

## Recombinant Production of Alginate Lyase for Improved Stress Resistance in Plants

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Three alginate lyase producing strains of *Klebsiella* sp., and one strain of a *Paenibacillus* sp. were isolated from soil and water samples. The alginate lyase gene *algK* from *Klebsiella* sp. 20-3 and *algM* from *Paenibacillus* sp. 8-5 were cloned into *Escherichia (E.) coli* and expressed. The activity of recombinant AlgK (1640.1 U/mg) was 40 × higher than that of recombinant AlgM (36.7 U/mg). Sodium alginate was degraded by AlgK into alginate oligosaccharides. The AOs with a degree of polymerization value of 13.9 were found to stimulate the accumulation of phytoalexin in wounded soybean cotyledon. The 3 g/L concentration of AOs with DP 13.9 displayed the best effect on promoting germination and root development of rice seedlings, as well as a greater advantage for the rice seedlings to adapt to drought resistance and cadmium stress. The AOs with DP 13.9 were analysed by electrospray mass spectroscopy (ESI-MS), and the main component was trimer.

**Key words:** Alginate lyase, Alginate oligosaccharides, Plant resistance, Drought stress, Cadmium stress.

Alginate is a linear heteropolysaccharide consisting of polyuronic acid consisting of 1, 4 linked  $\beta$ -D-mannuronic acid (M) and  $\alpha$ -L-guluronic acid (G). The glycosidic bond in alginate can be broken by physical, chemical or enzymatic methods, forming alginate oligosaccharides (AOs). The biological role of alginate oligosaccharides on plants is depended upon the molecular weight, structure and composition of the oligosaccharide<sup>1-2</sup>. Alginate oligosaccharides can be produced as described by Holtan<sup>3</sup> at 95°C and a pH of 2.8. However, oligosaccharides produced in this method form a wide range of molecular weight oligosaccharides as a result of non-specific reactions which are difficult to control. The enzymatic production of alginate oligosaccharides using alginate lyases are more specific and can be produced using lower energy and chemical inputs<sup>4</sup>.

Alginate lyase are classified by cleaving the polymer chain via a  $\beta$ -elimination mechanism and organized based on primary structure into seven polysaccharide lyase families. They are ubiquitous in nature and have been described in bacteria, fungi, algae, invertebrates and bacteriophages<sup>5-9</sup>. Alginate lyases from bacteria most commonly belong to polysaccharide lyase (PL) families 7, which has conserved sequences of R-X-E-L-R-E-M and Y-F-K-A-G-X-Y<sup>10-11</sup>. *Sphingomonas* sp. A1 produces three alginate lyases A1-I, A1-II, and A1-III<sup>12</sup> that have been expressed in *E. coli*. The activity of A1-I, A1-II, and A1-III when produced in *E. coli* cells were found to have 10-fold higher activity over *Sphingomonas* sp. A1 wild type strain expressed enzymes. The heterologous expressed enzyme results in alginate lyases with a significantly higher specific activity that is useful for the industrial of AO's.

Environment conditions such as drought and heavy metal pollution are having an impact on the planting agriculture. There have been a number

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of papers which describe the use of AOs for stimulating the accumulation of phytoalexin in soybean cotyledon<sup>13</sup>. They have also been shown to effect plant seed germination, root growth and shoot elongation<sup>14-17</sup>. The discovery of the effects of AO's on plant resistance to abiotic stress<sup>18-20</sup> is an active area of research by a number of groups. The potential of AOs on improving plant resistance to abiotic stresses makes them being focus of concern but the effects and mechanisms still need to be studied in detail. And how to prepare the AOs with exact activities is the essential to promote the application of them into agriculture. In order to search for new alginate lyase, the environmental samples from soil, river and sludge from a kelp processing pool were screened by enrichment culture in this study. After re-screening, several strains with high levels of activity were obtained. The gene encoding alginate lyase was cloned and expressed. This article also examines the effect of AOs generated by AlgK on plant growth and stress resistance, providing basis for the applications of alginate lyase and AOs in the agricultural and environmental governance.

