Screening of High-performance Flocculant-producing Bacteria and Optimization of the Conditions for Flocculation of Wheat Distillery Wastewater

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This study aimed to screen high-performance flocculant-producing bacteria for flocculating suspended matter in wheat distillery wastewater. After the preliminary and secondary screening, a high-performance flocculant-producing bacteria strain was screened from the activated sludge of wheat distillery wastewater. Single factor and orthogonal experiments were used to optimize the culture and flocculent conditions for the flocculant-producing bacteria. A superior strain of *Klebsiella* M1 was screened and identified by 16S rDNA. The initial flocculating degree was up to 72%. Based on single-factor tests, the optimum flocculent conditions were a resting time of 30 min, 8%(v/v) culture medium dosage, and 3%(v/v) $CaCl_2$. The optimum culture conditions were an incubation temperature of 30 °C for 48 h at pH 4.5 and rotation speed of 150 rpm. Under the optimum conditions, the flocculating degree was up to 82%. The best fermentation medium components were 15 g/L glucose, 2 g/L peptone, 1 g/L KH_2PO_4, and 2.5 g/L K_2HPO_4. A high flocculating degree was also achieved with the low-cost medium. The *Klebsiella* M1 bacteria strain can be used as a good bioflocculant produced bacteria for wheat distillery wastewater.

Keywords: Bioflocculants; Bioresources; Wheat distillery wastewater; Klebsiella spp.; Screening; Optimization

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INTRODUCTION

Anhui Ruifuxiang Food Co. Ltd is the largest enterprise in China that produces alcohol from wheat. The daily wastewater output is approximately 3,000 tons. The concentration of the chemical oxygen demand in the wastewater is up to 50,000 mg/L. The suspended solids (SS) content is almost 10,000 mg/L, and the total nitrogen concentration is about 1,000 mg/L. The main components of SS are 6% to 7% total suspended solids, approximately 3% protein, 1.5% to 2.5% pentosan, and 2% to 3% yeast, at a pH of 3.2 to 4. Some characteristics of the wastewater are a high temperature, high acidity, high concentration, high viscosity, and small number of suspended particles. Presently, this company mainly uses chemical flocculants, such as polyacrylamide (PAM), which are combined with air floatation and anaerobic fermentation to treat the wastewater. The amount of PAM used is approximately 10 ppm to 15 ppm, and the cost of the flocculant treatment is approximately 0.2 yuan/ton of wastewater. The PAM treatment effect is more effective than other chemical flocculants. However, the residual acrylamide monomer is a neurotoxin, which can cause neurotoxicity and cancer; affected individuals may display

symptoms of weakness and ataxia (Yokoi *et al.* 1997; Rudén 2004). Therefore, it is necessary to develop a safe and efficient green flocculant that does not produce the secondary pollution. Because of the innocuity, high efficiency, and green environmental-friendly nature of microbial flocculants (bioflocculant), they have been attracting major interest from the scientific research community (Liu *et al.* 2013). Bioflocculants, which are obtained through fermentation with bacteria or fungi under unique culture conditions, extraction, and refinement, is a metabolic product produced by microorganisms or their secretions. A bioflocculant consists of polymers possessing flocculating activity that can combine with pollutants in wastewater and then generate sediment to be filtered (Vijayalakshmi and Raichur 2003). Most studies have shown that the primarily components of bioflocculant are polysaccharides and proteins (Liu *et al*. 2009). Most of them are polysaccharides (Luo *et al*. 2014). Bioflocculants have been used to treat many types of wastewater, such as food, printing and dyeing, minerals, and milk (Wang *et al.* 2007; Okaiyeto *et al.* 2015). However, the study of microbial flocculating bacteria on flocculating wheat distillery wastewater has not been conducted. Because the flocculating ability of bacteria can show strong specificity for different types of sewage, it is necessary to consider various flocculant-producing bacteria. (Agunbiade *et al.* 2017; Li *et al.* 2017). High-performance flocculant-producing bacterial strains were screened to produce bioflocculant from wheat distillery wastewater, which was selected as the raw material, and the conditions of flocculation and culturing were optimized to develop a highly efficient green purification process for wheat distillery wastewater.

Many factors influence the production and flocculant activity of bioflocculants, such as the composition of the culture medium, bacteria culture conditions, and flocculating conditions (Luo *et al.* 2014). The optimization of the above conditions is beneficial to the flocculating effect of the bioflocculant. Kurane *et al*. (1986) reported that when the optimum culture temperature was 30 °C, the amount of bioflocculants and cell growth were two times higher than at 25 °C and 37 °C. When the initial pH value was 9.5, the cell growth rate was higher than at a pH of 7. Zhao and Liu (2008) showed that under optimum culture conditions (pH = 12, culture temperature = 30 °C, rotation speed = 150 rpm, and incubation time = 74 h), the amount of bioflocculant was the highest, up to 95.0%, which was higher than that of the control group). Yang *et al.* (2009) showed that the added bioflocculant amount, pH, coagulant aid ($CaCl_2$), and mixing speed have a substantial influence on the flocculating degree. Carbon, nitrogen, and inorganic salts are key factors to determine the cost of the culture medium. Obtaining the optimum carbon and nitrogen sources and the best phosphate ratio in a flocculating bacteria culture affects the cell growth, bioflocculant production, and leads to a lower cost of the culture medium (Chen *et al.* 2013; Murthy and Praveen 2013; Zhao *et al.* 2013b).

This study optimized the conventional culture conditions, carbon source, nitrogen source, and phosphate ratio. The optimum carbon source was determined using glucose, sucrose, lactose, maltose, and starch. The optimum nitrogen source was determined using single or multiple nitrogen combinations of beef extract, sodium nitrate, urea, ammonium sulphate, and peptone. The optimum inorganic phosphate dosage was obtained by determining the best ratio of potassium dihydrogen phosphate (KH_2PO_4) and potassium hydrogen phosphate (K_2HPO_4).

EXPERMENTAL

Materials

Isolation source of the strain and culture media

Anhui Ruifuxiang Food Co. Ltd (HeFei City, Anhui Province, China) provided the alcohol distillery residue and activated sludge, which were used for the screening materials.

The fermentation medium was composed of 20 g of glucose (Aladdin Industrial Corporation, Shanghai, China), 0.5 g of yeast extract (Beijing AOBOX Biotechnology Corporation, Beijing, China), 5.0 g of K_2HPO_4 (Xilong Scientific Corporation, Guangdong, China), 2.0 g of KH_2PO_4 (Xilong Scientific Corporation, Guangdong, China), 0.2 g of $MgSO_4$ (Aladdin Industrial Corporation, Shanghai, China), 0.1 g of NaCl (Hangzhou Microbial Reagent Co., LTD, Hangzhou, China), 0.1 g of ammonium sulphate (Aladdin Industrial Corporation, Shanghai, China), and 0.5 g of urea (Xilong Scientific Corporation, Guangdong, China).

