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Purification, characterization and sequence analysis of Alginate Lyase Gene Bacterium

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Bacterial isolate MAK4 were isolated from brown algae collected from coastal area of Red sea, Hurghada, Egypt. And confirmed for alginate lyase production by halo formation around the colonies after flooding with cetylpyridenium chloride or Gram's iodine. The isolate MAK4 were identified according to morphological, biochemical and phylogenetic identification through 16s rRNA. The 16s rRNA gene was sequenced and deposited to Gene Bank. The bacterial isolate was identified as *Martelella sp*. Strain was named MAK4. It is aerobic, mesophilic, Gram –ve, none spore forming none motile, rod-shaped organism and produces catalase and oxidase. PCR was performed for alginate lyase gene using two pairs of degenerative primers. The alginate lyase gene was sequenced and also deposited to the Gene Bank. The alginate lyase enzyme were isolated and purified from the culture medium by ammonium sulfate precipitation, sephadex G-100 and DEAE-Cellulose chromatography. The isolated enzyme has specific activity 62.9 u/mg, 6.15 purification folds and has 35 kDa molecular weight. The alginate lyase enzyme showed highest activity at 37 °C and pH 7. The enzyme was stable over pH range 6-9 and retained 80% of activity after incubation at 40 °C for 90 minutes. The enzyme was active in absence of metal ions but the activity was enhanced by addition of NaCl, KCl and Ca+2. And the activity was lost with addition of EDTA. The enzyme activity was strongly decreased with Cu+2, Zn+2, Co+2, Hg+2 and Mn+2 but the enzyme activity is slightly affected with Mg+2, Fe+2 and Ba+2. Novel bifunctional alginate lyase could be used in eradication of resistant bacterial biofilm in clinical samples and production of alginate oligosaccharides in industry.

Keywords: Alginate, Alginate lyase, *Martelella sp.*

INTRODUCTION

Alginates are an anionic linear polysaccharides consists of α-Lguluronic acid (G) and β-D manuronic acid (M) which are linked together through1,4-Oglycosidic linkage. so alginates 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.(Lee and Mooney 2012) (Pawar and Edgar 2012). Alginates are widely found in nature in brown seaweeds (Phaeophyceae). Alginate were also produced by few bacterial species as Azotobacter and many species of Pseudomonas. (Kam et al. 2011). 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. (Kawamoto et al .2006). The acetylated form of alginate produced by bacteria as in case of Pseudomonas aeruginosa and Azotobacter vinelandii as the main part of bacterial biofilm (Gimmestad et al. 2009). 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. There for alginate lyase enzyme could be helpful when combined with antibiotics in treatment of chronic lung infection in cystic fibrosis patient (Islan et al. 2013). . 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 produce oligosaccharides with non-reducing terminals like 4-deoxy-L-erythro-hex-4-enopyranosyluronic acid .(Zhang et al. 2004) (Dong et al. 2014)(Parket al. 2014) (Mikami et al. 2012). algLs are classified according to substrate specificity to poly G- specific lyases which degrade G blocks (Hu et al. 2006) (Kim, et al., 2009), poly M- specific lyases which degrade M blocks (Sawabe et al. 1997)and poly MG-specific lyases (called bifunctional lyase) which degrade both M and G blocks (Yamasaki et al. 2004). AlgLs are producd 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 (Russell and Gacesa., 1988). 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 (Gimmestad et al. 2009). 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 it's 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

Bacterial isolates 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:

Culture

The filtrate is serially diluted and directly spread on plates containing mineral salt medium of 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 .plates are incubated at 37 °C for 96 hrs.(Zhu et al., 2015)

Enrichment culture

One ml of the filtrate is inoculated in 25 ml of liquid medium of previously mentioned composition at 37 °C/24 hr .on 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 hr.

Screening for alginate degrading bacteria:

In order to visualize and detect alginate degrading bacteria. Plates were flooded with Gram's iodine(Sawant et al. 2015**)** or 10% of cetylpyridenium 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 are kept in slants containing mineral salt medium with 1% alginate and stored at 4°C.

Determination of alginate activity

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 hr) in minimal alginate concentration in the media.

sofast growing bacterial isolate in medium with 0.4 % alginate was selected for further studies. This bacterial isolate was named as MAK4.

