Optimization of Microbial Flocculant-Producing Medium for *Bacillus subtilis*

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Abstract This study aimed to improve microbial flocculant production by optimizing the components of a *Bacillus subtilis* CZ1003 culture medium. Using the flocculation rate as the dependent variable, single-factor experiments were performed and beef extract at a concentration of 9 g/L was found to be the optimal nitrogen source, while glucose at a concentration of 20 g/L was the optimal carbon source. KCl, MgCl₂, NaCl, and CaCl₂ at concentrations of 0.75, 2.5, 0.5, and 5.0 g/L, respectively, were the optimum inorganic salts, in order of flocculant production activity. Orthogonal experimental demonstrated that KCl played a dominant role for *Bacillus subtilis* production of bioflocculants, followed by NaCl and CaCl₂. Optimization experiments demonstrated that the optimal combination of the two salts was 0.75 g/L KCl and 0.5 g/L NaCl, resulting in a flocculation rate of 36.2% when included together at these concentrations. The final optimized medium consisted of 20 g/L glucose, 9 g/L beef extract, 0.75 g/L KCl, and 0.5 g/L NaCl. Compared with the initial medium, the optimized medium enhanced the flocculation activity from 12.1 to 36.2%, which equates to an increase of 199.17%. Meanwhile, the flocculant yield was increased from 0.058 g/L to 0.134/L, an increase of 131.03%. The optimized medium could be used to improve microbial flocculant production and provides a basis for further exploration.

Keywords Microbial flocculant · Medium optimization · *Bacillus subtilis* · Flocculation activity

Introduction

Microbial flocculants (bioflocculants) are organic polymers related to microbial metabolism that possess interesting activities that are safe, non-toxic, environmentally friendly and can be efficiently produced. These features make them an ideal substitute for inorganic and organic synthesized coagulants and flocculants, and they have great potential for use in a range of applications [1, 2]. Early experiments by our group found that one microbial flocculant produced by *Bacillus subtilis* was able to remove chemical oxygen demand (COD), color, ammonia, and total nitrogen from tannery wastewater [3, 4]. However, the flocculation activity must be improved for its true potential to be realized.

In previous studies on the culture medium used for bioflocculant-producing microorganisms, flocculation activity was found to be closely related to medium composition, primarily to the carbon and nitrogen sources (and inorganic salts) present, and the optimal composition differs for different strains [5]. The carbon source selected should be beneficial to both cell growth and flocculant production, and should ideally use abundant raw materials that are easily obtained at low cost, such as soluble starch, sucrose, glucose, ethanol, lactose, maltose, and mannitol [6, 7]. Similarly, the nitrogen source, which is needed for protein synthesis, is also a significant factor that determines the yield and quality of the resultant bioflocculant. Studies have shown that nitrogen sources such as yeast, beef extract and urea are beneficial for bacterial propagation and bioflocculant production [8, 9]. In terms of inorganic salts,

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some secreted bioflocculants are greatly influenced by metal ions in the medium. Generally, Ca^{2+} improves the bioflocculant activity. However, certain ions improve the bioflocculant activity at low concentrations but inhibit the activity and yield at higher concentrations. For example, 0.25% KNO_3 , 0.01% $\text{Al}(\text{SO}_4)_3$, and low concentrations of CaCl_2 , CaCO_3 , and MgSO_4 promote bioflocculant production in the C-62 strain, but when the concentration is higher than 0.5%, the flocculating activity decreases. To date, NaCl , CaCl_2 , MgCl_2 , K_2HPO_4 , and MgSO_4 have been studied in this regard [10, 11].

