High Flocculation of Coal Washing Wastewater Using a Novel Bioflocculant from *Isaria cicadae* GZU6722

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Abstract

Chanhua (*Isaria cicadae*) was known as a rare entomogenous fungus with various pharmacological activities since a long time ago in China, which has attracted considerable attention. However, less knowledge was maintained about its products as potential bioflocculants. In this work, a bioflocculant IC-1 produced by *Isaria cicadae* GZU6722, consisted mainly of protein (4%) and polysaccharides including neutral sugars (52.75%) and galacturonic acid (38.14%), was characterized. It presented high efficiency in flocculating coal washing wastewater, and the flocculating efficiency could reach 91.81% by addition of 24 mg l\(^{-1}\) IC-1 compared to the addition of 60 mg l\(^{-1}\) APAM (anionic polyacrylamide) under the same treatment conditions. The highest flocculating efficiency reached 95.8% in the presence of 2% CaCl\(_2\). Compared to APAM, the flocculating efficiency of coal washing wastewater by IC-1 varied little with the increasing dosage. Although the flocs in the APAM-assisted sediment were larger than that in the IC-1-assisted sediment after 1 min of sedimentation, few flocs were still found in the supernatant of both treated samples after 10 min of sedimentation. More interestingly, it was observed under the microscope that the flocs in the IC-1-assisted sediments were more compact than that in the APAM-assisted sediments, suggesting that polymer bridging might take place after IC-1 was added into the coal washing wastewater. The evaluation of costs indicated that the use of IC-1 to treat the coal-washing wastewater may be an economical and feasible way to avoid the extra cost for post-treatment of conventional flocculants.

Key words: coal washing wastewater, *Isaria cicadae*, bioflocculant, APAM

Introduction

Coal is one of the most important global energy resources and comprises the majority of China’s energy economy. However, coal mining and its use in energy applications have resulted in considerable ecological issues. Coal washing wastewater is the tailwater of wet coal washing in coal processing, which contains large amounts of silt and slime with a typical characteristic of dark black color (Kirk et al. 2003; Yan et al. 2012). The vicinity of the coal mine has caused serious environmental pollution, and coal washing wastewater is one of the main sources of pollution in the coal industry, and increases public environmental attention (Lemly 2018). Coal washing wastewater is a colloidal suspension that can remain stable for months. It contains small particles that do not undergo gravitational sedimentation and their surface negative charge prevents them from colliding with each other and forming bigger flocs, which can settle easily (Yan et al. 2012; Thiruvenkatachari et al. 2016; Kapse et al. 2017). Consequently, the recycling and reuse of post-treatment effluent can be an efficient approach to reduce the volume of effluent that is discharged into environments. China is one of the largest...
 coal-producing countries in the world (IEA 2018), and is facing with the intractable problem of remediating coal washing wastewaters produced daily. The removal of suspended solids (SS) from wastewaters could relieve environmental burdens from discharge, and also help to increase coal production (Duong et al. 2000). Coal washing wastewaters require massive amounts of flocculants (Wang et al. 2017), thus the efficient and environmentally friendly bioflocculants would be of significant advantage.

Several methods have been used to treat such wastewaters, including ion exchange, filtration, flotation, flocculation, and solvent extraction (Geremias et al. 2003). Among these technologies, flocculation that includes coagulation/flocculation, and direct flocculation is an effective and easily applied method in the removal of insoluble particles from wastewaters (Bolto and Gregory 2007; Teh et al. 2016). Flocculation removes organic compounds along with suspended solids and also decolorizes waste streams. In particular, coagulation/flocculation with poly aluminum chloride (PAC) and anionic polyacrylamide (A-PAM) can be used to reduce significantly the abundance of suspended solids of coal washing wastewaters. However, the dosages and concentrations of coagulants used are proportional to the waste material levels in the wastewaters (Duong et al. 2000; Li et al. 2010; Luo et al. 2011; Wang et al. 2017). Furthermore, conventional coagulants including PAM and PAC are expensive, corrosive, and biologically harmful. Specifically, PAM monomers (acrylamide) are strongly neurotoxic (Friedman 2003; Hariri et al. 2015) and would raise a significant danger to the environment and human health. Therefore, an effective, chemically non-invasive, and low-cost coagulant alternative is needed to meet the stringent environmental regulations expected for discharged effluent or solid waste qualities (Zeng et al. 2007; Luo et al. 2011).

