzeri* subgroup.

Purification of alginate lyase produced from Pseudomonas stutzeri *MSEA04*

Ion-exchange chromatography on DEAE-Cellulose A-52

Combined culture supernatants containing the crude enzyme were at first partially purified by fractional precipitation with different agents. Seventy percent ethanol fraction showed the highest alginate lyase activity as it gave about 133.3 U/mg specific activity, which was 3.6 fold higher than that obtained from the crude enzyme (data not shown). This fraction was evaporated and concentrated. Nine mg protein of the enzyme was dissolved in 15 ml of 0.05 M tris buffer (pH 7.5) and was loaded on DEAE-Cellulose A-52 column. It has contained 65 fractions as shown in Figure 2. The protein recovered by the obtained fractions reached about 83% of the applied sample. It was noticed that the protein was separated into 3 components in the column. The first protein component was a minor one and was covered by fractions 8 to 15. It represented about 27% of the total recovered protein from the column. The second protein peak was a major one and was covered by fractions 16 to 24. The protein recovered by this peak represented about 48.2% of the total protein recovered. The third one was a minor and was covered by fractions 25 to 30. It represented about 24.89% of the total recovered protein. The total activity of alginate lyase recovered

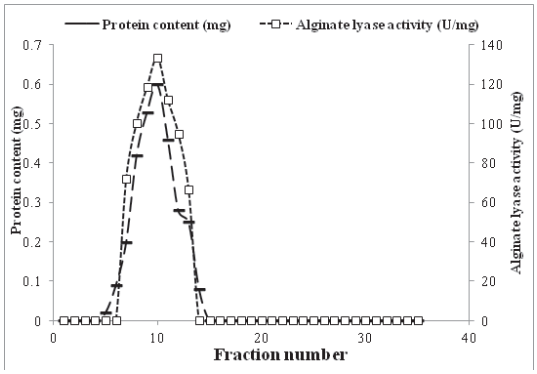


Fig. 3. Purification of the major active alginate lyase component obtained from Ion-exchange chromatography using gel filtration on Sephadex-G100 (one protein peak; alginate lyase is also located in one peak)

from the anion exchange column reached about 92.7% of the activity of the sample applied to the column. The first peak eluted by fractions 1 to 7 with no protein peak and it was estimated to be reducing sugars. The total alginate lyase activity was calculated to be 450 U/ml and was fractionated in the column into 2 peaks. The first peak was a major one and was present with the second protein component. It eluted at step-gradient 0.1–0.2 M NaCl in 0.5 M tris buffer (pH 7.5) and was covered by fractions 16 to 24. The major enzyme peak showed approximately an activity of 79.4% of the total recovered activity from the column. The second alginate lyase activity peak was a minor one and the activity recovered represented about 20.63% of the total alginate lyase activity recovered. The specific activity of the fractions obtained from the column reached 113.3 U/mg showing about 3.06 fold purification of the crude enzyme preparation (culture extract).

Gel filtration in Sephadex-G100

The collected fractions obtained from ion exchange chromatography were evaporated and concentrated. Three mg protein of the enzyme was dissolved in 10 ml of 0.05 M tris buffer (pH 7.5) and was loaded on a Sephadex-G100 column. The results indicated in Figure 3 that the gel filtration of the purified alginate lyase yielded 35 fractions. The protein recovered by the obtained fractions reached about 97.6% of the applied sample. The total alginate lyase activity recovered from the Sephadex G-100 column represented about 86.53% of the original activity. Also, the protein was separated into one component in the column which was covered by fractions 5 to 14. The alginate lyase activity was fractionated in the Sephadex column into one major peak covering fractions from 7 to 12. The specific activity of the fractions from column reached 116 U/mg showing about 3.13 fold purification of the crude enzyme preparation. A summary of the purification steps of alginate lyase enzyme is shown in Table 1.