The bacterial culture medium (Lactic acid bacteria culture medium: MRS) contained 10.0 g of peptone (Aladdin Industrial Corporation, Shanghai, China), 10.0 g of beef extract (Hangzhou Microbial Reagent Co., LTD, Hangzhou, China), 5.0 g of yeast extract (Beijing AOBOX Biotechnology Corporation, Beijing, China), 2.0 g of citric acid diammonium hydrogen (Xilong Scientific Corporation, Guangdong, China), 20.0 g of glucose (Aladdin Industrial Corporation, Shanghai, China), 1.0 mL of Tween 80 (Tianjin Zhiyuan Chemical Reagents Co., Ltd., polyoxyethylene (20) sorbitan monooleate), 5.0 g of sodium acetate (Xilong Scientific Corporation, Guangdong, China), 2.0 g of $K_2HPO_4 \cdot 3H_2O$ (Xilong Scientific Corporation, Guangdong, China), 0.58 g of $MgSO_4 \cdot 7H_2O$ (Xilong Scientific Corporation, Guangdong, China), 0.25 g of $MnSO_4 \cdot H_2O$ (Xilong Scientific Corporation, Guangdong, China), and 18.0 g of agar (Hangzhou Microbial Reagent Co., LTD, Hangzhou, China). The pH ranged from 6.2 to 6.6.

The bacterial culture medium (Nutrient Broth:NB) contained 5 g of beef extract (Hangzhou Microbial Reagent Co., LTD, Hangzhou, China), 5 g of sodium chloride (Xilong Scientific Corporation, Guangdong, China), and 10 g of peptone (Aladdin Industrial Corporation, Shanghai, China) , and had a neutral pH.

The fungus culture medium (Potato Dextrose Agar Medium: PDA) was composed of 200 g of potato (Supermarket, Hefei, China), 20 g of glucose (Aladdin Industrial Corporation, Shanghai, China), and approximately 15 g to 20 g of agar (Hangzhou Microbial Reagent Co., LTD, Hangzhou, China), and had a neutral pH.

Methods

Screening of flocculant-producing bacteria

The preliminary screening: 1 g activated sludge and 1 g alcohol distiller residue from the wheat alcohol production enterprise were sampled and then added to aseptic distilled water with several sterilized glass beads to disperse the bacterial microcells into single cells. 1 mL single cell suspension was added to the 99 mL enrichment culture medium of the liquid MRS, NB, and PDA and incubated at 30 ℃ and 37 ℃, respectively, rotated at 150 rpm for 48 h. The liquid media were diluted with distilled water. The petri dishes with fungus and bacterial separation agar media were coated with 0.1 mL of dilute fluid. The separation media were cultured for 48 h, and the colony characteristics were observed. Smooth, large, and sticky colonies were selected and purified by using the streak plate technique (Guo 2013). The screened strains were stored in a refrigerator at 4 °C.

The secondary screening: the strains obtained from the primary screening were inoculated in 100 mL of standard fermentation medium and cultured at the corresponding screening temperature and rotation speed of 150 rpm for 24 h. The flocculant performance was measured by testing the flocculating degree of the wheat distillery wastewater (Yang *et al.* 2015), and then the high efficiency microbial flocculant-producing strains were selected.

Species identification by molecular biology

The screened bacterial strains were sequenced by 16S rDNA. The primer synthesis and sequencing work were completed by Sangon Biotech Co., Ltd. (Shanghai, China). According to the sequenced 16S rDNA results, the nucleotide sequence was BLAST aligned with the NCBI database, and the screened species was identified. The 16S rDNA gene sequence of some of the strains were extracted from Genbank and the phylogenetic tree of the strain was drawn using the N-J method in MEGA 4 software (Center of Evolutionary Functional Genomics Biodesign Institute, Arizona State University, Tempe, AZ, USA).

Culture preservation

Five to six branches were filled with 6 mL of fresh medium to form slope culture, and as many strains as possible were coated on the entire slope for the harvesting strains. Packaged bacteria were mailed to the China Type Culture Collection Center (Wuhan, China) for preservation.

Determination of the growth curve of the strain

The purified strain was inoculated into 150 mL culture medium, and the absorbance value at 600 nm (OD_{600}) was measured. The culture broth pH, biomass, and OD_{600} values were determined at the screening temperature of 37 °C and at a constant rotation speed of 150 rpm. Samples were collected every 6 h. A non-inoculated medium was used as the control group. The growth curve was drawn with the culturing time as the abscissa and the OD_{600} value as the ordinate (Xing *et al.* 2010).

Determination of the flocculating degree

The flocculating activity was determined by measuring the flocculating degree of the wheat distillery wastewater. The method of determining the flocculating degree: 5 mL culture broth was added to triangular flasks with 100 mL of wheat distillery wastewater for mixing at room temperature. The triangular flask was rapidly oscillated for 3 min (120 rpm), slowly oscillated for 2 min (50 rpm), and then was rested for 10 min. Finally, the supernatant was collected to measure the absorbance value (OD_{780}). Distilled water was used as the control group instead of the culture broth. The flocculating degree of the sewage was calculated with the following equation (Li *et al.* 2017),

$$FR = \frac{(B-A)}{B} \times 100\% \quad (1)$$

where FR is the flocculating degree (%), A is the supernatant absorbance value after adding the culture broth, and B is the absorbance value of the supernatant after adding distilled water.

Single factor test of the flocculating conditions

The culture broth (fraction 5% v/v) 5 mL and $CaCl_2$ (mass fraction 1% solution m/v) 1 mL were added to 100 mL of wheat distillery wastewater. The mixture was rested for 10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 70 min, 80 min, and 90 min. The optimum flocculent resting time was found by measuring the flocculating degree of wheat distillery wastewater. Based on the above results, 2 mL, 4 mL, 6 mL, 8 mL, 10 mL, 15 mL, and 20 mL strain culture broths were added to 100 mL of wheat distillery wastewater and the optimum concentration was determined.

One mL, 2 mL, 3 mL, 4 mL, and 5 mL coagulant aid ($CaCl_2$ mass fraction 1% solution) was respectively added to 100 mL of wheat distillery wastewater, and the optimum amount of the coagulant aid was determined on the base of measuring the flocculating degree.

Distribution of the flocculating active substances in the strain

The experiment was divided into four groups: 50 mL M1 bacteria culture broth was centrifuged at 8000 rpm for 15 min, and the supernatant was collected as the supernatant liquid group (the first group). The precipitate was washed three times with distilled water and volume fixed to 50 mL as the cell suspension group (the second group). The other precipitate was crushed by sonication (KF-QC, Nanjing Kenfan Electronic Technology. Co., LTD, Nanjing, China) and used as the cell broken solution group (the third group). The fourth group was 50 mL M1 bacteria culture broth. Then the flocculating activity of the above four samples was determined by the flocculating degree to confirm the distribution of the flocculating active substances (Yan 2013).

Single factor optimization of the culture conditions for the flocculant-producing bacteria

The medium with an initial pH 7 was inoculated with 1% strain culture broth and incubated at 37 °C and 150 rpm. Samples were collected every 6 h for 72 h. The optimum culturing time was confirmed on the basis of flocculating degree.

