# Optimized Extraction Process and Compositional Analysis of Bioflocculant Produced by *Klebsiella* M1

Huan Diao,[a,b] Lvmu Li,[a,c,*] Jun Liang,[a] Chen Lu,[c] Xiaoling Ding,[c] and Wenjie Guo [d]

The extraction process was optimized and compositional analysis was carried out for bioflocculant produced by *Klebsiella* M1. Single factor and response surface tests were adopted to optimize extraction of the bioflocculant. Physical and chemical tests were performed to qualitatively and quantitatively determine the chemical components of the bioflocculant. The molecular structure was analyzed by ultraviolet spectroscopy, Fourier transform infrared spectroscopy, gas chromatography, nuclear magnetic resonance spectroscopy, gel chromatography, and scanning electron microscopy. The optimum extraction conditions for the flocculant produced from *Klebsiella* M1 bacteria were as follows: anhydrous ethanol as the extractant, a material to liquid ratio of 1.54:1, pH of 9.06, and an extraction time of 12 h. The flocculant yield was up to 3.91 g/L. The bioflocculant was composed mainly of polysaccharides (65.9%) and proteins (19.7%). The polysaccharide molecular weight was $4.78 \times 10^6$ D, and it was comprised of L-rhamnose, L-arabinose, L-fucose, D-xylose, D-mannose, D-glucose, and D-galactose at the ratio 0.29:0.36:1:0.31:0.47:0.57:1.01. The polysaccharide contained hydroxyl and oxygen-containing functional groups ($\delta$ = 3.5 to 4.5 ppm) and aromatic groups ($\delta$ = 7 ppm). Thus, the bioflocculant produced by M1 bacteria was a polysaccharide type and anhydrous ethanol was a suitable extractant.

*Keywords: Klebsiella M1; Bioflocculant; Response surface optimization; Bioresources; Polysaccharide*

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## INTRODUCTION

A microbial flocculant is a type of active flocculant substance that is produced by certain microorganisms under specific culture conditions. It is considered to be a new, efficient, and inexpensive water treatment agent, which is produced by microbial fermentation, extraction, and purification processes (Wei *et al.* 2006). Compared with traditional inorganic and organic polymer-based synthetic flocculants, microbial flocculants possess the characteristics of biodegradability, safety, non-toxicity, non-secondary pollution, a wide range of applications, good thermal stability, acid and alkali adaptability, strong turbidity removal efficiency, and good decolorizing performance (Jia and Yu 2012).

Wastewater from food processing enterprises contains a large amount of organic matter and suspended matter (Cao *et al*. 2005). Similarly, tannery wastewater contains a large number of organic pollutants and hard-to-degrade substances such as tannin, and it was also a high chromaticity (Zhu 2015).

Dipdye wastewater also contains heterocyclic organic compounds such as phenols and aromatic hydrocarbons, which are usually characterized by high chromaticity, complex composition, high alkalinity, and obvious biological toxicity (Ma 2013). However, the composition of wheat alcohol wastewater is also very complex. The chemical oxygen demand concentration (COD) is up to $5 \times 10^4$ mg/L, the suspended solid concentration is $1 \times 10^4$ mg/L, and the concentration of total nitrogen is approximately 1000 mg/L. Therefore, these types wastewater typically have complex components, high chroma, high COD, and they are difficult to treat.

Currently, such wastewater is treated mainly by a chemical flocculant-polyacrylamide combined with air flotation and anaerobic fermentation. The polyacrylamide dosage used is approximately 10 ppm to 15 ppm, the cost of the treatment is approximately 0.2 ¥/t, and the treatment works well at Anhui Ruifuxiang Enterprise. However, polyacrylamide can be retained by the human body, and residual monomeric acrylamide is a neurotoxic agent that may cause cancer. After poisoning, the body shows weakness, dyskinesia, and other symptoms (Yokoi *et al*. 1997; Rudén 2004). In addition to polyacrylamide, chemical flocculants such as aluminium sulfate (alum) and iron salts are often used as coagulants in wastewater treatment, but there are some deficiencies.

Therefore, it is necessary to develop a safe, efficient, and environmentally compatible flocculant to treat wheat distillery wastewater. Recently, microbial flocculants have been the subject of much research activity (Liu *et al*. 2013) because of their non-toxic and environmentally compatible characteristics. Bioflocculants have been successfully used to treat many different wastewaters from food processing, printing and dying, mineral extraction, and dairy processes (Wang *et al*. 2007; Okaiyeto *et al*. 2015), but no research has been conducted on wheat distillery wastewater (Agunbiade *et al.* 2017; Li *et al.* 2017). The authors recently screened the flocculant-producing bacterium M1 (preserved number CCTCC M 2018098) from a sedimentation tank at Anhui Ruifuxiang Co. Ltd. (China). The experimental results showed that M1 belongs to the genus *Klebsiella*. It has a high flocculating activity for wastewater and its flocculation degree was up to 82.0% (Diao *et al*. 2018).

The flocculation was affected not only by the extractant, extraction temperature, pH, and extraction time, but also by the interaction of those factors (Liao *et al*. 2018). Therefore, an optimized extraction process is important for improving the product yield (Chen 1996; Chow *et al*. 2003). Additionally, the differences in the compositions and structures of bioflocculants can lead to different flocculating characteristics. Two such examples are polysaccharide flocculants, which are composed mainly of polysaccharides and have a good tolerance to temperature, enzymes (Hu and Gao 2007; Li *et al.* 2013), and protein flocculants, which are composed mainly of proteins and have a poor tolerance to temperature and enzymes (Takeda *et al*. 1992). Therefore, it is important to study the physicochemical properties and structural compositions of flocculants (Li *et al.* 2010; Zhang *et al*. 2013).

This study aimed to optimize the extraction process of the flocculant produced by *Klebsiella* M1, examine its physicochemical properties to obtain a higher bioflocculant extraction yield, determine its structural composition, and establish a scientific basis for the study of the flocculation mechanism.

## EXPERMENTAL

### Materials

*Preparation of the extraction liquid and crude extraction of the bioflocculant*

The *Klebsiella* M1 seed solution (Anhui Xinhua University Laboratory 613, HeFei, China) was first inoculated in a glucose medium (1% inoculation volume) and then fermented at 30 °C at 150 rpm for 48 h. Then, following the method of Luo *et al.* (2005), the precipitate was extracted and lyophilized to yield the crude bioflocculant.

### Optimization Methods for the Bioflocculant Extraction Process

*Single factor optimization*

Single factor optimization was performed by selecting various extractants and optimizing the process with respect to liquid ratio, extraction time, and pH value. Organic solvents were adopted as extractants and included anhydrous ethanol, acetone, methanol, ethyl ether, trichloromethane, and petroleum ether.

The M1 culture solution was centrifuged for 15 min at 8000 g, and the supernatant liquid was collected. It was then mixed with an extractant at double the volume. This was repeated three times for each solvent. After sitting for 24 h at 4 °C, each sample was centrifuged for 20 min at 8000 g. The supernatant was then discarded, and the precipitate was freeze-dried. In this way, the crude extract product of the bioflocculant was obtained. It was then weighed and the yield was calculated:

$$\textit{Yield of extracted crude microbial flocculant}\ (\mathrm{g/L}) = \textit{dry weight of the crude extract}\ (\mathrm{g}) / \textit{supernatant volume}\ (\mathrm{mL}) \times 1000 \quad (1)$$

To optimize the material to liquid ratio, the optimized extractant results were used. The ratios of the material to extractant were 0.5:1, 0.75:1, 1:1, 1.25:1, 1.5:1, 1.75:1, and 2:1. The other steps were the same as that used for optimization of the extractant.

To optimize the extraction time (h), the optimized results of the material to liquid ratio were employed. The optimum extractant ratio was selected, and the extraction yield was measured for the extraction durations 0 h, 12 h, 24 h, 36 h, and 48 h.

To optimize the pH, the optimized results of the extraction duration were used. The extraction yield was determined for the pH values 5, 6, 7, 8, 9, and 10.

*Response surface methodology for optimization*

Based on the single factor experiments, response surface methodology was used to optimize the extraction process. The classic three factor and three level Box-Behnken experimental design (Design-Expert 8.0.5.0, Stat-Ease Inc., USA) was adopted. The three factors selected to optimize the conditions were the ethanol to extractant ratio (material to liquid ratio; code *A*), pH (code *B*), and extraction duration (code *C*). The three variable levels used were -1, 0, and +1. The test factors and level combinations are shown in Table 1. The bioflocculant yield was the response value obtained by the center combination design.