Characterization of bacterial isolate:

Biochemical test

The bacterial isolate were grown in plate containing mineral salt agar with 1 % Alginate at 37°C. Gram stain procedure (Barile., 2012)and the following biochemical tests are 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.(Ling et al.2001)**.**

Genomic DNA extraction and sequencing of alginate lyase gene:

Genomic DNA was isolated from bacteria using cell lysis method (Sambrook and Russell, 2006) 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-1). The agarose solution was poured into a gel casting try .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.(Dou et al.,2013)

Construction of plasmids 27F and 1525R

16S rRNA gene from chromosomal DNA of strain MAK4 was amplified using (Biometra thermo cycler, Germany) and sequenced usinguniversal 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 determin the identity of the isolated strain.(Yang et al.,2016)

The phylogenetic tree of the isolated strain was constructed using the MegAlign software.

Production of alginate lyase enzyme

Seed culture

The purified bacterial isolate MAK4 was inoculated in 50 ml of the screening medium containing (g/l) sodium alginate, 5 ; $(NH_4)_2SO_4$,5; K_2HPO_4, 2; $FeSO_4 \cdot 7H_2O$, 0.01; $MgSO_4 \cdot 7H_2O$, 1; NaCl, 30; at pH 7. and shaken at 180-200 rpm at 37°C.(Yagi et al.2016)

Batch fermentation to produce alginate lyase enzyme:

Old culture broth (5ml) 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 – 40cm) 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 filteration 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. (Chen et al. 2018).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 reucing terminal through β-elimination reaction. Protein assay is carried out by measuring absorbance at 280 nm using standard protein (bovin serum albumin).

Protein and enzyme assay.

Protein 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.

Detection of protein concentration

Protein is assayed according to Bradford by dye binding assay (Huang et al.2019) 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.

Bradford reagent preparation:

Dissolve 50 mg of Coomassie Brilliant Blue G-250 in 50 ml of methanol and add 100 ml 85% (w/v) phosphoricacid (H_3PO_4). Add the acid solution mixture slowly into 850 ml of H_2O (final volume 1 liter) and let the dye dissolve completely. Filter to remove the precipitates just before use. Store in a dark bottle at 4 °C.

Alginate Lyase Assays.

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 (Zhu et al. 2016).

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 (OD_{600}) 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.(Wang et al.,2017)

Determination of Optimum alginate concentration for growth and alginate lyase activity

The bacterial isolate 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 wl 600 nm and result used to draw growth curve .alginate lyase activity are detected from the media by colorimetric method.

Determination of optimum NaCl concentration for growth and lyase activity

The bacterial isolate 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 were 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. Total reaction mixture 2 ml.(chen et al.2016)

Thermal stability for alginate lyase enzyme:

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

Optimum pH for aginate 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 . Total reaction mixture 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, $MgCl_2$, $CaCl_2$, $BaCl_2$, $ZnCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_3$, Hg^{2+}, $Mn^{2+}$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) as a 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 .then enzyme activity was detected.

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.

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)
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-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.(Uchimura et al., 2010)

PCR product were sent to(Macrogen, Korea) to get the sequence for alginate lyase gene.

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 ; $(NH_4)_2SO_4$,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 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 as shown in figure (1).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 hrs and growth in minimal concentration of alginate in the culture medium .we named it MAK4 figure (5). 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.

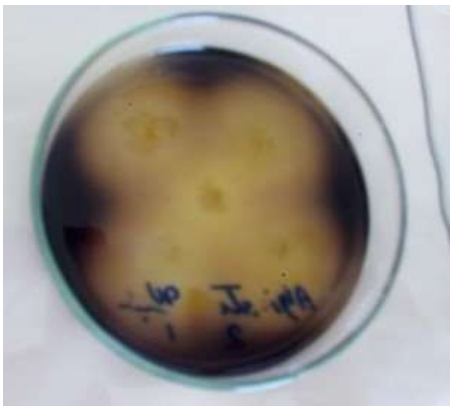

Figure 1: isolate MAK4

Figure2: Isolate MAK4

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 in table (2) carried out by BioMerieuxVITEK 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	-

3-16S rRNA Gene amplification and sequencing:

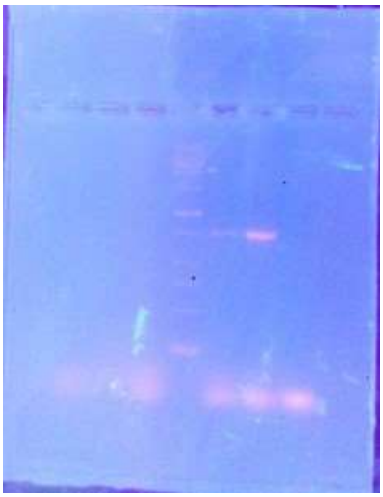

Figure 3: PCR result for 16s rRNA Gene.