The experimental method remained constant with various culture temperatures of 25 °C, 30 °C, 35 °C, 37 °C, and 40 °C. The samples were cultured for 48 h, and the optimum culture temperature was determined in the light of flocculating degree (Luo *et al.* 2014). The initial pH values of the culture medium were 5, 6, 7, 8, and 9. Then, 1% culture broth was inoculated to the standard medium with different pH and cultured at 37 °C and 150 rpm for 48 h. The optimum pH value was measured on the basis of the higher flocculating degree. The strains were cultivated with different rotation speeds, such as 90 rpm, 120 rpm, 150 rpm, 180 rpm, and 210 rpm in order to find the optimum speed.

Orthogonal optimization of the culture conditions for the flocculant-producing bacteria

An orthogonal design experiment was performed on the culturing conditions of the flocculant-producing bacteria. Four factors and three levels of the L_9 (3^4) orthogonal test were designed on the basis of the single factor experiment. The orthogonal test design conditions are presented in Table 1.

Single factor optimization of medium experiment

On the basis of the common culture medium, the experimental method remained constant with various carbon sources, which included glucose, sucrose, lactose, maltose, and starch (Murthy and Praveen 2013; Zhao *et al.* 2017). The concentration was up to 20

g/L. The flocculating degree was calculated according to Eq. 1, and the optimum carbon source was determined after culturing for 72 h at 37 °C.

Table 1. L_9 (3^4) Orthogonal Experiments Design Table of the M1 Flocculant-producing Bacteria Culture Conditions

No.	Factors			
	A - Culture Temperature	B - Initial pH	C - Rotation Speed	D - Culture Time
1	1 (27 °C)	1 (4.5)	1 (135 rpm)	1 (42 h)
2	1	2 (5)	2 (150 rpm)	2 (48 h)
3	1	3 (5.5)	3 (165 rpm)	3 (54 h)
4	2 (30 °C)	1	2	3
5	2	2	3	1
6	2	3	1	2
7	3 (33 °C)	1	3	2
8	3	2	1	3
9	3	3	2	1

Yeast extract, peptone, beef extract, sodium nitrate, urea, and ammonium sulphate were used as the nitrogen sources for the medium. One, two, and three nitrogen sources were used for the design combination (Chen *et al.* 2013), and the nitrogen content in the medium was 1.2 g/L. The flocculating degree was calculated according to Eq. 1, and the optimum nitrogen source was measured after culturing for 72 h at 37 °C.

The ratios of potassium hydrogen phosphate to potassium dihydrogen phosphate were 0.5 g/L: 1.25 g/L, 1 g/L:2.5 g/L, 2 g/L:5 g/L, 3 g/L:7.5 g/L, and 4 g/L:10 g/L (Guo *et al.* 2015). The flocculating degree was calculated according to Eq. 1, and the optimum phosphate dosage ratio was determined after culturing for 72 h at 37 °C.

Orthogonal optimization of the culture medium

On the basis of the single factor experiments, the orthogonal experiments of three factors and four levels of carbon sources, nitrogen sources, and phosphate ratios were performed (Table 2). After culturing for 72 h at 37 °C, the optimum medium compound of the M1 bacteria was selected in the light of the flocculating degree.

Table 2. Factors and Levels in the Single Experiments

Level	Factor		
	A - Glucose (g/L)	B - Peptone (g/L)	C - KH_2PO_4:K_2HPO_4
1	15	0.5	0.75:1.875
2	20	1.2	1:2.5
3	25	2.0	1.25:3.125
4	30	2.5	1.5:3.75

Determination of wheat distillery wastewater quality parameters

The wastewater quality parameters such as SS, COD, BOD, turbidity, and pH were determined before and after flocculating test. Suspended solids (SS): gravimetric method was adopted (GB/T 11901-89). The pH value was determined by pH meter. COD: measured by dichromate method (GB/T 11914-89). BOD_5: used five days biochemical oxygen demand determination method (GB/T 7488-87). Turbidity was determined by spectrophotometric method (GB /T 13200-91).

RESULTS AND DISCUSSION

Screening and Identification of the Strains

The colonies of five strains that exhibited a viscous, smooth surface and had flocculant activities were isolated at 30 °C from the bacterial screening medium, and 10 strains were isolated at 37 °C from the same medium. Another five strains, which had the same characteristics, were screened from the PDA medium at 30 °C, and two strains were isolated at 37 °C.

Table 3. Isolation Sources of the Bacteria and Surface Characterization of the Colony

No.	Sample Source	Incubation Temperature (°C)	Colony Morphology
N1	Precipitated sludge	37	Irregular edge, almond white, smooth and humid
N2	Precipitated sludge	37	Small colony, round, whey
N3	Precipitated sludge	37	Small colony, round, milky white, silk
N4	Precipitated sludge	37	Small colony, round, milky yellow
N5	Precipitated sludge	37	Medium colony, round, whey
M1	Precipitated sludge	30	Transparent, smooth, moist, sticky, whey, drawing
M2	Precipitated sludge	30	Circular, convex, yellowish white
M3	Precipitated sludge	30	Small colony, round, whey
M4	Alcohol distiller grain	30	Milky white, smooth, moist and sticky
N6	Alcohol distiller grain	30	White, fold, edge radiate, sticky
M5	Precipitated sludge	37	Small colony, oval, whey
M6	Precipitated sludge	37	Oval, milk white, sticky
M7	Precipitated sludge	37	Oval, milk white, drawing
N7	Alcohol distiller grain	37	Round, milky, smooth and humid surface
N8	Alcohol distiller grain	37	Oval, irregular, smooth and moist surface
M8	Precipitated sludge	30	Round, small colony, sticky, white
M9	Precipitated sludge	30	Oval, reddish, smooth and humid
M10	Alcohol distiller grain	30	Oval, zigzag edge, milky white
N9	Alcohol distiller grain	30	Oval, zigzag edge, white, sticky
N10	Alcohol distiller grain	30	Irregular shape, smooth and moist, slightly red
M11	Precipitated sludge	37	Round, protruding, milky, smooth and moist
M12	Precipitated sludge	37	Round, different size, wax like, sticky

After the preliminary and secondary screening, a novel bioflocculant-producing bacterium with a high and stable flocculating activity, named M1, was isolated from the activated sludge, and the initial flocculating degree was up to 72.1%. The M1 strain colonies in the solid medium were transparent, viscous, lawn-shaped, and silk-like. The sequencing results showed that the 16S rDNA gene sequence length of the M1 strain was 1389 bp. According to blasting with sequences from the NCBI database, the homology with *Klebsiella pneumoniae* was up to 99% (KR905685.1). Therefore, strain M1 was identified as *Klebsiella* sp. (Pang *et al.* 2016).

Table 4. Flocculating Degree of the 22 Strains

No.	Average flocculating degree (%)
N1	2.81
N2	12.39
N3	17.22
N4	11.87
N5	7.85
M1	72.09
M2	30.45
M3	42.08
M4	61.39
N6	4.17
M5	8.62
M6	6.05
M7	22.38
N7	4.45
N8	34.59
M8	44.22
M9	1.72
M10	8.78
N9	44.64
N10	44.00
M11	8.97
M12	7.64

The MEGA 4 software was used to construct the phylogenetic tree (Fig. 1). The strain was preserved at the China Center for typical culture collection, and the preserved number was CCTCC M 2018098. The 16S rDNA gene had been registered at GenBank (National Center for Biotechnology Information, NCBI, Maryland, USA) and its login number was MG987011.