**Table 1.** Design of the Experimental Factors and Codes

| Variable | Code | Level | | |
|---|---|---|---|---|
| | | -1 | 0 | +1 |
| Ethanol:Extract (Material to Liquid Ratio) | A | 1.375:1 | 1.5:1 | 1.625:1 |
| pH | B | 8.5 | 9 | 9.5 |
| Extraction Duration (h) | C | 9 | 12 | 24 |

## Identification of the Physicochemical Properties of the Bioflocculant

The dry sample of the crude M1 bioflocculant was ground into a fine powder in a mortar. Approximately 0.2 g of the bioflocculant was added to a beaker containing 100 mL of distilled water. The contents were mixed and kept in a water bath at 80 °C about 1 h to dissolve them, and then cooled to room temperature. The protein residues were measured qualitatively by the ninhydrin chromogenic reaction (Xiong *et al.* 2010). The presence of free or binding sugars was detected by the Molisch reaction (Guo 2005). Iodine solution (potassium iodide as the cosolvent) was used to check the starch content of the sample (Mondragón *et al*. 2004). The total sugar content was measured by the phenol-sulphuric acid method (Chaplin and Kennedy 1994), and the Coomassie Brilliant Blue method was used to determine the protein content (Bradford 1976).

## Purification of the Polysaccharides

In this study, the Sevage reagent was used to remove protein. The salt, small amount of organic solvents, and small biological impurities were removed by dialysis. Samples were poured into dialysis bags, tightened, placed in containers filled with ultra-pure water as dialysate, and the dialysate was replaced every four hours to ensure that the small molecular substances were separated. Then, gel chromatography was used to further purify the crude polysaccharides. The gel used was Sephadex G-200 (Beijing Ruida Heng Hui Technology Co. Ltd., Beijing, China), and the chromatographic column used had the dimensions of 40 cm × 25 cm. Approximately 25 g of Sephadex G-200 was boiled in a large beaker for more than 10 h to swell it completely. After loading, 0.9% NaCl was used as the eluent to balance the column. The flow rate of the eluent was 0.6 mL/min, and the sample was collected with an automatic collector (10 mL per tube). The collected samples were concentrated by rotary evaporation and then freeze-dried to obtain the pure polysaccharides. The sulfuric acid-phenol method was used to determine the sugar concentration in the collected sample.

## Structural Analysis of the Polysaccharides

*UV spectral analysis*

The purified polysaccharides were dissolved in distilled water to make a 10 mg/L solution. The ultraviolet (UV) spectral analysis was performed with an enzyme-labeled instrument (Spectra MAX 190, Molecular devices, California, USA) over a wavelength range of 200 nm to 900 nm. The wavelength of the absorption peak was used to determine if the sample contained protein, nucleic acid, and other chemical components.

*FTIR spectroscopy*

Fourier transform infrared (FTIR) spectroscopy (Nicolette is50, Thermo Scientific, New York, USA) was used to analyze the characteristic bonds of the polysaccharides.

Approximately 1.0 mg of pure *Klebsiella* bioflocculant was compressed with KBr to form pellets and the KBR pellets were scanned from 4000 $cm^{-1}$ to 450 $cm^{-1}$.

## Determination of the Monosaccharide Components in the Polysaccharides

*Preparation of the monosaccharide derivatives*

Hydrolysis of the bioflocculant polysaccharide was conducted in accordance with Zhang (2014), and then the derivatives were prepared. The preparation was divided into three groups and each group had one replicate:

(1) Blank group: 10 mg of hydroxylamine hydrochloride and 0.5 mL of pyridine were added to a 5-mL Xilin bottle with a glue plug;

(2) Standard group: 10 mg of each monosaccharide (D-glucose, D-xylose, D-mannose, D-galactose, D-fructose, L-rhamnose, L-fucose, and L-arabinose sugars) were added to make the standard group, and then hydroxylamine hydrochloride (10 mg) and pyridine (0.5 mL) were added;

(3) Test group: hydroxylamine hydrochloride (10 mg) and pyridine (0.5 mL) were added to a Xilin bottle with a rubber plug, and then pure polysaccharide hydrolysate (10 mg) was added.

The above samples were plugged and stirred well to dissolve them. A capping machine was used to seal the bottles with an aluminum lid, the bottles were then placed in a constant temperature water bath at 90 °C for 0.5 h, and then they were cooled to room temperature. Approximately 0.5 mL of acetic anhydride was then added. The water bath and cooling process was repeated, and the samples were membrane filtered (pore size = 0.22 μm). When the filtrate was placed in a 2-mL sample bottle, the monosaccharide derivatization process was complete.

*Standard curve preparation*

The prepared monosaccharide standard samples were added to 0.1-mL sampling bottles, and pyridine (0.9 mL) was added. The monosaccharides were prepared at the concentrations 0.05 mg/mL, 0.1 mg/mL, 0.2 mg/mL, 0.4 mg/mL, 0.5 mg/mL, and 1.0 mg/mL, and used for gas chromatography (GC) analysis. To draw a linear regression diagram, the concentration of each monosaccharide was taken as the abscissa and the corresponding GC peak area was the ordinate. The regression equation and linear correlation coefficient were calculated.

*Conditions of gas chromatography*

The separation column used was a DB-5 (Shimadzu, Kyoto, Japan) with the dimensions 30 m × 0.32 mm × 0.25 μm. The $H_2$ flow rate was 40 mL/min, and the airflow was 400 mL/min. The flow rate of the carrier gas was 19.7 mL/min, the shunt ratio was 20:1, the sample size was 1.0 μL, the inlet temperature was 250 °C, and the flame ionization detector temperature was 290 °C.

*Calculation of the monosaccharide mole ratio*

The concentrations of the monosaccharides in the polysaccharides were calculated using a linear regression equation, and the molar numbers of the monosaccharides were calculated according to Eq. 2 (Bai and Zhang 2011),

$$\mu = (C \times L) / M \quad (2)$$

where *C* is the measured concentration of the monosaccharide (mg/mL), *L* is the volume before sampling (μL), and *M* is the molecular weight of the monosaccharide (g/mol), μ is the number of moles of monosaccharides (mol).

*Molecular weight determination of the polysaccharides*

The molecular weight of the bioflocculants was determined by gel permeation chromatography-refractive index-multi angle light scattering (GPC-RI-MALS) analysis. The detector was a RI/MALS (GPC-RI-MALS, Shimadzu, Kyoto, Japan). The mobile phase was $NaNO_3$ and the flow rate was 0.4 mL/min. The column temperature was 60 °C, the analysis column model was Ohpak SB-806 HQ (Beijing Jingjing times Technology Development Co., Ltd. Beijing, China), and the sampling amount was 100 μL (polysaccharide aqueous solution).

*Observation of the bioflocculant morphology by scanning electron microscope*

The surface morphology of the bioflocculants produced by M1 bacteria was observed with a Hitachi S-4800 scanning electron microscope (Hitachi, Tokyo, Japan), and the characteristics were analyzed to provide a basis for identification of the molecular structure.

*Nuclear magnetic resonance analysis of the polysaccharides*

An Agilent DD2 600 MHz nuclear magnetic resonance (NMR) spectrometer (Palo Alto, USA) was used to obtain the $^1$H-NMR spectra of the polysaccharides. The NMR conditions were as follows: an $^1$H-NMR resonance frequency of 600 MHz, magnetic field intensity greater than or equal to 14.09 T, frequency range of 20 MHz to 600 MHz, and frequency resolution less than or equal to 0.1 Hz to 0.001 Hz.

Approximately 0.05 g of purified bioflocculant polysaccharide was weighed and dissolved in 1.0 mL of $D_2O$ using a hot water bath for 1 h. Then, the solution was transferred to a standard 5.0-mm tube for NMR analysis using tetra-methyl silane (TMS) as the internal standard.

