

# Alginate Lyase of a Novel Algae Fermentation Strain



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A novel algae fermentation strain was obtained in our previous work. This strain can produce alginate lyase and alcohol dehydrogenase used for the ethanol fermentation from algae. This research investigated the fermentation, separation and purification of alginate lyase, and the molecular weight of alginate lyase was determined. The optimum conditions for enzyme fermentation were as follows: fermentation medium with 20 g L<sup>-1</sup> alginate, initial pH 6.0, and temperature 35 °C. The flasks were cultured in a shaking incubator at 120 rpm for 96 h. The enzyme was purified using the method of salting out, dialysis, and gel chromatography. After purification, the SDS-PAGE method was used to determine the molecular weight of the protein. The molecular weight of alginate lyase was 30–35 KDa. This research contributes to algae biodegradation and fuels production from algae.

## Keywords:

ethanol, brown algae, alginic acid

## Introduction

Bio-ethanol has long been attracting worldwide attention and many efforts have been made to search for alternative biomass sources for the production of bio-ethanol, such as corn<sup>1</sup>, wood<sup>2</sup>, sugarcane<sup>3</sup>, switch grass<sup>4</sup>, rice straw<sup>5</sup>, corn straw<sup>6</sup>, and wheat straw<sup>7</sup>. Today, about 30 % of corn is used for ethanol production, and more corn is required to meet the increasing demand for bio-ethanol<sup>8</sup>. The higher amounts of corn used for biofuel production could generate devastating effects on food supply around the world. Ethanol production from lignocellulose is a promising alternative, but the current technologies for lignocellulose fermentation face the problems of high cost and complex processes required to release simple sugars from recalcitrant polysaccharides<sup>9</sup>.

Marine algae are attractive renewable energy resources due to their abundance, high photosynthetic efficiency, and high production rate<sup>10</sup>. Algae do not contain toxins, and are easy to degrade biologically. Therefore, they are likely to become one of the most promising biomass fuels in the future<sup>11</sup>. Brown algae, as the second most abundant marine biomass, have several key features of an ideal feedstock for biofuel production. Brown algae do not need arable land, fertilizer, or fresh water; they pos-

sess high photosynthetic efficiency and have a high production rate, are free of land management and have beneficial impacts on food supplies<sup>12</sup>, making them an attractive alternative resource.

Brown algae contain about 30 %–67 % carbohydrate by dry weight, and the main components of polysaccharides are alginate, laminaran, and mannitol<sup>13</sup>. Laminaran and mannitol can be easily used by microbes and converted into bio-ethanol, but industrial microbes cannot degrade alginate as carbon source. The potential of using brown algae to produce bio-ethanol cannot be fully exhibited<sup>14–17</sup>. To gain full use of alginate, a series of lyases are required, such as alginate lyase. In order to solve the problem of biological degradation of brown algae, the utilization of alginate is necessary.

Alginate lyase has a variety of sources, including algae, marine animals and microorganisms. The microbial source of alginate lyase is rich, including *Pseudomonas aeruginosa*<sup>18</sup>, *Klebsiella pneumoniae*<sup>19</sup>, *Vibrio* sp.<sup>20</sup>, *Azotobacter chroococcum*<sup>21</sup>, *Alteromonas* sp.<sup>22</sup>, *Sphingomonas* sp.<sup>23</sup> and *Flavobacterium* sp.<sup>24</sup> In our previous research, a novel algae fermentation strain was obtained, and it was 99 % identical to *Meyerozyma guilliermondii*<sup>25</sup>. The experiment was conducted on the ethanol fermentation of this strain. In addition, this strain can produce alginate lyase and ethanol dehydrogenase. At present, the research on the fermentation conditions, separation, and purification of the alginate lyase of *Meyerozyma guilliermondii* is still lacking. The re-

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search of alginate lyase will promote the research of algae biodegradation and fuels production from algae. In addition, alginate oligosaccharides are the products of alginate after degradation by alginate lyase. In recent years, much attention has been paid to the physiological effects of alginate oligosaccharide and its medicinal activity.

In this research, the fermentation conditions of *Meyerozyma guilliermondii*, the separation and purification process of alginate lyase, and the molecular weight determination of alginate lyase were investigated.