The most of alternative flocculants are bioflocculants, which have attracted recent research and industry interest due to their high flocculation capacities, eco-friendly properties, and biodegradability. Moreover, some of these new flocculants can be produced from biological or agro-industrial wastes (Shahadat et al. 2017). Over 100 species of bioflocculant-producing microorganisms have been described and investigated with respect to their flocculation performance in different wastewater types, for example, the bioflocculant IH-7 produced by Aspergillus flavus used in the kaolin suspension (Pu et al. 2014; Aljuboori et al. 2015; Guo and Chen 2017), the polysaccharide produced by Paenibacillus mucilaginosus WL412 for the flocculation of coal washing wastewater (Xu et al. 2017), the potential of a bioflocculant in sludge dewatering (Pu et al. 2014; Aljuboori et al. 2015; Guo and Chen 2017), and a novel bioflocculant from Rhizopus sp. used in potato starch wastewater (Pu et al. 2014; Aljuboori et al. 2015; Guo and Chen 2017). Thus, it is necessary to screen out more and more bioflocculants with higher efficiency and without secondary pollution, which could be applied effectively into the treatment of coal washing wastewater.

In our previous work, a novel bioflocculant-producing fungus strain I. cicadae GZU6722 was identified and a bioflocculant named as IC-1 was purified. Although IC-1 may represent an attractive new bioflocculant, more investigations are still needed to achieve high flocculation efficiencies and maintain low environmental risks during the application of IC-1. The purpose of the present study was to provide an essential characterization of the novel bioflocculant and to evaluate the potential application of IC-1 in the treatment of the coal washing wastewaters. We focused on the flocculation performance of IC-1 in coal washing wastewater, and the determination of the environmental conditions for the optimal flocculation performance, including the dosage of IC-1 and CaCl₂, the mixing time, and the sedimentation time. In addition, the flocculation activity of IC-1 and APAM was investigated in the same conditions. More specifically, the sediments after treatment of IC-1 and APAM were examined by electron microscopy to explore the flocculating mechanism of IC-1 based on the experimental results. Cost evaluation and the feasibility of IC-1 were discussed regarding the application for the treatment of coal washing wastewater.

**Experimental**

**Materials and Methods**

Purification and determination of IC-1. Isaria cicadae GZU6722 was a fungus strain with a high potential of bioflocculation, which was persevered in Guizhou Agricultural College (Registration ID: GZU1FR6722). It was maintained on Potato Dextrose Agar (PDA) slant at 4°C, and the medium for slant consisted of (g l⁻¹) potato extract, 4; glucose, 20 and agar, 15. The medium for subculture consisted of (g l⁻¹): glucose, 20; peptone, 5; KH₂PO₄, 2; MgSO₄ • 7 H₂O, 0.5; and CaCl₂, 0.5. Meanwhile, the initial pH was adjusted as 6.5 ± 0.2. In order to carry out the experiment, the spore suspension was resuspended to the desired concentration of 1 × 10⁷ spores ml⁻¹ by flooding the slant with sterile water after the 5d of cultivation at 22°C.

For purifying the bioflocculant, the culture broth was centrifuged at 6000 rpm for 10 min after seven days of incubation, then followed by the collection of the supernatant and was concentrated to one-third of the initial volume at 50°C. Threefold volumes of cold ethanol (at 4°C) were poured into and kept for 24 h at 4°C. After centrifugation at 6000 rpm for 15 min again, the
Precipitate was washed with ethanol, freeze-dried, and assigned as IC-1 (Fig. 1A, 1B).