## RESULTS AND DISCUSSION

### Optimized Single Factor Test of the Bioflocculant Extraction

*Effect of different extraction agents on the yield of bioflocculants*

This experiment explored the extraction effects of various extractants, such as anhydrous ethanol, acetone, methanol, *etc.* The results (Table 2) showed that the extracting effects of ethanol, acetone, and methanol were better than those of the other organic solvents studied. The extracting effect of acetone, where the yield was up to 4.27 g/L, was the best, followed by anhydrous ethanol at 3.74 g/L. The yield from methanol was comparatively poor, at only 2.61 g/L. The yields from trichloromethane, ether, and petroleum ether were zero, which made them unsuitable for extraction. Although the ethanol extraction rate was slightly lower than that of acetone, ethanol was more economical and safer. Considering the economic and safety factors, ethanol was more suitable as an extractant.

**Table 2.** Effects of the Different Extractants on the Bioflocculant Yield

| Extractant | Bioflocculant Mass (g) | Yield (g/L) |
|---|---|---|
| Anhydrous ethanol | 0.1868 | 3.74 |
| Acetone | 0.2134 | 4.27 |
| Methanol | 0.1307 | 2.61 |
| Ether | 0 | 0 |
| Trichloromethane | 0 | 0 |
| Petroleum ether | 0 | 0 |

*Effect of different material to liquid ratios on the bioflocculant yield*

Figure 1 shows the results of the material to liquid ratio single factor test on the bioflocculant yield. The yield increased gradually as more ethanol was added, and the greatest yield was obtained at the 1.5:1 ratio.

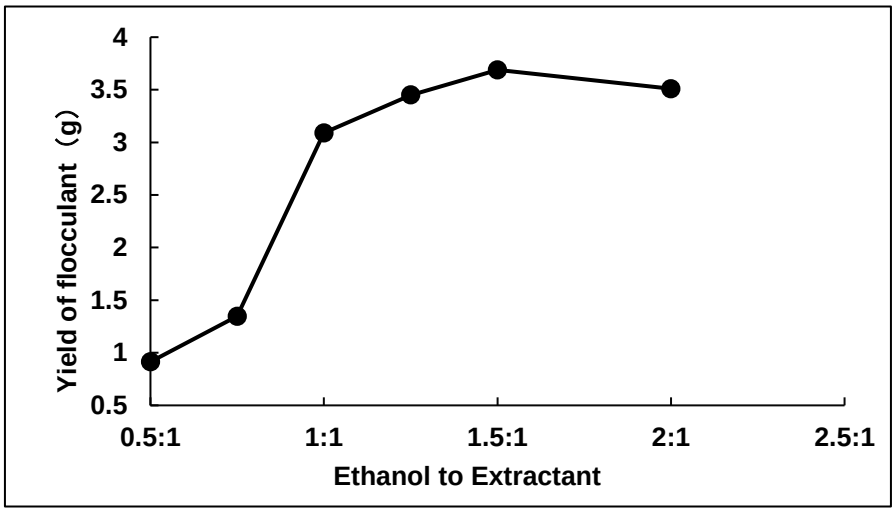



**Fig. 1.** Effect of the material to liquid ratio on the bioflocculant yield

*Effect of the extraction time on the bioflocculant yield*

Figure 2 shows the results of the extraction time single factor test on the bioflocculant yield. The yield was highest when the extraction time was 12 h, and the yield decreased as the extraction time increased.

*Effect of the pH on the bioflocculant yield*

Figure 3 shows the results of the pH value single factor test on the bioflocculant yield. As the pH value increased, the bioflocculant yield increased gradually. The maximum bioflocculant yield was obtained at a pH of 9.

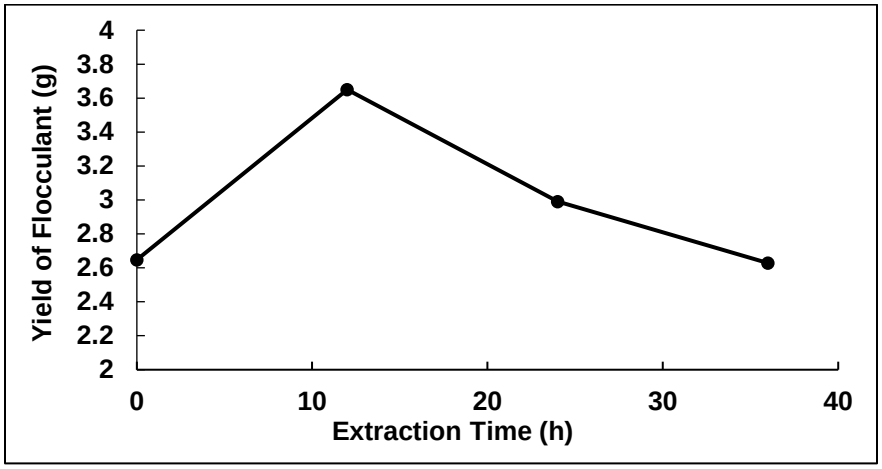



**Fig. 2.** Effect of the extraction time on the bioflocculant yield

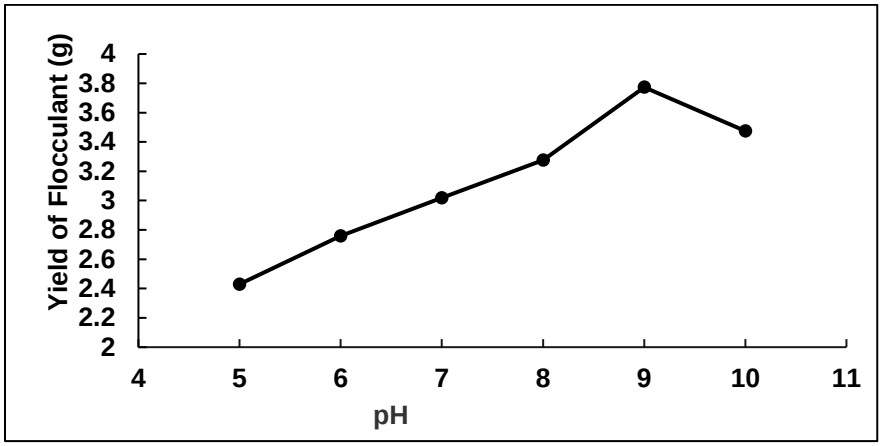



**Fig. 3.** Effect of the pH on the bioflocculant yield

## Response Surface Methodology Optimization for Extracting Bioflocculant

*Formation and analysis of the regression model*

The response surface results of the bioflocculant yield is shown in Table 3. The results were analyzed by a Box-Behnken design, and a regression model of the variance analysis was obtained. The results are shown in Tables 4 and 5. Table 4 shows that the one-term effects $A$ of the model and the two-term effects $A^2$ and $B^2$ were significant. The contribution rate of each factor was as follows: $A > B > C$ ( Extractant > pH > extraction time).

The two polynomial equations for the bioflocculant yield and variables (material to liquid ratio, pH, and extraction time) were obtained by multivariate regression fitting analysis,

$$Y = 3.87 + 0.24A + 0.089B - 0.046C - 0.098AB - 0.051AC + 0.033BC - 0.34A^2 - 0.24B^2 - 3.900E - 003C^2 \quad (3)$$

where $E$ describes "Scientific Numbering" of 2-digit decimals.

**Table 3.** Box-Behnken Response Surface Design and Results

| No. | Code | | | Bioflocculant Yield (g/L) |
|---|---|---|---|---|
| | *A* | *B* | *C* | |
| 1 | 0 | -1 | -1 | 3.616 |
| 2 | +1 | 0 | +1 | 3.702 |
| 3 | +1 | +1 | 0 | 3.483 |
| 4 | -1 | +1 | 0 | 3.246 |
| 5 | -1 | -1 | 0 | 2.894 |
| 6 | 0 | +1 | +1 | 3.699 |
| 7 | 0 | 0 | 0 | 3.973 |
| 8 | 0 | 0 | 0 | 3.899 |
| 9 | -1 | 0 | -1 | 3.242 |
| 10 | 0 | 0 | 0 | 3.973 |
| 11 | +1 | -1 | 0 | 3.522 |
| 12 | -1 | 0 | +1 | 3.276 |
| 13 | +1 | 0 | -1 | 3.872 |
| 14 | 0 | -1 | +1 | 3.432 |
| 15 | 0 | 0 | 0 | 3.916 |
| 16 | 0 | 0 | 0 | 3.583 |
| 17 | 0 | +1 | -1 | 3.75 |