## Materials and methods

### Materials

All chemicals of analytical reagent grade were purchased from Beijing Chemical Factory (Beijing, China). All the biochemical reagents were bought from Beijing Biological Technology Factory (Beijing, China). The strains were stored in the tube culture at 4 °C in glycerol solution<sup>26</sup>. General biological equipment was purchased from Shanghai Precision Instrument Co., LTD (Shanghai, China).

### Fermentation experiments

The enzyme fermentation experiments were carried out in 150-mL flasks with 30 mL working medium. The composition of fermentation culture medium was as follows: 5.0 g L<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.0 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 2.5 g L<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O, 5.0 g L<sup>-1</sup> brewer's yeast extract, and different concentrations of alginate. All the flasks were cultured in a shaking incubator. The stored bacterial solution was firstly poured into the liquid medium (1 % peptone, 1 % alginate, and 1 % brewer's yeast extract), and cultured for 48 h to obtain the seed liquid. The seed liquid was then inoculated into fermentation medium with the inoculum amount of 5 %. Investigation was performed on the effects of temperature, initial pH, initial alginate concentration, shaking speed, and fermentation time on enzyme activity. Experiments were performed in the fermentation medium with temperature ranging from 20 °C to 40 °C, initial pH from 5 to 9, initial alginate concentration from 5 g L<sup>-1</sup> to 25 g L<sup>-1</sup>, shaking speed from 0 to 200 rpm, and fermentation time from 0 to 120 h.

### Separation of alginate lyase

The fermentation liquid was treated with J2-HS Beckman high-speed refrigerated centrifuge at 4 °C, 7168 g-force for 20 min. The supernatant fluid was crude enzyme and kept at 4 °C.

### Purification of alginate lyase

The crude enzyme obtained in the previous step was then put in an ice bath, and ammonium sulfate was added into the enzyme solution to reach a saturation varying from 20 % to 70 % under the action of magnetic stirrer. After standing for 10 min, the solution was centrifuged at 16128 x g for 20 min. The precipitate was collected and then dissolved in 1 mL phosphate buffer saline (PBS), and transferred to 1.5-mL tube. The enzyme mixture was then treated by dialysis method. The dialysis bags were cut into short pieces of desired length (10–20 cm), and put in 1 mmol L<sup>-1</sup> EDTA (pH 8.0) solution, and boiled for 10 min. Later, the bags were cooled to 4 °C and the enzyme solution was then placed in the dialysis bag. The dialysate was PBS solution (pH 6.0 40 mmol L<sup>-1</sup>) and was regularly replaced by fresh solution after 4 h, 8 h, and 14 h. After the last replacement, the dialysis continued for 2 h.

An amount of 400 mL enzyme solution after salting out and dialysis was prepared. The enzyme liquid then passed through the gel column for purification with the column material of G-100 Sephadex, and elution solution was PBS solution (pH = 6, c = 40 mmol L<sup>-1</sup>) with the flow rate of 1 mL min<sup>-1</sup> controlled by a peristaltic pump, then the fractions of different elution times (5–15 min, 15–30 min, 30–45 min, 45–60 min, 60–90 min, 90–120 min) were collected.

Sephadex G-100 gel dry particles were suspended in fivefold to tenfold the amount of elution solution (pH 6.0, 40 mmol L<sup>-1</sup> PBS) for full swelling, and the mixture was then heated in a boiling water bath for 1–2 h to achieve the full expansion of the gel.

### Analytical methods

Alginate lyase was obtained and the enzyme activity was measured according to the previous work<sup>27</sup>. An amount of 0.5 mL enzyme solution was added to a test tube containing 0.5 mL 0.5 % sodium alginate substrate (sodium alginate was dissolved in 0.05 mol L<sup>-1</sup> phosphate buffer solution, pH of 7.5), then mixed and bathed in water at 40 °C. The enzyme solution in the control group was replaced by deionized water and the increase in reducing sugar in the reaction solution was taken as the index of activity. A unit of enzyme activity is defined as the amount of enzyme required to produce 1 mg reducing sugar per minute under the above conditions. Each enzyme activity test was repeated three times.