The total sugar content of IC-1 was determined according to the phenol sulfuric acid method using glucose as standard, and the total protein content was determined by the Bradford method with bovine serum albumin as the standard (Guo et al. 2014). The neutral sugar and the galacturonic acid content of IC-1 were measured by an anthrone reaction and the carbazole-sulphuric reaction, respectively.

**Optimization of coal-washing wastewater treatment conditions by IC-1.** The main factors in the treatment conditions including the dosage of IC-1, mixing time, sedimentation time, and the dosage of CaCl2 were used to optimize the treatment conditions, and the flocculating efficiency of coal washing wastewater was used as an optimizing index. One of the factors was adjusted under the condition that the other selective factors were independently optimal for maximum flocculating efficiency. The optimization experiments were conducted using 50 ml of wastewater (Pu et al. 2014), and the experiments were conducted in triplicate.

**Assay of the flocculation performance of IC-1 and APAM.** Coal washing wastewater was provided
by a coal washery plant in Liupanshui City, Guizhou Province, China, and its pH ranged from 7.5 to 8.5. Anionic polyacrylamide (APAM, Mw 3.0 × 10^7 Da) was purchased from the Erli Environ-Technology (Shanghai) Co., Ltd., China.

The flocculating efficiency was investigated by the following procedure. Briefly, 1 g l^-1 of IC-1 (Fig. 1B) or APAM was prepared and then added into 50 ml coal washing wastewaters at a different dosage. The mixture was stirred slowly for few minutes by employing a magnetic stirrer at 60 rpm and was stood for a short time according to the optimized mixing and sedimentation times. Then, the optical density (OD) of the supernatant at 550 nm was measured using a spectrophotometer (GENESYS 10 UV, Thermo Scientific, USA). The flocculating efficiency was then calculated using the following equation:

\[
\text{Flocculating efficiency (\%) = \left( \frac{A_{550} - B_{550}}{A_{550}} \right) \times 100}
\]

where \( A_{550} \) and \( B_{550} \) were the OD550 (optical density at 550 nm) of the treated sample and the control sample supernatants, respectively. Control samples were the untreated coal washing wastewater. All experiments were conducted in triplicate.

**Morphosis of the flocs treated with IC-1 and APAM.** The sediment was collected after 10 min of sedimentation. Then, the flocs were imaged by the inverted phase-contrast microscope (ECLIPSE Ti-U, Nikon, Japan). The raw sample without adding any flocculant was used as a control (CK).

**Statistical analysis.** Statistical analysis was conducted using the SPSS statistical package (version 16.0 for Windows, SPSS Inc., USA), and all plots were produced using SigmaPlot (version 10.0 for Windows, Systat Software Inc., USA). Mean data values were calculated from triplicate measurements.

**Results and Discussion**

**Characterization of IC-1 produced by I. cicadae GZU6722.** The thermal stability of bioflocculant is considered to be dependent on the major component of a bioflocculant (Chaisorn et al. 2016). The chemical composition of IC-1 from I. cicadae GZU6722 was analyzed by chromogenic reactions, and the results were shown in Fig. 1C. The main components of bioflocculant were identified as polysaccharides, including neutral sugars (52.75%) and galacturonic acid (38.14%) rather than proteins (4%). The structure was similar to the sporoderm-broken spore powders produced by *Cordyceps cicadae (= I. cicadae)* using the infrared spectroscopic analysis (Sun et al. 2017). It could be attributed to the better thermal stability of the bioflocculant as the protein denatured easily when heated.

Based on its main components, it could be assumed that IC-1 might be of high molecular weight anionic polysaccharide due to the presence of galacturonic acids, whose carboxyl groups would be probably dissociating around neutral pH. The molecular weight of these fungal polysaccharides was approximately estimated in the range of from 1.1 × 10^4 to 1.48 × 10^7 Da that is quite similar to the molecular weight of biopolymers from *C. cicadae* (1.1 × 10^7 Da) (Sun et al. 2017), the molecular weight of IH-7 from *Aspergillus flavus* was 2.574 × 10^4 Da (Aljuboori et al. 2015). In contrast, the molecular weight of POS412 from *P. mucilaginosus* WL412 was up to 1.48 × 10^7 Da (Xu et al. 2017).

