

# A novel fungus strain (*Isaria cicadae* GZU6722) with high potential of bioflocculation

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Research

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## CITATION

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## ABSTRACT

**BACKGROUND:** *Isaria cicadae* was well known as a rare entomogenous fungus with various pharmacological activities in traditional Chinese medicine, but less attention was paid to its other biological characteristics rather than the medical applications.

**METHODS:** In the present study, the bioflocculation ability of total 36 *Isaria cicadae* strains were investigated. For the first time, *Isaria cicadae* GZU6722 was screened as a novel fungus strain that shows high potential of bioflocculation. From the time course of bioflocculant production, the bioflocculant was assumed a kind of the secondary metabolite. Then, the bioflocculant named as IC-1 produced by *Isaria cicadae* GZU6722 was purified and mainly consisted of protein (4%) and polysaccharides (91%), which contained 52.75% of neutral sugar and 38.14% of uronic acid.

**RESULTS:** IC-1 showed high flocculating rate in kaolin suspension in a wide range of temperature, suggesting its storage potential in cold and hot conditions and a potential application for the wastewater treatment. Furthermore, the cation addition could enhance the flocculating rate, even up to 96.08% in the case of addition of CaCl<sub>2</sub>. In the present work, IC-1 demonstrated its thermo-stability over wide range of temperatures, and also suggests its storage potential in cold and hot conditions.

**CONCLUSIONS:** In the present work, IC-1 from *Isaria cicadae* GZU6722, has exhibited its excellent flocculating performance under various conditions, and also suggests its storage potential in cold and hot conditions. These findings imply that the application potential of this novel bioflocculant for wastewater bioremediation.

**Key Words:** Cation-dependent; Flocculating activity; *Isaria cicadae*; Microbial flocculant.

## 1. INTRODUCTION

Flocculating process is widely used in various fields of industries, such as food production, fermenting process, water purification and wastewater treatment <sup>[1]</sup>. In this process, the flocculants are classified into convent chemical flocculants and natural occurring flocculants such as bioflocculants <sup>[2]</sup>. Usually, the inorganic flocculants includes polyaluminum chloride (PAC) and the synthetic organic flocculants includes polyacrylamide derivatives. However, the excessive use of these chemical flocculants can cause health and environmental problems <sup>[2]</sup>. For example, a residual aluminum from PAC and acrylamide monomers from polyacrylamide was reported to be neurotoxic and carcinogenic toward humans <sup>[3]</sup>. Recently, more and more researchers have paid much attentions to bio-flocculants which could be an alternative for the chemical flocculant for their high flocculation performance, ecofriendly biodegradability <sup>[4]</sup>.

Bioflocculants are mainly composed of macromolecular substances, such as polysaccharide and protein <sup>[5, 6]</sup>. The composition and properties of bioflocculants depend by the factors such as the type of bioflocculant-producing microorganisms (BPMs), composition of media and environmental conditions. The differences in the composition and properties of polysaccharides and proteins lead to differences in the flocculation ability and the application of bioflocculant <sup>[7-9]</sup>. Therefore, it is very important to screen the more eco-friendly strains with high bioflocculability <sup>[2, 10]</sup>.

*Isaria cicadae*, an entomogenous fungus, has been used as a therapeutic dietary in traditional Chinese medicine, which contains various pharmacological activities and applied as bio-control agents recently <sup>[11-13]</sup>. To date, much attention was paid to its medical applications, but there is still a lack of comprehensive and deep studies on its applications in environmental pollution remediations. In our previous work, *Isaria cicadae* has shown its good flocculation performance <sup>[14]</sup>, and yet little was known about its flocculating properties and optimized condition for its better improvement.

Therefore, the aim of the present study is to screen

the *Isaria cicadae* strains with high flocculating activity, and to investigate the optimal culture conditions including the variations of culture medium. Furthermore, the bioflocculant produced by the strain was purified, and its composition and properties were identified as well for its potential application in the wastewater treatment, even in the drinking water purification with its low risk.