The SDS-PAGE method was used to determine the molecular weight of protein. The chromogenic agent was 0.05 % Coomassie brilliant blue (R-250). The samples were added to the sample chamber by

microinjector. After covering the lip, the electrophoresis apparatus switch was turned on. Before the sample reached the gel, the current was controlled at 15–20 mA for about 15–20 min, and when the blue indicator reached the gel, the current was kept stable at 30–45 mA until the end of the electrophoresis process. When the blue indicator moved to the edge of the frontier, the electrophoresis was stopped. The biomass of yeast was measured according to the previous work<sup>28</sup>. The protein concentration was measured by the Bradford method. All experiments were repeated three times.

## Results and discussion

### Fermentation parameters of alginate lyase

In this research, different fermentation parameters, such as temperature, initial pH, initial alginate concentration, shaking speed, and fermentation time were varied in the production of alginate lyase.

The control of fermentation temperature during the alginate lyase production process was particularly important. Microorganisms' metabolism enzymes are sensitive to temperature<sup>17</sup>. In the appropriate temperature range, higher temperature could enhance the enzyme activity, thus increasing the production rate and improving the final yield, but too high temperature would destroy the structure of enzymes and other biological macromolecules, resulting in a decrease in the reaction rate. The fermentation temperature also affected other fermentation parameters, such as the concentration of dissolved oxygen. In this fermentation experiment, different temperatures were set, ranging from 20 °C to 40 °C. An amount of 20 g L<sup>-1</sup> alginate was used as the carbon source, and the initial pH of the fermentation medium was 5. All the flasks were cultured in a shaking incubator at 120 rpm for 72 h. As shown in Fig. 1, temperature directly affected the enzyme production. At the temperature of 30–35 °C, the strain had stronger enzyme activity, and beyond this range, the enzyme activity decreased sharply. At 35 °C, the highest activity was reached at 45.2 U mL<sup>-1</sup>. This optimal temperature was different from the optimal temperature of the yeast growth and the optimum temperature of ethanol fermentation<sup>25</sup>.

Carbon source is very important in the production of enzyme, and is the provider of cell carbon and metabolism energy<sup>29</sup>. In the fermentation process, carbon source helps build microbial cell and provides energy for the activity of microbial life. Carbon source can also act as enzyme inducer. In this experiment, *Meyerozyma guilliermondii* was isolated from the natural source using alginate as the only carbon source. The growth and metabolism of the strains were closely related to the content of alginate. During the process of ethanol fermenta-

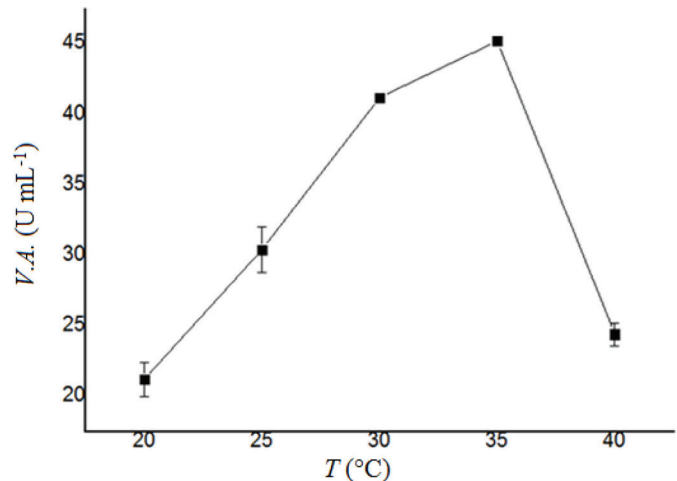


Fig. 1 – Effect of temperature on the enzyme activity (with pH 5, alginate concentration 20 g L<sup>-1</sup>, rotation rate 120 rpm, and fermentation time 72 h)

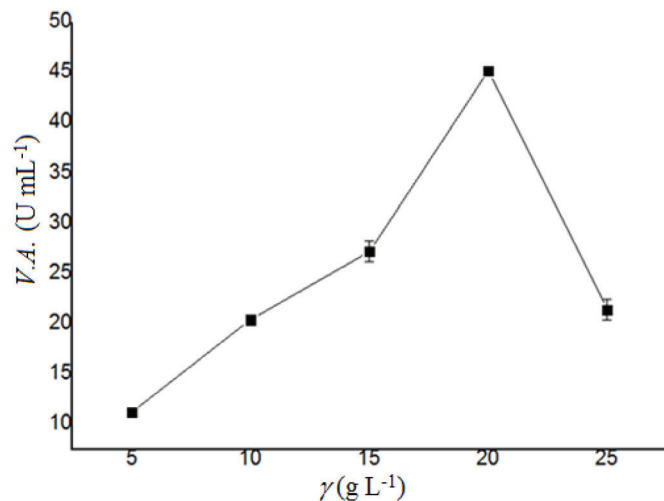


Fig. 2 – Effect of alginate concentration on enzyme activity (with pH 5, temperature 35 °C, rotation rate 120 rpm, and fermentation time 72 h)