## MATERIALS AND METHODS

### Isolation and Screening of alginate lyase producing strains

Alginate lyase producing strains were isolated from a variety of different environmental sites in China and included soil, water and sludge from a kelp processing pool located in Zhejiang province. Strains were isolated by adding a 1 g sample to a 500 ml flask containing 100 ml enrichment medium consisting of 0.5% sodium alginate, 0.2% K<sub>2</sub>HPO<sub>4</sub>, 0.5% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01% FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.2% MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.0% NaCl, 0.1% glucose, pH 7.0. The samples were incubated at 150 rpm, 30 °C to enrich the growth of alginate lyase producing bacteria. After 24 hours the cultures were diluted in saline and 100 ml aliquots plated onto a medium composed of 0.5% sodium alginate, 0.2% K<sub>2</sub>HPO<sub>4</sub>, 0.5% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01% FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.2% MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.0% NaCl, 1.5% agar (pH 7.0). The plates were incubated at 30 °C for 3 days and colonies were subcultured on the same media in order to obtain pure cultures.

### Detection of alginate activity

Isolates were screened for alginate lyase

activity by flooding the plates with cetylpyridinium chloride (CPC) following the method of Gacesa<sup>21</sup>. Colonies demonstrating a clear halo were considered as enzyme producing candidates. Positive strains were inoculated into screening liquid medium and cultured at 150 rpm, 30 °C. After 3 d the cells were removed by centrifugation at 10,000 g, 4 °C for 5 min. 400 µl 10% CPC was added into aliquots of 2 ml supernatant, replacing with 2 ml fresh medium as control.

### Alginate lyase activity assay

Wild candidate strains were inoculated into screening liquid medium and cultured at 150 rpm, 30 °C for 3 d. The cells were harvested by centrifugation at 10,000 g, 4 °C for 5 min, and the supernatant was the extracellular crude enzyme. The cell pellets obtained were washed in 0.01 M Phosphate Buffer Solution (PBS), then resuspended in the same buffer to disrupt them by ultrasound (Insonator, Kubota Model 201M, Tokyo, Japan) at 0 °C, 200 Hz for 40 min. The clear supernatant obtained after centrifugation at 10,000 g, 4 °C for 20 min was considered as intracellular crude enzyme.

The alginate lyase activity of extracellular and intracellular crude enzyme was measured according to Nakada's method<sup>22</sup>. A suitably diluted crude enzyme sample was mixed with 0.2% (w/v) sodium alginate, 0.01 M PBS to a total volume of 1 ml and then the reaction was incubated for 5 min at room temperature. The release of reducing sugars from sodium alginate was reflected as the increase of absorbance at wavelength of 235 nm. One unit of lyase activity is defined as the amount of enzyme that increases absorbance at 235 nm by 0.01 in 1 min.

### Strain identification by 16S rDNA sequence

The 16S rDNA was amplified using the common primers 27F and 1492R for bacteria. The PCR products were then sequenced by BGI (Beijing genomics institute, Beijing, China) and blasted online (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Multiple sequence alignment was accomplished using the CLUSTAL-W program.

### Cloning and expression of alginate lyase in *E. coli*

The forward primer 5' - CGTTCTGAACTGCGCGAGATG-3' and the reverse primer 5' - GGTTATAGACACCGCTTTGAAAT-3' were

designed from two highly conserved motifs R-X-E-L-R-E-M and Y-F-K-A-G-X-Y of the PL family 7 found in closely related species. The PCR products were purified and then sequenced. After alignment, the primers for the full length alginate lyase genes were designed according to the reported genes which have the highest sequence identity. The genomic DNA was extracted by the Genome DNA extraction kit (Generay, China) and used as the template for PCR. The PCR products with expected size were then inserted to the expression vector pET-28a(+) and expressed in *E. coli* BL21 (DE3) by isopropyl- $\beta$ -D-1-thiogalactopyranoside (IPTG) induction as general methods.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) carried out as described<sup>23</sup> using 12% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS. Supernatant of culture, cell lysate supernatant and cell debris were sampled and loaded on SDS-PAGE to analyze the locations of recombinant enzyme.