**Table 4.** Analysis of Variance of the Experimental Results of the Response Surface

| Source | Sum of Squares | df | Mean Square | *F*-Value | *p*-value | Significance |
|---|---|---|---|---|---|---|
| Model | 1.38 | 9 | 0.15 | 9.44 | 0.0037 | Significant |
| *A* | 0.46 | 1 | 0.46 | 28.5 | 0.0011 | ** |
| *B* | 0.064 | 1 | 0.064 | 3.94 | 0.0476 | |
| *C* | 0.017 | 1 | 0.017 | 1.06 | 0.3368 | |
| *AB* | 0.038 | 1 | 0.038 | 2.36 | 0.1682 | |
| *AC* | 0.01 | 1 | 0.01 | 0.64 | 0.449 | |
| *BC* | 4.42E-03 | 1 | 4.42E-03 | 0.27 | 0.6173 | |
| $A^2$ | 0.49 | 1 | 0.49 | 30.41 | 0.0009 | ** |
| $B^2$ | 0.24 | 1 | 0.24 | 15.07 | 0.006 | ** |
| $C^2$ | 6.40E-05 | 1 | 6.40E-05 | 3.96E-03 | 0.9516 | |
| Residual | 0.11 | 7 | 0.016 | | | |
| Lack of Fit | 6.75E-03 | 3 | 2.25E-03 | 0.085 | 0.9649 | Not significant |
| Pure Error | 0.11 | 4 | 0.027 | | 0.0037 | |
| Total Variation | 1.49 | 16 | - | | 0.0011 | |

Note: * indicates significance at $p < 0.05$; ** indicates significance at $p < 0.01$

The variance analysis of the above model equations showed that this experimental model had a p less than 0.01, which indicated that the two regression models were significant. Table 5 shows that the linear relation between the dependent and independent variables of the regression equation was significant, and the coefficient of determination ($R^2$) was 0.9239, which meant that the model can explain 92.39% of the response value. Also, the coefficient of variation was lower (3.54%), which suggested the repeatability of the test was better.

**Table 5.** Analysis of Variance of the Quadratic Model

| Item | Number | Item | Number |
|---|---|---|---|
| Std. Dev. | 0.13 | $R^2$ | 0.9239 |
| Mean | 3.59 | Adj. $R^2$ | 0.8261 |
| C.V. (%) | 3.54 | Pred. $R^2$ | 0.8156 |
| PRESS | 0.27 | Adeq. Precision | 10.348 |

Std. Dev. – standard deviation; C.V. – coefficient of variation; Adj. $R^2$ – adjusted coefficient of determination; and Pred. $R^2$ – predicted coefficient of determination

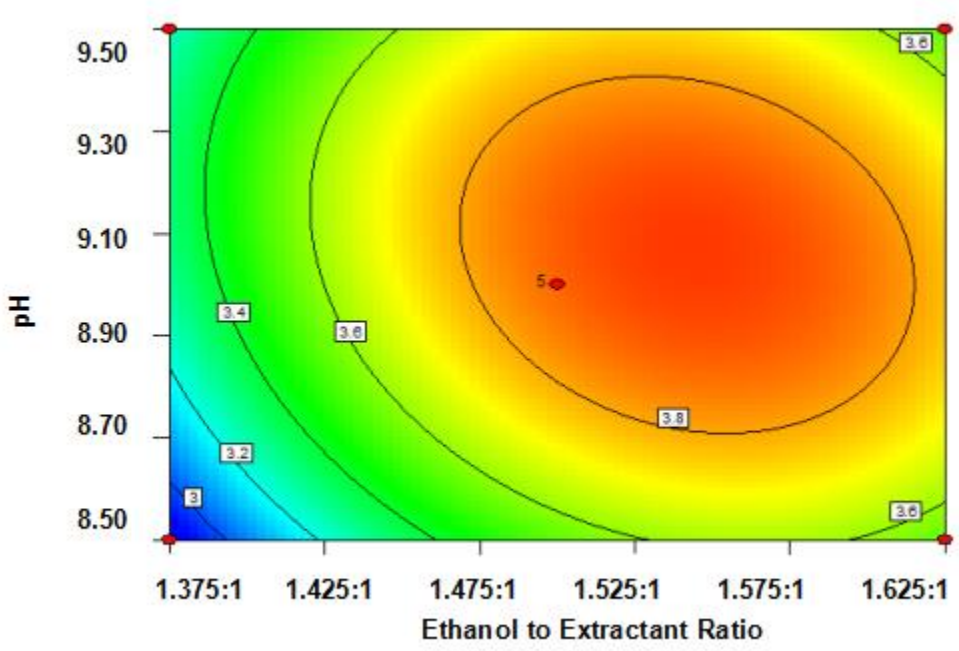
According to the analysis of variance (Table 4), the lack of fit was not significant ($p = 0.9649 > 0.1$), which indicated that the model had a good fitting degree with the actual test, and there was a high correlation between the predicted and actual values. There was no anomaly in the data, the model was appropriate, and the analysis result was reliable. Therefore, it can be applied to the theoretical prediction of the bioflocculant yield.

*Analysis of the response surface optimization*

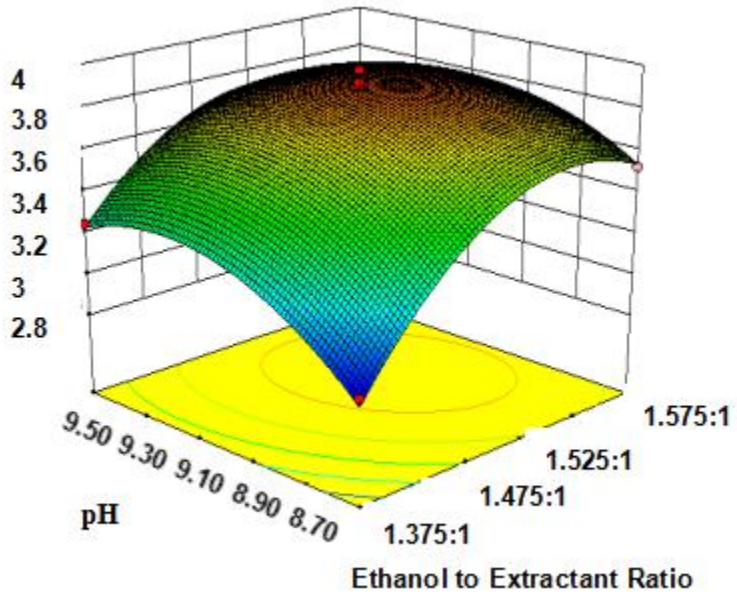
The regression model was analyzed. According to the regression equation, contour map, and figure from the response surface analysis, the bioflocculant yield was predicted by the interaction effect of the three factors (material to liquid ratio, pH, and extraction time), as is shown in Figs. 4 to 6. These figures show the influence of two factors on the bioflocculant yield when the third factor was maintained at the 0 level.

A response surface diagram and contour map made by a quadratic polynomial regression equation can directly reflect the effect of the bioflocculant extraction conditions on the yield. The shape of the contour map can reveal the strength of the interaction among various factors. An ellipse indicates that the interaction between two factors is significant, whereas a circle indicates the opposite. Figure 4 shows that the bioflocculant yield increased with an increase in the material to liquid ratio, but it increased first and then decreased with an increase in the pH. The shape of the contour map was elliptical, which indicated that the interaction of these factors was significant. Figure 5 shows that with an increasing material to liquid ratio and extraction time, the bioflocculant yield increased first and then decreased. The contour map was oval, which indicated that this interaction was significant. Figure 6 shows that with an increasing pH and extraction time, the bioflocculant yield increased first and then decreased. The shape of this contour map was elliptical, which indicated that the interaction of these factors was also significant.

The material to liquid ratio, pH, and extraction time each had an extreme value point. The optimum level of each factor was determined by the dynamic graph of this group. According to the analysis of the optimized test results and the principles of selecting a high yield, lower consumption, and shorter time consumption, the optimum extraction conditions obtained were a material to liquid ratio of 1.54:1, pH of 9, and extraction time of 12 h. Under these conditions, the final yield was up to 3.914 g/L.