tion, alginate lyase was the first key enzyme in the metabolic process. Alginate concentration was set as 5 g L<sup>-1</sup>, 10 g L<sup>-1</sup>, 15 g L<sup>-1</sup>, 20 g L<sup>-1</sup>, and 25 g L<sup>-1</sup>, respectively, at the fermentation temperature of 35 °C. The initial pH of the fermentation medium was 5. All the flasks were cultured in a shaking incubator at 120 rpm for 72 h. The results are shown in Fig. 2. It was observed that alginate substrate had a significant effect on enzyme production. The highest enzyme activity was found at the substrate concentration of 20 g L<sup>-1</sup>, but at the substrate concentration of 25 g L<sup>-1</sup>, the enzyme activity weakened. The high concentration of sodium alginate could have thickened the culture medium, thus affecting the mass transfer and cell growth, which further affected the enzyme production<sup>30</sup>. On the other hand, the enzyme activity was directly related to the alginate concentration, indicating that the alginate lyase was inducible enzyme<sup>31</sup>.

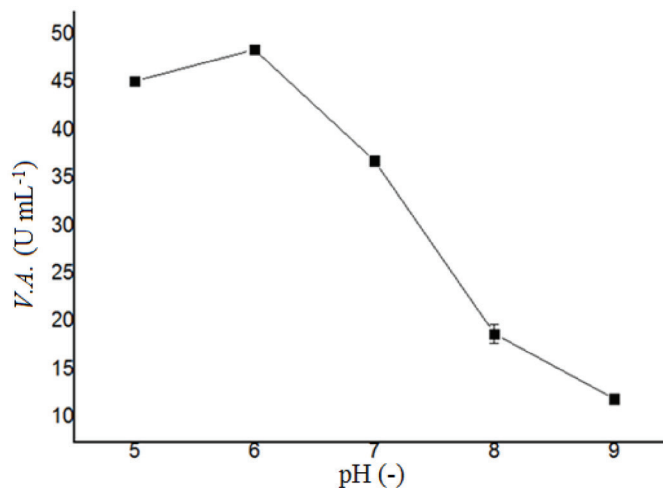


Fig. 3 – Effect of pH on enzyme activity (with temperature 35 °C, alginate concentration 20 g L<sup>-1</sup>, rotation rate 120 rpm, and fermentation time 72 h)

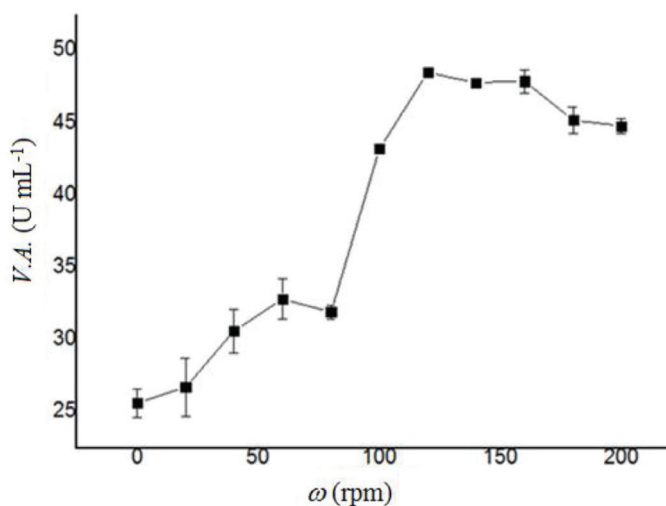


Fig. 4 – Effect of rotation rate on enzyme activity (with pH 6, alginate concentration 20 g L<sup>-1</sup>, temperature 35 °C, and fermentation time 72 h)