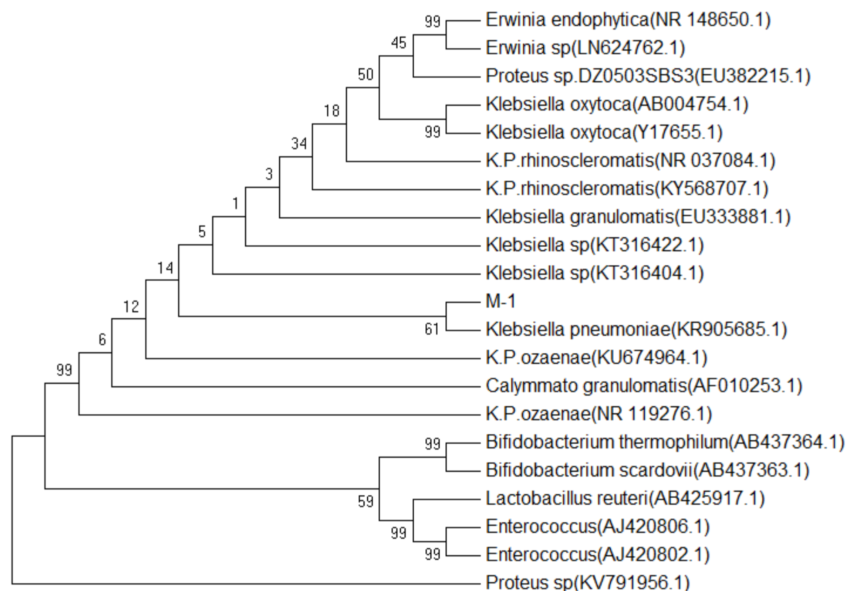

Fig.1. Phylogenetic tree of the M1 strain

Growth Curve of the Strain

The M1 strain entered the logarithmic growth phase after 2 h of inoculation and entered the stationary phase after 18 h, which was followed by a noticeable decline after 48 h. The complete growth curve is shown in Fig. 2. The pH value also showed a gradual decreasing trend with the bacteria growth, but during the bacteria growth in the stationary phase and afterwards, the pH value of the bacterial fluid increased (Xing *et al.* 2010).

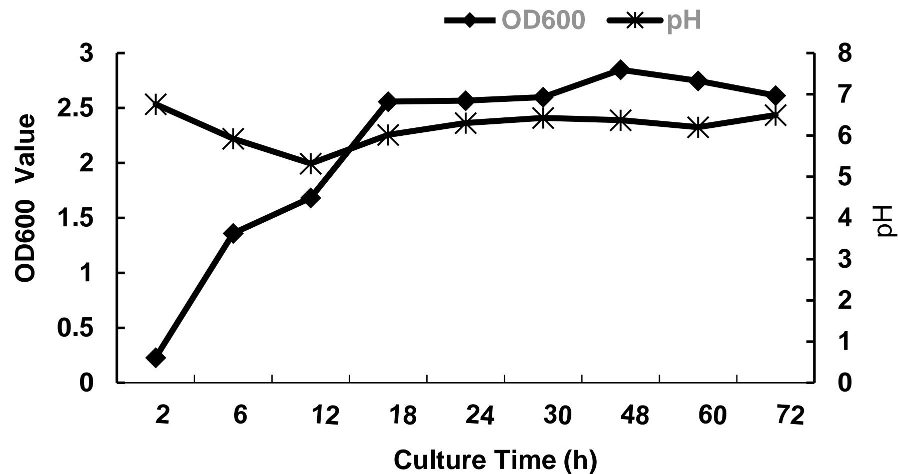

Fig. 2. Growth and pH curves of the M1 strain

Single Factor Optimization of the Flocculating Conditions

The single factor test results showed that there was a higher flocculating degree with longer resting times. However, the flocculating degree was not noticeably changed for resting time less than 30 min. According to test efficiency, the optimum resting time was determined to be 30 min. The results are shown in Fig. 3. The results of resting time test showed that the flocculating degree was increased with extending of resting time, due to long time of resting provides multiple opportunities for particles in wastewater suspension to aggregate and increased the flocculating degree (Al-Shamrani *et al.* 2002).

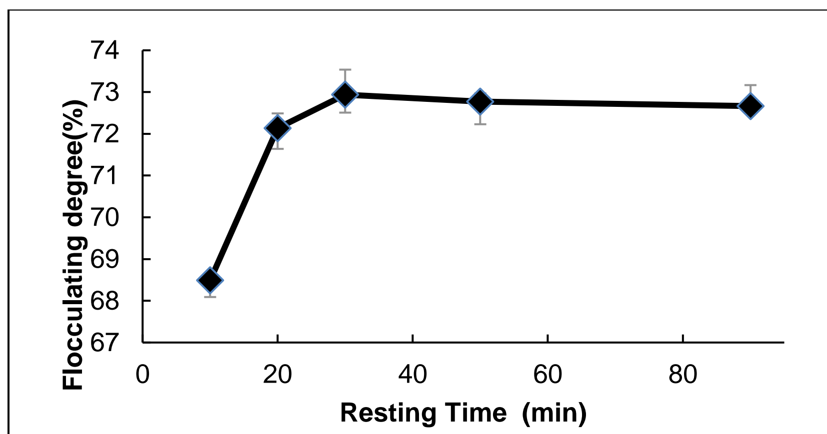

Fig. 3. Effect of the resting time on the flocculating degree. Values represent the means and standard deviations, n=3.

Due to the particularity of the experimental materials for the wheat distillery wastewater, the amount of added fermentation broth dosage was relatively large. The flocculating efficiency was a bit weaker at lower concentrations (less than 8 mL). When the dosage reached approximately 8 mL to 10 mL, the flocculating degree was slightly higher. Afterwards, the flocculating degree no longer increased with adding the dosage. The optimum additive amount of the fermentation broth was 8 mL (8% v/v), which is displayed in Fig. 4. Further increase in bioflocculant dosage resulted in decline of flocculating degree. Because insufficient dosage of bioflocculant will hinder or affect bridging mechanism formation of flocs and over dosage will result into strong repulsion between the excessive flocculants to inhibit floc formation. Hence, establishing the optimum fermentation broth dosage is important in flocculation. The relationship between dosage and flocculating activity of M1 bioflocculant was similar to that of the bioflocculant produced by other pure strains (Gong *et al*. 2008; Zheng *et al.* 2008).