Purification of alginate lyase:

The purification of alginate lyase frome the crude supernatant by ammonium sulfate precipitation and DEAE-Cellulose chromatography provide lyase with 5 fold purification and 16% yield and the final step of Sephadex G-100 column chromatography provide lyase with specific activity 63unit/ µ g protein with 6 fold of purification. Table (3) summarized all purification steps.

Table 3: Purification of alginate lyase from strain MAK4

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

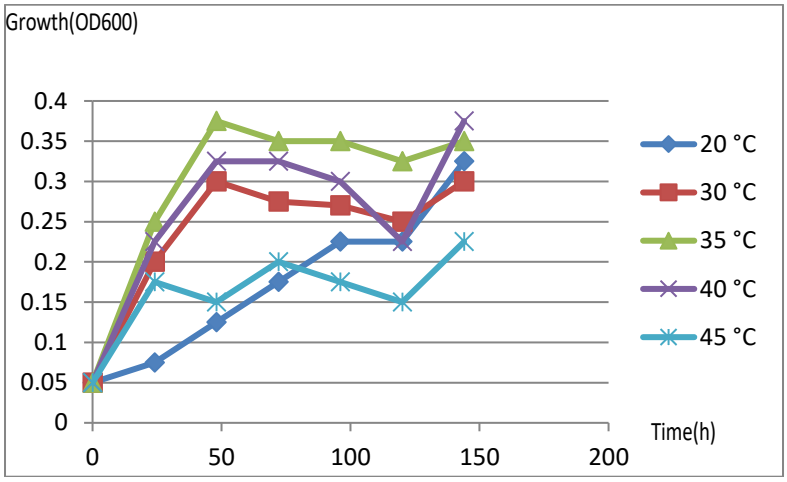

Figure 4: Strain MAK4 16S rRNAs gene, partial sequence
>AA000000.1 *Martelella sp.* strain 1334-033 16S ribosomal RNA gene, partial sequence

Table 4: protein and amino acid composition of alginate lyase gene.

Amino Acid	Number	Mol%
Ala A	37	10.57
Cys C	1	0.29
Asp D	32	9.14
Glu E	10	2.86
Phe F	15	4.29
Gly G	19	5.43
His H	11	3.14
Ile I	16	4.57
Lys K	22	6.29
Leu L	32	9.14
Met M	7	2.00
Asn N	20	5.71
Pro P	16	4.57
Gln Q	20	5.71
Arg R	18	5.14
Ser S	19	5.43
Thr T	18	5.14
Val V	11	3.14
Trp W	11	3.14
Tyr Y	15	4.29

Protein: alglyase AA
Length = 350 amino acidsMolecular Weight = 39963.63 Daltons

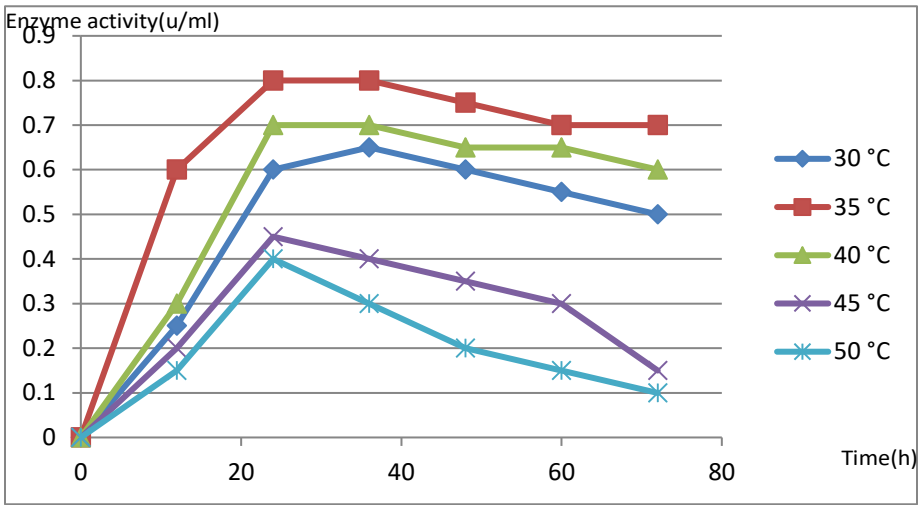

Figure 5: Optimization temperature for alginate lyase production

Optimization conditions for growth and production of alginate lyase:

A-optimum temperature for growth and lyase production:

Strain MAK4 show optimum growth between 35-40 °C after 48 hours of incubation as in figure(5 a) and lyase production reach maximum concentration after 24 hours as in figure (5b) under temperature range35-40 °C .

B-optimum alginate concentration growth of strain MAK4 and alginate lyase production:

The growth rate of strain MAK4 and lyase production increased with the increase in substrate level. this effect is noticed when using different alginate concentration from 0.2% to 0.8% alginate .it show optimum growth level and alginate lyase production at 0.8 % alginate as provided in figure (6a) and figure (6b).

C-Effect of NaCl concentration on growth and alginate lyase production:

Using various concentration of NaCl from 0.1-0.5 % indicated that the concentrations 0.2 M and 0.3 M of NaCl improved the growth of the isolate MAK4 and alginate lyase production as compared with other concentration 0.1 M .0.4 M and 0.5 . this results indicated that NaCl is required at certain concentration for alginate lyase activity which enable the bacteria to use alginate as sole carbon source. result in figure (7 a, 7b).

Characerization of alginate lyase extracted from Martelella sp.strain MAK4

Optimum temperature for alginate lyase:

The alginate lyase optimal temperature was determined using various temperatures from 20-55 °C .the optimal temperature is 37 °C. Figure (8).

Alginate lyase thermal stability:

As shown in figure (9) alginate lyase still active at 40°C for 90 min. and at 50 °C for almost 1hour.the alginase activity is significantly declined at 60°C and 70°C

Optimization PH and PH stability of alginate lyase

Alginate lyase optimal PH is 7 and the enzyme is relatively atable in PH range from 6 to 9 and still retaining about 80 to 70 % of alginase activity respectively .figure (10).

Effect of metal ions and EDTA on alginate lyase activity:

Divalent cations as Zn2+ ,Mn2+,Cu2+,Mg+2 ,Ba+2 Co+2 ,Hg+2, Fe+2, inhbited alginate lyase with different degree as shown in figure(11) while EDTA is completely inhibited alginate lyase activity. K+, Na+ and Ca+2 increased the alginase activity .

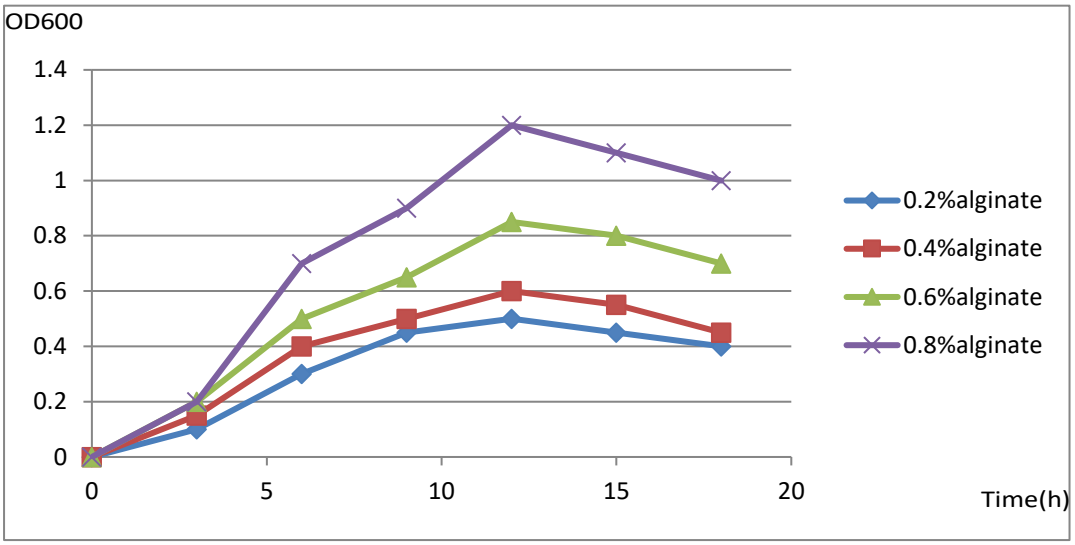

Figure 6: Optimization alginate concentration for growth of isolate MAK4.

