

Purification, Characterization and Sequencing of Alginate Lyase from *Martelella* sp. strain MAK4

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ABSTRACT

Bacterial isolate MAK4 was isolated from brown algae collected from the coastal area of the Red sea, Hurghada, Egypt and confirmed for alginate lyase production by halo formation around the colonies after flooding with cetylpyridinium chloride or Gram's iodine. Isolate MAK4 was identified according to morphological, biochemical and phylogenetic identification through 16s rRNA. The bacterial isolate was tentatively identified as *Martelella* sp. strain MAK4. It is aerobic, mesophilic, Gram-ve, non-spore forming nonmotile, rod-shaped organism and produces catalase and oxidase. PCR was performed for alginate lyase gene using two pairs of degenerate primers. The alginate lyase enzyme was isolated and purified from the culture medium by ammonium sulfate precipitation, Sephadex G-100 and DEAE-Cellulose chromatography. The isolated enzyme has a specific activity of 62.9 u/mg, 6.15 purification folds and has a molecular weight of 35 kDa. The alginate lyase enzyme showed highest activity at 37 °C and at pH 7. The enzyme was stable over the pH range 6 to 9 and retained 80% of its activity after incubation at 40 °C for 90 min. The enzyme was active in the absence of metal ions, but the activity was enhanced by the addition of NaCl, KCl and Ca²⁺. The activity was lost with the addition of EDTA. The enzyme activity was strongly decreased in the presence of Cu²⁺, Zn²⁺, Co²⁺, Hg²⁺ and Mn²⁺. This novel bifunctional alginate lyase has the potential to be used in the eradication of resistant bacterial biofilm in clinical samples and production of alginate oligosaccharides in industry.

INTRODUCTION

Alginates are anionic linear polysaccharides consists of α -Lguluronic acid (G) and β -D manuronic acid (M) which are linked together through 1,4-Oglycosidic linkage. Alginates are composed of homopolymeric region of polyguluronic acid called poly G, plymanuronic acid region called poly M and heteropolymeric region of both guluronic and mannuronic acid region called poly MG [1,2]. Alginates are widely found in nature in brown seaweeds (*Phaeophyceae*). Alginate is also produced by few bacterial species as *Azotobacter* and many species of *Pseudomonas* [3]. alginate obtained from brown algae are eventually not acetylated in contrast with alginate produced by bacteria are O-acetylated in the 2 and/or 3 position of D-mannuronate [4]. The acetylated form of alginate produced by bacteria as in case of *Pseudomonas aeruginosa* and *Azotobacter vinelandii* as the main part of bacterial biofilm [5]. the biofilm in mucoid bacteria as *Pseudomonas aeruginosa* which cause chronic lung infection in patient act as virulence factor which facilitate the adherence of bacteria to the target cells due to it's

alginate content and make bacterial cells resistant to be eradicated with antibiotics treatment as it degrade the alginate biofilm preventing bacterial cells from adherence and facilitate the diffusion of the aminoglycoside antibiotic into the bacterial cells. Therefore, alginate lyase enzyme could be helpful when combined with antibiotics in treatment of chronic lung infection in cystic fibrosis patient [6].

The alginate lyase (algL) catalyze alginate degradation through β -elimination mechanism. In which glycosidic bond between alginate monomers is broken and a double bond at C4-C5 carbons in the sugar ring is formed. This reaction produces oligosaccharides with non-reducing terminals like 4-deoxy-L-erythro-hex-4-enopyranosyluronic acid [7–10]. AlgLs are classified according to substrate specificity to poly G- specific lyases which degrade G blocks [11,12], poly M- specific lyases which degrade M blocks [13] and poly MG-specific lyases (called bifunctional lyase) which degrade both M and G blocks [14]. AlgLs are produced by both alginate synthesizing bacteria and non-alginate synthesizing bacteria. it has a role in both

biosynthesis and biodegradation of alginate. In biosynthesis it has a role to control the length of alginate polymer and polymerization reaction optimization [15]. alginate lyase have a role in spreading bacteria as in case of *Pseudomonas aeruginosa* by degrading alginate biofilm and in case of *Azotobacter vinelandii* evolved in cyst germination by degrading alginate protecting cyst coat [5]. Considering the role of algL as an adjuvant therapeutic agent in treatment of resistant infections caused by *Pseudomonas aeruginosa*. In this study a search for a novel alginate lyase and its encoding gene from marine source and optimize the conditions for growth and alginate lyase production in order to increase the yield of algL from *Martelella* sp. MAK4.

