Original Research Paper

Isolation and Identification of a Bioflocculant-Producing Strain and Application in Rice Vinegar Clarification

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Abstract: A bioflocculant-producing bacterium S-3# was isolated from fresh sand bones 40-50 cm underground in the desert region of China's Ningxia Province. It was identified as *Paenibacillus* sp. based on 16S rDNA sequence along with its biochemical and morphological characteristics. Chemical analysis found that the bioflocculant from S-3# is mainly composed of extracellular polysaccharide. Researchers observed no noticeable change (above 91%) in its flocculating activities in pH range 2.0-9.0 in kaolin suspensions and hypothesized that it could be useful in clarification of rice vinegar (pH 2.9-3.5). Using one-factor-at-a-time experiments, dosage of bioflocculant, temperature of flocculation system and metal cations were investigated for their efficacy on rice vinegar clarification. The Box-Behnken design, an experimental design for Response Surface Methodology (RSM), was also used to create a series of 17 runs to optimize the operating conditions. Quadratic regression models with estimated coefficients were developed to describe the suspended particle removals of rice vinegar. Results showed that the optimal flocculation conditions were 3.94 mg/L bioflocculant, 0.32 mM $CaCl_2$ and flocculation system temperature of 41.5°C, respectively. These findings were in reasonable agreement with the modeled values. Under these conditions, the increase rate of transmittance (λ = 900 nm) of rice vinegar ranged over 240.63% under these conditions.

Keywords: Bioflocculant, *Paenibacillus* sp., Extracellular Polysaccharide, Vinegar Clarification

Introduction

Flocculants are widely used in industrial processes, from drinking water purification, wastewater treatment, to downstream processes in fermentation processing (Yang *et al*., 2016; Gassara *et al*., 2015; Zhang *et al*., 2020). They can be divided into three categories: Inorganic flocculants, organic flocculants and bioflocculants (Ji *et al*., 2010; Polasek 2009; Salehizadeh *et al*., 2018). While the first two categories are widely used due to their effectiveness and low cost. However, these may include the possibility of fatal diseases resulting from extensive usage of alum and the generation of large amounts of sludge, thus introducing new environmental challenges in treatment, regeneration and reuse. In addition, evidence has shown that the second category of polyacrylamide derivatives is a strong carcinogen and has neurotoxic to humans (Semla *et al*., 2017). Bioflocculants produced by

microorganisms have attracted extensive attention due to their nontoxic, biodegradable and benign properties, still more the degradation intermediates will not cause secondary pollution (Salehizadeh *et al*., 2018; Ajao *et al*., 2018). Given these considerations, the use of bioflocculants is expected to increase in various fields as a promising substitute for inorganic and chemically synthetic flocculants.

Vinegars have been used as condiment for many years. They are nutritious and have lots of bioactive compounds including amino acids, polyphenols and tetramethyl pyrazine (Ho *et al*., 2017). These compounds contribute vinegars its unique flavors and also play important roles in preventing and treating human diseases, such as regulating blood lipid levels, weight loss, antifatigue and antitumor (Petsiou *et al*., 2014; Baba *et al*., 2013). However, the richness in proteins and polyphenols can induce haze formation in rice vinegar

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storage, seriously affecting the quality of the product. Currently, the main methods used for vinegar clarification include membrane filtration and flocculation (Lopez *et al*., 2005; Yin *et al*., 2019). Among these methods, flocculation is more widely used given its simple process and low cost. Many different types of flocculations have been reported in vinegar clarification, such as bentonite and chitosan (Xiao *et al*., 2007; Jiang *et al*., 2013).

Many bacteria have been found to produce bioflocculants and employed in industrial processes (Shahadat *et al*., 2017). However, maybe due to the limitation of the optimal pH range, there is no previous report on the application of bioflocculants in vinegar clarification (pH 2.9-3.5). Sand bone, made up of sand grains have been found in Ningxia Province, China. The sand bone growing like the roots of tree was 40-60 cm below the ground. It was first reported and named by our research group. In this study, we isolated and identified the novel bioflocculant-producing strain S3# from it and established its application in rice vinegar clarification. The findings from this work are positive and provide useful information for downstream processes in brewing food.

