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ANTIOXIDANT ACTIVITY OF ALGINATE-HYDROLYSATES PRODUCED BY ALGINATE LYASE DERIVED FROM MARINE BACTERIUM *MICROBULBIFER AGARILYTICUS* ALGLP5

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Abstract

Alginate lyases have attracted attention due to their application in saccharification of alginate for production of alginate oligosaccharides with promising biological activities. Herein, an alginate lyase-producing strain ALGLP5 was isolated from decayed seaweeds collected from the coastal zone of the Gulf of Suez, Egypt. Based on 16S rRNA analysis, the isolate was identified to be *Microbulbifer agarilyticus*. Extracellular alginate lyase was partially purified by ammonium sulfate precipitation and enzyme exhibited a specific activity of 2.70 units/mg protein. Alginate-hydrolysates produced by the partially purified alginate lyase showed promising antioxidant properties with 84% free radical scavenging activity. These results suggest that produced alginate oligosaccharides have great potential to employed as antioxidants in food and pharmaceutic fields.

Key words: Alginate lyase, Oligosaccharides, Antioxidant, DPPH, *Microbulbifer*

Introduction

Macroalgae are plentiful in marine ecosystems growing at rates that far exceed those of terrestrial plants and contain no lignin so simple biorefinery processing can efficiently produce sugars from these materials (Takagi *et al.,* 2016). Alginate constitutes up to 40% of the dry weight of some algal biomass and consists of β-D-mannuronate (M) and its C5 epimer α-L-guluronate (G) as monomeric units (Thomas *et al.,* 2013; Sari-Chmayssem *et al.,* 2016). These units are linked together with 1, 4-O-glycoside bonds to form a linear polysaccharide arranged as a polyM-block, a polyG-block, and an alternating or random polyMG-block (Vera *et al.,* 2011). Alginate lyases are a group of enzymes that can degrade alginate through β-elimination of the glycosidic bond yielding various oligosaccharides (Sawant *et al.,* 2015).

The hydrolysates of alginate, alginate oligosaccharides (AOs), have attracted increasing attention due to their biological activities with a variety of bioactive functions that can be applied in food and pharmaceutical industry, therapeutics and biotechnology (Zhu and Yin 2015; Zhu *et al.,* 2016a). The degradation products of alginate exhibit a variety of biological activities such as decreasing plasma LDL-cholesterol levels (Yang *et al.,* 2015), antimicrobial activity (Tøndervik *et al.,* 2014), antioxidant activity (Zhu *et al.,* 2016b), immunomodulation (Xu *et al.,* 2014), induction of defense responses in plants (Zhang *et al.,* 2015) and promotion of plant growth (Wang *et al.,* 2016). It has been reported that alginate lyase and alginate oligosaccharides (AOs) have antibacterial and antibiofilm properties and reinforces the activity of selected antibiotics against multi-drug resistant bacteria since they perturb multi-drug resistant (MDR) bacteria by interfering biofilm formation and reducing resistance to antibiotics (Germoni *et al.,* 2016; Bugli *et al.,* 2016). Also, AOs have antifungal properties due to their ability to disturb fungal growth and escalate conventional antifungal agents against various fungal pathogens like *Candida* and *Aspergillus* spp (Tøndervik *et al.,* 2014). Besides, alginate lyases have potential use as key biocatalysts for application in renewable sources of biochemicals and biofuels by saccharification alginate-rich seaweeds and facilitating the subsequent fermentation processes (Kim *et al.,* 2011; Sawant *et al.,* 2015). The present study addresses the isolation of alginate lyase-producing marine bacterium *Microbulbifer agarilyticus* ALGLP5 for

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production of the potential antioxidant, alginate-hydrolysates.