MATERIALS AND METHODS

Isolation, purification and preservation of bacterial isolate

Samples

Bacterial isolates able to produce alginate lyase enzyme were isolated from brown algae samples which collected from Hurghada coast, Red sea Egypt. the algal sample cut into small pieces and added to 10 mL of sterilized saline solution. shake and filtered. The filtrate then cultured by two methods:

Direct plating method

The filtrate is serially diluted and directly spread on plates containing mineral salt medium of the following composition (g/l) sodium alginate, 10; (NH₄)₂SO₄, 5; K₂HPO₄, 2; FeSO₄·7H₂O, 0.01; MgSO₄·7H₂O, 1; NaCl, 30; agar 15, and final pH was adjusted to 7. Plates were incubated at 37 °C for 96 h [16].

Enrichment method

One mL of the filtrate is inoculated in 25 mL of liquid medium of previously mentioned composition at 37 °C/24 hr on an orbital shaker (130 r/min). One mL is serially diluted and then spread on plates of the mineral salt medium and incubated at 37 °C / 96 h.

Plate screening for alginate degrading bacteria

In order to visualize and detect alginate degrading bacteria. Plates were flooded with Gram's iodine [17] or 10% of cetylpyridinium chloride. Clear zone or white halo around the colonies indicate alginate degrading activity due to production of alginate lyase. (Gram's iodine forms bluish black complex with alginate but not with hydrolyzed alginate)

Purification of the isolate

After screening for alginate degrading activity. The colonies with known alginolytic activity were isolated using standard dilution plating method. The isolate is kept in slants containing mineral salt medium with 1% alginate and stored at 4°C.

Election of most potent bacterial isolate

Bacterial isolates with alginate degrading activity from screening method were cultured in agar plates containing different alginate concentration 0.2,0.4,0.6 and 0.8 % (w/v). The selection depends on fast growing (24 h) in minimal alginate concentration in the media. so fast-growing bacterial isolate in medium with 0.4 % alginate was selected for further studies. This bacterial isolate was named as MAK4.

Storage

The most potent bacterial isolates were purified and confirmed for alginate degradation were stored in LB broth containing g/l (peptone 10, sodium chloride 10, yeast extract 5g) and 15% glycerol and stored at -80 °C [18].

Characterization of bacterial isolate

Phenotypic and biochemical characterization

The bacterial isolate were grown in plate containing mineral salt agar with 1 % alginate at 37°C. Gram stain procedure and the following biochemical tests were carried out; oxidase ,catalase, hydrogen sulfide and indol production, hydrolysis of starch ,gelatin ,phenylalanine and casein , Amino acid utilization and other biochemical tests. biochemical tests were carried out by BioMerieux VITEK 2 identification compact system [19].

Phylogenetic characterization of the bacterial isolate

Genomic DNA extraction

Genomic DNA was isolated from bacteria using cell lysis method [20] from the isolate and obtained using DNA extraction kits (Viogene, Taiwan).

Agarose gel electrophoresis

Agarose gel electrophoresis of genomic DNA was performed using horizontal slab gel electrophoresis. 0.8 g of agarose was dissolved in 100 mL of TBE electrophoresis buffer by heating till the agarose completely dissolved. The agarose suspension was cooled to 45°C and ethidium bromide (EtBr) was added (0.5 µg/mL). The agarose solution was poured into a gel casting tray left to cool for 30 min. the gel solidified. 10 µl of genomic DNA sample the mixed with 6 X gel loading dye and loaded into the well. The electrophoresis is carried out at 80 V. after electrophoresis, the DNA was visualized by UV trans-illuminator [21].

Amplification of 16S rDNA by PCR

16S rRNA gene from chromosomal DNA of strain MAK4 was amplified using (Biometra thermo cycler, Germany) and sequenced using universal primers forward 27F (AGAGTTTGATCMTGGCTCAG) and reverse 1525R (AAGGAGGTGATCCAGCC). Amplification is carried out by polymerase chain reaction (PCR) with the following cycle program : Initial denaturation at 95°C (5 min) followed by 35 cycles of denaturation at 95°C (30 s), annealing at 55°C (1 min) and extension at 72°C (1 min) followed by a final cycle of extension at 72°C (10 min). PCR products were shipped for sequencing to (Macrogen, Korea). The result of sequencing was compared with the database available in NCBI GenBank using BlastN program to determine the identity of the isolated strain [22]. The phylogenetic tree of the isolated strain was constructed using the MegAlign software.

Alginate lyase gene isolation and sequencing

Based on the sequences of alginate lyase gene (NCBI Reference Sequence: NC_002516.2) it is used as a template to design forward and reverse Primers for alginate lyase gene (Table 1)

Table 1. Primers used for amplification of alginate lyase gene.