Table 1
Purification steps of alginate lyase enzyme produced from *Pseudomonas stutzeri* MSEA04

Purification step	Total protein (mg)	Total activity (U)	Alginate lyase activity (U/mg)	Purification fold	Yield (%)
Crude enzyme	214.00	7918.00	37.00	1.00	100.00
Ethanol precipitation (70%)	9.00	450.00	50.00	1.35	5.68
Ion-exchange chromatography	3.00	340.00	113.30	3.06	4.29
Sephadex-G100	2.50	290.00	116.00	3.13	3.66

Characterization of the alginate lyase produced by Pseudomonas stutzeri *MSEA04*

Optimal activity

The effect of substrate concentration on the activity of the purified alginate lyase enzyme showed that the rate of the reaction was directly correlated with the substrate concentration. The optimum substrate concentration for the purified enzyme was 8 mg/ml reaction mixture, giving the highest specific activity (121.8 U/mg). The K_m and V_{max} values of the purified enzyme were found to be 1.07 mg alginate/ml and 128.2 U/mg protein, respectively (Fig. 4). Influence of pH and temperature of the reaction using a substrate concentration of 8 mg/ml reaction mixture were studied. The purified enzyme exhibited the highest activity at pH 7.5 giving specific activity (128 U/mg), showing a relative high activity in a pH range from 5.5 to 7.5. Moreover, alginate lyase activity increased gradually by increasing the reaction temperature

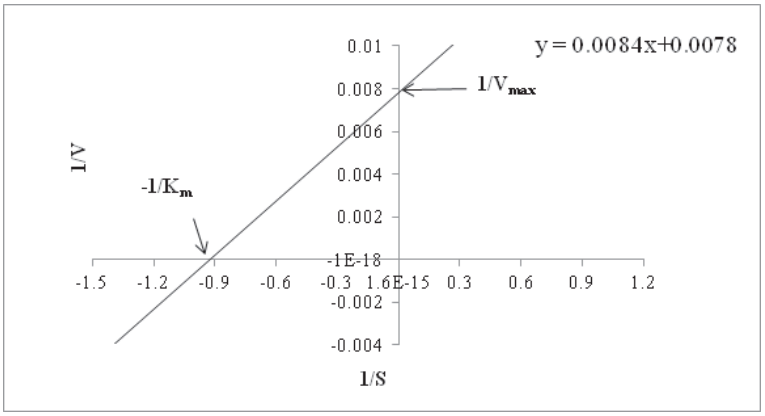
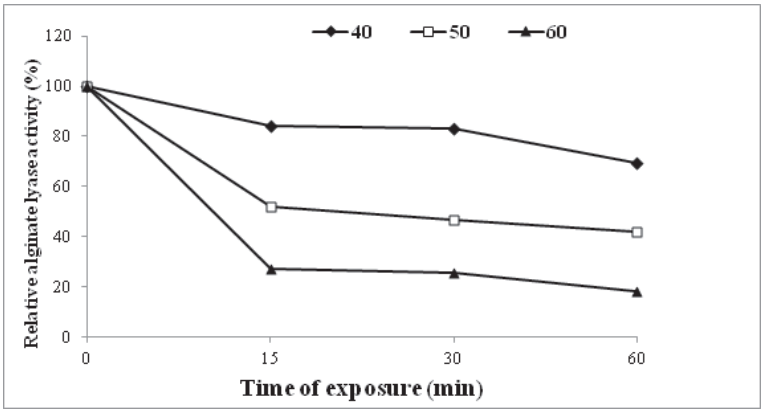


Fig. 4. Lineweaver-Burk plot for evaluation of kinetic constants (K_m and V_{max}) of purified alginate lyase from *Pseudomonas stutzeri* MSEA04. Enzyme activity was determined at different alginate concentrations of 1–10 mg/ml