#### **Biochemical characterization of recombinant enzyme**

The activity of the alginate lyase at different temperatures (0-70 °C) in 0.01 M PBS (pH 7.4) was measured to investigate optimum temperature. Similarly, optimum pH was determined by assaying the enzyme at different pH. The buffers used were 0.1 M citric acid-phosphate buffer (pH 5.0-8.0), Tris-HCl buffer (pH 7.0-8.5), glycine-NaOH buffer (pH 8.5-10.5). Thermostability and pH stability were measured by pre-incubating the recombinant enzyme at different temperature (0-70 °C) and pH (5.0-10.5) for 30 min respectively and the residual activity was measured. In addition, the effects of different metal ions, chelators and concentrations of Na<sup>+</sup> on alginate lyase activity had been studied in the reaction mixture as described above.

#### **Preparation of AOs by alginate lyase**

AOs were produced using sodium alginate as the substrate. 4 ml crude alginate lyase (cell lysate supernatant of recombinant strain pET 28a-*algK*/BL21(DE3)) was mixed with 200 ml 3% (w/v) alginate and incubated at 37 °C for 15 to 360 min. Samples were collected at intervals and incubated in a boiling water bath for 10 min to stop enzymatic reaction. Reducing sugar of the alginate-degraded products was determined by the dinitrosalicylic acid (DNS) method<sup>24</sup>. The total

sugar was determined by adding several drops of 6 M HCl into the 3% alginate solution and boiling it in water bath for 2 h to fully hydrolysis the sodium alginate into reducing sugar. The average degree of polymerization (DP) value of the AOs was calculated by dividing the reducing sugar into the total sugar.

#### **Effect of AOs on the soybean cotyledon bioassay**

The activity of AOs on inducing the accumulation of phytoalexins in soybean tissue was determined using the soybean cotyledon bioassay<sup>25</sup>. Tissue sections, 1 mm deep and 6 mm long, were removed from the surface of the cotyledons. Wounded cotyledons were arranged cut-side up on moist filter papers in sterile Petri dishes (ten cotyledons per dish). A 10  $\mu$ l aliquot of sample solution was applied to the entire wound surface of each cotyledon. Three dishes, each containing ten cotyledons, were used for each sample analyzed. The cotyledon dishes were incubated in the dark at 25 °C for 24 h. Each group of ten cotyledons were transferred to a beaker containing 10 ml of distilled water and shaken for 1 min. The optical density of these solutions was read at 286 nm. The negative control was performed by replacing AOs samples with sterile distilled water.

#### **Effect of AOs on the germination of rice seed**

Rice seeds were surface sterilized with 0.5% sodium hydrochloride for 5 min and then rinsed with sterile distilled water. After soaking in different concentrations of AOs (DP 13.9) for 24 h, rice seeds were rinsed with sterile distilled water, and placed in Petri dishes with sterile sand. The seedlings were watered daily with tap water. The plants were grown at 25 °C with 12/12 light/dark regime. After 9 days growth in incubator, 30 rice seedlings were collected from each treatment, washed gently, and dried in an oven at 85 °C for 12 h. Dry weight of the rice roots and shoots were determined for evaluating the effect of growth-promotion.

#### **Effect of AOs on inducing drought resistance in rice**

After surface sterilizing, rice seeds were soaked in sterile distilled water for 24 h and then placed in Petri dishes with sterilized sand. The seedlings were watered daily with 10 ml tap water and the culture condition was the same as described above. When the rice seedling grew

three-leaves, watering was stopped. Twenty-four hours after the drought, 3 g/L of AOs (DP 13.9) solutions (10 ml for each seedling) were sprayed homogeneously onto the surface of the seedling leaves. Samples were then taken for subsequent biomass and various bio-chemical analyses after the 4th spray. Those seedlings without drought resistance and AOs were used as control.

Leaves (0.1 g) were homogenized in a mortar and pestle, with 5 ml of 0.1 mM ice-cold phosphate buffer (pH 7.8) containing 0.1 mM EDTA. The homogenate was centrifuged at 12,000 g for 20 min at 4 °C. The supernatant was used as a crude enzyme extract for assays of the activities of catalase (CAT), superoxide dismutase (SOD), peroxidase (POD). All the operations were carried out at 4 °C. The assays were performed in three replicates for each treatment. CAT activity (EC 1.11.1.6) was assayed according to the method of Zhang and Kirkham<sup>26</sup>. POD activity (EC 1.11.1.7) was assayed according to the method of Zhang and Kirkham<sup>26</sup>. SOD activity (EC 1.15.1.1) was determined by measuring its capacity of inhibiting the photo-reduction by nitro blue tetrazolium (NBT) according to the method of Beauchamp and Fridovich<sup>27</sup>.