## 2. MATERIALS & METHODS

### 2.1 Microorganism and culture conditions

The strains of *Isaria cicadae* were isolated from the soils and the host from China and Korea, and were preserved at Institute of Fungal Resources, Guizhou University. The strains were maintained on Potato Dextrose Agar (PDA) slant at 4°C. The medium for slant was consisted of (g L<sup>-1</sup>): potato extract, 4; glucose, 20 and agar, 15; and the medium for subculture as consisted of (g L<sup>-1</sup>): glucose, 20; peptone, 5; KH<sub>2</sub>PO<sub>4</sub>, 2; MgSO<sub>4</sub>•7H<sub>2</sub>O, 0.5; and CaCl<sub>2</sub>, 0.5. Meanwhile the initial pH was 6.5 ± 0.2. In order to carry out the experiment, the spore suspension was resuspended to the desired concentration of 1×10<sup>7</sup> ml<sup>-1</sup> by flooding the plate with distilled water after the slant cultivation at 22°C for 5 d. The biomass samples were filtered and dried at 80°C in an oven for 4 h. Distilled water was used to prepare all medium solutions and the media were sterilized at 121°C for 30 min.

### 2.2 Screening of bioflocculant-producing microorganisms

A kaolin suspension was used to determine the flocculating rate of the bioflocculant in culture broth. Two gram of Kaolin clay (Merck, Germany) was suspended in 1 l of deionized water.

After 7d incubation, 1 ml culture broth were added into 100 ml kaolin suspension (5 g l<sup>-1</sup>) in 250 ml beaker, and the flocculating activity of the active fractions that could flocculate the kaolin suspension was measured. One ml of culture broth was added to 99 ml of kaolin suspension in a 400 ml beaker. The mixture was stirred slowly stirred at 80 rpm for 5 min. The optical density (OD) of the supernatant was

measured with a spectrophotometer (GENESYS 10 UV, Thermo Scientific, USA) at 550 nm. In the control experiment, 1 ml of culture broth was replaced with 1 ml of fresh culture medium. The flocculating rate was calculated according to the following equation:

$$\text{Flocculating rate (\%)} = (A_{550} - B_{550}) / A_{550} \times 100$$

where  $A_{550}$  and  $B_{550}$  were the OD<sub>550</sub> (optical density at 550 nm) of control and sample supernatant, respectively.

Bioflocculant-producing strain with the highest flocculating activity was screened to investigate the optimization of culture conditions.

For exploring the flocculating activity of different active fraction, the culture broth was centrifugated at 6000 rpm for 10 min, and the flocculating rates of the supernatant (A), mycelium in the same volume of distilled water (B), cell suspended after being washed in the same volume of distilled water (C), and the culture broth without centrifugation (D) were measured, respectively.

### 2.3 Purification and composition of the complex bioflocculant

The culture broth was centrifugated at 6000 rpm for 10 min, and then the supernatant was concentrated to one third volume at 50°C. Three volumes of cold ethanol (at 4°C) were added and kept for 24 hours at 4°C. Then, the mixture was centrifugated at 6000 rpm for 15 min again, and the precipitate was washed with ethanol and freeze-dried.

The polysaccharide in the bioflocculant was determined by Molish reaction and anthrone reaction, while the protein and amino acid were determined by ninhydrin reaction, biuret reaction and protein yellow reaction<sup>[15]</sup>.

The total sugar content of bioflocculant was determined according to the phenol sulfuric acid method using glucose as standard. The total protein content was determined by the Bradford method with bovine serumalbumin as standard<sup>[15]</sup>.

### 2.4 Factors affecting the flocculating rate

The factors including carbon source, nitrogen source, culture temperature, rotating speed, initial pH, inoculum size and metal ions were investigated. To determine the effect of carbon and nitrogen sources on bioflocculant production, glucose was replaced with sucrose, maltose, starch, fructose (20 g L<sup>-1</sup> for each type of carbon source), and peptone was replaced with NaNO<sub>3</sub>, NH<sub>4</sub>NO<sub>3</sub>, beef extract, yeast extract and urea nitrogen source (5 g L<sup>-1</sup> for each type of nitrogen source). The initial pH of production media were adjusted at 2–10. Temperature of production media were adjusted at 25–35°C and the rotating speed of the shaker was adjusted at 110–200 rpm. Accordingly, the inoculum size was used as 1, 2, 3, 4, 5, 6 ml spore suspension.