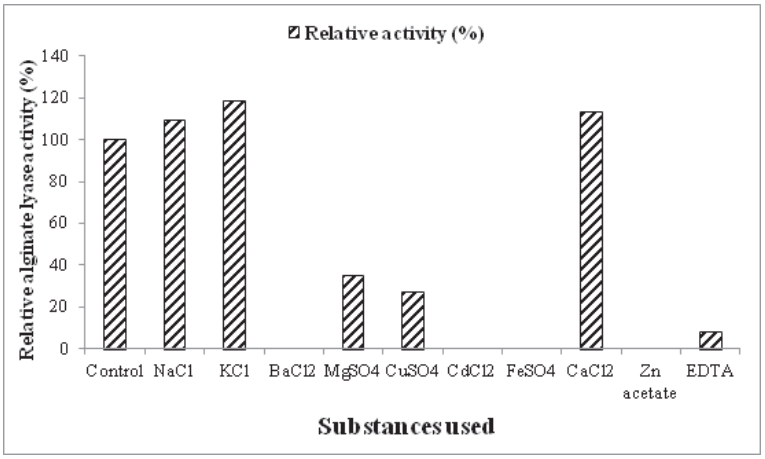


Fig. 5. Thermal stability of the purified alginate lyase from *Pseudomonas stutzeri* MSEA04. The enzyme was incubated at 40 °C, 50 °C and 60 °C for different periods of time (15, 30, 60), and then the residual activity toward alginate was assayed at 37 °C. The relative activity was defined as the percentage of activity determined with respect to the maximum alginate lyase activity

Fig. 6. Effect of some metal ions on the activity of the purified alginate lyase from *Pseudomonas stutzeri* MSEA04. 100 mM of each metal ion (or EDTA) was incubated with the enzyme at 37 °C, and then the activity of the enzyme toward sodium alginate was measured. The activity of the enzyme without any metal ion in the reaction mixture was taken as control

reaching its maximum value (128 U/mg) at 37 °C. However when the upper limit was approached, the activity diminished indicating thermal denaturation (data not shown).

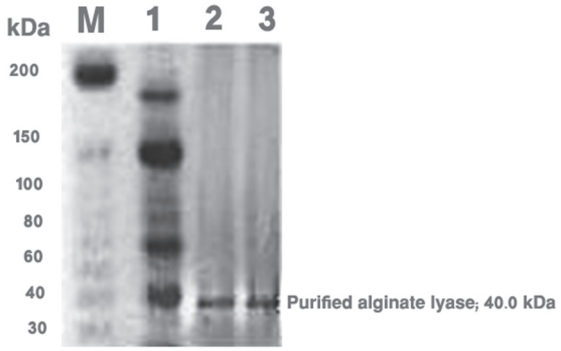


Fig. 7. Purity and molecular mass of the alginate lyase analyzed by 10% SDS-PAGE. The standard protein markers for SDS-PAGE are: Phosphorylase-b, 97.0 kDa; Bovine serum albumin, 67.0 kDa; egg albumin, 45.0 kDa; glyceraldehyde-3-phosphate, 36.0 kDa; Beta-Lactoglobulin, 18.4 kDa; 30 µg proteins were loaded in each lane. Lane M, molecular weight marker; Lane 1, crude enzyme; Lane 2, fraction from ion exchange chromatography; Lane 3, fraction from Sephadex-G100. The molecular mass of the enzyme was estimated to be 40.0 kDa

Thermal stability

The purified enzyme was partially stable after exposing to 40 °C for up to 30 and 60 minutes, respectively (Fig. 5). A further increase in temperature to 50 °C resulted in a further decrease in the stability of the enzyme. A treatment at a temperature of 60 °C showed a loss of 74.1% and 82% of the original activity after 30 min and 60 min of exposure, respectively.

Effect of some metal ions (activators and inhibitors) on the activity of the purified alginate lyase

According to our results, Na^{+}, K^{+} and Ca^{+2} activated the enzyme by 9.37, 18.75 and 13.28% respectively (Fig. 6), while Mg^{+}, Cu^{+2} and EDTA strongly inhibited the enzyme by 64.6%, 73% and 92.2%, respectively. On the other hand, Ba^{+2}, Cd^{+2}, Fe^{+2} and Zn^{+2} completely inhibited the enzyme.

Gel electrophoresis

The purity of the alginate lyase enzyme was examined and the molecular weight of it was determined by gel electrophoresis. The results shown in Figure 7 indicate that alginate lyase enzyme obtained by ion exchange column and the gel filtration chromatography gave a single band on SDS-PAGE gel, indicating the purity of the isolated alginate lyase with a molecular weight equal to 40.0 kDa.

DISCUSSION

Semipurified alginate lyase was obtained by DEAE-Cellulose A-52 column followed by Sephadex-G100 column. The specific activity of the fractions from Sephadex-G100 column reached 116 U/mg. Purification of partially purified alginate lyase from *Streptomyces* species A5 and *Aspergillus oryzae* have been studied using DEAE-Cellulose column and Sephadex G-100 column and the purified enzyme showed a specific activity of 101.6 U/mg and 67.24 U/mg, respectively [3, 21]. In addition, purification of alginate lyase from *Vibrio* sp. YKW-34 strain has been achieved by using DEAE-Sepharose FF column, a Phenol Sepharose FF column and a Sephacryl S-100 HR column for gel filtration [5].