#### Effect of AOs on rice tolerance to cadmium stress

After surface sterilizing, rice seeds were soaked for 24 h in 3 g/L of AOs (DP 13.9) solution or distilled water. Then the seeds were rinsed with sterile distilled water, and placed in Petri dishes with sterilized sand. After germination, the seedlings were cultured in the same condition as described above.

When the third leaf of rice seedlings was expanded, CdCl<sub>2</sub> solution of 1 mM was applied into the sand. After 5 of cadmium (Cd) treatment, crude enzymes were extracted at 4 °C from about 0.1 g tissue of plants of each treatment group, using a mortar and pestle, with 5 ml of extraction buffer. The extraction was used for activity assay of CAT, SOD and POD as crude enzyme extract. The methods of activity assay of these enzymes were the same as described above.

#### Determine the molecular mass of AOs

The AOs were desalted on a Sephadex G-25 column, eluted with deionized water. The samples were collected as 0.3 ml per tube and the absorbance of each tube was determined at 235 nm. The sample which had top absorbance at 235 nm was diluted

in MeOH-H<sub>2</sub>O (1:1) and analyzed by electrospray mass spectroscopy (ESI-MS) (negative ion mode)<sup>28</sup>.

## RESULTS

### Screening and identification of alginate lyase producing strains

After 10% CPC was spread on each screening plates, the clear hydrolytic halos around some strain were visible (Fig 1a). The strains with hydrolytic halos were picked out and cultured as candidates. The supernatant of culture was added by CPC solution to see if the reaction would turn into clear (Fig 1b). Four strains namely strains 8-5, 20-3, 20-15, 20-25, were selected out of 200 wild isolates in this step. The alginate lyase activities of the extracellular and intracellular crude extraction of the 4 strains were measured (Fig 1c, 1d). The extracellular alginate lyase activity of strain 8-5 is 30.0 U/ml (Fig 1c), higher than the other 3 strains. As for the intracellular alginate lyase activity of strains 20-3, 20-15, 20-25, they were in similar level as 844, 774, 761 U/ml (Fig 1d) individually. The intracellular lyase activity of 8-5 only was 61 U/ml.

Alignment of 16S rDNA gene sequences showed that strain 8-5 had 100% identity to many *Paenibacillus* sp. strains. Strains 20-3, 20-15, 20-25 are encapsulated, gram-negative bacteria and all showed 100% identity to many *K. pneumoniae* strains in their 16S rDNA sequences. Therefore, strain 8-5 should belong to the genus of

**Table 1.** Effect of different metal ions and chelator on the activity of AlgK

Modulators	Relative activity (%) <sup>b</sup>
Mg <sup>2+</sup>	114.4 ± 6.06
Ca <sup>2+</sup>	91.9 ± 4.14
Mn <sup>2+</sup>	108.1 ± 1.89
Co <sup>2+</sup>	62.1 ± 2.51
Ni <sup>2+</sup>	36.4 ± 1.34
Cu <sup>2+</sup>	40.6 ± 6.51
Zn <sup>2+</sup>	54.1 ± 9.86
Ba <sup>2+</sup>	97.3 ± 9.74
EDTA	8.2 ± 0.28
Control <sup>a</sup>	100 ± 0.98

<sup>a</sup> The relative activity assayed in the absence of the metal ions was regarded as 100%.

<sup>b</sup> Data were given as means ± SD, n=3.

**Table 2.** Effect of Na<sup>+</sup> concentrations on the activity of AlgK.

Modulators	Relative activity (%)								
	1 M <sup>c</sup>	0.9 M	0.7 M	0.5 M	0.3 M	0.2 M	0.1 M	0.05 M	0.03 M
Na <sup>+</sup>	29.4 ± 4.26	36.1 ± 0.88	56.5 ± 0.65	85.6 ± 7.37	116.7 ± 9.93	137.6 ± 2.56	117.7 ± 5.56	124.4 ± 9.89	119.1 ± 1.31

<sup>c</sup>The concentration is the final concentration of the reagents in the enzyme activity assay system

*Paenibacillus* and strain<sup>20-3, 20-15, 20-25</sup> may be same species from genus of *Klebsiella*.