In addition, the effects of metal ions (CaCl<sub>2</sub>, KCl, NaCl, MgCl<sub>2</sub> and MnSO<sub>4</sub>) were measured at the 1% (w/w) dosage. All experiments were conducted in triplicate.

### 2.5 Molecular phylogenetic analysis

The fungal isolates were identified by morphological traits and fungal ITS1-5.8S-ITS2, EF1a and β-tubulin region sequence analysis. DNA was extracted from fungal isolates using a DNA Extraction Kit. The ITS1-5.8S-ITS2 region was amplified and sequenced using fungal-specific primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATT GATATGC-3') as previously described<sup>[16]</sup>. The EF1a region was amplified and sequenced using fungal-specific primers EF1a-f (5'-ATGACACCGACAGCGACGGTCTG-3') and EF1a-r (5'-GCCCCCGGCCATCGTGACTTCAT-3')<sup>[17]</sup>. And the β-tubulin region was amplified and sequenced using fungal-specific primers Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and Bt2b (5'-ACCCTCAGTG TAGTGACCCTTGCC-3')<sup>[18]</sup>. The sequences of these regions were aligned with the sequence of *Isaria farinosa* using CLUSTAL X, and a phylogenetic tree was constructed using the neighbor-joining algorithm (MEGA version 5.2) with the bootstrap analysis of 1,000 replicates.

## 2.6 Statistical analysis

Statistical analysis was carried out using the SPSS statistical package (version 17.0 for Windows, SPSS Inc., USA), and all the plots were analyzed by Sigma Plot (version 11.0 for Windows, Systat Software Inc., USA). Mean values were calculated from 3 replicates. Least significant difference (LSD) analysis was conducted to determine the differences among the treatments at the 0.05 probability level.

## 3. RESULTS AND DISCUSSION

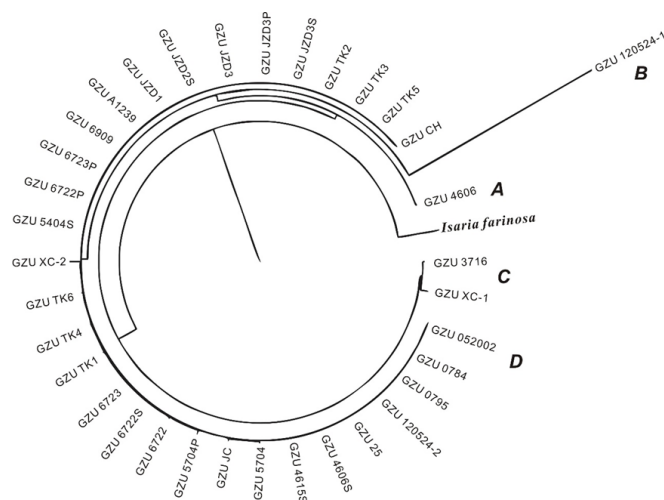
### 3.1 Screening of strains with high flocculating ability

Table 1 summarized the flocculating activity of 36 strains of *Isaria cicadae* in the institute. The highest flocculating rate of the bioflocculant produced by the GZU6722 strain reached a flocculating rate of 85.46% at the dosage of 1 ml culture broth per liter kaolin suspension. Although belonged to the same species, these strains exhibited the diverse flocculating rate, ranging from 13.38% to 85.46%. We observed that the strains with higher flocculating rate were isolated from Mount Qingcheng and Mount Emei in Sichuan Province of China, more than 50% of flocculating rate, but the strains with lower rate isolated from Jeju island of Korea, less than 41% of flocculating rate. This finding implied that the flocculating ability may be related with the natural habitat where the strain was collected and isolated. In order to identify the effects of the various factors on the of flocculating ability, *Isaria cicadae* GZU6722 strain was used in the following experiment.

To provide an insight into phylogenetic information of the flocculating activity, DNA were extracted from

36 strains and then the molecular analysis was performed. In Fig. 1, the phylogenetic tree was constructed con based on rDNA ITS1–5.8S–ITS2, EF1 $\alpha$ , $\beta$ -tublin sequences, revealing that all the 36 strains could be classified into four main groups with multiple subclusters as group A, group B, group C and group D.