Optimal conditions for the enzyme activity have been studied and compared with other studies as shown in Table 2. The optimal substrate concentration, pH and temperature toward alginate lyase activity were 8 mg/ml, pH 7.5 and 37 °C, respectively. The K_m and V_{max} values of the purified enzyme were found to be 1.07 mg alginate/ml and 128.2 U/mg protein. K_m and V_{max} values of alginate lyase from *Aspergillus oryzae* were 21.52 mg/ml and 222.68 U/mg proteins, respectively [21]. Several reports indicated that the optimum activity of alginate lyase was obtained between pH 7.0 and 8.0 from, *Streptomyces* species A5, and *Microbulbifer* sp. ALW1, respectively [3, 26]. Similarly, it was found that the alginate lyase activity was optimal at 37 °C like that purified from *Bacillus* species, *Escherichia coli and Streptomyces* species A5, respectively [3, 14, 15].

Alginate lyase from *Pseudomonas stutzeri* MSEA04 can be considered more thermostable than the alginate lyase produced from *Escherichia coli,* which lost 50% of its activity after 10 min at 45 °C [14], and alginate lyase obtained from *Aspergillus oryzae* which lost completely its activity at 50 °C [21]. However, it was still considered to be less stable than the alginate lyase obtained from *Bacillus* species which retained 60% and 50% of its activity after treatment for 30 min at 20 °C and 60 °C, respectively [15].

The present study on the effect of metal ions on alginate lyase from *Pseudomonas stutzeri* MSEA04 showed an activity enhancement through Na^{+}, K^{+} and Ca^{+2}. While, Mg^{+}, Cu^{+2} and EDTA strongly inhibited the enzyme. Moreover, Ba^{+2}, Cd^{+2}, Fe^{+2} and Zn^{+2} completely inhibited the enzyme activity. Purified alginate lyase from *Microbulbifer* sp. ALW1 was inhibited by Ba^{+2}, Zn^{+2}, Cu^{+2}, Fe^{+3} and Al^{+3}, and Na^{+} at 1 mM stimulated the activity [26]. Moreover, the activity of the purified alginate lyase produced by *Bacillus* species was completely inhibited by EDTA, partially inhibited by Cu^{+2}, and activated by Ca^{+2} ions [15]. Molecular masses of the purified alginate lyases produced from *Vibrio* sp. QY105, *Bacillus* sp. ATB-1015 strain, and *Pseudomonas aeruginosa* were in the range (37–41 kDa) [15, 22, 25] similarly to that reported by the investigated alginate lyase obtained from *Pseudomonas stutzeri* MSEA04 (40 kDa).

Table 2
Comparative studies on properties of new alginate lyase from *Pseudomonas stutzeri* MSEA04