Materials and Methods

Materials

The UNIQ-10 column DNA extraction kit (SK1201), DNA maker (SM0337) and DNA gel recycle and purification kit were purchased from Sangon Biological Engineering Technology, Shanghai, China. The forward primer sequence 7f (5-CAGAGTTTGATCCTGGCT-3) and reverse primer sequence 1540r (5-AGGAGGTGATCCAGCCGCA-3) were synthesized by Sangon Biological Engineering Technology, Shanghai, China. Sucrose, sodium nitrate, magnesium sulfate, dipotassium phosphate, hexadecyl pyridinium chloride and other reagents were of analytical grade. Raw rice vinegar produced by submerged fermentation was provided by Zhejiang Wuweihe Food Co., Ltd (Zhejiang, China).

Isolation and Culture of Strain S-3#

Bioflocculant-producing strain S-3# was isolated from fresh sand bones 40-50 cm underground in the desert region of Ningxia Province, China (Fig. 1 and 2). The enriched medium composition was: 20 g sucrose, 30 g sand (selected form sampling site), 0.5 g $MgSO_4$ $\cdot 7H_2O$, 1.0 g $NaNO_3$, 1.0 g K_2HPO_4, 0.005 g $FeCl_2$, 20 g agar, 1000 mL distilled water, with a total pH of 7.0-7.5. Using a sterilized needle, the brown material in the hollow part of the fresh sand bones was picked out and then was added into the 250 mL Erlenmeyer flasks

containing 50 mL enriched medium and then incubated at 180 rpm for 72 h at 28°C. After that, the sample was spread on the selected agar after a serial of dilution. The composition of the selected agar was: 30 g sucrose, 0.5 g $MgSO_4$ $\cdot 7H_2O$, 2.0 g $NaNO_3$, 1.0 g K_2HPO_4, 0.005 g $FeCl_2$, 18-20 g agar, 1000 mL distilled water, with a total pH of 7.0-7.5. Strains with high viscosity colony were picked out and inoculated into 250 mL flasks containing 50 mL isolation medium, then incubated at 180 rpm for 48 h at 28°C. 1 mL culture solution was used to measure the flocculating activity.

Fig. 1: Sand bone in natural environment (underground 40 cm)

Fig. 2: The cross section of sand bone

Identification of Bioflocculant-Producing Strain S-3#

The cell forms and the colony characteristics of strain S-3# on selected agar were observed after 48 h of incubation. The strain was identified by 16S rDNA sequencing. The genomic DNA of S-3# was extracted, then PCR amplification of the 16S rDNA was carried out using universal primers. The PCR conditions were: Denaturation at 98°C for 180 s, 98°C for 25 s with 30 cycles, 55°C for 25 s, 72°C for 60 s and a final extension at 72°C for 600 s. PCR products were purified and sequenced. The synthetic sequence was submitted to the GenBank and analyzed using BLAST. Clustal X 2.1 was used to the alignment of relevant sequences. With the neighbour-joining and maximum-parsimony algorithms using MEGA 5, Phylogenetic trees were constructed (Yang *et al.*, 2015).

Determination of Flocculating Activity and pH Stability

To evaluate the flocculating activity of strain S-3#, kaolin clay suspension was used. 4 g of kaolin clay was dissolved in 1 L distilled water as the Kaolin suspension. 100 mL of this kaolin suspension was added into a 250 mL conical flask which has 1 mL of cell-free supernatant and 3 mL of $CaCl_2$ (1%, w/v). The mixture was shaken for 30 s and then gently poured into a 100 mL cylinder and stand for 5 min. The clarifying solution was collected and measured its OD_{550}. The control group was done similarly, except that the cell-free supernatant was replaced by the production medium. The Flocculating Activity (FA) was calculated as following (Agunbiade *et al.*, 2018):

$$FA(\%) = \frac{B-A}{B} \times 100 \tag{1}$$

where, *A* and *B* are the absorbance of the sample and control group at 550 nm, respectively.