Interestingly, although some strains belonged to the same group in Fig. 1, the flocculating activity of these strains are very different from each other. For example, the strain GZUXC-1 and GZU3716 were in group C, but the flocculating rate of GZUXC-1 was more than twice that of GZU3716. It was concluded that the flocculating activity maybe not be related directly with the nuclear genes [19]. Furthermore, previous experiments have shown that the active substance was mainly the polysaccharide, which could not be directly coded by the nuclear genes [20, 21].



**Fig.1** Phylogenetic tree based on rDNA ITS1–5.8S–ITS2, EF1 $\alpha$  and  $\beta$ -tublin sequences

**Table 1.** Flocculanting rate of *Isaria cicadae* strains from Institute of Fungal Resources

Strains	Flocculanting rate %	Isolated from
GZU 5704	64.39±20.83	Mount Emei, Sichuan Province, China
GZU 5704P	64.84±17.79	Mount Emei, Sichuan Province, China
GZU6723P	50.50±1.08	Mount Qingcheng, Sichuan Province, China
GZU 5704S	48.78±1.15	Mount Qingcheng, Sichuan Province, China
GZU6723	54.34±13.36	Mount Qingcheng, Sichuan Province, China
GZU6723S	54.70±1.74	Mount Qingcheng, Sichuan Province, China

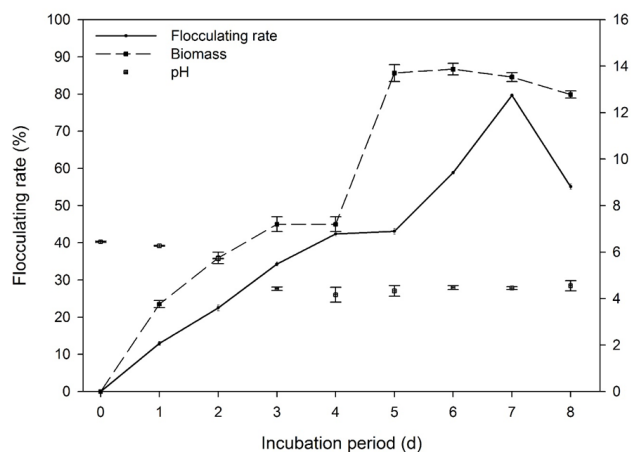
GZU 6722P	59.78±5.28	Mount Qingcheng, Sichuan Province, China
GZU 6722S	61.01±3.03	Mount Qingcheng, Sichuan Province, China
GZU 3716	61.39±19.68	Mount Qingcheng, Sichuan Province, China
GZU6722	85.46±3.38	Mount Qingcheng, Sichuan Province, China
GZUXC-1	26.12±22.92	Xicheng County, Sichuan Province, China
GZU 052002	64.54±8.46	Mount Yandang, Zhejiang Province, China
GZUCH	36.17±6.04	Guiyang City, Guizhou Province, China
GZU 120524-2	41.50±6.40	Guiyang City, Guizhou Province, China
GZU 120524-1	42.11±12.01	Guiyang City, Guizhou Province, China
GZU 0795	43.91±4.39	Guiyang City, Guizhou Province, China
GZU 0784	46.81±9.17	Guiyang City, Guizhou Province, China
GZU4615S	49.91±13.06	Guiyang City, Guizhou Province, China
GZUTK2	26.28±9.67	Leshan County, Guangxi Province, China
GZUTK6	42.75±9.60	Leshan County, Guangxi Province, China
GZUTK1	49.81±9.38	Leshan County, Guangxi Province, China
GZUTK3	53.73±7.29	Leshan County, Guangxi Province, China
GZUTK4	57.44±10.70	Leshan County, Guangxi Province, China
GZUTK5	63.14±7.26	Leshan County, Guangxi Province, China
GZU 6909	59.82±5.83	Libo County, Guizhou Province, China
GZU 25	27.38±1.97	Mojiang County, Yunnan Province, China
GZU 4606	41.22±4.92	Puer County, Yunnan Province, China
GZU 4606S	59.17±11.23	Puer County, Yunnan Province, China
GZU XC-2	64.89±5.89	Xicheng County, Sichuan Province, China
GZU A1239	29.97±3.52	
GZUJC	57.15±6.35	
GZUJZD-3P	13.38±2.38	Jeju island, Korea
GZUJZD-3	22.16±22.47	Jeju island, Korea
GZU JZD-3S	34.88±25.83	Jeju island, Korea
GZU JZD-1	36.35±5.30	Jeju island, Korea
GZU JZD-2S	40.76±4.56	Jeju island, Korea