During the fermentation process, the yeast cell was very sensitive to the pH of the medium<sup>32</sup>. In our previous research<sup>25</sup>, pH 5 was optimal for *Meyerozyma guilliermondii* fermentation to produce ethanol. In general, yeasts prefer slightly acidic environment. Nevertheless, the optimum pH values of enzyme fermentation and cell growth are often different. In order to optimize the pH value in the enzyme fermentation process, fermentation experiments were conducted under the following conditions: inoculum amount of 5 %, fermentation temperature of 35 °C, and initial sugar concentration of 20 g L<sup>-1</sup>. The pH was increased from 5 to 9. All the flasks were cultured in a shaking incubator at 120 rpm for 72 h. The influence of pH on enzyme activity is shown in Fig. 3. As may be seen from Fig. 3, the pH value of 5.0–6.0 was found to be suitable for the enzyme fermentation, which was simi-

lar to ethanol fermentation<sup>25</sup>. The pH value could affect the enzyme activity, which could improve the fermentation process. The enzyme activity of alginate lyase was related to the yield of ethanol fermentation.

The shaking intensity is related to the mass transfer efficiency within the fermentation broth, the efficiency of oxygen transfer, and the efficiency of heat transfer. Oxygen is very important for the growth and reproduction of cells<sup>33,34</sup>. In order to optimize the shaking speed in the enzyme fermentation process, fermentation experiments were conducted with the inoculum amount of 5 %, fermentation temperature of 35 °C, and initial sugar concentration of 20 g L<sup>-1</sup>. The initial pH of the fermentation solution was 6. All the flasks were cultured in a shaking incubator for 72 h. The rotation speed was set at 0 to 200 rpm. The influence of shaking intensity on enzyme activity may be found in Fig. 4. Enzyme activity was determined after the microorganism fermentation. As may be seen from Fig. 4, when the shaker rotated at 120–160 rpm, the enzyme activity was relatively strong. However, there were no great differences in enzyme activity after fermentation at different shaking speeds. Thus, the rotation speed of the shaker might not be a prominent factor. In addition, in this experiment, the volume of the fermentation solution in the flask was small, so the rotation speed had little effect on the efficiency of enzyme production. In the following experiments, the rotation speed was set to 120 rpm.

During the microbial fermentation process, a constant decrease was observed in the concentration of nutrient in the culture medium. The microbial fermentation product was obtained, and then the concentration reached a maximum value. However, with the trend of aging cell autolysis, the production capacity was reduced accordingly. The fermentation time was also very important for improving the utilization rate of the equipment<sup>30</sup>. In this experiment, in order to optimize the fermentation time, the experiments were conducted under the following conditions: inoculum amount of 5 %, fermentation temperature of 35 °C, and initial sugar concentration of 20 g L<sup>-1</sup>. The initial pH of the fermentation solution was 6. The rotation speed was set at 120 rpm. The fermentation time was from 24 h to 120 h. The effect of fermentation time on enzyme activity is shown in Fig. 5. Enzyme activity was determined after microorganism fermentation. As may be seen from Fig. 5, the highest enzyme activity was observed at the fermentation time of 96 h. The enzyme activity increased with fermentation time increasing from 24 h to 96 h, and for even higher fermentation time, a decline was observed in activity because of the higher substrate consumption.

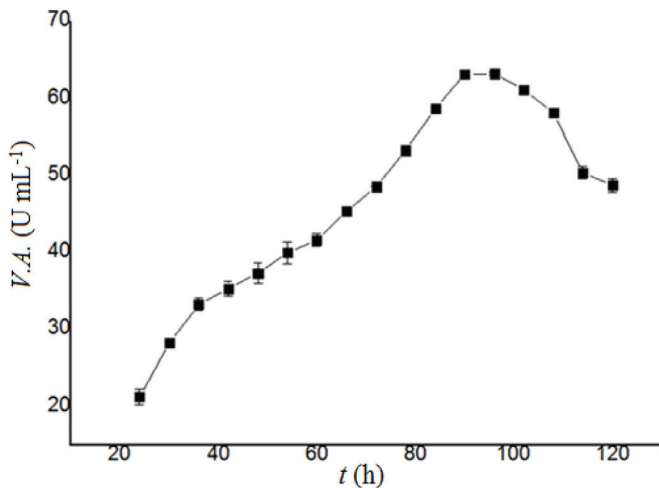


Fig. 5 – Effect of fermentation time on enzyme activity (with pH 6, alginate concentration 20 g L<sup>-1</sup>, rotation rate 120 rpm, and temperature 35 °C)