To figure out the effect of pH on the flocculating activity, the pH of kaolin clay suspension was adjusted to 2-10. The initial kaolin suspension (pH 7.5) was used as a control group (Pu *et al.*, 2020).

Bioflocculant Purification

To purify the bioflocculant, an equal volume of distilled water was added into the broth and then centrifuged at 14 000 g for 30 min to remove the cells. Triple volumes of ethanol (4°C) were added to the supernatant and then the crude bioflocculant precipitate was dried overnight in a desiccator. Then the crude bioflocculant was dissolved in distilled water and 2% (w/v) Cetylpyridinium Chloride (CPC) was added until that the insoluble CPC-bioflocculant complex was no

longer formed. The precipitate was re-dissolved in saline solution (0.6 mol/L NaCl), Finally, three volumes of ethanol were added to the supernatant and finally was lyophilized to obtain a purified bioflocculant (Salehizadeh and Shojaosadati, 2002). The total sugar content and protein content of the bioflocculant were determined according to the cited literature (Luo *et al.*, 2005; Liu *et al.*, 2010).

Optimization of Flocculating Conditions

Rice vinegar produced by submerged fermentation was collected and transported to the laboratory using a cooler box containing ice-pack. Transmittance was chosen as the index to measure the flocculating efficiency of the purified bioflocculant in rice vinegar (Xiao *et al.*, 2007). 1 mL the purified bioflocculant solution was added into 100 mL of rice vinegar. The mixture was agitated for 2 min and was allowed to standing for 10 min and then the supernatant was taken for analysis. The transmittance of the supernatant was measured using at 900 nm. The control group was prepared by same procedure except that the distilled water was replaced by the sample solution.

The Increase Rate of Transmittance (IRT) was calculated as follows:

$$IRT(\%) = \frac{B-A}{A} \times 100 \tag{2}$$

where, *A* initial value; *B* value after the flocculation treatment.

The bioflocculant dosage, the temperature of the flocculation system and the concentration of cations were the key factors affecting the flocculation efficiency. To get the optimal flocculating conditions of the bioflocculant applied in clarification of rice vinegar, Single factor experiments were used to examine the effects of bioflocculant dosage and various salts and temperature of the flocculation. Based on the above works, these factors were further optimized using a Box-Behnken design. The trials were conducted three times and the average IRT at each run was used as the response variable. The data obtained from RSM was analyzed according to the second-order polynomial equation with the Design-Expert 10.0 software. ANOVA and diagnostics analysis were used to evaluate the quality of analysis model (Wang *et al.*, 2013).

Results and Discussion

Isolation and Identification of Bioflocculant-Producing Strain

One bacterium with high viscosity colony (named S-3#) was obtained in the assessment with the limited conditions. The colony of strain S-3# was found to be

smooth, round, transparent, thick and colorless after 48h of aerobic incubation (Fig. 3). Its biochemical and physiological characteristics are: Gram stain (+), peroxidise (-), catalase (-), the Voges-prokauer test (-), Gelatin hydrolysate (-), urease (-), amylolysis (+), hydrogen sulfide (-), indole test (-). Microscopic examination showed that S-3# was a bacilliform, with thick capsules in its surroundings. The capsule size was 10-20 times larger than their body (Fig. 4).

The 16 s rDNA sequences of strain S-3# was determined to be 1458 bp long after the PCR amplification (Fig. 5). Figure 6 was the phylogenetic tree. Based on the morphological characteristics and 16s rDNA sequence analysis, strain S-3# was identified as *Paenibacillus* sp.