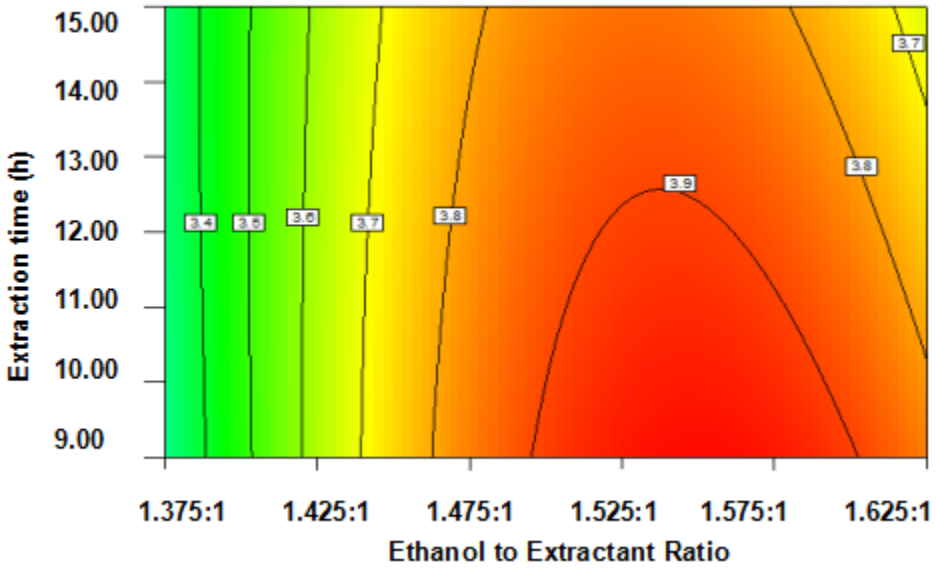



**(a)**

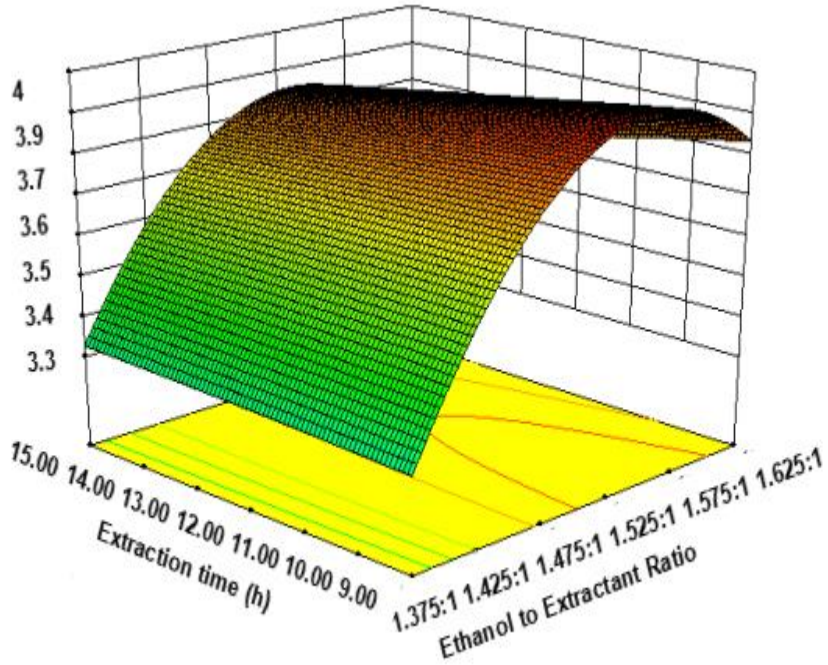



**(b)**

**Fig. 4.** Response surface (a) and contour graph (b) of the effects of the material to liquid ratio and pH on the bioflocculant yield

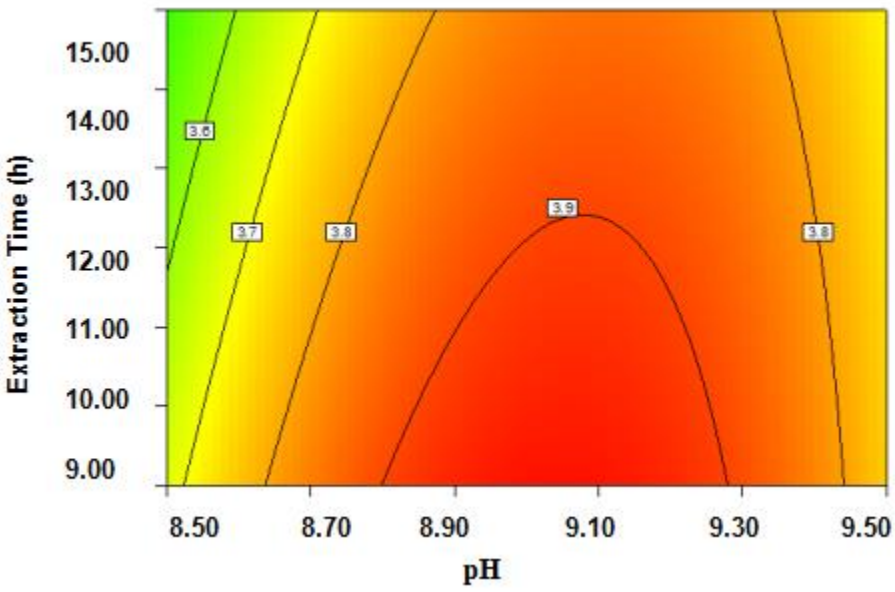



**(a)**

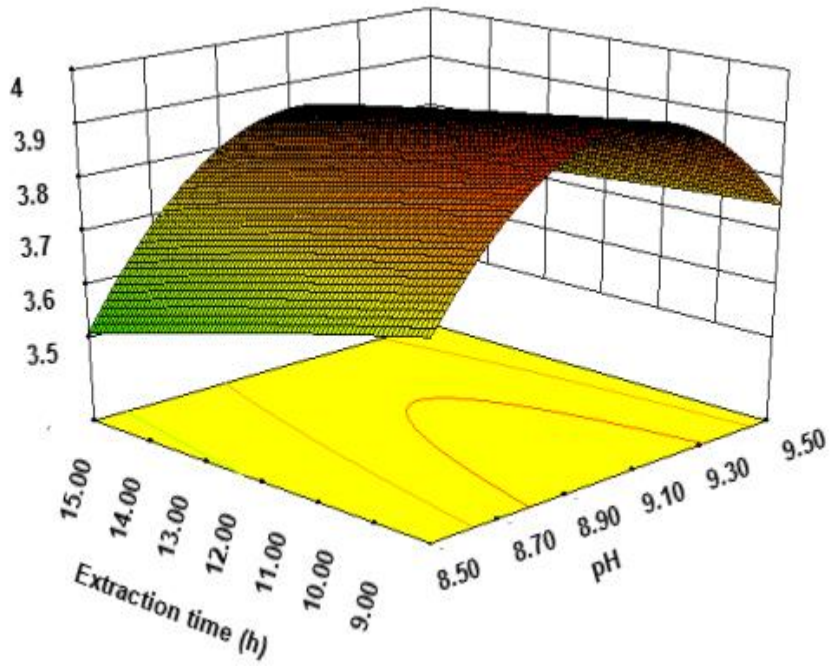



**(b)**

**Fig. 5.** Response surface (a) and contour graph (b) of the effects of the material to liquid ratio and extraction time on the bioflocculant yield




**(a)**




**(b)**

**Fig. 6.** Response surface (a) and contour graph (b) of the effects of the pH and extraction time on the bioflocculant yield

*Validation of the model*

The optimum conditions predicted by the model were adopted for bioflocculant extraction, and the tests were repeated three times. The conditions were an ethanol to extractant ratio of 1.54:1, pH of 9.06, and time of 12 h. The average bioflocculant yield was 4.00 g/L, and the relative error of the predicted value was approximately 2.1%, which indicated that the constructed model could predict the response surface of the bioflocculant value well.

*Qualitative study of the bioflocculant components*

The experimental results from the physical and chemical properties of the bioflocculant components are shown in Table 6. It was clear that there were protein and polysaccharide components in the bioflocculant, but no starch.

**Table 6.** Physicochemical Properties of the Bioflocculant

| No. | Measured Parameter | Test Method | Test Result | Conclusion | Content (%) |
|---|---|---|---|---|---|
| 1 | Protein amino acid | Chromogenic reaction of Ninhydrin | Blue-purple appeared under heating | Protein, but small amount | 19.74 |
| 2 | Polysaccharide | Molisch reaction | Purple red appeared between two layers of liquid | Containing polysaccharides | 66.64 |
| 3 | Starch | I-KI reaction | No blue | No starch | -- |

## Purification of Polysaccharides

Figure 7 shows that there was an obvious main peak in the chromatogram. The other peaks were not noticeable because the amount of polysaccharides or impurities was small.