Enzyme properties	*Pseudomonas stutzeri* MSEA04	*Vibrio sp.* YWA (23)	*Pseudoalteromonas* sp. SM0524 (9)	*Vibrio sp.* YKW-34 (5)	*Microbulbifer* sp. ALW1(26)	*Aspergillus oryzae* (21)
Optimum pH	7.5	7	8.5	7	7	6.5
Optimum temp (°C)	37	25	50	40	45	35
K_m	1.07 mg alginate/ml	72.73 g/L	No available data	No available data	No available data	21.52 mg/mL
V_{max}	128.2 U/mg protein	No available data	No available data	No available data	No available data	222.68 U/mg proteins
Thermal stability	The enzyme was partially stable after exposure to 40 °C for up to 30 and 60 minutes, respectively	No available data	The half life period of the enzyme was 41 min at 40 °C and 20 min at 50 °C, indicating that it has low thermal stability.	The enzyme was stable at temperature below 50 °C.	The enzyme was stable till 40 °C.	The enzyme decreased by almost 20% at both 25 °C and 40 °C, while its activity was completely lost at 50 °C.
Effect of metal ions	Na^+, K^+ and Ca^{+2} activated the enzyme, while Mg^+, Cu^{+2} and EDTA strongly inhibited the enzyme. On the other hand, Ba^{+2}, Cd^{+2}, Fe^{+2} and Zn^{+2} completely inhibited the enzyme.	The enzyme was enhanced by EDTA and Zn^{2+}, but inhibited by Ba^{2+}.	Na^+, K^+, Mg^{2+}, Ca^{2+}, Co^{2+} Ba^{2+}, Ni^{2+} and Sr^{2+}, had activated the enzyme. Zn^{2+} had no effect, and Cu^{2+} and Sn^{2+} had slight inhibitory effect on the activity.	The optimal activity exhibited in 0.1 M of Na+ or K+. This enzyme was resistant to denaturing reagents (SDS and urea), reducing reagents and chelating reagents (EGTA and EDTA).	The activity increased by adding 0.5 M NaCl	Zn^{2+}, Mn^{2+}, Cu^{2+}, Mg^{2+}, Co^{2+} and NaCl were found to enhance the enzyme activity, while (Ca^{2+}, Cd^{2+}, Fe^{2+}, Hg^{2+}, Sr^{2+}, Ni^{2+}), glutathione, and metal chelators (ethylenediaminetetraacetic acid and ethylene glycol tetraacetic acid) suppressed the activity.
Molecular weight (kDa)	40	62.5	32	60	26	No available data

Comparative studies on properties of new alginate lyase from *Pseudomonas stutzeri* MSEA04 and other microorganisms.

REFERENCES

1. Badur, A. H., Jagtap, S. S., Yalamanchili, G., Lee, J. K., Zhao, H., Rao, C. V. (2015) Alginate Lyases from Alginate-Degrading *Vibrio splendidus* 12B01 are endolytic. *Appl. Environ. Microbiol. 81*, 1865–1873.
2. Beltagy, E. A., Youssef, A. S., El-Shenawy, M. A., El-Assar, S. A. (2012) Purification of kappa (k)-carrageenase from locally isolated *Cellulosimicrobium cellulans. Afri. J. Biotechnol. 11*, 11438–11446.
3. Cao, L., Xie, L., Xue, X., Tan, H., Liu, Y., Zhou, S. (2007) Purification and characterization of alginate lyase from *Streptomyces* species strain A5 isolated from banana rhizosphere. *J. Agric. Food Chem. 55,* 5113–5117.
4. El-Katatny, M. H., Hetta, A. M., Shaban, G. M., El-Komy, H. M. (2003) Improvement of cell wall degrading enzymes production by alginate encapsulated *Trichoderma* spp. *Food Technol. Biotechnol. 41,* 219–225.
5. Fu, X. T., Lin, H., Kim, S. M. (2007) Purification and characterization of a Na+/K+ dependent alginate lyase from turban shell gut *Vibrio* sp. YKW-34. *Enz. Microbiol. Technol. 41,* 828–834.
6. Gimmestad, M., Sletta, H., Ertesvåg, H., Bakkevig, K., Jain, S., Suh, S.-J., Skjåk-Bræk, G., Ellingsen, T. E., Ohman, D. E., Valla, S. (2003) The *Pseudomonas fluorescens* AlgG protein, but not its mannuronan C-5-epimerase activity, is needed for alginate polymer formation. *J. Bacteriol. 185,* 3515–3523.
7. Kim, H. S., Lee, C. G., Lee, E. Y. (2011) Alginate Lyase: Structure, Property, and Application. *Biotechnol. Bioprocess Eng. 16,* 843–851.
8. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227,* 680–685.
9. Li, J. W., Dong, S., Song, J., Li, C. B., Chen, X. L., Xie, B. B., Zhang, Y. Z. (2011) Purification and Characterization of a Bifunctional Alginate Lyase from *Pseudoalteromonas* sp SM0524. *Mar. Drugs 9*, 109–123.
10. Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res. 25,* 3389–3402.
11. Lineweaver, H., Burke, D. (1934) The determination of enzyme dissociation constants. *J. Am. Chem. Soc. 56,* 658–666.
12. Lowry, O. H., Rosbrough, N. J., Farr, A. L., Randall, R. J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem. 193,* 265–275.
13. Michaud, P., Da Costa, A., Courtois, B., Courtois, J. (2003) Polysaccharide lyases: Recent developments as biotechnological tools. *Crit. Rev. Biotechnol. 23,* 233–266.
14. Miyake, O., Hashimoto, W., Murata, K. (2003) An exotype alginate lyase in *Sphingomonas* sp. A1: overexpression in *Escherichia coli*, purification, and characterization of alginate lyase IV (A1–IV). *Protein Expr. Purif. 29,* 33–41.
15. Nakagawa, A., Ozaki, T., Chubachi, K., Hosoyama, T., Okubo, T., Iyobe, S., Suzuki, T. (1998) An effective method for isolating alginate lyase-producing *Bacillus* sp. ATB-1015 strain and purification and characterization of the lyase. *J. Appl. Microbiol. 84,* 328–335.
16. Osawa, T., Matsubara, Y., Muramatsu, T., Kimura, M., Kakuta, Y. (2005) Crystal structure of the alginate (poly alpha-L-guluronate) lyase from *Corynebacterium* sp. at 1.2 A resolution. *J. Mol. Biol. 345,* 1111–1118.
17. Park, D., Jagtap, S., Nair, S. (2014) Structure of a PL17 Family Alginate Lyase Demonstrates Functional Similarities among Exotype Depolymerases. *J. Biol. Chem. 289,* 8645–8655.
18. Park, H. H., Kam, N., Lee, E. Y., Kim, H. S. (2012) Cloning and characterization of a novel oligoalginate lyase from a newly isolated bacterium *Sphingomonas* sp. MJ-3. *Mar. Biotechnol. 14*, 189–202.
19. Peterson, E. A., Sober, H. A. (1962) Column chromatography of protein: Substituted cellulases. In: S. Colowich, N. Kapllan (eds) *Methods in Enzymology.* Vol. 5 New York. 3–27.