GENE	PRIMER SEQUENCE
ALG-K1	5'-GGG GAT CCA TGA ACT CAA TAC CTC GCC GAG GTT -3' (33MER)
ALG-K2	5'-GGG GAT CCT TAG CGG TTA TCC CTG ACC TCT GAC -3' (33MER)
ALG2-F	5'- GGG GAT CCA TGA AAC AAT TCT CCG GGC TCT GGC -3' (33MER)
ALG2-F2	5'- GGG GAT CCT TCG CCT GGT TTA TCT GGC GTG AC -3' (32 MER)
ALG2-R	5'-GGG GAT CCT CAA ATG CCG CTT TCC GGG AGC GG -3' (32 MER)
ALG2-R3	5' -GGG GAT CCG TCG TAA TGG TCA TGC TCG CCG CC-3' (32 MER)

PCR amplification is carried out for the extracted genomic DNA with the following cycle program. initial denaturation at 95°C (5 min) followed by 35 cycles of denaturation at 95°C for 30 s. annealing at 55 °C for 1 min and extension at 72°C for 1 min and followed by final cycle of extension at 72°C for 10 min [23]. PCR product were sent to Macrogen, Korea to get the sequence for alginate lyase gene. The resulting 16s rRNA partial sequence (GenBank accession number pending) is shown in **Supplementary Figure S1**.

Production of alginate lyase

Seed culture

the purified bacterial isolate MAK4 was inoculated in 50 mL of the screening medium containing (g/l) sodium alginate, 5; (NH₄)₂SO₄, 5; K₂HPO₄, 2; FeSO₄·7H₂O, 0.01; MgSO₄·7H₂O, 1; NaCl, 30; at pH 7. and shaken at 180-200 rpm at 37°C [24].

Batch fermentation to produce alginate lyase enzyme

About 5 mL of the 24-hour old culture broth were used as initial inoculum for batch fermentation to produce alginate-degrading enzyme and cultivated for 48 h under the same conditions as the inoculum preparation. The culture broth was centrifuged at 10000 rpm for 20 min under cooling condition and the resultant supernatants were considered as a source of crude enzyme.

Isolation and purification of alginate lyase

The crude supernatant from the previous step was precipitated by slowly adding solid ammonium sulfate with continuous stirring at cold temperature 4 °C until 80% saturation. the precipitate obtained was isolated by centrifugation 12000 rpm at 4 °C for 30 min. the precipitate obtained was dissolved in equal volume of 50 mM phosphate buffer (PB) pH 7 containing 0.1 M NaCl. then loaded to PEAE-Cellulose column (2.5 – 40 cm) previously equilibrated by 10 mM PB pH 7 for further purification by anion exchange chromatography. the enzyme was then eluted with linear gradient of NaCl concentration (0.2-0.3 M) in total volume 500 mL with flow rate 30 mL/h. five mL fractions are collected and tested for protein and enzyme activity.

The active fractions are collected together. the collected fractions were further purified by Gel filtration through Sephadex G-100 column (2.5-40cm) previously washed with 10 mM PB. The enzyme was then eluted with PB pH 7 with total volume 500 mL with flow rate 30 mL/hr. five mL fractions are collected and tested for protein and enzyme activity. The active fractions are then pooled and concentrated and used as purified enzyme [25]. The alginate lyase activity was assayed using alginate as a substrate by measuring the increase in absorbance at 235 nm. that increase is resulting from double bond formation between C-4 and C-5 at the non-reducing terminal through β-elimination reaction. Protein assay is carried out by measuring absorbance at 280 nm using standard protein (bovine serum albumin).

Protein and enzyme assay

Protein standard curve

In order to construct protein standard curve Bovine Serum Albumin (BSA) is used as standard protein. A stock solution of 1 mg/mL was used to prepare series of concentrations 0, 2, 4, 6, 8, 10 and 12 µg /mL. sample volume 100 µl. measure the absorbance at 595 nm. The absorbance then plotted against concentration. Protein is assayed according to Bradford by dye binding assay [26] which based on the binding of the protein molecules to Coomassie dye under acidic conditions results in a color change from brown to blue. 100 µl was used as a sample

and the absorbance is measured at 595 nm against blank of the same volume prepared from sample buffer.

Alginate lyase Assay

Assays carried out for biochemical characterization using 0.1 mL of purified enzyme supernatant then added to 1 mL of 0.3 % alginate in 50 mM phosphate buffer pH 7 and kept at 37°C for 20 min. quantitative assay for alginate degrading enzyme were carried out by measuring the increase in absorbance at 235 nm because of the release of 4-deoxy-L-erythro-hex-4-ene pyranosyl uronate at the non-reducing terminal of the produced oligomers. Enzyme unit (U) of enzyme activity is the amount of enzyme that cause an increase of 0.01 of optical density units for min. Quantitative measurement of lyase activity is carried out by thiobarbituric acid method The enzyme specific activity is estimated by one unit of enzyme is the amount of enzyme needed to produce 1 mol of β-formyl- pyruvic acid per minute [27].