Materials and Methods

Isolation of alginate lyase producing bacteria

Decayed seaweeds samples were collected from the coastal zone of the Gulf of Suez, Egypt. Under aseptic conditions, ten grams of the collected samples were homogenized in 100 mL of sterile seawater. The homogenates were diluted up to 10^{-6} using sterile seawater, and 0.1 mL of each dilution was spread on the surface of the isolation medium plates (1% sodium alginate, 3% NaCl, 0.5% $(NH_4)_2SO_4$, 0.1% $MgSO_4 \cdot 7H_2O$, 0.2% K_2HPO_4, 0.02% $CaCl_2$ and 0.002% $FeSO_4.7H_2O$) containing alginate as the sole carbon source. The inoculated plates were incubated at 28°C for 5 days. Afterwards, single colonies with clear hydrolytic zones were picked and re-streaked several times on the same medium until pure cultures were obtained. Then the isolated strains were re-screened for alginate lyase activity by flooding the plates with 10% (w/v) cetyl pyridinium chloride (CPC) for 30 min. The strain designated ALGLP5 exhibiting the maximum clearing zone against an opaque white background was selected for further investigations.

Phylogenetic analysis

The most potent bacterial strain ALGLP5 was identified via amplifying of 16S rRNA gene by polymerase chain reaction (PCR) using 27F and 1492R universal primers. The amplified PCR product was purified and sequenced at Macrogen (Seoul, South Korea). The BLASTn search program (http://www.ncbi.nlm.nih.gov) was used to look for nucleotide sequence homology. The 16S rRNA gene sequence of the most promising strain was submitted to GenBank and accession number was assigned. The sequence obtained was then aligned by ClustalW using MEGAX software (Kumar et al. 2018) and a neighbor-joining (NJ) tree with bootstrap value 1000 was generated using the software.

Culture conditions for alginate lyase production

For the production of alginate lyase, Zobell Marine Broth 2216 (Himedia, India) supplemented with 1% sodium alginate as the sole source of carbon was used as the production medium. The inoculated medium was incubated aerobically at 28°C on an orbital shaker at 180 rpm for 48 h. After incubation, bacterial cells were removed by centrifugation at 16000 rpm for 15 min and cell-free supernatant was subjected to partial purification using ammonium sulfate.

Solid Ammonium Sulfate Precipitation

The supernatant was brought to 80% (w/v) saturation by slow adding of solid ammonium sulfate and left at 4°C overnight. Then, the precipitate was collected by centrifugation at 20,000 rpm for 30 min, resuspended in 1.5 ml of 20 mM Tris-HCl, and dialyzed against the same buffer at 4°C for 2–3 days. The dialysate was assayed as the partially purified enzyme.

Enzyme assay

The activity of alginate lyase was assayed using 1% sodium alginate in 20/ mM NaH_2PO_4-Na_2HPO_4 buffer (pH 8.0) for 5/ min/ at 35°C. The reaction was terminated by heating in a boiling water bath for 10/ min. The released reducing sugars were estimated by using the dinitrosalicylic acid (DNS) method (Miller 1959). One unit of enzyme activity was defined as the amount of enzyme required to release 1/µmol reducing sugar (measured as D-glucose) per minute. The protein concentration was determined using the Bradford assay (Bradford 1976).

Preparation of alginate-oligosaccharides

Partially purified alginate lyase (100 U) was added to 100 ml of 50mM Tris–HCl buffer (pH 8.0) containing 1% (w/v) sodium alginate and incubated at 40°C for 12 h and then was stopped by heating in a boiling water bath for 10 min. Two-fold ethanol was added to the reaction mixture to remove the high-molecular-mass polysaccharides. After centrifugation, the supernatant containing water-soluble fraction was lyophilized. The amount of total sugars was determined by the method of phenol-sulfuric acid (Dubois *et al.*, 1956).