Flocculating Activity and pH Stability Assessment

Flocculating activity of culture broth from the strain S-3# was tested using the Kaolin suspension method, which was 92.8% after 48 h of fermentation. It is much higher than the *Klebsiella* sp. (86.9%) (Liu *et al*., 2014) and the *Bacillus megaterium* (83.8%) (Luo *et al*., 2016). This suggests that the strain S-3# is able to synthesize bioflocculant effectively. The pH was a key factor that might affect the flocculating activity by changing the ionization degrees of the bioflocculant (Salehizadeh and Yan, 2014). Therefore, different kinds of bioflocculant may have different optimum pH due to their different compositions. The pH stability of S-3# was investigated (Fig. 7). The highest flocculating activity was 95.5% at pH 5.0. The bioflocculant was quite stable with the pH between 2.0 and 9.0, more than 91% flocculation was achieved at this pH range. This result was similar to a polysaccharide-based B40 produced by *Bacillus velezensis* B40, which maintained a 90% flocculation efficiency at pH 1.0-10.0 (Zaki *et al*., 2013). However, the optimal pH of most bioflocculants ranged between 5.0 and 8.0, such as *Achromobacter* sp. (pH 6.0) (Subudhi *et al*., 2014), *Aspergillus flavus* sp. (pH 7.0) (Aljuboori *et al*., 2013) and *Halomonas* sp. (pH 7.0-8.0) (Shen *et al*., 2015).

The high flocculating activity achieved in a wide pH range suggests that this bioflocculant could be applied in acidic neutral and alkalescency conditions. But when the pH was higher than 9.0, its flocculating activity dramatically decreased, this may be due to the bioflocculant having different electric states at different pH (Pan *et al*., 2009).
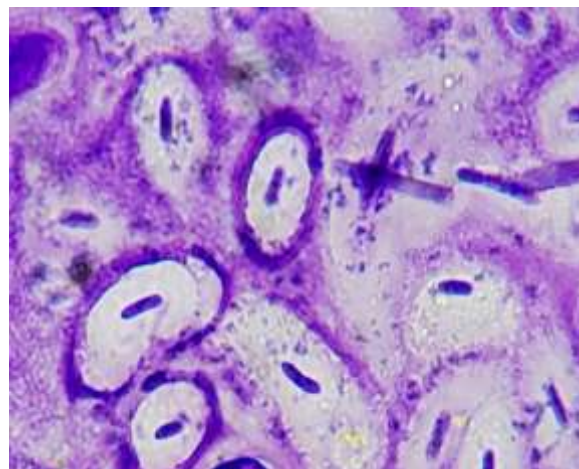