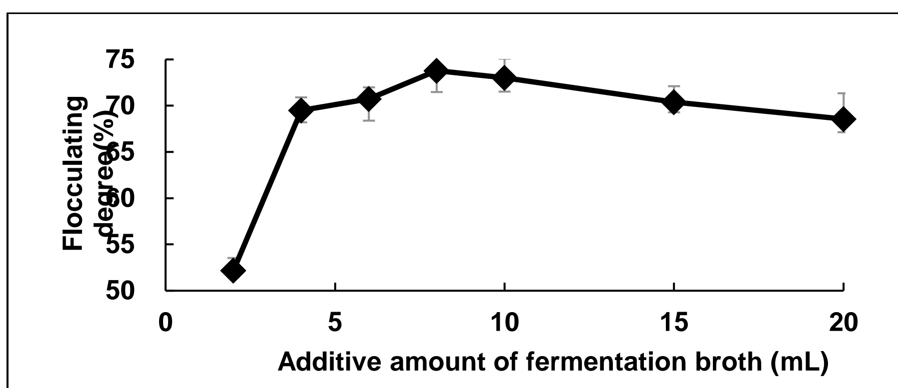

Fig. 4. Effect of the fermentation broth amount on the flocculating degree. Values represent the means and standard deviations, *n*=3

$CaCl_2$ liquid was chosen as coagulant aid. As shown in Fig. 5, increasing the added amount resulted in higher flocculating degree. The optimum addition amount was 3%. Adding calcium ions during flocculation can help to increase flocculating activity. This is mainly because Ca^{2+} can draw close to the suspended particles with negatively charged particles through Coulomb attraction, forming a Ca^{2+}-suspension complex, and thus promoting the occurrence of flocculating precipitation (Li *et al.* 2008).

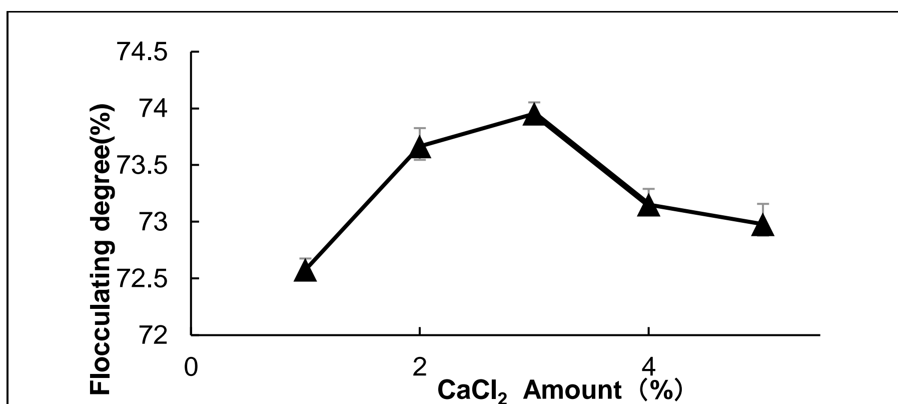

Fig. 5. Effect of the coagulant aid on the flocculating degree. Values represent the means and standard deviations, *n*=3.

Increasing the dosage can compress the colloid double layer, reduce the electrostatic repulsion, and thereby promote flocculating degree and adsorption. If the dosage is insufficient, the coagulation aid effect is not obvious, but excessive Ca^{2+} can inhibit the bridging effect and neutralization of flocculating polymer (Zhao *et al.* 2013a). In this experiment, 3% addition was found to be a suitable dosage.

Distribution of the Flocculating Active Ingredients in the Strains

Figure 6 shows that the flocculating degrees of the supernatant and fermented raw liquid were significantly higher (65.4% and 71.9%, respectively) than that of the cell suspension and broken cell solution groups (6.78% and 11.55%, respectively). This phenomenon showed that the flocculating active component of the M1 strain was predominantly concentrated extracellularly and was the extracellular metabolite.

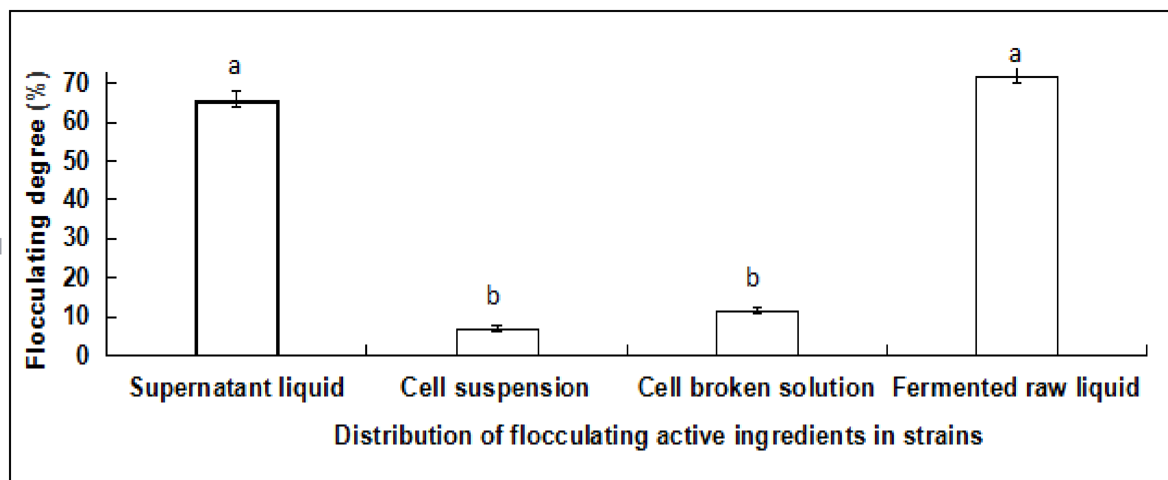

Fig. 6. Distribution of the flocculating active ingredients in the M1 strain. Values represent the means and standard deviations, *n*=3; Values in a column with different superscripts are significantly different (p<0.01).

Optimization of the Culture Conditions

Single factor optimization of the culture conditions

With an increase in the culture time, the flocculating degree of the M1 bacteria gradually increased.

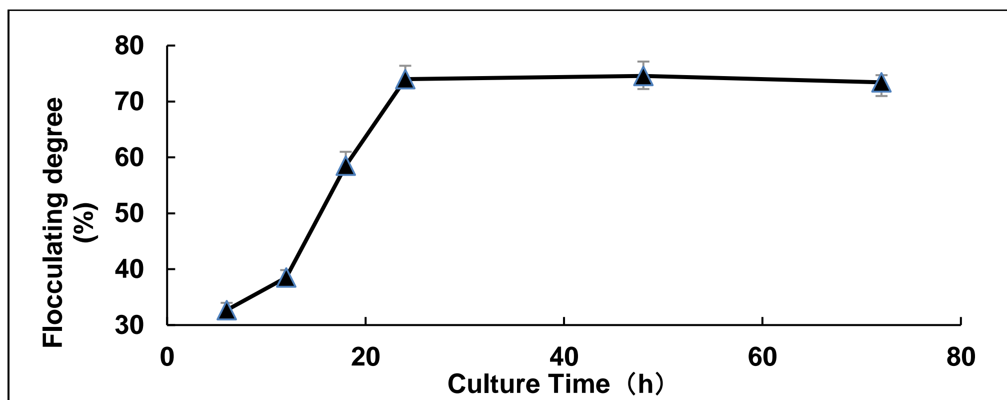

Fig. 7. Effect of the culture time on the flocculating degree. Values represent the means and standard deviations, *n*=3.