SDS-PAGE analysis of alginate lyase

SDS-PAGE is carried out either for detection the molecular mass of the purified enzyme and the purity of the purified enzyme protein. To perform SDS-PAGE we do it according to the method of Laemmli [28,29] with 12.5% running gel and 5% stacking gel, under denaturation conditions (3 min, 90 °C) in the presence of SDS and β-mercaptoethanol in Tris-HCl buffer. The gel was run at a constant 20 mA for 1 h at 25°C and dyed with 0.25% Coomassie Blue R-250 in 50% methanol and 10% acetic acid for 1 h, followed by destaining in 25% methanol and 7% acetic acid overnight until the gel background was clear.

Optimization of the culture conditions for growth and alginate lyase production

Determination of optimal temperature for the growth and alginate lyase production

The alginate lyase-excreting bacteria were cultivated in the liquid screening medium at series temperatures, from 20 °C to 45 °C with an interval of 5 °C, and 180 rpm for 4 days. Timing sampling was done during the cultivation. The bacterial density in each sample was measured at a wavelength of 600 nm (OD₆₀₀) to draw a growth curve. Each sample was centrifuged at 12,000× g, 4 °C for 12 min, and the supernatant was collected to measure the alginate lyase activity at its optimal temperature. The optimal temperature for alginate lyase activity was determined by measuring the enzyme activity from 10 °C to 60 °C with an interval of 10 °C [30].

Determination of optimum alginate concentration for growth and lyase activity

The bacterial isolates were grown in liquid screening medium containing alginate in concentration range from 0.2 to 0.8 % (w/v) at 37 °C and 180 rpm for 18 h. During incubation (every 3 h) bacterial density were determined at 600 nm and result used to draw growth curve. Alginate lyase activity is detected from the media by colorimetric method.

Determination of optimum NaCl concentration for growth and lyase activity

The bacterial isolates were grown in the liquid screening medium containing different concentration of NaCl from 0.1 to 0.5 M at 37 °C and 180 rpm for 18 h. bacterial density was detected by measuring optical density at 600 nm every 3 h. and result used to draw growth curve. alginate lyase activity is detected from the media by colorimetric method.

Characterization of purified alginate lyase

Optimum temperature for alginate lyase activity

To detect optimum temperature for alginate lyase. 10 µg of purified enzyme was incubated in 20 mM Tris-HCl buffer (pH 7) with 0.2-% (w/v) alginate solution for 30 min at detected temperature from 20-60°C. with 0.2 % alginate solution and a total reaction mixture of 2 mL [31].

Thermal stability for alginate lyase enzyme

Thermal stability of the lyase was detected by incubating the purified enzyme 10 µg at 40, 50, 60 and 70 °C for different periods 15, 30, 60 and 90 min. Then the solution was cooled for 10 min and the residual activity was assayed at 37 °C and at pH 7. The relative activity is the percentage of detected activity and the maximum alginate lyase activity.

Optimum pH for alginate lyase enzyme

The optimum pH was determined by incubating the purified enzyme for 30 min. at 37 °C in various pH using different buffers (50 mM citrate buffer pH 3-6.5, 50 mM borate buffer pH 6.5-8.5, 50 mM tris-HCl buffer pH 8-9.5 and 50 mM Gly-NaOH buffer pH 9-11) and 0.2 alginate solution. The total reaction mixture was 2 mL.

pH stability for alginate lyase enzyme

The purified enzyme is incubated for 1 h. at 4°C in different pH using the previously mentioned buffers. The residual activity is then detected after adding 0.2 alginate solution in 50 mM phosphate buffer pH 7 at 37 °C for 30 min.

Effect of metal ions and EDTA on alginate lyase activity

To detect the effect of metal ion on the purified alginate lyase activity, 2 µg of the purified enzyme was incubated with 2-mM salt solution (NaCl, KCl, MgCl₂, CaCl₂, BaCl₂, ZnCl₂, CoCl₂, CuCl₂, FeCl₃, Hg²⁺, Mn²⁺ and EDTA) in 0.5 mL of 20-mM Tris-HCl buffer (pH 7) for 10 min and then incubated with 0.5 mL of 0.4-% (w/v) sodium alginate substrate at 37 °C for 10 min. alginate lyase activity assayed. the lyase activity in absence of any metal ion or chemical considered as 100 %.

Substrate specificity of alginate lyase

To detect substrate specificity for alginate lyase. 10-µg of purified enzyme and 0.2-% (w/v) of different substrates (sodium alginate, poly-MG, poly-M and poly-G) at the final concentration in 20-mM Tris HCl buffer (pH 7) were incubated in 1-mL reaction mixture for 10 min at 37.5 °C, and then, the reaction mixture was boiled for 10 min to inactivate the enzyme. The enzyme activity was then determined.

RESULTS

Bacterial isolate

Bacterial isolates were grown in mineral salt medium containing alginate as sole carbon source as selective screening medium with the following composition (g/l) sodium alginate, 10 ; (NH₄)₂SO₄, 5; K₂HPO₄, 2; FeSO₄·7H₂O, 0.01; MgSO₄·7H₂O, 1; NaCl, 30; agar, 15 and final pH was adjusted to 7.5. plates are incubated at 37 °C. isolates with alginate degrading activity are recognized by formation of clear zone around colonies (white halos) because of degradation of alginate after flooding the medium with Gram's iodine.