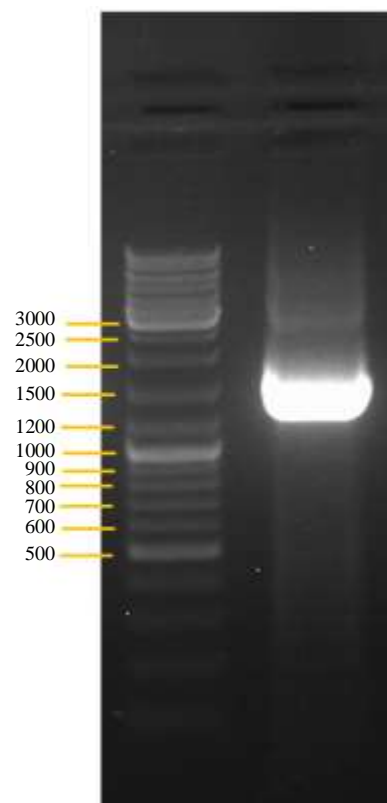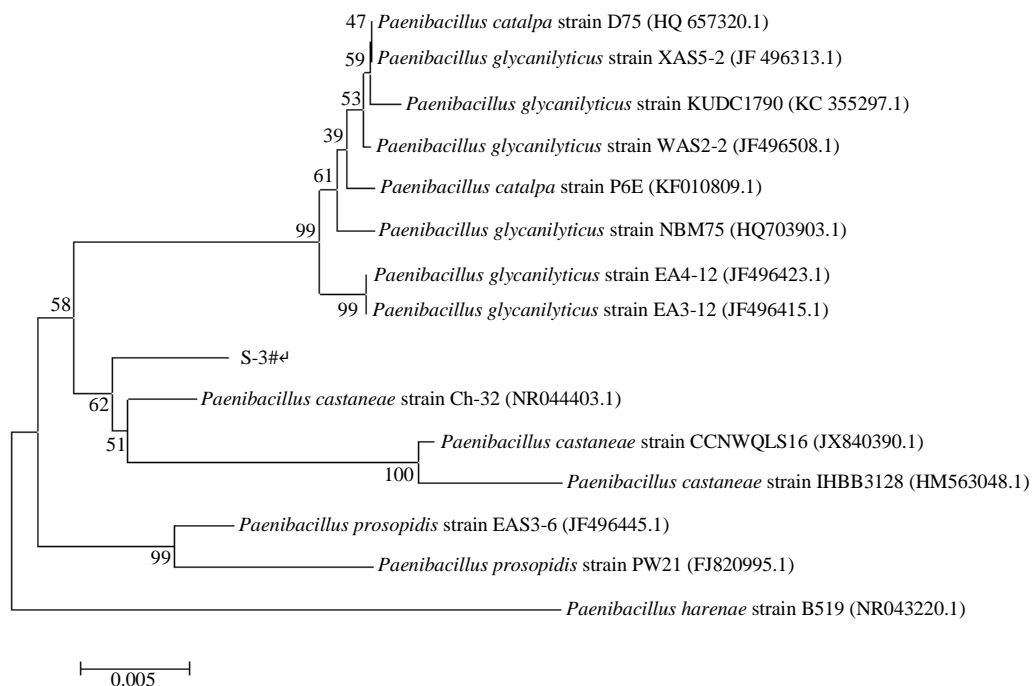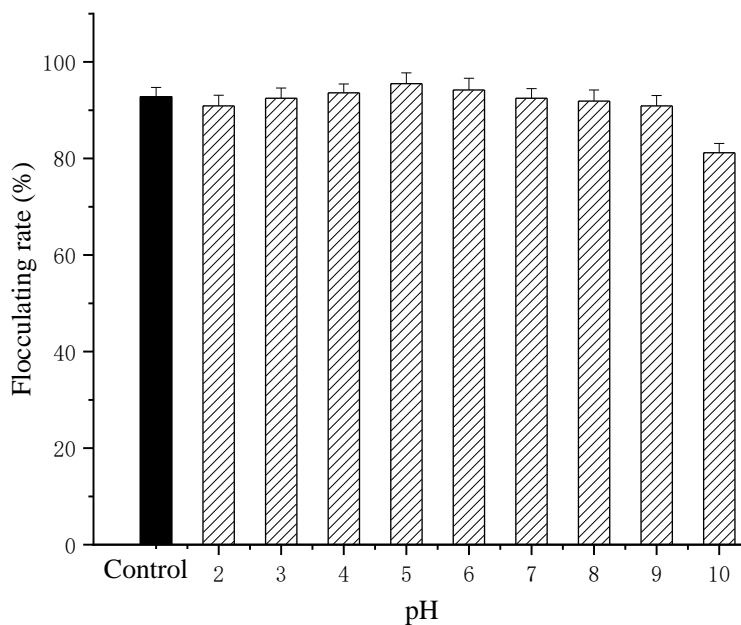**Fig. 4:** Microscopic characteristic of strain S-3# (×1000)

Fig. 5: Result of the PCR product in agarose gel electrophoresis. (Left lane: DNA marker; Right lane: S-3# PCR product)

Fig. 3: Colony characteristic of strain S-3#

Fig. 6: Neighbour-joing tree based on 16S rDNA sequences showing relationship between strain S-3# and related strains

Fig. 7: Effect of pH on the flocculating rate in kaolin clay suspension

Extraction of the Composition of S-3#

About 11.02 g of purified bioflocculant was obtained from 1 L of culture broth. It appeared as a white and hygroscopic powder, with 89.1% (w/w) polysaccharide and 5.8% (w/w) protein. Results revealed that it was a polysaccharide-based bioflocculant and the protein content was very low. To evaluate the effect of polysaccharide and protein on the flocculating activity, the Sevag reagent treatment was used to remove the protein in the bioflocculant and the flocculation activities did not significantly declined. This confirmed that extracellular polysaccharides were the key effective component for the flocculation ability.