**Flocculation performance of IC-1 under various conditions.** The parameters used for the evaluation of the performance of wastewater treatment include the dosage of IC-1, mixing time, sedimentation time, and the dosage of CaCl_2_. In Fig. 2A, 91.06% of flocculating efficiency was obtained at the IC-1 dosage of 40 mg l^-1_, which is marginally higher than that observed after the addition of IC-1 at the dosage of 60 mg l^-1_. However, the suspended substance removal was little facilitated with rising of the dosage of IC-1, resulting in a correspondingly increasing cost owing to the higher IC-1 consumption.

The mixing test was conducted by a magnetic stirrer and the effect of the mixing time was investigated. In Fig. 2B, the flocculating efficiency showed a slight downward trend with the increasing mixing time. But the sedimentation time led to little effect on the flocculation efficiency, as shown in Fig. 2C.

In addition, Ca^{2+} was the most favorable metal ion for the flocculation in kaolin suspensions (Li et al. 2017). Fig. 2D showed a similar effect like that for the flocculating efficiency in the treatment of coal washing wastewater. An additional 2% CaCl_2 _ dosage enhanced rapidly flocculating efficiency up to 95.8%. However, only minor enhancements of flocculation were observed with higher CaCl_2 _ dosages. As a result, the optimal CaCl_2 _ dosage was estimated at 2%. Table I summarized the comparison of the flocculation performance of IC-1 with other bioflocculants for the coal wastewater, either under the condition with the additives or not. Nevertheless, it might be not assured that inorganic coagulants would be necessary for the bioflocculant, such as POS412 produced by *P. mucilaginosus* WL412. The flocculating efficiency was more than 95% for coal washing wastewater without the assistance of inorganic coagulants (Xu et al. 2017; Yang et al. 2019).

It was well known that the coal particle surface shows a strong negative charge (Yan et al. 2012; Xu et al. 2017). Few cations are commonly applied to neutralize the negative charges of cation-dependent bioflocculants and kaolin particles, thereby allowing increased adsorption of bioflocculant onto kaolin particles (Dermilim
Isaria cicadae with high bioflocculation

Moreover, the charges and radius of the cations could affect the flocculating activity, and a bigger radius led to larger bonded particles which could be easily settled (Sousa et al. 1992). Our results showed that the flocculating activity of the bioflocculant was Ca\(^{2+}\)-dependent, and the flocculating activity was about 95.8% at the CaCl\(_2\) dosage of 2%. This result was agreed with the report that many microbial flocculants were cation-dependent, such as a bioflocculant from *Serratia ficaria* (Gong et al. 2008), bioflocculants from *Ochrobactrum ciceri* W2 (Wang et al. 2013).

Comparison between IC-1 and APAM in flocculating activity. Regarding the possible competition of IC-1 in the treatment of the wastewater, APAM was selected for comparison under the same optimal conditions as maintained above. Figure 3 showed that the flocculating efficiency of both IC-1 and APAM in the coal washing wastewater exhibited dose-dependent patterns. The highest flocculating efficiency of coal washing wastewater reached 91.81% by adding 24 mg l\(^{-1}\) IC-1, whereas the addition of 60 mg l\(^{-1}\) APAM produced the maximum value of 91.41% which was maintained by 16 mg l\(^{-1}\) IC-1 dosage. Compared with IC-1, the flocculating efficiency of coal washing wastewater by APAM increased with the rising dosage. It means that changes in the concentration of APAM have played a more vital role.

![Diagram](image_url)

**Fig. 2.** The flocculating performance under various conditions. (A) Different dosage of IC-1; (B) Different mixing time; (C) Different sedimentation time; (D) Different dosage of CaCl\(_2\) as a coagulant. Error bars indicate standard deviation of triplicate experiments. Significant differences among different treatments are indicated by lowercase (\(p<0.05\)).