Three isolates having alginate degrading activity among them we selected the most potent isolate according to the diameter of the clear zone, rapid growth after 48 h and growth in minimal concentration of alginate in the culture medium and we named this isolate MAK4. And reconfirmed for alginate lyase production. Isolate MAKE4 were used for further evaluation for its potential in mass production of alginate lyase and optimization the condition for growth and alginate lyase production.

Identification of the isolate MAK4

Isolate MAK4 Gram -ve, short rods, grow aerobically. non motile. The colonies are almost circular, cream colored, opaque with no pigment. with the following biochemical characters (Table 2) as carried out by BioMerieux VITEK 2.

Table 2. Morphological, physiological and biochemical characteristics for strain MAK4.

Test	Results
shape	Short rods
Gram stain	-
motility	-
Aerobic growth	+
Spore forming	-
Colony color	cream
catalase	+
peroxidase	+
Nitrate reduction	+
amylase	-
Beta-galactosidase	+
Alpha glucosidase	-
Ala-phe-proarylamidase	-
cyclodextrin	-
L-aspartate arylamidase	-
Mannitol	+
D-mannose	+
D-malate	-
N-acetyl-D-glycosamine	+
Leucin-arylamidase	-
L-prolin arylamidase	-
Alphagalactosidase	-
L-pyrrolydonyl-arylamidase	-
Casein hydrolysis	+
Starch hydrolysis	+
Tyrosine arylamidase	+
D-Sorbitol	+
Urease	+
D-Galactose	-
POLymixin B resistance	-
Lactose	-
Novobiocin resistance	-
Growth in 6.5 % NaCl	+
Optochin resistance	+
Arginine dihydrolase 2	-
D-trehalose	+
Salicin	+
Saccharose/sucrose	+
ALPHA-mannosidase	-
Lactate Alkalinization	-
Phosphatase	+
Methyl-B-D-glucopyranoside	+
D-Raffinose	-
BETA-glucuronidase	-
Bacitracin resistance	+

Purification of alginate lyase

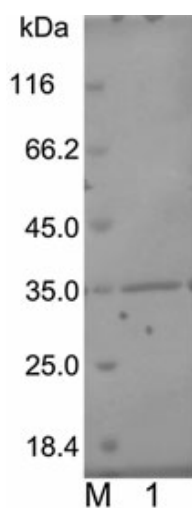
The purification of alginate lyase from the crude supernatant by ammonium sulfate precipitation and DEAE-Cellulose chromatography (Data published elsewhere) provide lyase with 5 fold purification and 16% yield and the final step of Sephadex G-100 column chromatography provide lyase with a specific activity of 63 unit/µg protein with 6 fold of purification (Table 3).

Table 3. Summary of the purification of alginate lyase from strain MAK4.

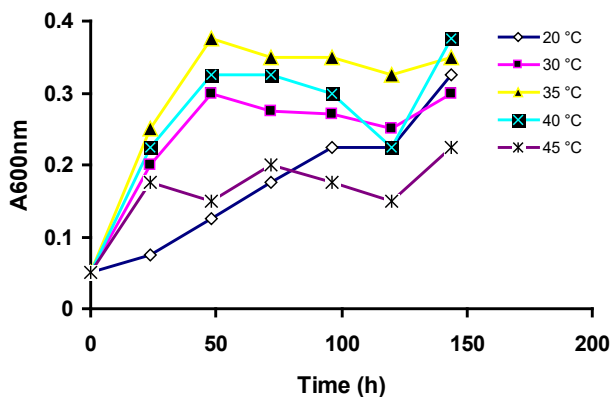
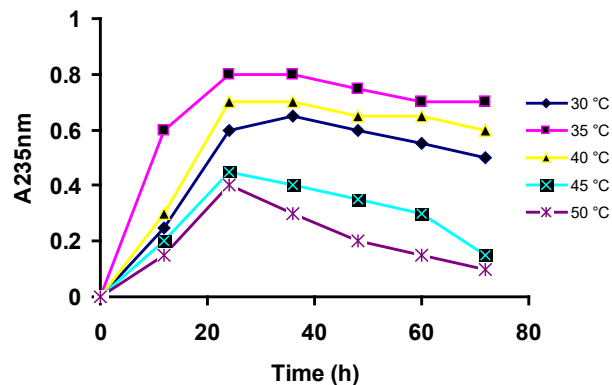
Fraction	Yield%	Purification fold	Specific activity (unit/ μ g of protein)	Total activity (unit)	Total protein (μ g)
Supernatant.	100	1	9.9	2130	215
Ammonium sulphate 25.5	3.9	3.9	38.30	544	14.2
80%					
DEAE-Cellulose	5.10	5.1	50.10	336	6.7
Sephadex G-100	8.3	6.15	62.9	176	2.8