#### Cloning and expression of alginate lyase

The conserved fragment of alginate lyase genes amplified from *Paenibacillus* sp. 8-5 exhibited high similarity with beta-D-mannuronate lyase from *Paenibacillus* sp. Y412MC10 (GenBank Accession Number: ACX67576.1). Meanwhile, the DNA amplified from *Klebsiella* sp.20-3, 20-15, 20-25 showed identity to Aly from *K. pneumoniae* (AAA25049.1). Therefore, in order to clone the alginate lyase gene (*algM*) from *Paenibacillus* sp. 8-5, the forward primer (*NdeI*) 5' - CATATGGTCAAACGAAGGTTTGCTT-3' and the reverse primer (*XhoI*) 5' - CTCGAGCTACGGATGCATGTGCGTT-3' were used. The forward primer (*NdeI*) 5' - TCTCATATGATGTTAAAAAGCGGCGT-3' and the reverse primer (*XhoI*) 5' - TCGCTCGAGCTATCGCTGCGCTCC-3' were used to clone the alginate lyase gene from *Klebsiella* sp<sup>20-3, 20-15, 20-25</sup> according to reported DNA sequences in Genbank.

The DNA sequences of *algK* gene amplified from the 3 strains of *Klebsiella* were totally the same as *aly* from *K. pneumoniae* (AAA25049.1), which confirmed these 3 strains belong to *Klebsiella* sp. The amino acid sequence of AlgM (JQ304790) sharing the highest identity (94%) to alginate lyase of *Paenibacillus* sp. Y412MC10 (ACX67576.1). The molecular weights of recombinant expressed proteins AlgM and AlgK were 47.5 kDa and 33.5 kDa respectively by SDS-PAGE (Fig 2). The activity of AlgM was 196 U/ml, and the specific activity was 36.7 U/mg. The activity of AlgK was 8245 U/ml, and its specific activity was 1640.1 U/mg, much higher than that of AlgM.

#### Biochemical characterization

The thermostability was investigated for AlgK. As shown in Fig 3a, the residual activity

was stable when the temperature was below 30 °C, but decreased rapidly as the temperature increased from 40 to 70 °C. About 50% of the relative activity was remained after pre-incubated at 40°C for 30 min but totally lost at 70°C. And the optional temperature is 30 °C.

The alginate lyase activity was measured at various pH buffers. Maximum activity was observed at pH 8.0 in 0.1 mM Tris-HCl buffer (Fig 3b). As for the pH stability, the enzyme activity was also most stable at weak base condition. When pH value was higher than 8.0 or lower than 7.0, the enzyme activity was decreased (Fig 3c).

The effects of the metal ions on the activity of AlgK were analyzed. The metal ions of Mn<sup>2+</sup> and Mg<sup>2+</sup> had activating effects on the activity of AlgK. Ba<sup>2+</sup> had no effect, and Ca<sup>2+</sup> had slight inhibitory effect on the activity of AlgK. The enzyme activity decreased more than 50% in comparison with control when Co<sup>2+</sup>, Ni<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup> existed in the reaction. The enzyme was strongly inhibited by EDTA as the activity lost nearly 80% (Table 1).The effect of the concentration of Na<sup>+</sup> on the enzyme activity was examined. AlgK showed the highest activity in presence of 0.2 M Na<sup>+</sup> (Table 2).