Factors Affecting the Bioflocculant on Rice Vinegar Clarification

In this study, we found that S-3# possesses high flocculating activity in kaolin suspensions and the flocculating activity has no remarkable change at pH from 2.0 to 9.0. Therefore, it could be used in rice vinegar clarification (pH 2.9-3.5).

The effect of bioflocculant dosage (Fig. 8) showed an over 48% increase rate of transmittance in the range of 1-11 mg/L, with the maximum IRT of 133.62% observed

at optimum bioflocculant dosage of 3.0mg/L. Figure 8 also showed that the dosage affected the flocculation efficiency. When bioflocculant is insufficient, the bridging phenomena cannot efficiently formed. In contrast, an abundance of negatively charged bioflocculant can cause the repulsion of negatively charged particles and similarly reducing effective volume leading to poorer stability. This is consistent with the flocculation characteristics of polysaccharide flocculants produced by other microorganisms (Gong *et al*., 2008; Kwon *et al*., 1996).
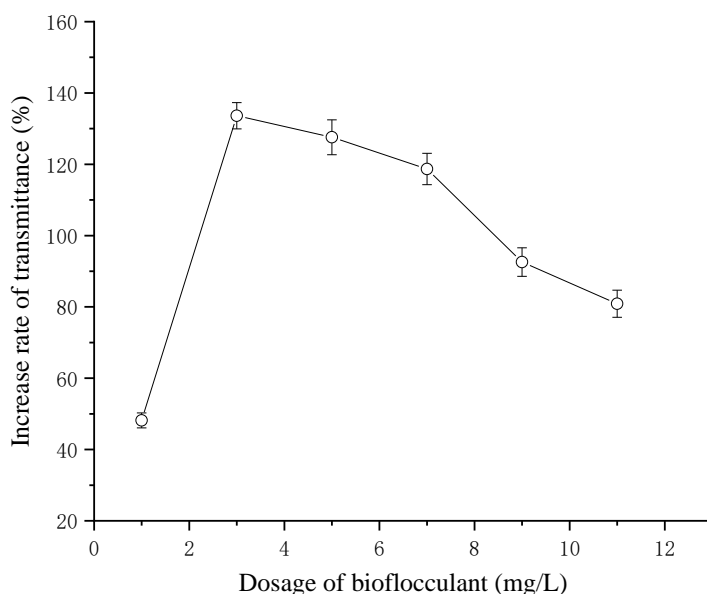

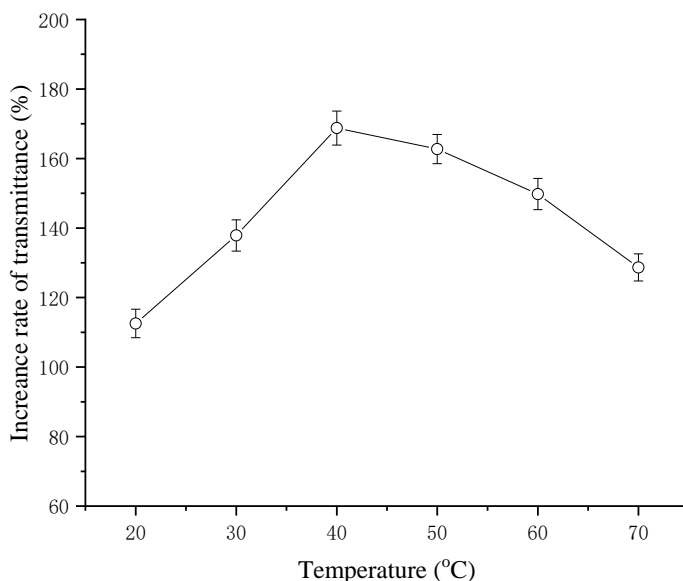**Fig. 8:** Effect of dosage of bioflocculant on the increase rate of transmittance