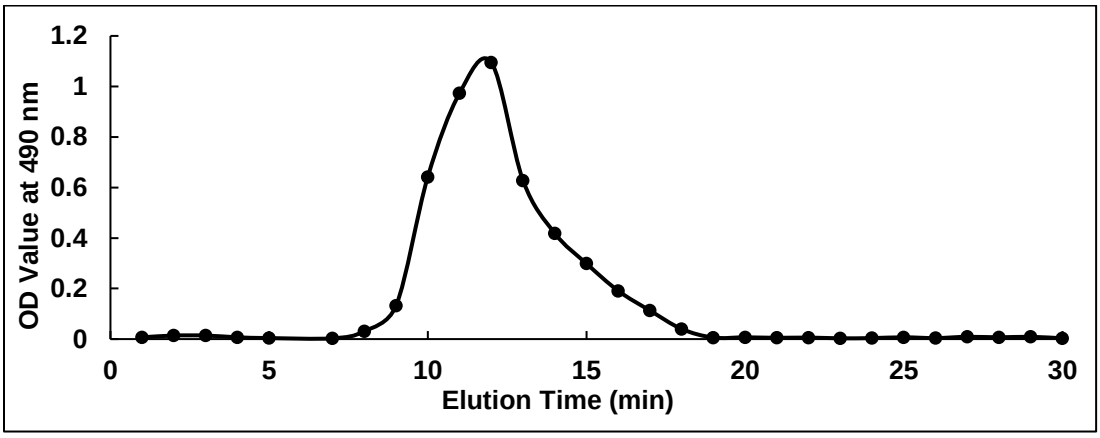



**Fig. 7.** Glucose gel chromatogram

## Analysis of the Bioflocculant Composition

*UV spectral analysis*

The UV spectroscopy results are shown in Fig. 8. The UV spectrum had a smooth curve in the wavelength range of 200 nm to 1000 nm and there were no characteristic peaks, such as protein and nucleic acid, present.

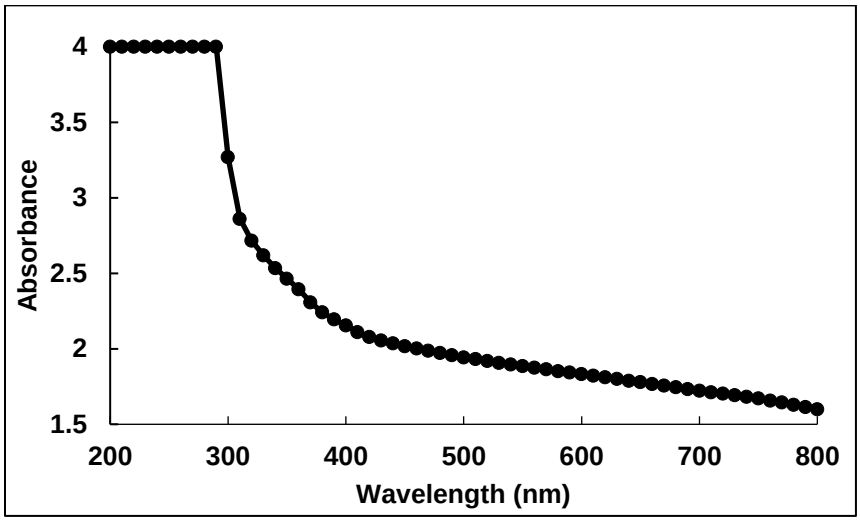



**Fig. 8.** UV full band scanning results of the pure bioflocculant

*Infrared analysis of the functional groups in the bioflocculant*

The infrared spectrum is shown in Fig. 9.

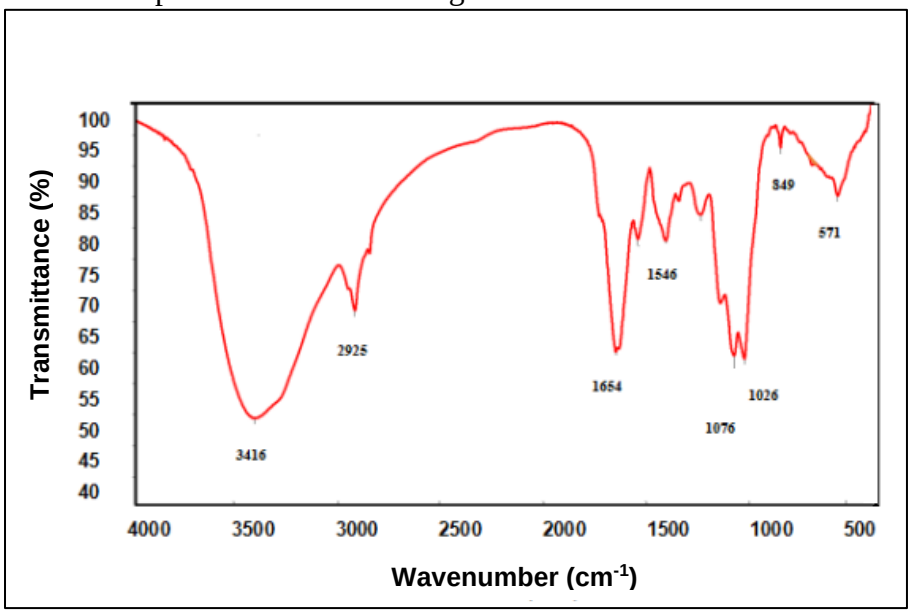



**Fig. 9.** Infrared spectrum of the bioflocculant

The peak at 3416.1 $cm^{-1}$ was the characteristic peak of telescopic vibrations of -OH bonds in the sugars. The peak at 2925.8 $cm^{-1}$ was characteristic of the expansion vibration of the saturated C-H bond. The peak at 1654.1 $cm^{-1}$ was the expansion vibration of the saturated -COOH bond, the absorption peak at 1540 $cm^{-1}$ was typical of C=C skeleton vibration, the peak near 1240 $cm^{-1}$ was classic C-O expansion vibration, and the peak near

1070 $cm^{-1}$ was from C-N telescopic vibration (protein characteristic peak). It was concluded that the bioflocculant produced by the M1 strain contained carbohydrates and few protein substances.

*Determination of the monosaccharide components in the bioflocculant*

Figure 10 shows, the peak time and order of elution of each monosaccharide in the standard sample containing L-rhamnose (21.297 min) and D-galactose (29.537 min).

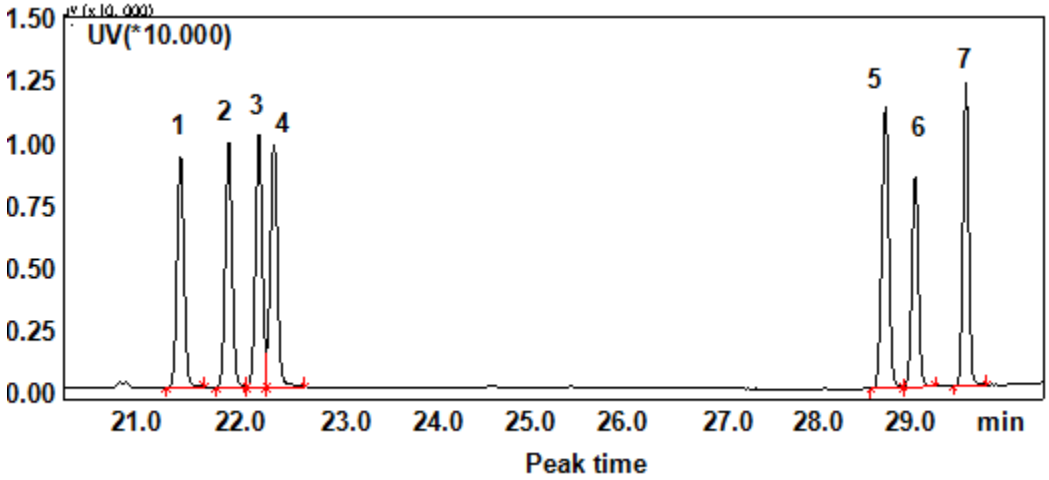



**Fig. 10.** Monosaccharide elution sequence: 1 – L-rhamnose; 2 - L-arabinose; 3 - L-fucose; 4 - D-xylose; 5 - D-mannose; 6 - D-glucose; and 7 - D-galactose

According to the regression equation and formula (Eq. 2), the concentration and molar number of each monosaccharide were calculated. The results from the GC analysis are shown in Table 7. Based on L-fucose, the mole ratio of the seven monosaccharides was calculated and found to be 0.29:0.36:1:0.31:0.47:0.57:1.01 (L-Rhamnose:L-Arabinose:L-Fucose:D-Xylose:D-Mannose:D-Glucose:D-Galatose).