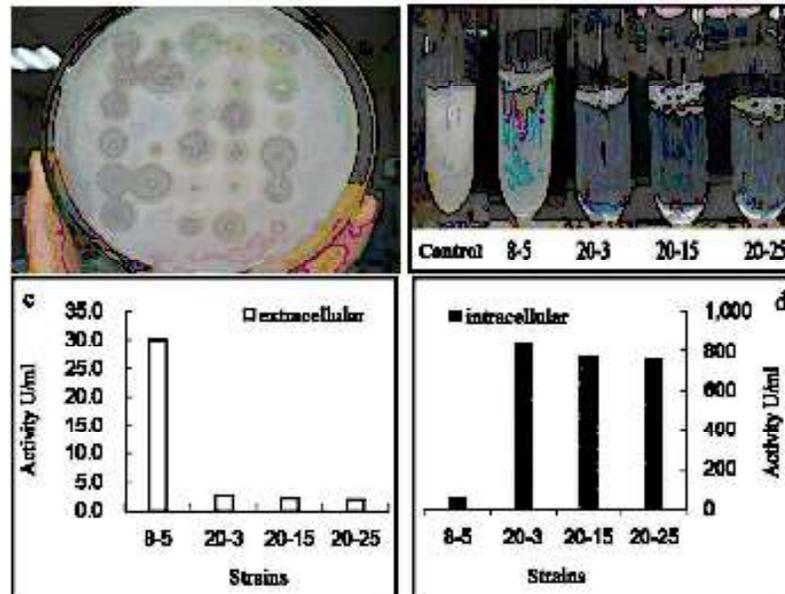
#### AOs activate plant defenses in response to wounding according to a soybean cotyledon bioassay

**Table 3.** Effect of AOs on the germination of rice seeds

Concentration of AOs (%)	Dry weight (mg · plant <sup>-1</sup> )	
	root	shoot
0.00	0.9 ± 0.25	1.5 ± 0.26
0.05	0.7 ± 0.18	1.4 ± 0.17
0.10	0.9 ± 0.23	1.5 ± 0.17
0.20	1.2 ± 0.19	1.6 ± 0.21
0.30	1.7 ± 0.12	2.3 ± 0.41
0.50	1.4 ± 0.22	1.7 ± 0.66

The AOs with different DP were obtained by degrading sodium alginate for different time using crude enzyme preparation of AlgK. Results of cotyledon bioassay showed, alginate-degraded products of different size could elicit the

accumulation of phytoalexin but the intact alginate with high molecular weight could not. AOs with DP 13.9 showed the maximal elicitor activity, which was used as the following examination (Fig 4).



(a) Screening alginate lyase producing strains by hydrolytic halos. (b) Screening alginate lyase producing strains by the supernatant of culture adding CPC. (c) The extracellular alginate lyase activity quantitatively analysis. (d) The intracellular alginate lyase activity quantitatively analysis.

Fig. 1. Screening alginate lyase producing strains



Lanes: M, Protein molecular weight marker (97.2, 66.4, 44.3, 29.0, 20.1, 14.3 kDa ); Lane 1: uninduced *E. coli* containing pET28a/algM; lane 2, induced *E. coli* containing pET28a/algM; lane 3, Supernatant of AlgM recombinant cells lysate; lane 4, pellet fraction of recombinant cells lysate; Lane 5: uninduced *E. coli* containing pET28a/algK; lane 6, induced *E. coli* containing pET28a/algK; lane 7, Supernatant of AlgK recombinant cells lysate; lane 8, pellet fraction of recombinant cells lysate

Fig. 2. SDS-PAGE analysis of recombinant AlgM and AlgK

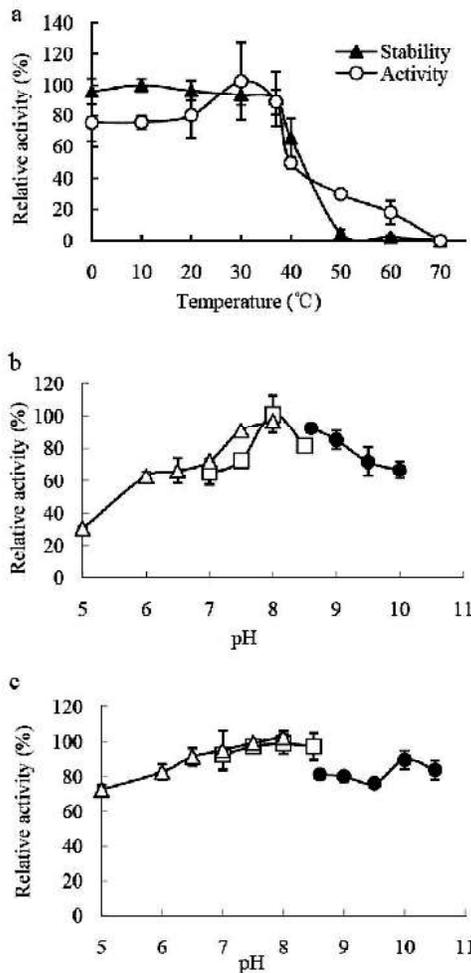


Fig. 3. (a) Effect of temperature on the activity and stability of AlgK. (b) Effect of pH on the activity of AlgK. (c) Effect of pH on the stability of AlgK