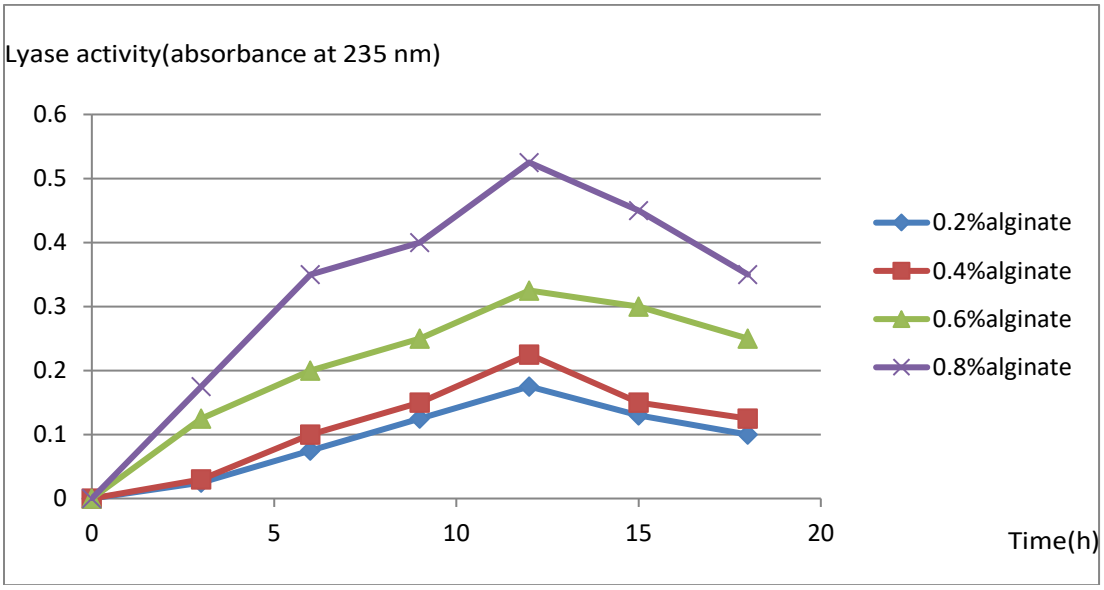

Figure7: Optimization alginate concentration for alginale lyase production.

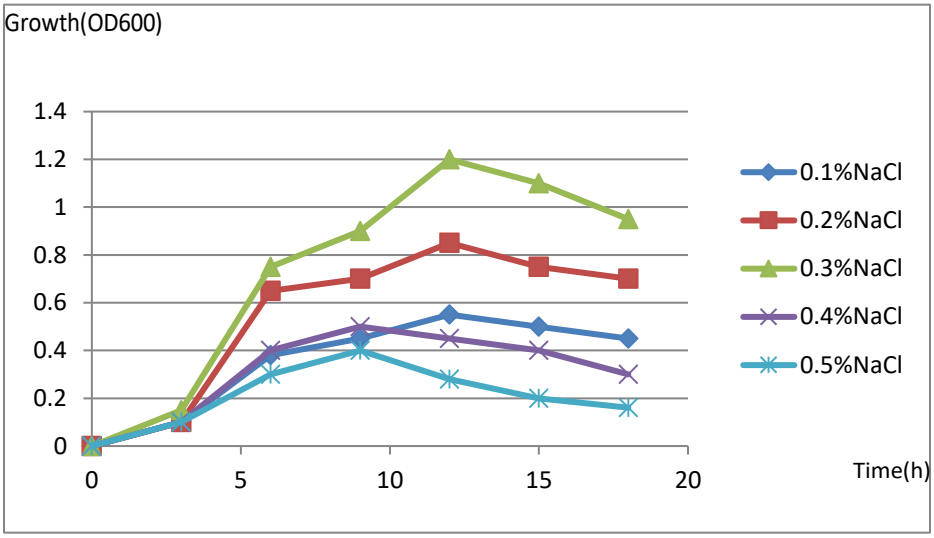

Figure 7a: Effect of NaCl conc. on growth of strain MAK4.