Antioxidant activity of the enzymatic hydrolysates

To investigate the antioxidant activity of the produced alginate-oligosaccharides, DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging assay was conducted (Yang *et al.*, 2006). The above-prepared hydrolysate powder was dissolved in distilled water and subjected to antioxidant activity assays. In brief, 1.0 ml of the hydrolysate sample solution (1mg/ml) was mixed with 2 ml of 0.2 mM DPPH dissolved in ethanol. After shaking for 30 min in the dark at room temperature, the absorbance was measured at 517 nm. The DPPH radical scavenging effect of the sample was calculated as follows: scavenging ability (%) = (1–absorption of sample/absorption of control)×100.

Results and Discussion

Isolation of alginate lyase producing bacteria

Twenty marine alginate-metabolizing strains were isolated from decayed seaweed samples collected from the Gulf of Suez, Egypt, using sodium alginate as the sole

carbon source. Based on the clear zone formation after flooding with 10% (w/v) CPC solution, the strain designated ALGLP5 exhibiting the highest alginolytic activity was selected for further investigations. In similar means, the alginolytic activity of many bacteria isolated from various habitats had been visualized by formation of a clear zone on an opaque background after staining plates with CPC solution (Kim *et al.*, 2013; Sawant *et al.*, 2015).

Phylogenetic analysis

The 16S rRNA gene sequence (1,341 bp) of strain ALGLP5 was deposited in GenBank (Accession No. MK314731). Phylogenetic analysis performed with partial and almost complete sequences of closely related species indicated that strain ALGLP5 is affiliated within the family *Microbulbiferaceae*, the class Gammaproteobacteria. BLAST analysis of 16S rRNA gene of the strain ALGLP5 revealed that it shares 99.7% similarity with *Microbulbifer agarilyticus* strain JAMB A3 (Accession No. NR_041001), 98.5% similarity with *M. salipaludis* strain SM-1 (Accession No. NR_025232) and 97.6% similarity with *M. elongatus* strain ATCC 10144 (Accession No. NR_112059), accordingly it was identified to be *Microbulbifer agarilyticus*. The NJ tree showing phylogenetic relationships between strain ALGLP5 and the closest related bacteria is presented Fig. 1. These results are consistent with findings indicated that many members of the family *Microbulbiferaceae*, which contains the genus *Microbulbifer* can degrade many complex polysaccharides including alginate (Wakabayashi *et al.*, 2012; Yang *et al.*, 2015; Jonnadula *et al.*, 2018; Jiang *et al.*, 2019). It has been suggested that the marine environment is a highly productive ecosystem with great microbial diversity (Farahat 2020). Recently, a wide range of alginate-degrading bacteria, including *Gilvimarinus*, *Vibrio* and *Pseudoalteromonas* spp. have been isolated from marine environments (Zhu *et al.*, 2018; Daboor *et al.*, 2019; Huang *et al.*, 2019).

Enzyme activity of crude and partially-purified alginate lyase

Alginate lyase activity was assayed using the DNS method for detection of the released reducing sugars. Results revealed that ALGLP5 secreted extracellular alginate lyase with a specific activity of 0.83 U/mg protein. The specific enzyme activity was increased from 0.83 units/mg protein to 2.70 units/mg protein after ammonium sulfate precipitation and dialysis. Three-fold increment in specific activity was observed using ammonium sulfate precipitation table 1. It has been reported that alginate lyase activity of *Microbulbifer* sp. ALW1 was found to be 1.94 U/mg (Zhu *et al.*, 2016b). In a similar study, a novel alginate lyase, AlyH1, from the

Table 1: Partial purification of alginate lyase by solid ammonium sulfate precipitation.