### 3.2 Time course of bioflocculant production by *Isaria cicadae* GZU6722 strain

As seen from the growth curve of the strain in hydrolyzate-containing cultivation medium in Fig.2, the cells were in logarithm growth phase during 0–5 d, with a rapid growth period occurring during 0–3 d, and reached stationary phase since 5th d. On 7th d and onward, the cells were in death phase. The maximum biomass is 14.02 g in the 6<sup>th</sup> d, whereas the highest flocculating rate reached 79.96% in 7th d, which is later than the peak of the biomass. It indicated that the bioflocculant could be a kind of the secondary metabolite, produced from the primary metabolites in the growth. Fig.2 also showed the pH de-

creased during 0–3 d, and is around 4.5 since 3 d, which might be due to the presence of organic acid components of the bioflocculant.

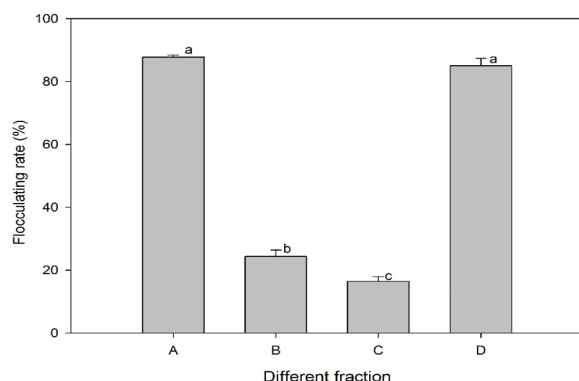
Our results showed that the cells produced bioflocculants along with their growth, and the bioflocculant quantity was increased rapidly with cultivation time and peaked at 7th d. Afterwards, the bioflocculant quantity was decreased, which may be due to substrate exhaustion and enzymatic activity decrease<sup>[22]</sup>. Restated, the production of the bioflocculant was positively associated with the biomass growth<sup>[23]</sup>. Consequently, a period of 7 d was chosen as the culture time for the subsequent experiments.



**Fig.2** Time course of bioflocculant produced by *Isaria cicadae* GZU6722 strain. Error bars indicate standard deviation of triplicate experiments.

### 3.3 Distribution of flocculating activity and composition analysis of the novel bioflocculant

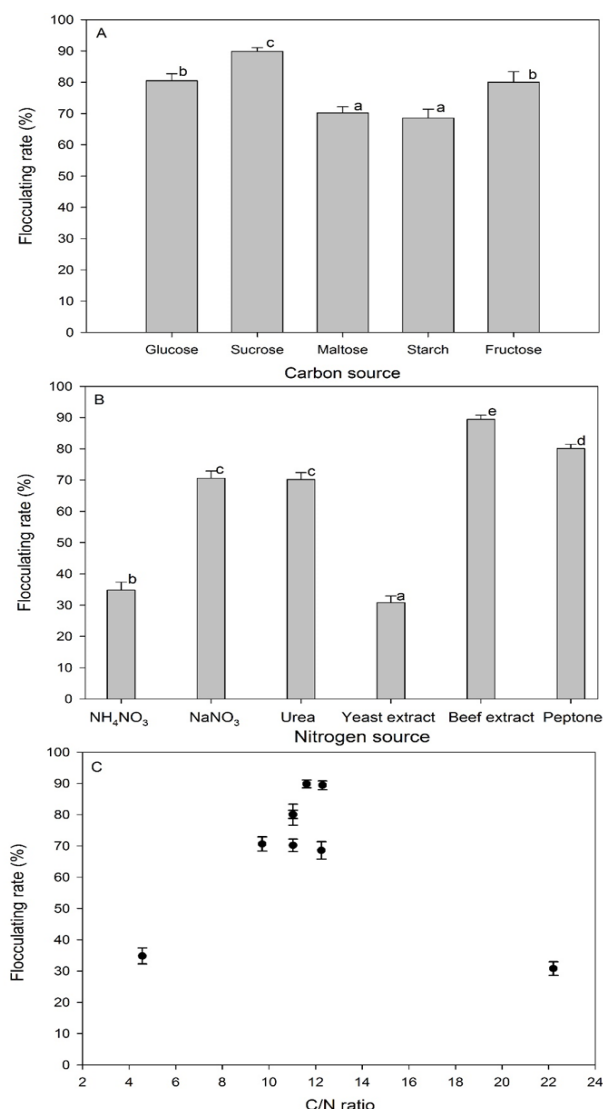
The flocculating rates of different fractions of the culture broth from *Isaria cicadae* GZU6722 were shown in Fig. 3. From Fig. 3, the supernatant had the highest flocculating rate, while the washed cells had the lowest, which confirmed that the flocculating substance mainly distributed in the supernatant. It was the active secondary metabolite produced by the fungus but not their mycelia that had the flocculating activity. This is similar to most strains capable of bioflocculating, such as *Serratia ficaria* [24] and *Rhizopus* sp. [25].