SDS-PAGE and Zymography analysis

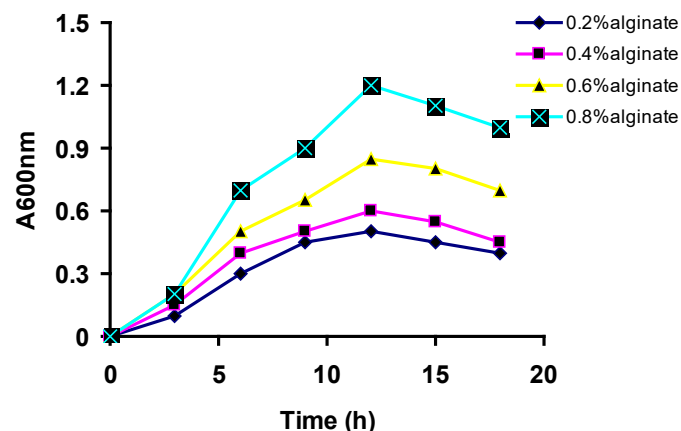
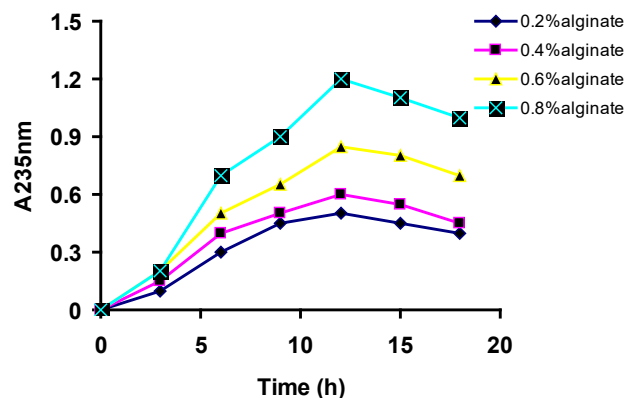
The SDS-PAGE analysis was carried out using 12% polyacrylamide gel to determine the molecular weight of the alginate lyase gene, which was estimated to be 35 kDa (Fig. 1), which was approximate to the calculated size of the enzyme via gene sequence analysis and composition (Supplementary Figs. S2 and S3 and Table S1).

**Fig. 1.** SDS-PAGE analysis of alginate lyase from isolate MAK4. Line M and 1 are markers and sample, respectively.**Optimum conditions for growth and production of alginate lyase**

Strain MAK4 show optimum growth between 35-40 °C after 48 h of incubation (Fig. 2) and lyase production reach maximum concentration after 24 h in between 35 and 40 °C as well (Fig. 3).

**Fig. 2.** Optimum growth temperature for isolate MAK4.**Fig. 3.** Optimum temperature for alginate lyase production.

The growth rate of strain MAK4 and lyase production increased with the increase in concentration of substrate. This effect is noticed when using different alginate concentration from 0.2% to 0.8% alginate with the optimum growth and alginate lyase production both occurring at 0.8 % alginate (w/v%) (Figs. 4 and 5).

**Fig. 4.** Optimum alginate concentration for growth of isolate MAK4.**Fig. 5.** Optimum alginate concentration for alginate lyase production.

Using various concentration of NaCl from 0.1-0.5 % indicated that the concentrations of 0.2 and 0.3 M of NaCl improved the growth of the isolate MAK4 and alginate lyase production as compared with other concentrations (Figs. 6 and 7).

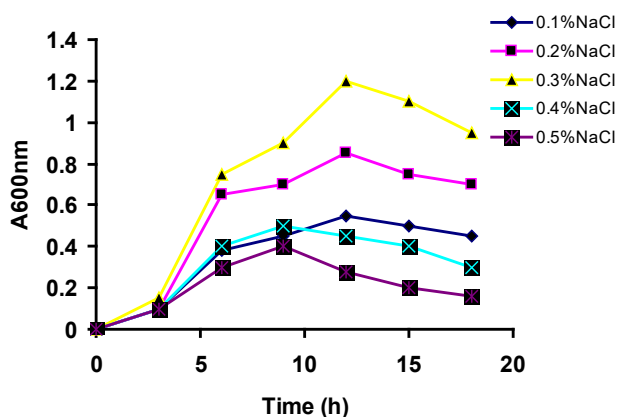


Fig. 6. The effect of NaCl concentration on the growth of strain MAK4.