In the present study, we aimed to optimize carbon and nitrogen sources and inorganic salts in the culture medium of *Bacillus subtilis* in order to improve the bioflocculant activity. Based on the initial medium (glucose 30 g, yeast extract 8 g, dipotassium hydrogen phosphate 2 g, magnesium sulfate 0.25 g, sodium glutamate 10 g, distilled water 1 L, pH 7.5), the type and concentration of the carbon and nitrogen source and inorganic salts were optimized. Yeast extract, beef extract, peptone, sodium nitrate, urea, ammonium chloride, ammonium sulfate, ammonium nitrate, and potassium nitrate were tested as nitrogen sources, glucose, sucrose, maltose, fructose, lactose, mannitol, soluble starch, and ethanol were tested as carbon sources, and dipotassium phosphate, magnesium sulfate, sodium glutamate, aluminum chloride, magnesium chloride, copper chloride, sodium chloride, potassium chloride, and calcium chloride were tested as inorganic salts. After optimization of all three parameters, the bioflocculant-producing capacity of the medium was improved significantly compared to the initial medium.

Materials and Methods

Experimental Strains and Media

Bacillus subtilis CZ1003 was sourced from our laboratory stocks. The initial bioflocculant-producing medium contained the following: 30 g glucose, 8 g yeast extract, 2 g dipotassium hydrogen phosphate, 0.25 g magnesium sulfate, 10 g sodium glutamate, distilled water to 1000 mL (pH 7.5) [3].

Strain Inoculation

Initial medium (50 mL) was transferred into a 250 mL conical flask and autoclaved at 121 °C for 20 min. After cooling, the strain was inoculated into the medium and incubated in a shaker at 35 °C and 180 rpm for 36 h.

Determination of Flocculation

The flocculation activity was determined by measuring the flocculation rate of kaolin suspension. In a 100 mL cylinder, a sample of 0.4 g kaolin, 80 mL distilled water, 5 mL 1% CaCl_2 and 2 mL sample were added and mixed. Then, distilled water was added to a total volume of 100 mL and the pH was adjusted to 7.0. After standing for 5 min, the absorbance was determined at 550 nm using a 7230G spectrophotometer (Beijing Purkinje General Instrument Co., Ltd, Beijing, China). As a control, distilled water was used to replace both CaCl_2 and the sample, and the absorbance was determined in the same way. The flocculation rate was calculated based on the absorbance difference using the following formula:

$$E = (B - A) \times 100 \% / B$$

where E is the flocculation rate (%), A is the absorbance of the kaolin suspension processed by the sample, and B is the absorbance of the control (blank kaolin suspension) [3].

Distribution of Flocculation

Medium (20 mL) was transferred into a 100 mL conical flask and autoclaved at 121 °C for 20 min. After cooling, 2 mL *Bacillus subtilis* culture was inoculated into the medium and incubated in a shaker at 35 °C and 180 rpm for 36 h. After incubation, 10 mL was centrifuged at 4192.5 g for 15 min, and the supernatant and pellet were collected. The precipitate was suspended in Tris–HCl buffer (pH 7.0) and sonicated using a UH-500A sonicator (Beijing Tayasaf Co., Ltd., China). After centrifugation, the supernatant was collected and suspended in 10 mL Tris–HCl buffer to generate to cell content sample. Flocculation rates were measured for both supernatant and cell content samples, and the distribution of the flocculation activity was determined by comparing these values [12].

Single Factor Optimization of the Nitrogen Source

Determination of the Optimum Nitrogen Source

The yeast extract in the initial medium was replaced with 8 g/L of beef extract, peptone, sodium nitrate, urea, ammonium chloride, ammonium sulfate, ammonium nitrate, or potassium nitrate (or water as a control). Media was autoclaved at 121 °C for 20 min. After cooling, 5 mL of *Bacillus subtilis* culture was inoculated into each medium and incubated in a shaker at 35 °C and 180 rpm for 36 h. After incubation, flocculation rates were measured with kaolin suspension and compared to determine the optimum nitrogen source.

Optimization of the Nitrogen Concentration

In 100 mL Erlenmeyer flasks containing 20 mL media, different amounts of the optimal nitrogen source were added to a final nitrogen concentration of 4, 6, 7, 8, 9, 10, and 12 g/L and autoclaved at 121 °C for 20 min. After cooling, 5 mL of *Bacillus subtilis* culture was inoculated into each medium and incubated in a shaker at 35 °C and 180 rpm for 36 h. After incubation, flocculation rates were measured with kaolin suspension and compared to determine the optimum nitrogen concentration.