**Fig.3** Flocculating rate of the different fraction. A is the supernatant after the centrifugated broth, B is mycelia suspended in the same volume of distilled water, C is mycelia suspended in the same volume of distilled water after being washed and D is the culture

broth without centrifugation. Significant differences among different treatment are indicated by lowercase ( $p < 0.05$ ).

The chemical composition of the bioflocculant by *Isaria cicadae* was analyzed by chromo-genic reactions, and the results were shown in Fig.4. The main component of bioflocculant was identified as polysaccharide (91%) rather than protein (4%), which could be attributed to the thermal stability of the bioflocculant because protein denatured easily in boiled water [26, 27].



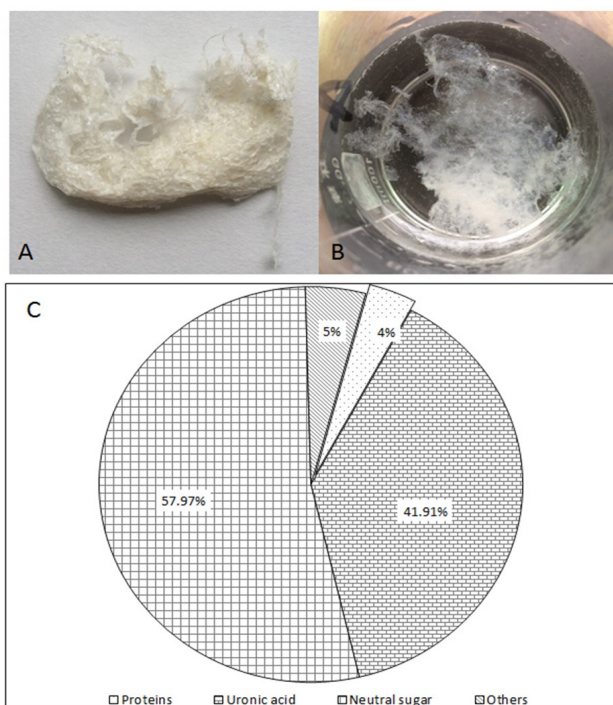
**Fig.4** Purified bioflocculant by *Isaria cicadae* GZU6722 strain and its composition. A is purified bioflocculant, B is the bioflocculant suspension, and C indicate the composition of the purified bioflocculant.



### 3.4 Flocculating activity under various culture conditions

#### 3.4.1 Effects of carbon, nitrogen sources and C/N ratio on the flocculating activity

Fig.5A showed the varied flocculating rate of the bioflocculant after 7th d of cultivation in media containing sucrose, fructose, maltose or starch instead of glucose. Sucrose, glucose and fructose were favorable for the bioflocculant production, while the production was relatively low when maltose and starch were used as the carbon source. The highest production was achieved in sucrose medium. Sucrose and glucose were also reported as the favorable carbon sources for *Aspergillus flavus* in the production of bioflocculant [28]. In this work, beef extract and peptone as organic nitrogen source were effectively used in the bioflocculant production, while  $\text{NaNO}_3$ ,  $\text{NH}_4\text{NO}_3$ , yeast extract and urea led to the poor production (Fig. 5B). At a fixed concentration of beef extract of  $5.0 \text{ g l}^{-1}$ , a rapid increase in the bioflocculant production was noticed when the C/N ratio was increased up to 12.3/1 (Fig. 5C), and a further increase in the C/N ratio caused a decrease in the production.