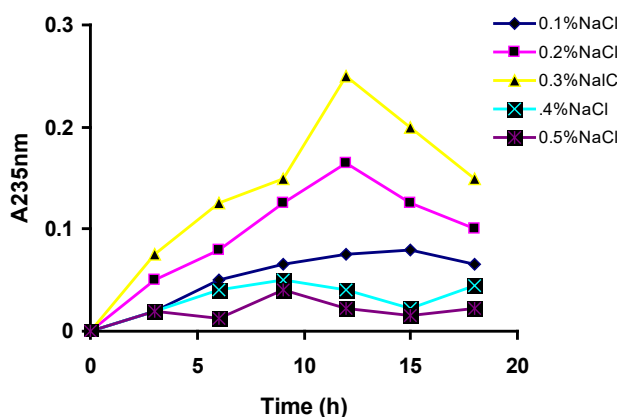


Fig. 7. The effect of NaCl concentration on alginate lyase activity.

Characterization of alginate lyase extracted from *Martelella* sp. strain MAK4

The alginate lyase optimal temperature were determined using various temperatures from 20-55 °C. The optimal temperature was at 37 °C (Fig. 8).

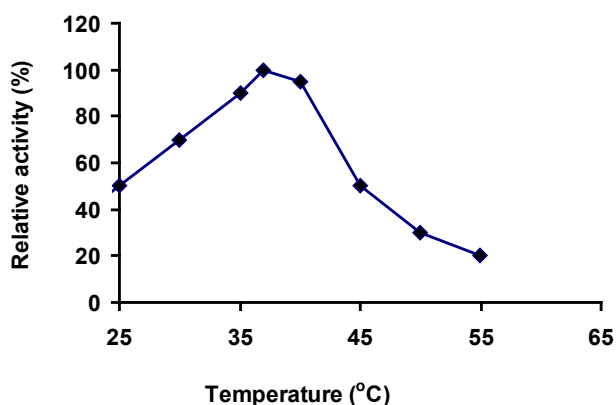


Fig. 8. The optimal temperature of alginate lyase activity.

Alginate lyase was still active at 40 °C for 90 min and at 50 °C for almost 1 h. The alginase activity was significantly declined at 60 and 70°C (Fig. 9).

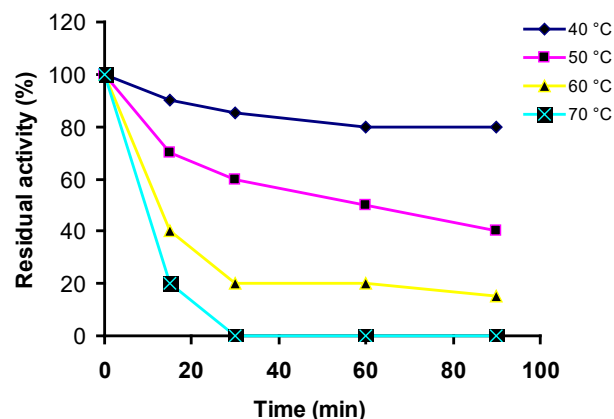


Fig. 9. Thermal stability of alginate lyase.

Alginate lyase optimal pH was 7 and the enzyme was relatively stable in the pH range from 6 to 9 and still retained about 80 to 70 % of alginase activity, respectively, at these pHs (Fig. 10).

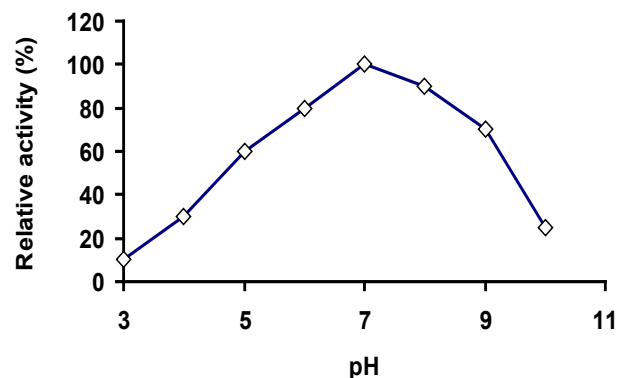


Fig. 10. Optimal pH of alginate lyase.

Divalent cations such as Zn^{2+} , Mn^{2+} , Cu^{2+} , Mg^{2+} , Ba^{2+} , Co^{2+} , Hg^{2+} and Fe^{2+} , inhibited alginate lyase to different degrees while EDTA completely inhibited alginate lyase activity. Ions such as K^+ , Na^+ and Ca^{2+} increased the alginase activity (Fig. 11).

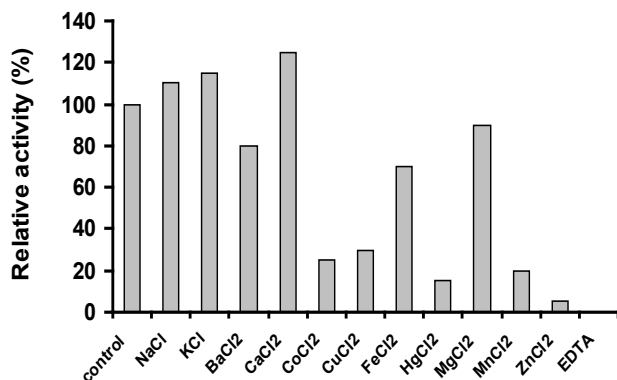


Fig. 11. The effect of metal ions and EDTA on alginate activity.