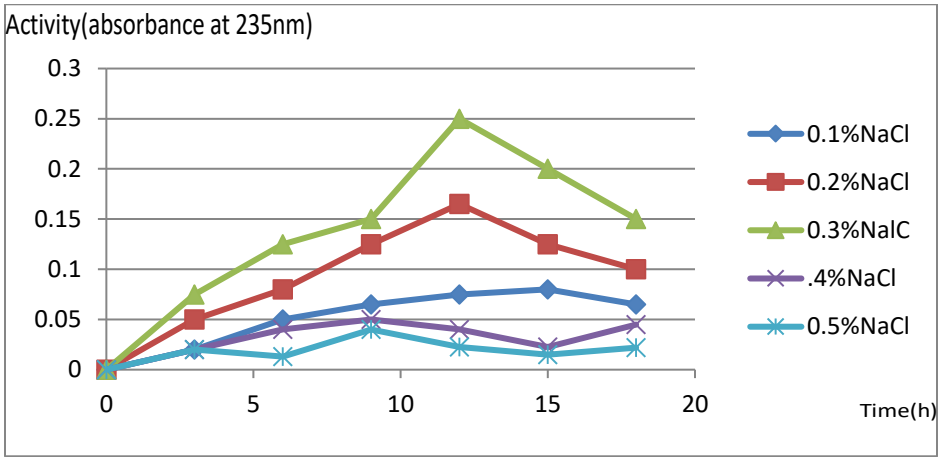

Figure 7b: Effect of NaCl conc. on alginate lyase activity.

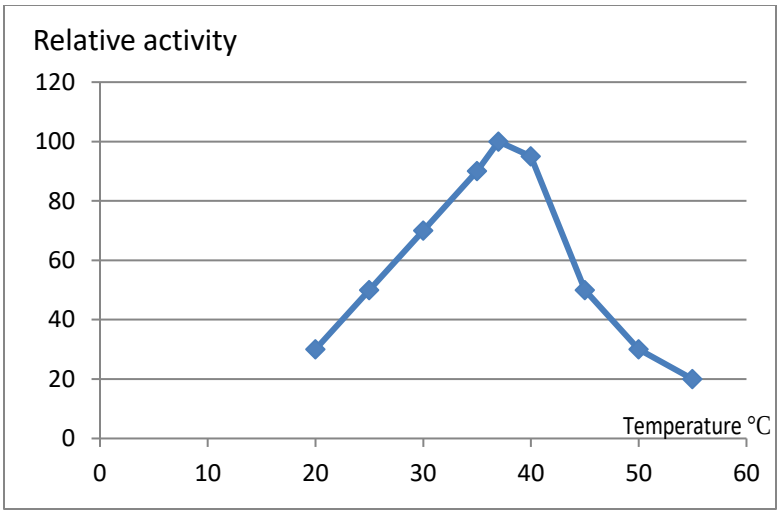

Figure8: Optimization temperature of alginate lyase.

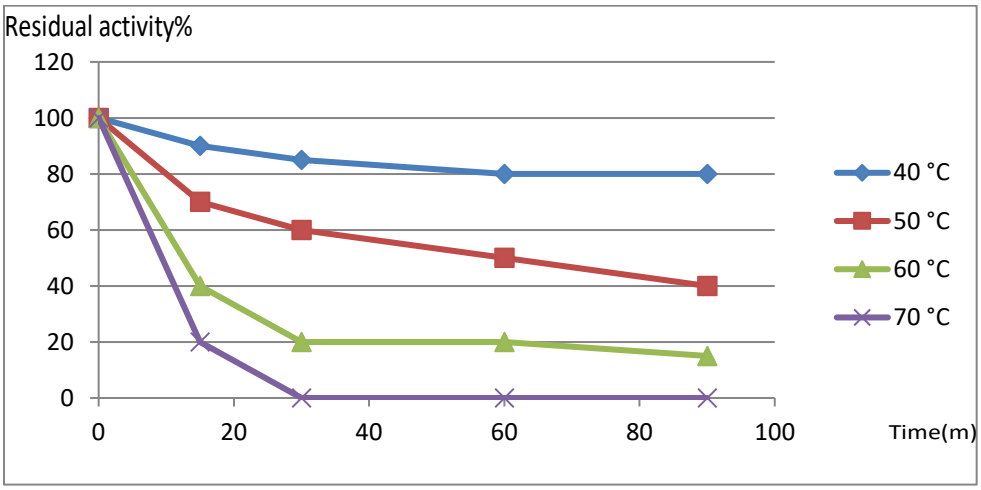

Figure 9: thermal stability of alginate lyase.