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**Table I**

Flocculation performance of IC-1 compared with other microbial flocculants for the coal wastewater treatment.

| Bioflocculant     | Origin                     | Maximum Flocculating Efficiency | Additives       |
|-------------------|----------------------------|---------------------------------|-----------------|
| IC-1              | *Isaria cicadae* GZU6722   | 95.8%                           | 2% CaCl\(_2\)   |
| POS412            | *Paenibacillus mucilaginosus* WL412 | 96.53% (Xu et al. 2017)      |                 |
| MBF-L918          | *Pseudomonas veronii* L918 | 95.51% (Liu et al. 2016)       |                 |
| A bioflocculant   | *Azotobacter chroococcum*  | 95.76% (Yang et al. 2019)      | 50 mg/l CTAB    |
role in the performance as compared to IC-1. For this reason, it could be suggested that IC-1 may have a great advantage over APAM in practice. But the flocculating efficiency with the rising IC-1 concentration decreased when the dosage was more than 32 mg L$^{-1}$, implying that there would be a threshold effect in the flocculation by IC-1 in accord with what was observed during the fungal bioflocculant application in the kaolin suspensions treatment and the coal washing wastewater treatment (Xu et al. 2017). Thus, it is likely that the flocculating mechanism of IC-1 differs from that of APAM, which might be related to charges of APAM.

There must be an optimal dosage for the performance of the different bioflocculants in various conditions. The flocculating activity should be highest under optimal dosages, with decreasing activity at suboptimal dosages though. Specifically, flocculating efficiencies would suffer from too low the bioflocculant concentration at lower than optimal dosages. In addition, higher than optimal dosage would also inhibit activity. At suboptimal bioflocculant levels, bridging can not effectively occur. In contrast, the excessive addition of negatively charged bioflocculant would lead to competition and repulsion of negatively charged coal particles (Yan et al. 2012), thereby leading to poor settling (Gong et al. 2008). In this scenario, colloid particles are surrounded by abundant polymers in wastewaters and are stabilized due to the absence of bridging (Pu et al. 2014). Thus, the over-dosing of IC-1 impacts adversely flocculation.

The goal of treating coal washing wastewaters is to accelerate the particle settling rate and reduce wastewater contaminants. Therefore, the settling time is an important parameter that is used to evaluate the behavior of wastewater treatment. Figure 4 showed the settling time difference among the untreated sample, APAM-assisted, and IC-1-assisted flocculation. In the untreated samples, coal particles remained suspended after 10 min of sedimentation, whereas in the case of the APAM-assisted and IC-1-assisted treatment,
coal particles were flocculated into flocs which settled easily (Fig. 4E). Remarkable changes happened after 1 min by adding APAM and IC-1. In Fig. 4A and Fig. 4C, a great number of flocs were formed in the APAM-assisted and IC-1-assisted treatment of coal washing wastewater and the flocs in APAM-assisted treatment were larger than that in IC-1-assisted treatment. Five minutes later, although the number of the flocs reduced greatly in the APAM-assisted and IC-1-assisted treatment of coal washing wastewater (Fig. 4B, 4D), the size of the flocs in the APAM-assisted treatment was still larger than that in IC-1-assisted treatment. It revealed that APAM may more easily accelerate the formation of flocs compared to IC-1.

Figure 5 shows the microscopic images of the fine particles in the samples treated by IC-1 and APAM.
shown in Fig. 5A and Fig. 5B, the fine particles in the untreated sample sediments were mostly scattered and kept suspended, which resulted in little change even after 10 min of sedimentation. However, the flocs in the treated samples were formed after adding APAM and IC-1, respectively in Fig. 5C and Fig. 5E, which implied that the addition of the flocculant may trigger the formation of large flocs during flocculating process. From Fig. 5D and Fig. 5F, the flocs in the IC-1-assisted sediments were more compact than that in the APAM-assisted sediments. It indicated that the sediments treated with IC-1 may be more stable than those treated with APAM. Polymer bridging and charge neutralization are two well-known mechanisms that contribute to flocculation (Li et al. 2017; Xu et al. 2017). The results described above revealed that polymer bridging could take place after adding IC-1, which might assist fine particles of coal to connect flocs together and form larger aggregates.