Single Factor Optimization of Carbon Source

Determination of the Optimum Carbon Source

In 100 mL Erlenmeyer flasks containing 20 mL media comprising the optimal nitrogen source, 30 g/L glucose, sucrose, maltose, fructose, lactose, mannitol, soluble starch, or ethanol was added (water was added as a control) and autoclaved at 121 °C for 20 min. After cooling, 5 mL of *Bacillus subtilis* culture was inoculated into each medium and incubated in a shaker at 35 °C and 180 rpm for 36 h. After incubation, flocculation rates were measured with kaolin suspension and compared to determine the optimum carbon source.

Optimization of the Carbon Concentration

To each 100 mL Erlenmeyer flask containing 20 mL media, different amounts of the optimal carbon source were added to a final carbon concentration of 4, 6, 7, 8, 9, 10, and 12 g/L and autoclaved at 121 °C for 20 min. After cooling, 5 mL of *Bacillus subtilis* culture was inoculated into each medium and incubated in a shaker at 35 °C and 180 rpm for 36 h. After incubation, flocculation rates were measured with kaolin suspension and compared to determine the optimum carbon concentration.

Optimization of Inorganic Salts

Single Factor Optimization of the Concentration of Inorganic Salts

Based on media formulations in numerous literature sources, as well as the known effects of metal ions and anions on bioflocculant-producing strains, a number of inorganic salts were selected for testing [5, 10, 11]. Specifically, calcium chloride, potassium chloride, sodium chloride, magnesium sulfate, dipotassium hydrogen phosphate, magnesium chloride, aluminum chloride, cupric chloride, and sodium glutamate were tested at the concentrations shown in Table 1.

In medium containing optimal nitrogen and carbon sources, inorganic salts were added to replace the salt in the original formulation and media were autoclaved at 121 °C for 20 min. After cooling, 5 mL of *Bacillus subtilis* culture was inoculated into each medium and incubated in a shaker at 35 °C and 180 rpm for 36 h. After incubation, flocculation rates were measured with kaolin suspension and compared to determine the optimum inorganic salt and the optimum concentration.

Orthogonal Combination of Inorganic Salts

Based on the optimal inorganic salt and the optimal concentration determined above, the four salts with the highest flocculation rates were chosen for further optimization. Four factor three level $L_9(3^4)$ orthogonal experiments were performed, and flocculation rates were measured to determine the effects of the four salts in combination with each other.

Optimization of the Combination of Inorganic Salts

Based on the results of the single inorganic salt experiments, the best four salts and the optimal concentration for each were selected for combination experiments that included two, three, and four combinations of the inorganic

Table 1 Concentration of inorganic salts in the medium (g/L)

Inorganic salt	Tested concentration									
Calcium chloride	0.05	0.10	0.25	0.50	1.0	2.5	5.0	10	20	
Potassium chloride	0.10	0.25	0.50	0.75	1.0	–	–	–	–	
Sodium chloride	0.05	0.10	0.50	1.0	2.5	5.0	10	20	–	
Magnesium sulfate	0.05	0.25	0.50	0.75	1.0	1.25	2.5	5.0	10	
Dipotassium phosphate	0.25	0.50	0.75	1.0	2.0	2.5	5.0	10	–	
Magnesium chloride	0.05	0.10	0.25	0.50	1.0	2.5	5.0	10	–	
Aluminum chloride	0.05	0.10	0.25	0.50	1.0	2.5	5.0	10	–	
Copper chloride	0.05	0.10	0.25	0.50	1.0	2.5	5.0	10	–	
Sodium glutamate	0.50	1.0	5.0	10	15	20	25	30	–	

Note: “–” indicates a concentration of zero

salts. The four selected salts were denoted as A, B, C, and D, and the combinations tested were AB, AC, AD, BC, BD, CD, ABC, ABD, ACD, BCD, and ABCD. The flocculation rate for each combination was measured and analyzed to determine the optimal combination.