When the incubation time reached 48 h, the flocculating degree of the culture solution with the wheat distillery wastewater was up to 74.6%. This result is shown in Fig. 7. The results indicated that the bioflocculant was a product of biosynthesis of M1, not that of cell autolysis (Lu *et al*. 2005). After flocculating activity reached the maximum, the flocculating activity presented a declining tendency. This may be associated with the decomposition of flocculant in the fermentation broth, or the lack of nutrients needed for the later microorganisms (Li *et al*. 2007).

It can be seen from Fig. 8 that the flocculating degree increased at the first temperature increase step (25 °C to 30 °C), and then tended to decrease as the temperature was further increased. The flocculating degree of the wheat distillery wastewater was relatively high, near 75.5%, at 30 °C. The flocculating degree decreased when the culture temperature was higher than 37 °C. Culture temperature not only affects microbial growth and metabolism, but it also affects enzyme activity in microbial cells (Zhang *et al.* 2002). High temperature leads to the decrease or even loss activity of enzyme in the strain, which affects the growth and metabolism of strains, and causes low flocculation degree of strain M1 fermentation liquid. Low temperature leads to a slowing down of the growth of a strain and reduced activity of an enzyme *in vivo*, and even results in a reduction of the flocculants and other metabolites. The time of the flocculation material synthesis and accumulation is thereby prolonged.

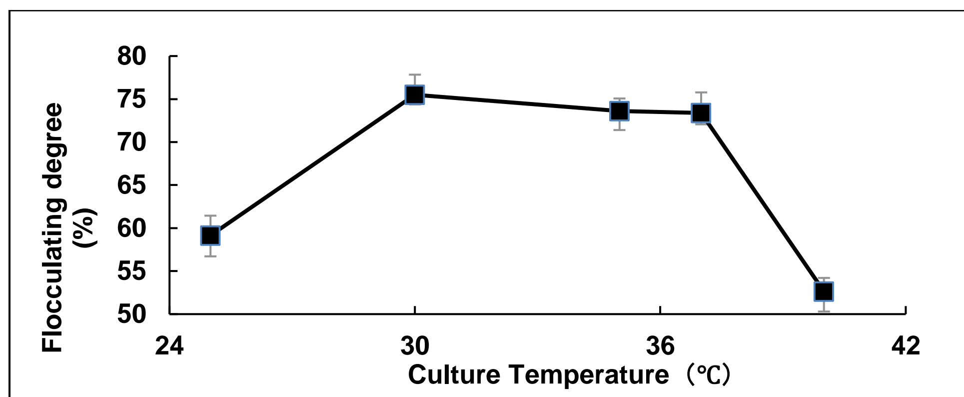

Fig. 8. Effect of the culture temperature on the flocculating degree. Values represent the means and standard deviations, *n*=3.

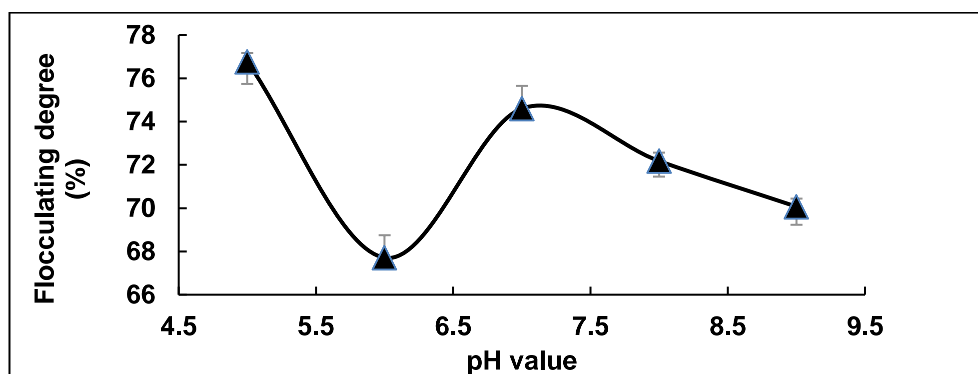

Fig. 9. Effect of the pH on the flocculating degree. Values represent the means and standard deviations, *n*=3.

The initial pH of the culture medium could affect the electrification state of microorganism cell and redox potential, and enzymatic reaction of microorganisms (Ho *et al.* 2010). At pH 5, the flocculating degree was at the maximum value. The rest value was decline, which may be caused by error. At less than pH 5, there may be a higher value; it is necessary to do further study in the future.

The flocculating degree increased at the first increase step for the rotation speed 90-150 rpm and then declined with a further increasing rotation speed. The flocculating degree was relatively high at 150 rpm, as is shown in Fig. 10. This result is consistent with the research findings of Chai *et al*. (2000). The rotational speed is not only related to the concentration of dissolved oxygen in the medium, but also affects the absorption of nutrients and intracellular metabolic reaction of the strain (Zhang *et al.* 2002). When the rotational speed is too high, the dissolved oxygen is sufficient in the medium, and the rapid proliferation of microorganism cells consumes a lot of nutrients, which leads to less formation of flocculation products. When the rotational speed is too low, the dissolved oxygen in the fermentation medium is insufficient and the microorganisms compete for oxygen, which affects the normal metabolic cell reaction and the absorption of nutrients.

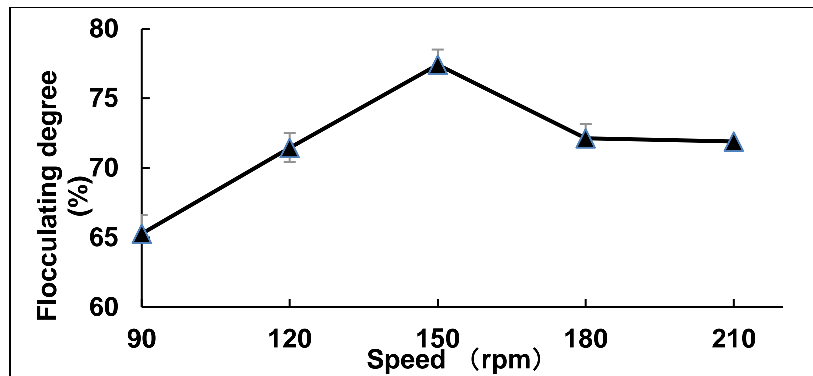

Fig. 10. Effect of the rotation speed on the flocculating degree. Values represent the means and standard deviations, *n*=3.