Fig. 9: Effect of temperature on the increase rate of transmittance

The effect of the temperature of flocculation system was also investigated (Fig. 9). Flocculating activity increased with temperature increase up to the maximum value of 40°C, thereafter slowly decreased. This could be due to the fact that the high temperature changed the spatial structure of the bioflocculant and induced the lower flocculation efficiency.

To achieve high flocculating activity, metal cations are often added as they may enhance the bridging and neutralization of bioflocculants in the process of flocculation (Sobeck and Higgins, 2002; Kim et al., 2013; Vimala et al., 2020). Figure 10 shows the effect of cations on flocculation. Apparently, trivalent ions (Al^{3+} and Fe^{3+}) and bivalent ions (Ca^{2+}, Mg^{2+} and Fe^{2+}) were more effective than monovalent ions (Na^+ and K^+). Similarly, the bioflocculant S-14 produced by *Serratiaficaria* sp. was effective with metal ions (Ca^{2+} and Mg^{2+}) (Gong et al., 2008).

Given high cost and other considerations (Fe^{2+} is easy to oxidize and bring color into the system, Al^{3+} is easy to lead to Alzheimer's disease and other disadvantages), Ca^{2+} appears to be the ideal coagulant aid. In Fig. 11, the high flocculation efficiency was achieved when Ca^{2+} concentration was 0.3 mM.
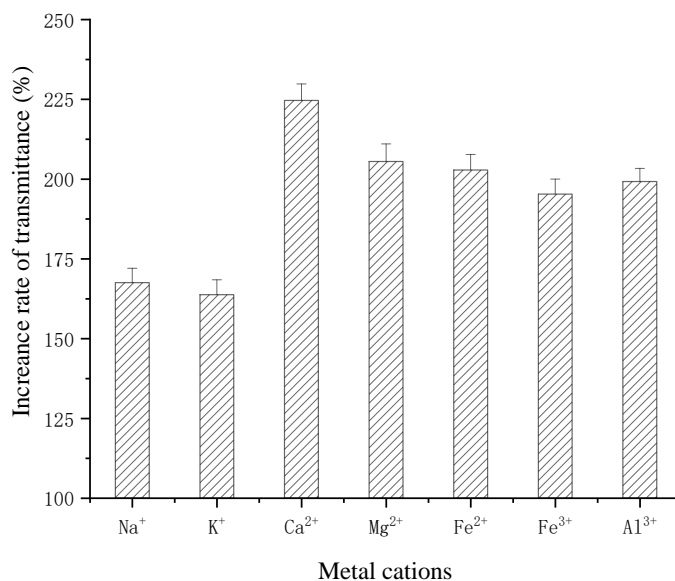

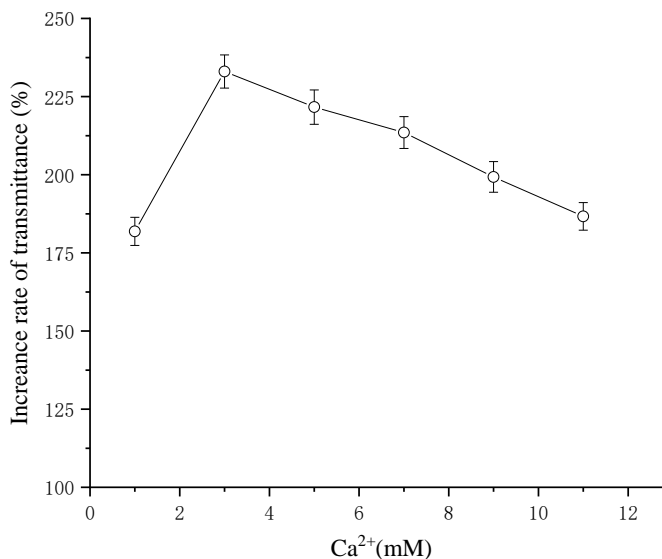**Fig. 10:** Effect of metal cations on the increase rate of transmittance