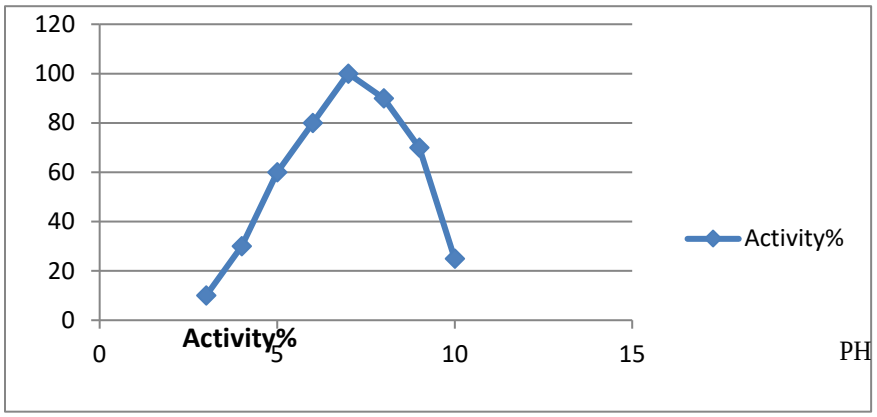

Figure10: Optimization PH of alginate lyase.

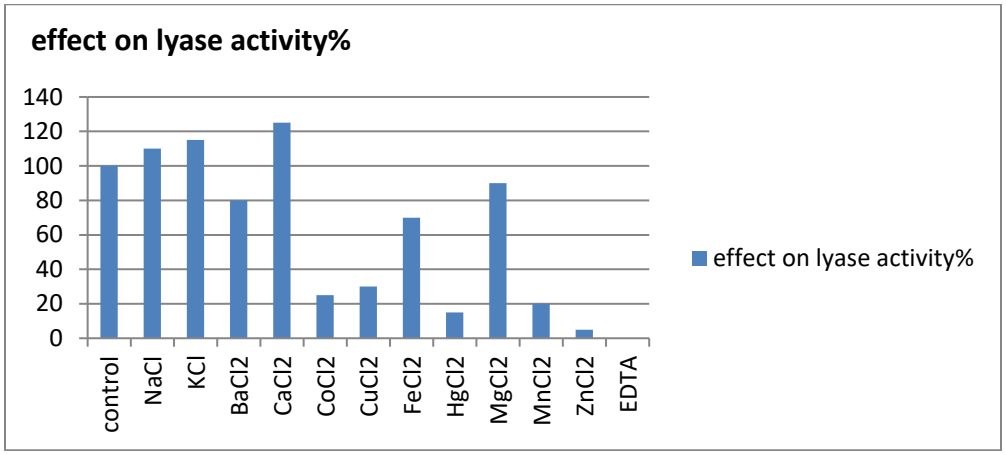

Figure11: Effect of metal ions and EDTA on alginase activity.

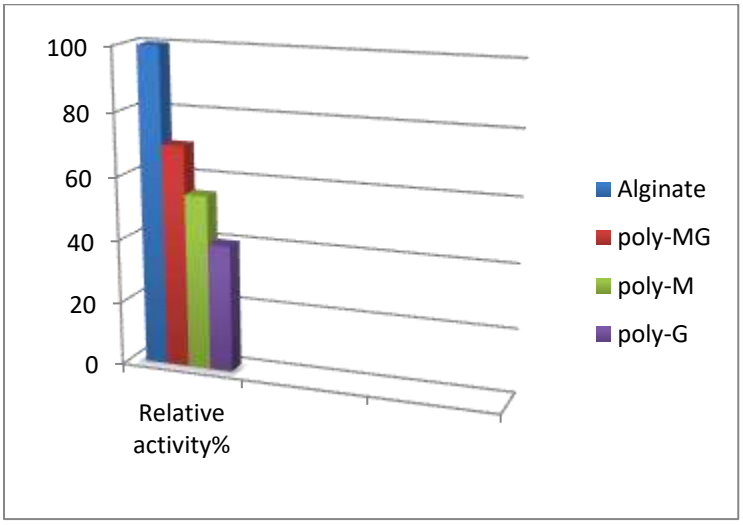

Figure 12: Alginate lyase substrate specificity

Substrate specificity of alginate lyase :

The purified alginate lyase was most active aginst poly-MG blocks and show lower activity toward poly-M .while the lowest activity is noticed toward poly-G as seen in figure (12).