Table 5. Orthogonal Test Results of the M1 Bacteria Culture Conditions

No./Factor	A - Culture Time	B - Culture Temperature	C - pH	D - Speed	Flocculating degree
1	1	1	1	1	64.82%
2	1	2	2	2	72.98%
3	1	3	3	3	62.93%
4	2	1	2	3	71.86%
5	2	2	3	1	74.79%
6	2	3	1	2	81.82%
7	3	1	3	2	76.90%
8	3	2	1	3	74.67%
9	3	3	2	1	67.37%
k1	2.0074	2.1358	2.2131	2.0699	
k2	2.2847	2.2244	2.1221	2.3170	
k3	2.1894	2.1212	2.1463	2.0946	
R	0.2774	0.1032	0.0668	0.2224	

Orthogonal experiment optimization results of the culture conditions

According to the range of values shown in Table 5, the order of the factors with the highest influence on the flocculating degree was as follows: A > D > B > C. The optimum culture conditions were A_2, B_2, C_1, and D_2, *i.e.*, culture time 48 h, culture temperature 30 °C, fermentation pH 5, and rotation speed 150 rpm. According to these conditions, the flocculating degree was up to 82.0%, and the highest orthogonal verification test result was approximately 81.8%. The results of orthogonal experiment showed that the influence of culturing temperature on flocculating degree was the greatest.

Results of the Medium Optimization

Single factor optimization experiment

Carbon source plays a significant role in cell growth and synthesis of metabolites. Different carbon sources had different influences on the flocculating degree of the bacteria. Figure 11 shows that the influences of lactose, maltose, and starch as the carbon sources on the production of the M1 bacteria had the same degree, whereas sucrose and glucose had more significant effects than the other carbon sources. The flocculating degree of the glucose group was the highest followed by the sucrose group. The carbon source is the most important nutrient source for microorganisms and an important element for the formation of the cytoskeleton. Carbon source is an important component of the medium. Cheap carbon sources can effectively reduce costs. Under the same effect, the lower cost carbon source is the better one. In this experiment, glucose and sucrose were similar in price, and glucose was chosen for subsequent experiments because it supported a slightly higher bioflocculation degree. Therefore, the glucose was judged to be the best carbon source for the M1 strain.

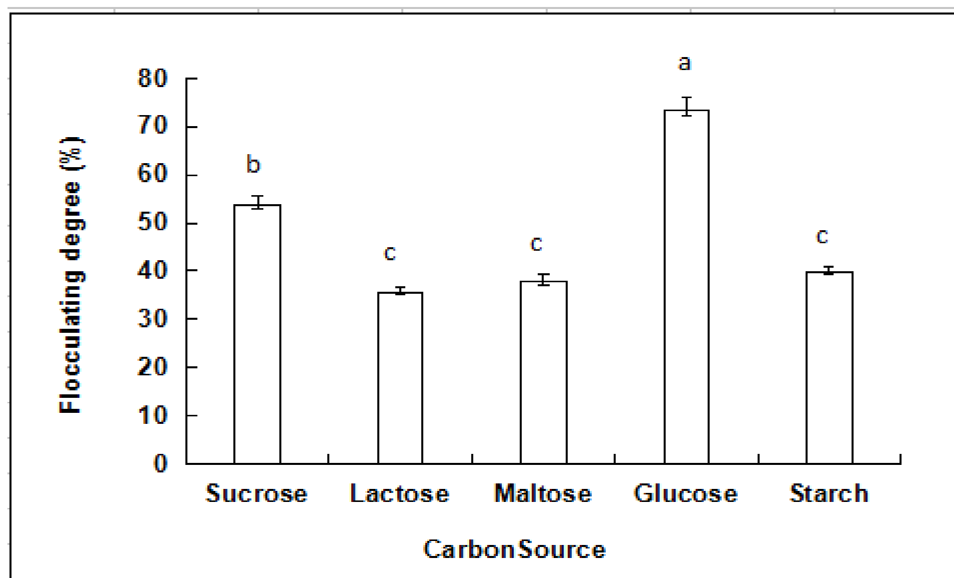

Fig. 11. Effect of the different carbon source on the flocculating degree. Values represent the means and standard deviations, *n*=3; Values in a column with different superscripts are significantly different ($p<0.05$).

The flocculating degree was measured with different nitrogen sources. Figure 12 shows that nitrogen source had a marked influence on the growth of the M1 bacteria. The peptone resulted in the highest flocculating degree as the only nitrogen source, and it was

up to 80.3%. This indicated that peptone was superior to the combination of two or three nitrogen sources. The selection principle of nitrogen source is the same as that of carbon source. The cost of the nitrogen source is also important. Based on a survey of the cost of various nitrogen sources currently available, and combined with its flocculation-promoting ability, peptone was the best nitrogen source for M1 strain. These results were consistent with the conclusion reported by Sekelwa *et al*. (2013). Peptone was therefore selected as the nitrogen source in the following experiments.

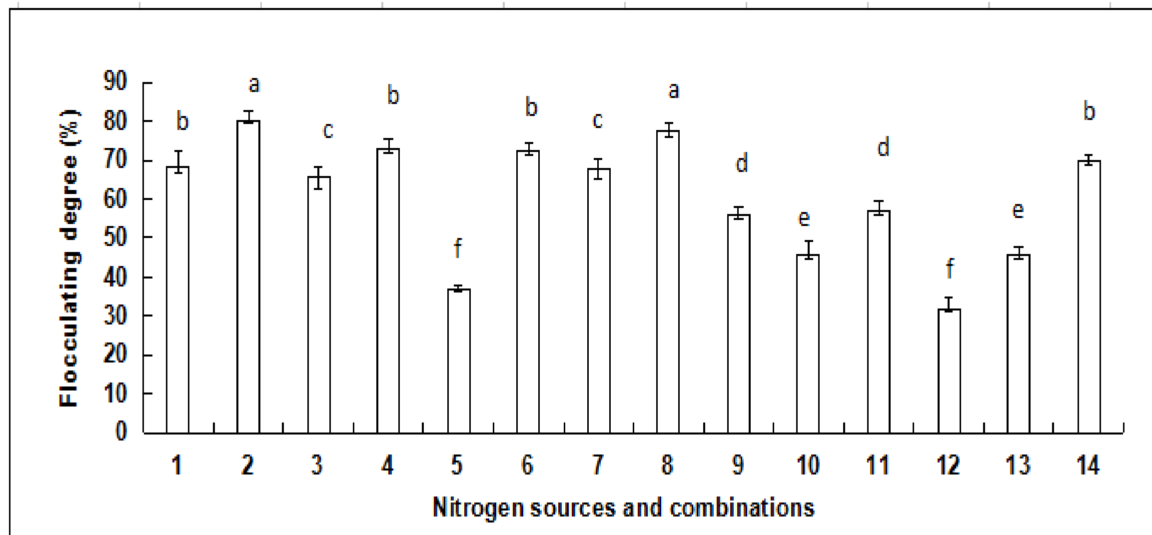

Fig. 12. Effect of the different nitrogen sources on the flocculating degree
Notes: 1 $NaNO_3$; 2 Peptone; 3 $(NH4)_2SO_4$; 4 Beef extract; 5 Urea; 6Yeast extract; 7 Peptone + yeast extract; 8 Urea + yeast extract; 9 Peptone + urea; 10 Yeast extract + ammonium sulfate; 11 Peptone + sodium nitrate + ammonium sulfate; 12 Yeast extract + peptone + ammonium sulfate; 13 Yeast extract + beef extract; 14 Yeast extract + ammonium sulfate + urea. Values represent the means and standard deviations, n=3; Values in a column with different superscripts are significantly different ($p<0.05$).