Table 1 – Gel chromatography results at different periods

Time (min)	Enzyme activity, $c_U$ (U mL <sup>-1</sup> )	Standard deviation
5–15	49.2	2.4
15–30	148.5	3.1
30–45	101.7	2.1
45–60	53.8	3.4
60–90	29.5	1.6
90–120	10.3	1.9

In the fermentation process, to obtain high-quality fermented products and lower production costs, there was a need to determine the optimal conditions for the growth and fermentation of the strains. In this research, according to the experiments, the optimum conditions for enzyme fermentation were as follows: fermentation medium of 20 g L<sup>-1</sup> alginate, initial pH of 6.0, and temperature of 35 °C. The flasks were cultured in a shaking incubator at 120 rpm for 96 h.

### Separation and purification of alginate lyase

In this research, the separation and purification of alginate lyase were also carried out.

The enzyme was purified using the method of salting out, dialysis, and gel chromatography. The crude enzyme solution obtained by centrifugation was put in the ice bath and ammonium sulfate was added to reach the saturation from 20 % to 70 % with respect to ammonium sulfate under the action of magnetic stirrer. After standing for 10 min, the solution was centrifuged at 16128 x g for 20 min. The precipitate was collected and then dissolved in 1 mL PBS, and transferred to 1.5-mL tube. Then the

enzyme mixture was treated by dialysis method. After the dialysis, measurement was performed on the enzyme activity for samples prepared under different saturation levels, and the samples produced at the saturation of 30 % to 60 % were collected for further investigation because high enzyme activity was observed within this concentration range. Under different saturation levels, different kinds of proteins precipitated. In other papers<sup>35</sup>, the saturation levels from 35 % to 60 % were selected for the salting out treatment of alginate lyase.

The residual liquid in the dialysis bag was used for the following purification process. Gel chromatography method is a kind of filtration based on molecular weight. The result of alginate lyase activity is shown in Table 1. The enzyme activity of the fraction eluting in 15–30 min was the strongest and reached 148.5 U mL<sup>-1</sup>. Through chromatography purification, the enzyme activity had increased by 2.35 times compared with the original one.

### Molecular weight determination of alginate lyase

The mobility of proteins in polyacrylamide gel electrophoresis is determined by many factors, such as charge, size and shape of molecules. In this research, the fermentation solution was treated by centrifugation to obtain crude enzyme, followed by the separation and purification of the enzyme. After purification, the SDS-PAGE method was used to determine the molecular weight of proteins. The molecular weights of protein standard Marker (MarkerMK034) were 18.4 kDa, 25 kDa, 35 kDa, 45 kDa, 66.2 kDa, and 116.2 kDa. It was found that the molecular weight of alginate lyase was 30–35 KDa, which was similar to the result in previous research<sup>36</sup>. The molecular weight of alginate lyase produced by marine microorganisms was 24–110 KDa, as reported in the previous work<sup>37</sup>. The alginate lyase produced by yeast fell within this range as well.

### Conclusion

Brown algae, as the second most abundant marine biomass, are an ideal raw material for bio-ethanol production. In order to solve the problem encountered in the ethanol production from brown algae, the utilization of alginate is necessary. Therefore, the research of alginate lyase is highly significant. In our previous research, a novel algae fermentation strain was obtained, and it was 99 % identical to *Meyerozyma guilliermondii*. This strain could produce alginate lyase and ethanol dehydrogenase at the same time. At present, the fermentation conditions, separation, and purification of the

alginate lyase of *Meyerozyma guilliermondii* requires in-depth investigation. Therefore, in this research, the fermentation, separation and purification of alginate lyase were investigated, and the molecular weight of alginate lyase was determined. According to the experiments, the optimum conditions for enzyme fermentation were as follows: fermentation medium of 20 g L<sup>-1</sup> alginate, initial pH of 6.0, and temperature of 35 °C. The flasks were cultured in a shaking incubator at 120 rpm for 96 h. The crude enzyme solution was then obtained by centrifugation of fermentation broth. The enzyme was purified using the method of salting out, dialysis, and gel chromatography. After purification, the SDS-PAGE method was used to determine the molecular weight of the protein. The molecular weight of alginate lyase by *Meyerozyma guilliermondii* was 30–35 KDa.

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