Table II. The estimated costs of APAM and IC-1 to treat 1 metric ton of coal washing wastewater at the same flocculating efficiency.

|          | Cost/t | Dosage/ g m⁻³ | Cost per metric ton of wastewater |
|----------|--------|----------------|----------------------------------|
| APAM     | US$ 2300 | 60            | US$ 0.13                         |
| IC-1*    | US$ 2500 | 24            | US$ 0.06                         |

* The cost of IC-1 was estimated according to the market price of the culture substrate, and the maximum biopolymer yield is around 14.02 g per 100 ml culture substrate.

Feasibility analysis of IC-1 in the coal washing wastewater treatment. Besides attaining high flocculating efficiency and toxic effect, the cost of flocculant is also a key factor to consider as a whole in the application of wastewater treatment. Indeed, the widespread application of these bioflocculants depends on lowering costs through the use of economical substrates and novel fermentation/recovery schemes (Shahadat et al. 2017). Costs can be approximately estimated based on the application of APAM or IC-1 at their respective dosages to maintain 91.81% of flocculating efficiency in the treatment of 1 ton of wastewater feed (Wong et al. 2006), and the results are shown in Table II. In our previous work, the maximum biopolymer yield was around 14.02 g per 100 ml of the culture substrate (Li et al. 2017). According to the substrate scheme mentioned above combined with the market price of the culture substrate in China, the cost of flocculating 1-ton coal washing wastewater by IC-1 is estimated to be equal to $0.06 at the large-scale production. Compared to IC-1, the cost with APAM is relatively higher (around $0.13 t⁻¹), almost two times as the cost by IC-1. For this reason, it is better to choose IC-1 as an economical bioflocculant in the treatment of the coal washing wastewaters.

The focus of the present work aimed to investigate the performance of IC-1 in the treatment of wastewater, not like other studies that relied on bioflocculant behavior via kaolin suspensions. As it has been shown, the results of the present study inferred that IC-1 from I. cicadae GZU6722 has exhibited satisfactory flocculating activity in practice, therefore, it would be a promising bioflocculant.

Like IC-1, other biodegradable bioflocculants have attracted the increasing scientific and technological consideration in wastewater treatment applications because they are harmless to human health and do not cause secondary pollution via the degradation of intermediate metabolites (Okaiyeto et al. 2014). As a traditional Chinese medicine, I. cicadae has been used for a long time in the form of powders or decoction (Wang et al. 2017). A few chemical constituents were isolated and purified from the mycelia and the liquid culture filtrate, which were mainly polysaccharides (Xu et al. 2018). Furthermore, the significance of I. cicadae as a potential therapeutic agent for immune diseases such as cancer, allergy, and parasitic disease has also been highlighted (Takano et al. 2005; Sun et al. 2017). According to the results mentioned, IC-1 mainly consists of biodegradable polysaccharides that explain its thermo-stability over a wide range of temperatures and also suggests its storage potential in cold or hot conditions. Hence, the application of IC-1 to flocculate coal washing wastewater can serve as an economical, proficient, and environmentally friendly approach, without the extra cost involved in the post-treatment of flocculant or the toxic effect when using conventional chemical flocculants like APAM.

Conclusion

In summary, IC-1 has shown to be an effective and alternative microbial flocculant for the coal washing wastewater treatment. The maximum flocculating efficiency was found to be 91.81% after the addition of IC-1 at the dosage of 24 mg l⁻¹ and even up to 95.8% when assisted by 2% CaCl₂. When compared to IC-1, the cost of the APAM application is relatively higher (around $0.13 t⁻¹), almost two times as the cost of the IC-1 application. In light of the results, the utilization of IC-1 for the treatment of coal wastewater would be a useful, economical, and environmentally friendly alternative to conventional flocculants like APAM when the extra cost has to be involved in the post-treatment.
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