Step	Volume (ml)	Total activity (U)	Total proteins (mg)	Specific activity (U/mg)	Purification (Fold)	Yield (%)
Culture supernatant	100	232	279	0.83	1	100
Ammonium sulfate	2	165	61	2.70	3.2	71.1

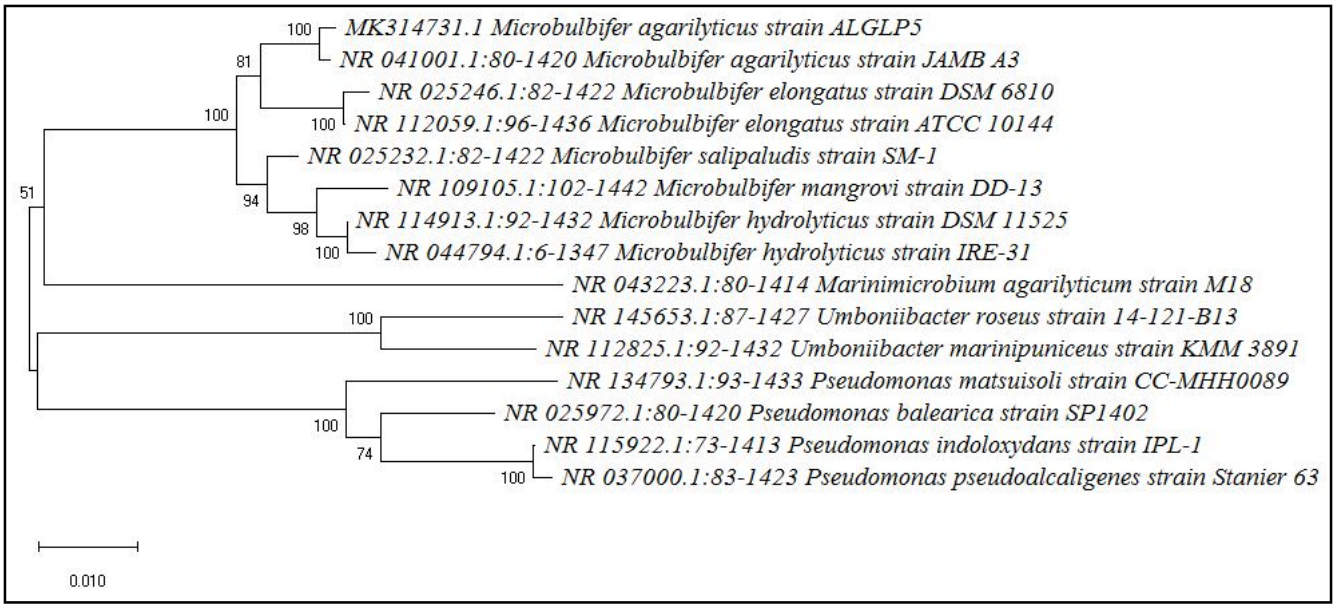

Fig. 1: Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between *M. agarilyticus* strain ALGLP5 and the most closely related species.

marine bacterium *Vibrio furnissii* H1 showed the specific activity of 2.40 U/mg (Zhu *et al.*, 2018).

Antioxidant activity of the enzymatic hydrolysates

The free radical scavenging activity of alginate-hydrolysates was estimated by using the stable free radical DPPH, results revealed promising antioxidative properties of oligosaccharides obtained by enzymatic hydrolysis of alginate by alginate lyase derived from strain ALGLP5. The enzymatic hydrolysis products of 12 h treatment had the inhibitory effects on DPPH by about 84%. In agreement with our findings, oligosaccharides have been prepared from alginate by alginate lyase from *Pseudoalteromonas carrageenovora* ASY5 exhibited DPPH radical scavenging activity of 81.5% (Zhang *et al.*, 2020). In a similar study, the oligosaccharides produced by enzymatic treatment of alginate with alginate lyase good antioxidant activities, wherein alginate oligosaccharides with smaller molecular weight would have better antioxidant activities (Xu-xia *et al.*, 2014).

Conclusion

In this study, *Microbulbifer agarilyticus* strain ALGLP5 was found to produce an extracellular alginate lyase enzyme that digests sodium alginate producing biologically active oligosaccharides with promising antioxidative properties. These results suggest that produced alginate oligosaccharides have great potential to employed as antioxidants in food and pharmaceutical fields.

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