Phosphate can form a good buffer system, which plays an important role in the regulation of pH during fermentation. The phosphate ratio test results are shown in Fig. 13.

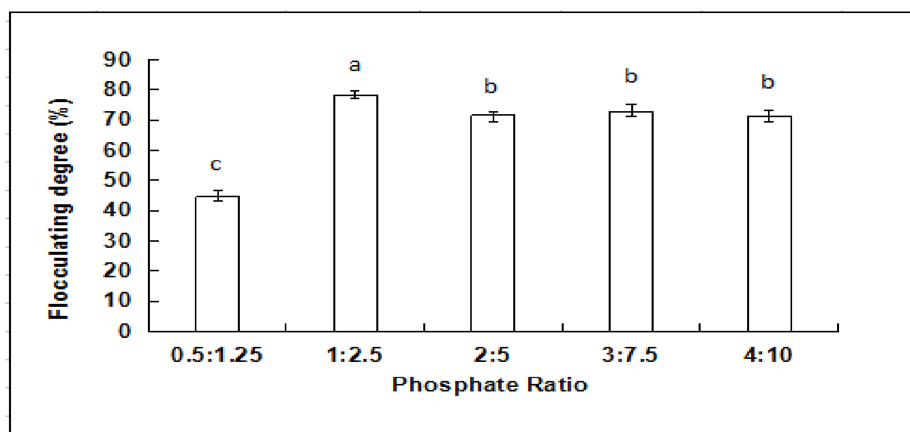

Fig. 13. Effect of the different phosphate ratios on the flocculating degree. Values represent the means and standard deviations, n=3; Values in a column with different superscripts are significantly different ($p<0.05$).

The flocculating degree of the M1 strain was the lowest when the phosphate dosage ratio was 0.5 g/L:1.25 g/L. The flocculating degree of the M1 strain was the highest when the phosphate dosage ratio was 1 g/L:2.5 g/L. Phosphorus is an important component of microbial cells, as it is the framework of proteins. Phosphate also regulates the acid-base balance in the culture medium. Therefore, it was necessary to study the effect of the phosphate dosage ratio on the flocculating degree of the M1 strain.

Orthogonal optimization of the medium composition

The orthogonal range was calculated according to the orthogonal experimental results. According to the degree of the ranges, the order of the factors with the highest impact was as follows: C > A > B. Table 6 shows that the order of the most important nutrient source was as follows: phosphate ratio > glucose > peptone. In Table 7, the analysis of variance showed that glucose and peptone did not have a significant difference ($P > 0.05$), while phosphate did have a significant difference ($P < 0.05$). In conclusion, the best combination was A_4, B_3, and C_2, *i.e.*, 30 g/L glucose, 2 g/L peptone, and 1 g/L KH_2PO_4 to 2.5 g/L K_2HPO_4. Table 7 shows that glucose at concentrations of 15 g/L and 30 g/L did not have a significant effect. Considering the cost, the conditions with the lowest cost and smallest dose should be chosen. Therefore, the optimum conditions were A_1, B_3, and C_2; 15 g/L glucose is the carbon source, 2 g/L peptone is the only nitrogen source, and the phosphate dosage ratio is 1 g/L KH_2PO_4 to 2.5 g/L K_2HPO_4.

Table 6. L_{16} (4^3) Orthogonal Test Data and Analysis of the Results

No./Factor	A	B	C	Flocculating degree
1	1	1	1	72.00
2	1	2	2	77.50
3	1	3	3	68.70
4	1	4	4	66.31
6	2	2	1	57.83
7	2	3	4	59.94
8	2	4	3	59.32
9	3	1	3	56.22
10	3	2	4	49.07
11	3	3	1	74.12
12	3	4	2	74.74
13	4	1	4	62.67
14	4	2	3	71.56
15	4	3	2	80.28
16	4	4	1	77.06
K1	284.51	267.41	281.01	
K2	253.61	255.96	309.04	
K3	254.15	283.04	255.8	
K4	291.57	277.43	237.99	
K1 - average value	71.1275	66.8525	70.2525	
K2 - average value	63.4025	63.99	77.26	
K3 - average value	63.5375	70.76	63.95	
K4 - average value	72.8925	69.3575	59.4975	
R (range)	9.49	6.77	17.76	

Table 7. Variance Analysis Table of the Orthogonal Tests of the Carbon Source, Nitrogen Source, and Phosphate Ratio in the M1 Culture Medium

Factor	Sum of Squares of Deviations	Freedom	F Comparison	F Critical Value	Significance
A	291.903	3	2.904	F0.01(2, 6) = 9.78	
B	70.227	3	0.699	F0.10(2, 6) = 3.29	
C	716.984	3	7.134	F0.05(3, 6) = 4.760	*
Error	201.00	6			

Water Quality Parameters of Wheat Distillery Wastewater

The SS, COD, BOD, turbidity and pH of wheat distillery wastewater are shown in Table 8. The pH value after flocculation was the same as the raw wastewater, and removal efficiency of SS, COD, BOD, and turbidity were up to 71.9%, 61.5%, 48.6%, and 72.7% respectively. It is thus clear that bioflocculant produced by M1 bacteria exhibited strong ability to remove SS and turbidity, and also has good removal effect on COD and BOD of wheat distillery wastewater.

Table 8. Determination of Wheat Distillery Wastewater Quality Parameters

Item	pH	SS（mg/L）	COD(mg/L)	BOD(mg/L)	Turbidity(NUT)
Raw water quality	3.41	5428	27310	16400	2580
Water quality after flocculation	3.45	1525	10514	8430	705
Removal efficiency (%)	/	71.9%	61.5%	48.6%	72.7%

CONCLUSIONS

1. A highly effective flocculant-producing bacteria, *Klebsiella* M1, was screened from activated sludge and could be used as a high-performance bioflocculant-producing bacterium for wheat distillery wastewater. The initial flocculating degree was up to 72.1%. The strain was preserved in the China Center for typical culture collection, and the number was CCTCCM 2018098. The 16S rDNA gene had been registered at GenBank and its login number was MG987011.
2. According to the single factor experiment, the optimum flocculating conditions were a resting time 30 min, 8% culture medium dosage, and 3% $CaCl_2$.
3. The optimum culture conditions for the M1 bacteria were culture time 48 h, culture temperature 30 °C, initial pH 4.5, and rotational speed 150 rpm. After the verification test, the flocculating degree reached 82.0%. The orthogonal optimization results indicated that the optimum culture medium of the M1 bacteria contained glucose as the carbon source (15 g/L), peptone as the sole nitrogen source (2 g/L), and a phosphate dosage ratio of 1 g/L KH_2PO_4 to 2.5 g/L K_2HPO_4. A high flocculating degree was achieved with a low-cost medium.

4. These experiments showed that the M1 strain could be used as a good bioflocculant-produced strain for wheat distillery wastewater. A new method was developed to purify wheat distillery wastewater with bioflocculants.

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