The purified alginate lyase was most active against poly-MG and show lower activity towards poly-M, while the lowest activity was towards poly-G (Fig. 12).

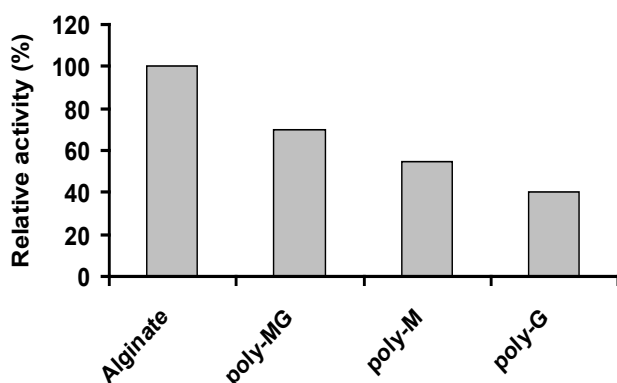


Fig. 12. Alginate lyase substrate specificity.

DISCUSSION

Marteella sp. Strain MAK4 was isolated from brown algae collected from coastal area in Egypt (Hurghada and Baltim beaches). The strain MAK4 was identified according to the methods in Bergeys manual of systemic Bacteriology and 16S rRNA Gene was sequenced and deposited to the GenBank. the *Marteella* sp. strain MAKE4 was aerobic, mesophilic, Gram negative, non-spore forming, non-motile, short rods shape organism. catalase and oxidase positive and hydrolyzing casein and starch. In this research successfully isolated and sequenced alginate lyase encoding gene after genomic DNA extraction from strain MAK4 using two primers forward Alg-K1 5'-GGG GAT CCA TGA ACT CAA TAC CTC GCC GAG GTT -3' and reverse Alg-K25'-GGG GAT CCT TAG CGG TTA TCC CTG ACC TCT GAC -3'. PCR amplification was performed, and PCR products was then analyzed in 1%(w/v) agarose gel, excised from the gel and purified. SDS-PAGE analysis was performed using 12% polyacrylamide gel to determine the purity and molecular weight of alginate lyase enzyme.

The molecular weight of *Marteella* sp. Strain MAK4 alginate lyase was 35 kDa. It was the same molecular weight as AlgC-PL7 produced by *Cobetia* sp. NAP1 [24] and higher than Alm isolated from *Agarivorans* sp. JAM-Alm. Of molecular weight 31kDa [32], and AlyV5 isolated from *Vibrio* sp. QY105

with molecular weight 33 kDa [30] but smaller than Alg7D isolated from *Saccharophagus degradans* with 63.2 kDa [12].

The alginate lyase isolated from *Marteella* sp. strain MAK4 has optimal temperature 37°C. The same as alginate lyase isolated from *Streptomyces* sp. A5 [33], lower than 40 °C recorded for The KJ-2 alginate lyase from *Stenotrophomas maltophilia* KJ-2 [1] and AlySY08 from *Vibrio* sp. Aly08 [34] and 50 °C for Cel32 from *Cellulophaga* sp. NJ-1 [35] and Oal17 from *Shewanella* sp.Kz7 [36] and higher than 30 °C for Alm isolated from *Agarivorans* sp. JAM-Alm [32].

The optimal pH for alginate lyase isolated from *Marteella* sp. Strain MAK4 is 7. The same as alginate lyase purified from marine *Microbulbifer* sp. ALW1. [16] and alginate lyase isolated from *Vibrio* sp. YKW-34 [37] and almost the same as pH 7.5-8 as in alginate lyase isolated from *Alteromonas* sp. Strain No. 272 [38] and higher than pH 6.5 reported for alginate lyase isolated from *Aspergillus oryzae* [39].

In conclusion, the *Marteella* sp. Strain MAK4 alginate lyase relatively stable after 90 minutes in 40°C. and about 50% loss in activity after 60 minutes in 50 °C. while the enzyme lost about 80 % of activity after 30 minutes at 60 °C and after 15 minutes at 70 °C. complete loss of activity occurred after 30 minutes at 70 °C. Metal ions have different effect on The activity of MAK4 alginate lyase. The activity was enhanced with K⁺, Na⁺, and Ca²⁺. in the other hand the activity is strongly inhibited by Cu²⁺, Zn²⁺, Co²⁺, Hg²⁺ and Mn²⁺. While the activity was lost completely with EDTA. the enzyme was inhibited in a lower extent with Ba²⁺, Fe²⁺ and Mg²⁺.

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