**Table 7.** Determination of the Pure Polysaccharides by Gas Chromatography

| Monosaccharide | Retention Time (min) | Peak Area (A) | Molecular Weight | Concentration (mg/mL) | Mole Number | Peak Area Ratio |
|---|---|---|---|---|---|---|
| L-rhamnose | 21.263 | 1134.6 | 164.16 | 0.0218 | $1.33 \times 10^{-4}$ | 0.01011 |
| Unknown peak | 21.599 | 6726.6 | —— | —— | —— | 0.05999 |
| L-arabinose sugar | 21.773 | 2108.2 | 150.13 | 0.0248 | $1.65 \times 10^{-4}$ | 0.0188 |
| L-fucose | 22.089 | 35706.7 | 164.16 | 0.0753 | $4.59 \times 10^{-4}$ | 0.3184 |
| D-xylose | 22.246 | 1159.4 | 150.13 | 0.0216 | $1.44 \times 10^{-4}$ | 0.01034 |
| D-mannose | 28.661 | 10512.6 | 180.155 | 0.0386 | $2.14 \times 10^{-4}$ | 0.09375 |
| D-glucose | 28.977 | 12094.5 | 180 | 0.0468 | $2.60 \times 10^{-4}$ | 0.1079 |
| D-galactose | 29.519 | 42687 | 180.16 | 0.084 | $4.66 \times 10^{-4}$ | 0.3806 |

Figure 11 and Table 7 show that the polysaccharide was composed mainly of L-fucose, D-mannose, D-glucose, and D-galactose. It also contained small amounts of L-rhamnose, L-arabinose, and D-ylose.

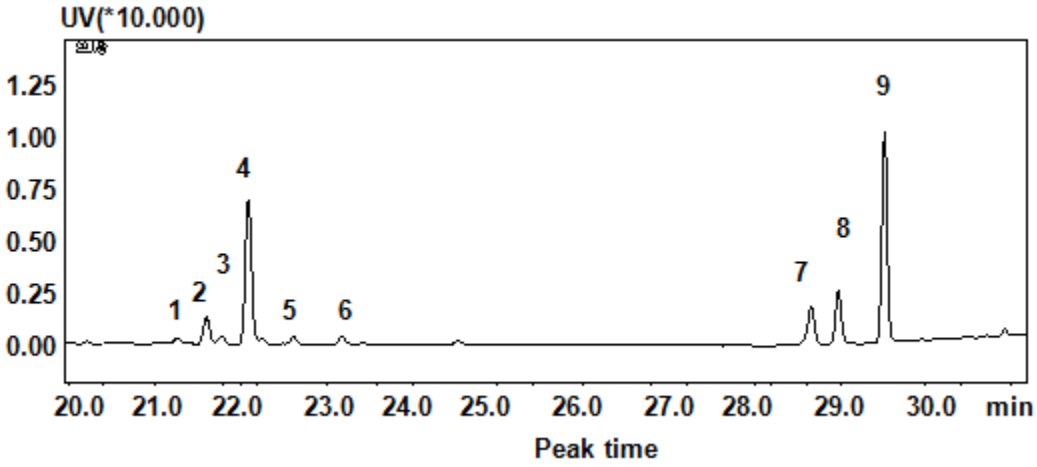



**Fig. 11.** Gas chromatogram of the pure polysaccharides: 1 — L-rhamnose; 2 — unknown peak; 3 — L-arabinose sugar; 4 — L-fucose; 5 — D-xylose; 6 — impurity peak; 7 — D-mannose; 8 — D-glucose; and 9 — D-galactose

*Determination of the bioflocculant molecular weight*

Figure 12 shows the results of gel permeation chromatography, which showed two peaks at 45.734 min and 57.789 min. The area of the latter peak was small relative to that of the former peak, which indicated that it was not a homogeneous polysaccharide. The molecular weight of the former peak was calculated to be $4.784 \times 10^6$ D, and the molecule was indicated to be a linear polymer with an irregular conformation.

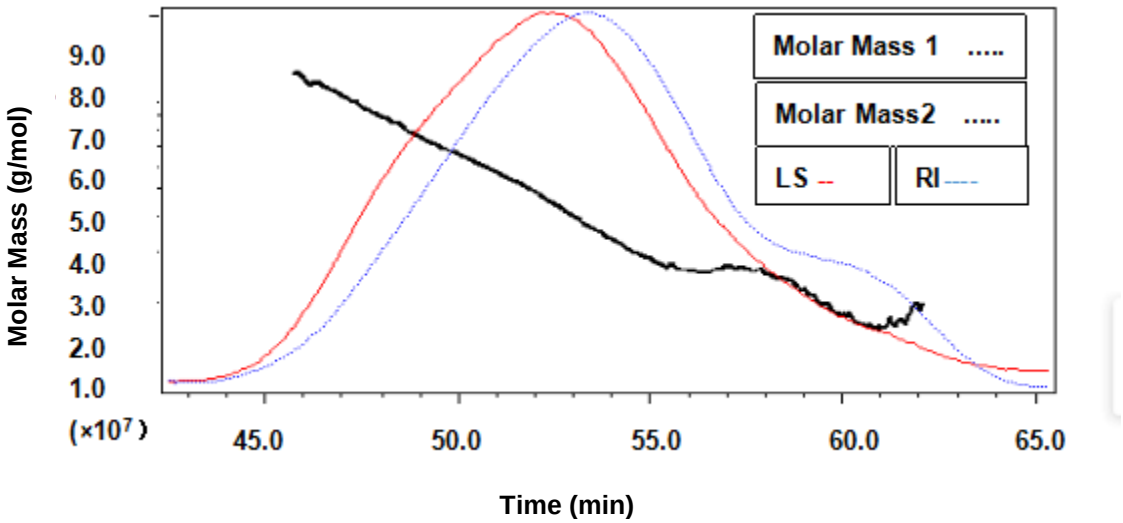



**Fig. 12.** Gel permeation chromatogram for the molecular weight determination

Rhamnose sugar *via* hydroxyl groups may bond with ether functionalities in the molecular chain and form branched chains. Also, amino acids, such as serine and threonine, could be linked to xylose to form a small number of amino acid branched chains. The

presence of carboxyl and carbonyl groups indicated that there were some open-chain monosaccharides in the molecule. Also, the presence of methoxyl groups revealed that there were some cyclic monosaccharides in the molecule.

*Scanning electron microscopy analysis of the bioflocculant*

The scanning electron microscopy analysis of the crude and pure bioflocculant product (Fig. 13) indicated that the surface of the pure bioflocculant sample was more exquisite, the choroid was clearer, the connection of the pores was closer, the pore density was larger, the cross linking of the chains was more obvious, and the network formed by cross linking was denser.