Figure (12) alginate lyase substrate specificity

7-Isolation and sequencing of alginate lyase gene

The genomic DNA of Martelella sp.strain MAK4 were extracted and purified then PCR is performed using primers designed for alginate lyase gene (previously mentioned in table (1) Figure (13) show PCR for alginate lyase gene.

Figure 13: PCR for alg. Lyase.

DISCUSSION

Martelella sp. Strain MAK4 was isolated from brown algae collected from coastal area in Egypt (hurghada and Baltim beach) . 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 martelella 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 *Martelella sp*. Strain MAK4 alginate lyase was 35 kDa. It was the same molecular weight as AlgC-PL7 produced by *Cobetia sp*.NAP1 (Yagi et al. 2016) and higher than Alm isolated from Agarivorans sp. JAM-Alm. Of molecular weight 31kDa (Kobayashi., 2009).and AlyV5 isolated from Vibrio sp. QY105 with molecular weight 33 kDa (Wang et al.2013) . But smaller than Alg7D isolated from Saccharophagus degradans with 63.2 kDa (Kim et al. 2012) .

The alginate lyase isolatd from Martelella sp. strain MAK4 has optimal temperature 37°C. The same as alginate lyase isolated from Streptomyces sp. A5 (CAO et al. 2007) , lower

Alginate lyase gene sequence figure (7a)forward and (7b) reverse sequence will be shown later in separate file

SDS-PAGE and Zymography analysis:

The SDS-PAGE analysis is carried out using 12% polyacrylamide gel to determine the molecular weight of the aginate lyase gene

Figure 14 SDS-PAGE analysis.

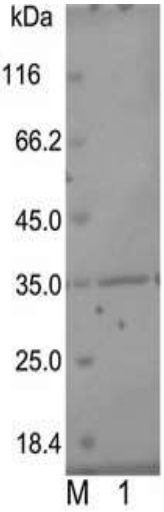

Figure 14:SDS-PAGE Zymogram.

16S rRNAs gene, partial sequence, Alginate lyase Gene Sequence And Protein Composition Of Alginate Lyase Enzyme

than 40 °C recorded for The KJ-2 alginate lyase from Stenotrophomas maltophilia KJ-2 (Lee et al., 2012) and AlySY08 from Vibrio sp. Aly08 (Li et al., 2017) and 50 °C for Cel32 from Cellulophaga sp. NJ-1 (Zhu et al., 2016) and Oal17 from Shewanella sp.Kz7 (Wang et al.,2015) and higher than 30 °C for Alm isolated from Agarivorans sp. JAM-Alm (Kobayashi,. 2009).

The optimal pH for alginate lyase isolated from *Martelella* sp. Strain MAK4 is 7 .the same as alginate lyase purified from marine *Microbulbifer* sp. ALW1. (Zhu, et al. 2015) andalginate lyase isolated from *Vibrio sp.* YKW-34 **.** (Fu et al., 2007) .almost the same as PH 7.5-8 as in alginate lyase isolated from Alteromonas sp. Strain No. 272 (IWAMOTO et al. 2001) . and higher than PH 6.5 reported for alginate lyase isolated from Aspergillus oryzae (Singh et al . 2011).

The martelella sp. Strin 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 withK+ , Na+, and Ca+2 .in the other hand the activity is strongly inhibited by Cu+2, Zn+2, Co+2,Hg+2 and Mn+2. While the activity was lost completely with EDTA .the enzyme was inhibited in lower extent with Ba+2, Fe+ and Mg+

CONCLUSION

Martelella sp. Strain MAK4 Producing alginate lyase. The martelella sp. Strin MAK4 alginate lyase relatively stable after 90 minutes in 40°C. The enzyme has specific activity 62.9 u/mg The molecular weight of *Martelella sp*. Strain MAK4 alginate lyase was 35 kDa.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

All authors contributed in manuscript. All authors read and approved the final version.

AUTHOR CONTRIBUTIONS

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