Comparison of the Initial and Optimized Media

Initial and optimized medium (500 mL) was autoclaved at 121 °C for 20 min. After cooling, 5 mL of *Bacillus subtilis* culture was inoculated into each medium and incubated in a shaker at 35 °C and 180 rpm for 36 h. After incubation, flocculation rates were measured and bioflocculants extracted and compared.

The bioflocculant extraction procedure was performed as follows: Cultures were centrifuged at 5000 rpm for 15 min and supernatants collected. Next, three volumes of pre-cooled (4 °C) ethanol was added to the supernatant and incubated at 4 °C overnight. Following centrifugation at 5000 rpm for 15 min, precipitates were collected and vacuum freeze-dried to obtain the bioflocculant product from the supernatant. For intracellular bioflocculants, the pellet from the initial centrifugation was sonicated, centrifuged, and the supernatant collected and added to three volumes of pre-cooled ethanol. After incubation at 4 °C for 48 h, the mixture was centrifuged at 5000 rpm for 15 min and the precipitate was collected and vacuum freeze-dried prior to bioflocculant measurement.

Results and Discussion

Distribution of Flocculation Activity in *Bacillus subtilis*

The flocculation rates of supernatants and cell contents were compared, and were 8.92, and 27.68%, respectively,

Fig. 1 Flocculation rates for different nitrogen sources. 1 yeast extract, 2 beef extract, 3 peptone, 4 sodium nitrate, 5 urea, 6 ammonium chloride, 7 ammonium sulfate, 8 ammonium nitrate, 9 potassium nitrate

suggesting the flocculation activity was primarily located within cells. Therefore, cell contents were used in all subsequent experiments for the determination of flocculation activity.

Optimization of Nitrogen Source

Determination of the Optimum Nitrogen Source

As shown in Fig. 1, when the nitrogen concentration was fixed at 8 g/L, beef extract was the optimal nitrogen source and had a bioflocculant rate of 20.20%, compared with 12.10% for the control (yeast extract). For comparison, the rate was 18.20% with ammonium nitrate and only 5.80% with peptone. In addition to its impact on bioflocculant production, 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, beef extract was the best nitrogen source for *Bacillus subtilis* bioflocculant production.

Determination of the Optimal Nitrogen Concentration

The flocculation rate with different concentrations of beef extract and the optimal nitrogen source are shown in Fig. 2. The flocculation rate reached a maximum value of 24.70% with 9 g/L beef extract, and this concentration was therefore chosen for subsequent experiments.

Optimization of Carbon Source

Determination of Optimum Carbon Source

Using the optimal nitrogen source type and concentration, the flocculation rate was tested for different carbon sources (Fig. 3). Glucose, sucrose, and fructose were comparable, with flocculation rates of 24.70, 24.45, and 25.25%, respectively. As discussed above for the nitrogen source, the cost of the carbon source is also important. Although

Fig. 2 Flocculation rates at different nitrogen concentrations

Fig. 3 Flocculation rates for different carbon sources. *a* glucose, *b* sucrose, *c* maltose, *d* fructose, *e* lactose, *f* mannitol, *g* soluble starch, *h* ethanol

fructose displayed good bioflocculation activity, it is much more expensive than the other carbon sources, and was therefore not chosen. Since glucose and sucrose were similar in price, glucose was chosen for subsequent experiments because it supported a slightly higher bioflocculation rate.

Determination of Optimal Carbon Concentration

Flocculation rates with different concentrations of the optimal carbon (Fig. 4) showed that a maximum value of 28.10% was reached with 20 g/L glucose. Although there was some variation, the flocculation rate was generally lower with a higher or lower glucose concentration, and 20 g/L was therefore considered optimal.