**AOs improve the germination and growth of the rice seed**

The AOs at different concentrations had different effects on the germination of the rice seed. At low concentration, the AOs treatment showed no different act on the germination of the rice seed as the control. With the increasing concentration of AOs, the stimulating effect on the root and shoot was enhanced, especially on the growth of the root. Maximum promotion was observed at concentration of 3 g/L (Table 3). When the AOs concentration reached 5 g/L, the stimulating function began to decline, but the root growth is still significantly stronger than the control.

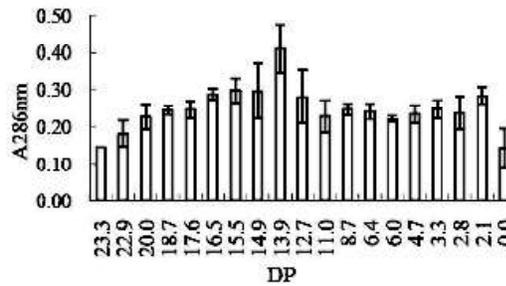


Fig. 4. Effect of AOs with different DP on the soybean cotyledon bioassay.

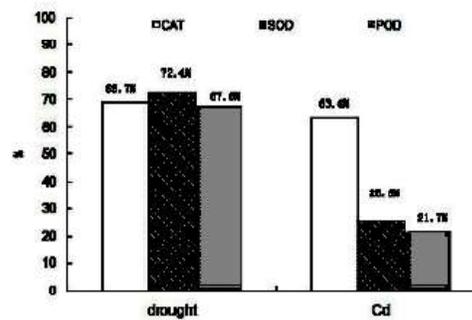


Fig. 5. Effects of AOs on the CAT, POD, SOD activity of rice seedling after drought stress and Cd stress treatment

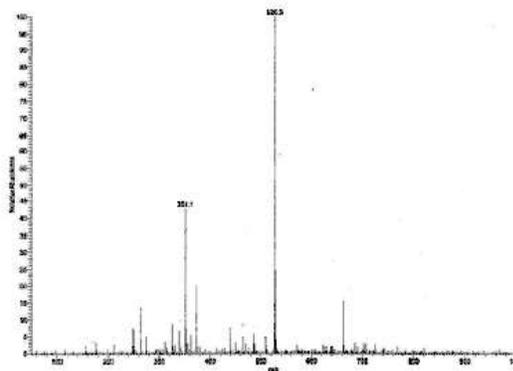


Fig. 6. ESI-MS analysis of AOs

**AOs increase CAT, POD and SOD levels of rice seedlings under drought stress and Cd stress**

Fig 5 shows the effects of AOs treatments on the CAT activities in rice seedlings during the experiment. During 4 d drought stress, the activity of CAT in treatment groups was higher than those in the control group. In the drought environment, AOs treatments were more conducive to rice

seedlings to produce CAT than distilled water treatment. 3 g/L AOs made the rice seedlings produce more 68.7% CAT than control group. The POD activities increased 72.4% than control group. 3 g/L AOs induced the rice seedlings to produce more POD to resist the poor environmental conditions (Fig 5). The AOs processing makes rice produce SOD 67.7% more than control group, and relieves seedlings from drought damage.

Cd stress induced higher CAT activity in AOs treatment groups than in control group. CAT activity increased 63.4% after Cd stress of 5 days compared with the control. AOs prevented damage of heavy metals to rice to some extent. With the 3 g/L AOs treatment, it was more advantageous for the rice seedlings to be able to adapt to the toxic environmental condition. 3g/LAOs treatments were more advantageous to the rice seedling in the production of POD. On the 5th day of Cd treatment, the POD activity and SOD activity of AOs groups increased 25.6% and 21.7% than those in the control group, which helped plants to adapt to heavy metal exposure.