**Fig.5** Effect of carbon sources, nitrogen sources and C/N ratio on flocculating activity of the bioflocculant. A indicates the effect of carbon sources on

flocculating activity with peptone used in the medium as nitrogen source. B indicates the effect of nitrogen sources on flocculating activity with glucose used in the medium as carbon source. C shows the effect of C/N ratio of the flocculating activity. Error bars indicate standard deviation of triplicate experiments. Significant differences among different treatment are indicated by lowercase ( $p < 0.05$ ).

#### 3.4.2 Effect of the initial temperature and pH on the flocculating activity

Physical conditions affect the microbial growth and productivities, as enzymatic activity of microorganism depends on temperature of culture medium [25, 28]. The production of bioflocculant rapidly increased as the temperature of culture medium increased from 20 to 25°C, and the highest flocculating rate was 93.8% at 25°C, then slightly decreased as the temperature reached 30°C (Fig. 6A). Nevertheless, the production of bioflocculant dropped significantly in culture medium temperature (35°C), as the enzymes of bioflocculant production are deactivated at 35°C [8]. The optimal temperature range for bioflocculant-producing microorganisms was reported to be between 25 and 30°C [7]. As was shown, the optimum temperature for the production of bioflocculant was 25°C, which was chosen for the following experimental condition.

For most microorganisms the microbial product such as the bioflocculant regularly increase between the minimum and optimum pH, and a corresponding regularly decrease in microbial product between the optimum and maximum pH. This reflects the effect of varying pH on enzymatic reaction rates and nutrient absorption. Fig. 6B showed the effect of initial pH of culture medium on the flocculant activity. The production of bioflocculant dramatically increased following pH variation from 2 to 4, and stable at pH range from 4 to 8, within which, the maximum flocculating rate was 93.83%. Thus, pH 8 was selected as the initial pH in the following experiments.

### 3.4.3 Effect of the air amount and the inoculum size on the flocculating activity

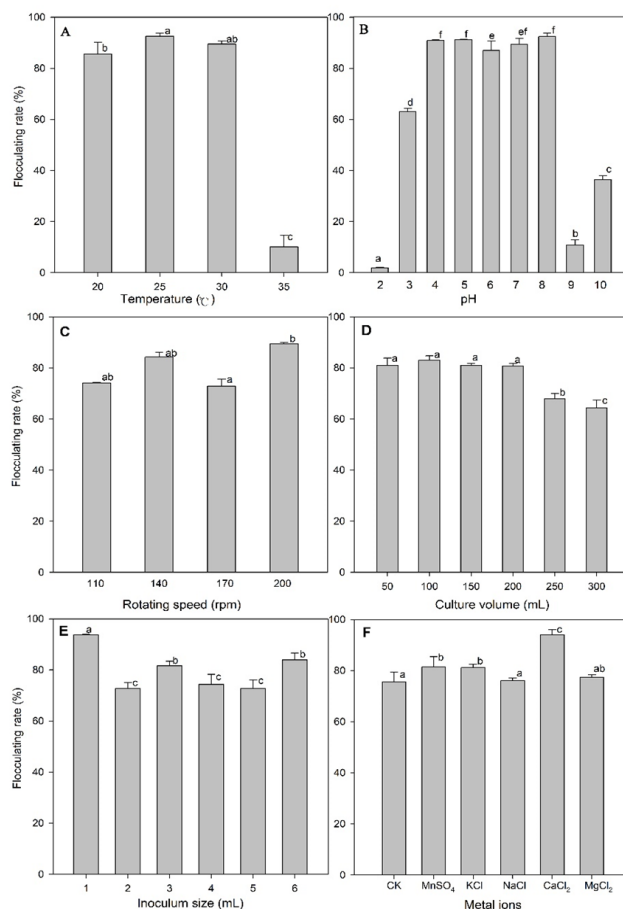
Microorganisms used in this study were all aerobic ones and large amount of air should be supplied during their growth. Accordingly, the culture medium volume in flask and the rotating speed can affect the air quantity directly, i.e., large amount of the medium in flask makes less air left, so that microorganisms will grow slowly under this condition, thus causing the decrease of the flocculating rate. Fig. 6C and Fig. 6D showed the flocculating rates of culture broth produced by cultivating at different rotating speed and various culture volume of medium in 500 ml flask, respectively. The results indicated that when the proportion of culture medium volume and flask cubage was under 1/2, the air quantity in the flask would maintain the growth of microorganisms under stirring function, which had little effect on flocculating activity of the produced bioflocculant.