Optimization of Inorganic Salts

Determination of the Optimal Concentration for Isolated Inorganic Salts

Based on the flocculation rates at different concentrations of each inorganic salt (Figs. 5, 6, 7, 8, 9, 10, 11, 12, 13), the top four salts were chosen for further investigation. Statistical analysis of the results (Table 2) revealed that of the

Fig. 4 Flocculation rates at different carbon concentrations

nine inorganic salts tested, KCl, MgCl_2 , NaCl, and CaCl_2 supported the highest flocculation rates. KCl supported the highest flocculation rate of all, which was 31.40% at 0.75 g/L, followed by MgCl_2 (29.50% at 2.5 g/L), NaCl (26.70% at 0.5 g/L), and CaCl_2 (21.80% at 5.0 g/L). As shown in Figs. 5, 6, 7 and 8, the flocculation rates fluctuated somewhat, but on the whole decreased above or below the optimum concentrations. The results indicated that KCl, MgCl_2 , NaCl, and CaCl_2 acted as effective coagulants and improved microbial flocculation by adsorbing and/or bridging between flocculant particles. Conversely, the

Fig. 5 Relationship between flocculation rate and potassium chloride concentration

Fig. 6 Relationship between flocculation rate and magnesium chloride concentration

Fig. 7 Relationship between flocculation rate and sodium chloride concentration

Fig. 8 Relationship between flocculation rate and calcium chloride concentration

Fig. 9 Relationship between flocculation rate and magnesium sulfate concentration

Fig. 10 Relationship between flocculation rate and copper chloride concentration

other five inorganic salts tested (MgSO_4 , CuCl_2 , sodium glutamate, AlCl_3 , and K_2HPO_4) failed to effectively improve bioflocculant production.

Orthogonal Combination of Inorganic Salts

Based on the above results, the top four optimal inorganic salts for supporting bioflocculant production in *Bacillus subtilis* were KCl, MgCl_2 , NaCl, and CaCl_2 . Next, we

Fig. 11 Relationship between flocculation rate and monosodium glutamate concentration

Fig. 12 Relationship between flocculation rate and aluminum chloride concentration

Fig. 13 Relationship between flocculation rate and dipotassium phosphate concentration

performed a $L_9(3^4)$, four-factor, three-level orthogonal experiment to test the effects of different combinations of these inorganic salts (Table 3). Salts were denoted as A, B, C, and D in reference to the four factors. For each factor, three different concentrations were chosen according to the respective optimal concentration, representing the three levels. The results are shown in Table 4.

Table 2 Single factor optimization of inorganic salts

Single inorganic salt	Optimal concentration (g/L)	Flocculation rate (%)
KCl	0.75	31.4
MgCl ₂	2.5	29.5
NaCl	0.5	26.7
CaCl ₂	5.0	21.8
MgSO ₄	1.25	17.4
CuCl ₂	1.0	17.4
Sodium glutamate	5.0	13.8
AlCl ₃	5.0	13.5
K ₂ HPO ₄	5.0	13.3

Table 3 Factors and levels in orthogonal experiments

Levels	Factors			
	A (KCl)	B (MgCl ₂)	C (NaCl)	D (CaCl ₂)
1	0.70	2.0	0.25	4.5
2	0.75	2.5	0.50	5.0
3	0.80	3.0	0.75	5.5

As shown in Table 4, the flocculation rates of the first level for factor A were summed up as K_1 then divided by three to obtain the mean k_1 . The range of k values for each factor across the three levels was calculated and represented by R . The larger the K value, the higher the flocculation rate for that level. The larger the R value, the

higher the effect of the factor on the flocculation activity. As shown in Table 4, the flocculation rate for each orthogonal combination was not particularly high. However, the R values revealed that the effects of the four factors were ordered $KCl > NaCl > CaCl_2 > MgCl_2$. KCl therefore played a dominant role in *Bacillus subtilis*-mediated flocculant production, followed by NaCl. Based on the k values, the optimal combination of inorganic salts was $A_1B_3C_3D_3$, corresponding to 0.70 g/L KCl, 3.0 g/L MgCl₂, 0.75 g/L NaCl and 5.5 g/L CaCl₂, which resulted in a flocculation rate of 15.0%.