20. Seiderer, L. J., Newell, R. C., Cook, P. A. (1982) Quantitative significance of style enzymes from two marine mussels (Choromytilus meridionalis Krauss and Perna perna Linnaeus) in relation to diet. *Mar. Biol. Lett. 3,* 257–271.
21. Singh, R. P., Gupta, V., Kumari, P., Kumar, M., Reddy, C. R. K., Prasad, K., Jha, B. (2011) Purification and partial characterization of an extracellular alginate lyase from *Aspergillus oryzae* isolated from brown seaweed. *J. Appl. Phycol. 23,* 755–762.
22. Wang, Y., Guo, E. W., Yu, W. G., Han, F. (2013) Purification and characterization of a new alginate lyase from a marine bacterium *Vibrio* sp. *Biotechnol. Lett. 35,* 703–708.
23. Wang, Y. H., Yu, G. L., Wang, X. M., Lv, Z. H., Zhao, X., Wu, Z. H., Ji, W. S. (2006) Purification and Characterization of Alginate Lyase from Marine *Vibrio sp.* YWA. *Acta Biochim. Biophys. Sinica 38*, 633–638.
24. Wenjun, H., Jingyan, G., Yuanyuan, C., Huihui, L., Yuezhong, L., Fuchuan, L. (2015) A Novel Alginate Lyase (Aly5) from a Polysaccharide-Degrading Marine Bacterium *Flammeovirga* sp. MY04: Effects of Module Truncation to the Biochemical Characteristics, Alginate-Degradation Patterns, and Oligosaccharide-Yielding Properties. *Appl. Environ. Microb*.; doi:10.1128/AEM.03022-15.
25. Yamasaki, M., Moriwaki, S., Miyake, O., Hashimoto, W., Murata, K., Mikami, B. (2004) Structure and Function of a Hypothetical *Pseudomonas aeruginosa* Protein PA1167 Classified into Family PL-7 A NOVEL ALGINATE LYASE WITH A β-SANDWICH FOLD. *J. Biol. Chem. 279*, 31863–31872.
26. Zhu, Y., Wu, L., Chen, Y., Ni, H., Xiao, A., Cai, H. (2015) Characterization of an extracellular bio-functional alginate lyase from marine *Microbulbifer* sp. ALW1 and antioxidant activity of enzymatic hydrolysates. *Microbiol. Res. 182,* 49–58.