#### The molecular mass of AOs

The AOs (DP 13.9) were desalted on Sephadex G-25 column. The collection of elution with the highest absorbance at 235 nm was diluted in MeOH-H<sub>2</sub>O (1:1) and analyzed by ESI-MS (Fig 6). The results demonstrated that the major peak was trimer. The peak of dimer on ESI-MS was too small. It could be deduced that trimer were the main products with DP of 13.9, and were the main component of AOs which lead to variety of improving effects on rice.

### DISCUSSION

Alginate lyase producing bacterial strains from diverse environmental samples were screened. Surprisingly, we found the strains which grow on the screening plate were not always the alginate lyase producing strains probably because sodium alginate is unstable and may be degraded into oligosaccharides during autoclaving. Experiments show that there was a small amount of reducing sugar produced in the screening medium after high-temperature autoclaving (data not shown). To identify the lyase activity, two kinds of re-screening methods were used, one by agar plate and one by liquid reaction. The liquid reaction method

screening for the candidates with lower turbidity by the naked eyes avoids the interference from agar degrading strains and some faint halos appeared on agar plate. So four alginate lyase producing bacterial strains were isolated and this method is more rapid and effective for screening.

The amino acid sequence of AlgK had the same amino acid sequence as Aly of *K. pneumoniae* subsp. *aerogenes* type 25. As Baron reported<sup>29</sup>, the gene of Aly was cloned, then connected to the plasmid pBluescript (SK-), and expressed into *E. coli* DH1. The molecular masses of protein Aly was 31.4 kDa. The activity of the Aly was 0.27 U/ml. In this study, the gene sequence of AlgK was ligated to the expression vector pET-28a with the strong promoter from T7 bacteriophage, then, transformed into host strain *E. coli* BL21 (DE3). The activity of recombinant AlgK was 8245 U/ml. The specific activity of recombinant AlgK was 1640.1 U/mg, much higher than that of recombinant HdAly<sup>30</sup> with 1190 U/mg and rA9mT<sup>10</sup> with 1401 U/mg under the same definition of enzyme specific activity.

The phytoalexin accumulation in soybean cotyledon is related to the average DP of AOs. In this study, the maximal phytoalexin production was observed by using AOs of DP 13.9 as elicitor. But in An's research<sup>13</sup>, AOs with DP 6.8 induced the maximal production of phytoalexin. Total sugar was determined by the phenol-sulfuric acid method in An's report. The different detection method of total sugar may account for certain difference.

The AOs produced by enzymatic method displayed a variety of benefits for the growth of plants in this study, including increasing germination rate and speed<sup>14</sup>, improving resistance to drought and heavy metal stress. This is consistent with other reports<sup>4,16</sup> which implies AOs of special structure may have important applications in farming agriculture. In fact, AOs also were reported to have many important roles to health including improving immunity, intestinal microbial community, anti-infection and anti-tumor<sup>31</sup>. So the demand of AOs for agriculture, pharmaceutical and other biotechnology uses will increase quickly considering their versatile biological roles. Alginate lyase is sure the key in the processing of huge alginate resource to AOs of highly characterized preparations.

This study obtained recombinant strain

which expressed alginate lyase much more than wild strain, and also examines the effect of AOs produced by the enzyme preparation on plant growth and stress resistance, providing basis for the applications of alginate lyase and AOs in the agricultural and environmental governance. It's useful for exploiting the green plant nutrient and growth regulator in the future.

### CONCLUSION

Alginate lyase gene *algK* and *algM* were cloned. The activity of recombinant AlgK (1640.1 U/mg) was higher than that of recombinant AlgM (36.7 U/mg). The AOs with DP 13.9 showed the best elicitor activity stimulating the accumulation of phytoalexin in wounded soybean cotyledon. The 0.30% concentration of AOs with DP 13.9 displayed the best effect on promoting germination and root development of rice seedlings, as well as a greater advantage for the rice seedlings to adapt to drought resistance and Cd stress. The main component of AOs with DP 13.9 was trimer.

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