Optimization of the Combined Inorganic Salts

The results of optimization of the combined inorganic salts are shown in Fig. 14.

As is evident in Fig. 14, KCl & NaCl (A&C) proved to be the best combination, with a flocculation rate of 36.2%, followed by KCl & NaCl & CaCl₂ (A&C&D) with a flocculation rate of 28.0%. It was also found that any combination including MgCl₂ resulted in a low flocculation rate, consistent with the effects of the four factors on the flocculation rate shown in Table 4.

By combining the single factor optimization of inorganic salts, the orthogonal experiments, and the combination experiments, the optimal combination of the inorganic salts was 0.75 g/L KCl and 0.5 g/L NaCl, and the final optimized medium for *Bacillus subtilis* bioflocculant production contained 20 g/L glucose, 9 g/L beef extract, 0.75 g/L KCl, and 0.5 g/L NaCl.

Table 4 Results of orthogonal experiments

No.	Factors				Results flocculation rate (%)
	A (KCl)	B (MgCl ₂)	C (NaCl)	D (CaCl ₂)	
1	1	1	1	1	8.6
2	1	2	2	2	7.5
3	1	3	3	3	15.0
4	2	1	2	3	5.0
5	2	2	3	1	8.3
6	2	3	1	2	4.9
7	3	1	3	2	3.2
8	3	2	1	3	4.0
9	3	3	2	1	−5.5
K_1	31.101	16.800	17.499	11.400	
K_2	18.207	19.800	6.999	15.600	
K_3	1.701	14.400	26.499	24.000	
k_1	10.367	5.600	5.833	3.800	
k_2	6.069	6.600	2.333	5.200	
k_3	0.567	4.800	8.833	8.000	
R	9.800	1.800	6.500	4.200	

Fig. 14 Flocculation rates with different combinations of inorganic salts. A KCl, B MgCl₂, C NaCl, D CaCl₂

Table 5 Comparison of the initial medium and optimized medium

Indexes	Medium		Increase (%)
	Initial medium	Optimized medium	
Flocculation rate (%)	12.1	36.2	199.17
Flocculant yield (g/L)	0.058	0.134	131.03

Comparison of the Initial Medium and the Optimized Medium

The flocculation rate and yield of the initial medium and the optimized medium were measured, and the results showed that the flocculation rate with the optimized medium was improved from 12.1 to 36.2%, which represents an increase of 199.17%. Furthermore, the flocculation yield from the optimized medium was increased from 0.058 g/L to 0.134 g/L, which represents an increase of 131.03% (Table 5). Both the flocculation rate and flocculation yield were therefore substantially improved compared to the initial medium. In addition, the optimized medium contained only four kind of components and were obviously less than the initial medium which make the optimized medium be simpler and cheaper. As bioflocculants are safe, non-toxic, environmentally friendly and can be efficiently produced, the bioflocculants which have been successfully optimized for the medium have great potential for use in a range of applications.

Conclusion

In order to improve the quality and yield of the bioflocculant resultant from *B. subtilis* CZ1003 for further wide applications, the carbon source, nitrogen source, and inorganic salts were optimized through single-factor experiments, orthogonal experimental and optimization of the combined inorganic salts. As a result, we successfully

improved the medium for *B. subtilis* CZ1003 flocculant production. The results presented that beef extract at a concentration of 9 g/L was found to be the optimal nitrogen source, and glucose at a concentration of 20 g/L was the optimal carbon source, while optimal combination of the salts was 0.75 g/L KCl and 0.5 g/L NaCl, resulting in a flocculation rate of 36.2%. The optimized medium contained 20 g/L glucose, 9 g/L beef extract, 0.75 g/L KCl, and 0.5 g/L NaCl. Compared to the initial medium, the flocculant activity of the optimized medium was increased by 199.17%, and the flocculant yield by 131.03%. The improved medium may prove beneficial for improving microbial flocculant production in wastewater treatment plants and other applications.

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