The flocculating rates of the bioflocculant obtained from cultures inoculated with 1–6 ml spore suspension were shown in Fig. 6E. The inoculation amount of 1 ml suspension in medium recorded the highest flocculating rate of 93.89%. It could be concluded that the flocculating activity would be the highest under optimal dosage, but decrease when the inoculation was more or less than the optimal one.

### 3.4.4 Effect of the metals on the flocculant activity

In addition, the bioflocculant production by *Isaria cicadae* was stimulated in the presence of different metal ions such as  $Mn^{2+}$ ,  $K^+$ ,  $Na^+$ ,  $Ca^{2+}$  and  $Mg^{2+}$  in culture medium with the flocculating rate of 81.37, 81.13, 76.00, 96.08 and 77.33%, respectively (Fig. 6F), and  $Ca^{2+}$  was the most favorable metal ion for the production. [29] reported that some metal ions ( $Ca^{2+}$ ,  $Mn^{2+}$ ,  $K^+$ ,  $Mg^{2+}$  and  $Na^+$ ) significantly stimulated the bioflocculant production by *Bacillus* sp [30]. Commonly, cations are applied to neutralize the negative charges of cation- dependent bio-flocculants and kaolin particles, thereby increasing the adsorption of bioflocculant onto kaolin particles [31]. The results showed that the flocculating activity of the bioflocculant was  $Ca^{2+}$ -dependent, if there were no  $Ca^{2+}$  added, the flocculating activity was about

76.65% at optimum condition. This agreed with the reports that many microbial flocculants were cation-dependent [23].



**Fig.6** Effect of various culture conditions on the flocculating activity of the bioflocculant. (A) Culture temperature, (B) Initial pH in the culture medium, (C) Rotating speed of the shaker, (D) Volume of the culture, (E) Inoculum size and (F) Metal ions. Error bars indicate standard deviation of triplicate experiments. Significant differences among different treatment are indicated by lowercase ( $p < 0.05$ ).

## 4. CONCLUSIONS

Currently most of bioflocculants have attracted research and industry interests, as alternative flocculant, due to their high flocculation performance, eco-friendly, and biodegradability. A number of investigations using different microbial flocculants for the treatment of different types of wastewater have been carried out, such as removing suspended solids (SS) of coal washing wastewater treatment, decolorization



for dye solution and improving performance of activated sludge. In the present work, a strain of *Isaria cicadae* GZU6722 with high flocculating activity was screened, and its bioflocculation characterization was investigated for the first time. This study showed that *Isaria cicadae* GZU6722 is an outstanding bioflocculant-producing strain, it produced bioflocculant named as IC-1. The bioflocculant optimally produced with sucrose and beef extract under wide range of environmental conditions. IC-1 showed excellent flocculating rate of kaolin suspension, especially with  $\text{CaCl}_2$  addition. According to the data, IC-1 mainly consists of polysaccharides that explain its thermostability over wide range of temperatures, and also suggests its storage potential in cold and hot conditions. The present work has demonstrated that IC-1 has a potential application for the wastewater treatment, and it can be used as an alternative to common chemical flocculants in actual treatments. The next step is to improve the application feasibility of the bioflocculant in the treatment of the wastewater.

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#### Conflict of Interest

All authors declare no conflict of interest.

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