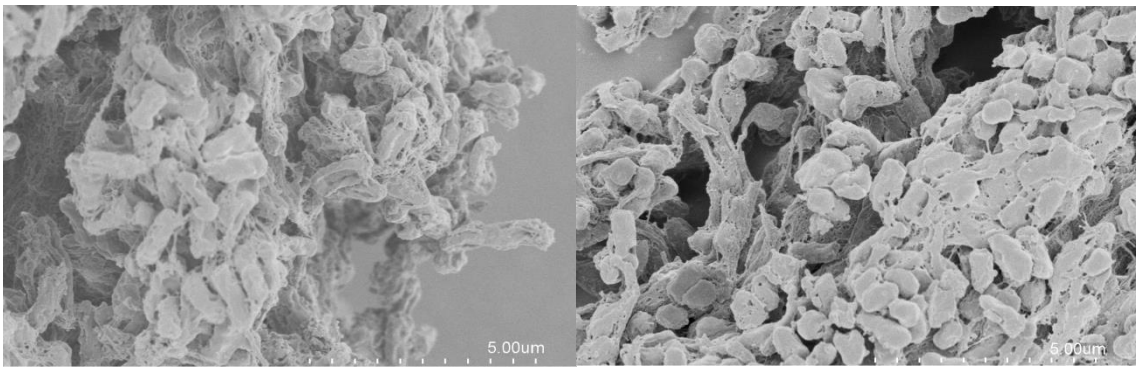



**Fig. 13.** Electron microscopic analysis of the crude (a) and pure (b) bioflocculant (10000x magnification)

*Nuclear magnetic resonance analysis of the bioflocculant*

The chemical $D_2O$ was used as a solvent in the NMR analysis. The $^1$H-NMR spectrum of the bioflocculant (Fig. 14) showed that the resonance signals were mainly concentrated at the chemical shift of 3.5 ppm to 4 ppm, which was an obvious characteristic region for carbohydrates. Because of the high molecular weight of the *Klebsiella* extracellular polysaccharide, the $^1$H-NMR signals were mainly concentrated in the high-field region. The peak at a $\delta$ of 4.54 ppm was a heavy water solvent peak and the peak at a $\delta$ of 0 ppm was the reference substance TMS. The stacked peaks at $\delta$ values of 0.5 ppm to 3.5 ppm were similar to those from polysaccharide structures. A cluster of peaks appeared in the $\delta$ range of 3.5 ppm to 4.5 ppm, which indicated that there were oxygen-containing groups in the sugar that were either alkoxy or hydroxyl groups. There was a small cluster peak near a $\delta$ of 7 ppm, which was the resonance peak of aryl hydrogens. This indicated that the polysaccharide chain was aromatic without any heteroatoms. If there had been a heteroatom, it would probably pertain to the N atom and would appear at a $\delta$ greater than 7 ppm. Therefore, the $^1$H-NMR spectrum showed that the bioflocculant had resonance signals, such as oxygen-containing groups (3.5 ppm to 4.5 ppm) and aromatic groups (~7.0 ppm).

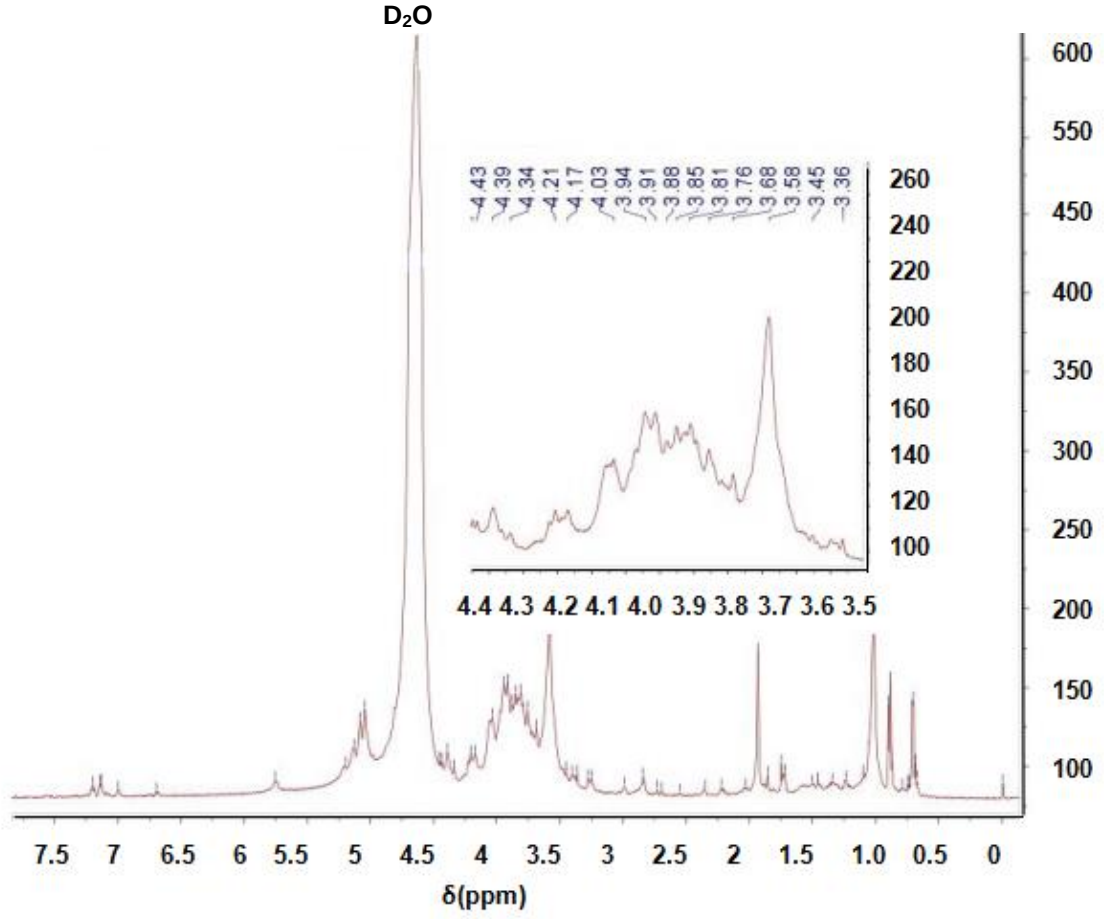



**Fig. 14.** $^{1}$H-NMR spectrum of the polysaccharides in the bioflocculant

## Discussion

In the single factor experiment on the bioflocculant extraction, the yield with acetone as the extractant was 0.532 g/L higher than that with ethanol. Acetone is more expensive than ethanol and is toxic; hence, it does not conform to the idea of green chemistry. After comparison and comprehensive consideration, ethanol was selected as the best extractant.

In this study, the scope was determined by the results of the single factor tests and the optimum levels and interactions of the various factors were also studied by response surface analysis. Two multiple mathematical models were established that pertained to the extraction rates of the bioflocculants. Currently, there are few reports on response surface optimization of the bioflocculant extraction process. The optimized conditions in the study were used to do experiments. The actual extraction rate was close to the theoretical value, which showed that the method had a practical application value.

The molecular weights and structures of polysaccharides are closely related to their functions (Bai and Zhang 2011). The molecular weight of the *Klebsiella* M1 bioflocculant in this experiment was $4.784 \times 10^6$ D, which was higher than that of some other known products. The bioflocculant produced by M1 bacteria had a greater molecular mass, more adsorption sites, a stronger bridge effect, and higher flocculation activity relative to those with a lower molecular weight (Salehizadeh and Shojaosadati 2001). The physical and chemical characteristics of the bioflocculants were studied by GC, gel chromatography, and NMR analyses. The polysaccharide bioflocculant produced by M1 was a linear macromolecule with a high molecular weight, as was determined by the different analysis

methods. This was consistent with the reports in the literature that stated that most products are polysaccharide types and the rich hydroxyl and carboxyl group contents play an important role in the flocculating effect (Zhu *et al.* 2008; Tang and Wang 2011). At the same time, the purification process was complex, but necessary because of the many impurities in the bioflocculant, such as pigments, proteins, and others, that can interfere with the separation of bioflocculant polysaccharides and damage the glucose gel column. It was necessary to remove impurities before separation, but it was difficult to remove them completely. Therefore, it was found that the pure product still contained protein in its infrared spectrum.

A group of unidentified peaks in the monosaccharide components was temporarily undetermined because the monosaccharides chosen were the common types, and so the unknown substance may be another rare monosaccharide or glucuronic acid. Compositional analysis of monosaccharides is an important part of sugar analysis and is important for studying the structure of polysaccharides. This study provides a reference for further study of the chemical structure of the *Klebsiella* polysaccharide.

The $^{1}$H-NMR spectrum of the polysaccharide can be used to speculate the configuration of the glycoside bonds. When the chemical shift of a polysaccharide proton signal is greater than 5 ppm, it is mainly an α-configuration. When the shift is less than 5 ppm, it is mainly a β-configuration. The results showed that the polysaccharide bioflocculant was composed mainly of β-glucosides.

## CONCLUSIONS

1. This study provides a theoretical basis for the application of bioflocculants produced by the *Klebsiella* M1 species.
2. The bioflocculant produced by *Klebsiella* M1 was a polysaccharide bioflocculant, and the products yield was greatly improved after optimization. Therefore, this bioflocculant should be studied further and applied in the biological treatment of sewage.
3. *Klebsiella* sp. has been used as an important type of environmental microbial resource and produces a bioflocculant with important practical applications.
4. The extraction of products from *Klebsiella* not only has great development value, but is also important for generating more polysaccharide bioflocculants with an abundant activity.

## ACKNOWLEDGMENTS

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