Fig. 11: Effect of Ca^{2+} on the increase rate of transmittance

Using RSM for Optimization

Bioflocculant dosage, flocculation system temperature and $CaCl_2$ were significant factors influencing flocculation. The following experiments using three-level, three-factor Box-Behnken design model with 17 experiments were performed to optimize these factors, the average of both increase rate of transmittance at each run was used as the response variable (Table 1), The statistical testing was carried out with the Fisher's statistical method for Analysis Of Variance (ANOVA) (Table 2). The actual response values from the ANOVA analysis were compared with predicated response values, showing a statistically valid low probability value of P (P_{model} = 0.0009) indicating that the equation is very reliable in predicting the flocculating efficiency. The lack of fit value was insignificant (p = 0.1292), indicating that the equation was adequate for predicting flocculating efficiency. The adequacy of the model was indicated by the determination coefficient (R^2 = 0.9504), which accounted for 95.04% of the response variability.

Table 1: Box-Behnken design and its measured values

Run	Dosage of bioflocculant (mg/L)	Ca^{2+}(mM)	Temperature (°C)	Increase rate of transmittance (%)
1	3	0.3	40	235.11
2	3	0.2	35	205.52
3	3	0.3	40	236.62
4	2	0.3	45	198.03
5	2	0.3	35	194.12
6	3	0.3	40	239.22
7	4	0.4	40	225.88
8	2	0.2	40	203.77
9	2	0.4	40	191.63
10	4	0.3	45	230.74
11	4	0.3	35	202.33
12	3	0.4	45	213.51
13	3	0.4	35	190.41
14	3	0.3	40	234.51
15	3	0.3	40	227.55
16	3	0.2	45	187.29
17	4	0.2	40	226.48

Table 2: ANOVA of flocculating condition

Source	SS	DF	MS	F	Probability value
Lake of fit	197.451	3	65.8169	3.4918	0.1292
Pure error	75.395	4	18.8489		
Model	5234.140	9	581.5711	14.9204	0.0009
Total	5506.986	16			

b

c
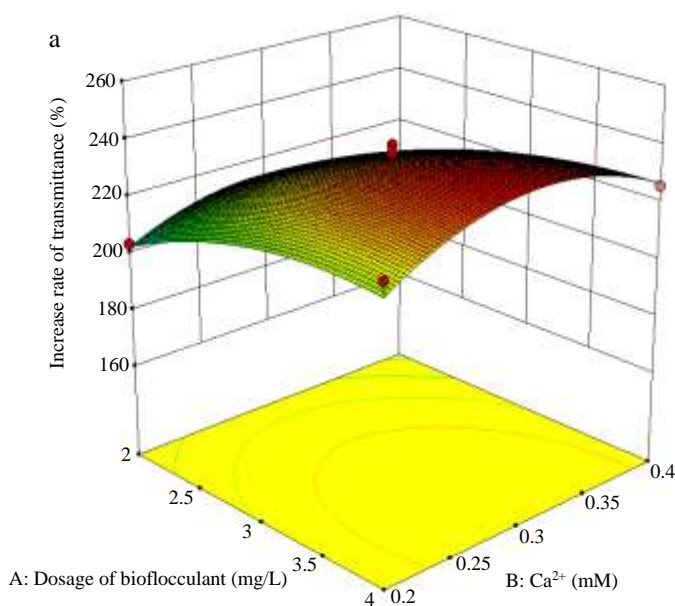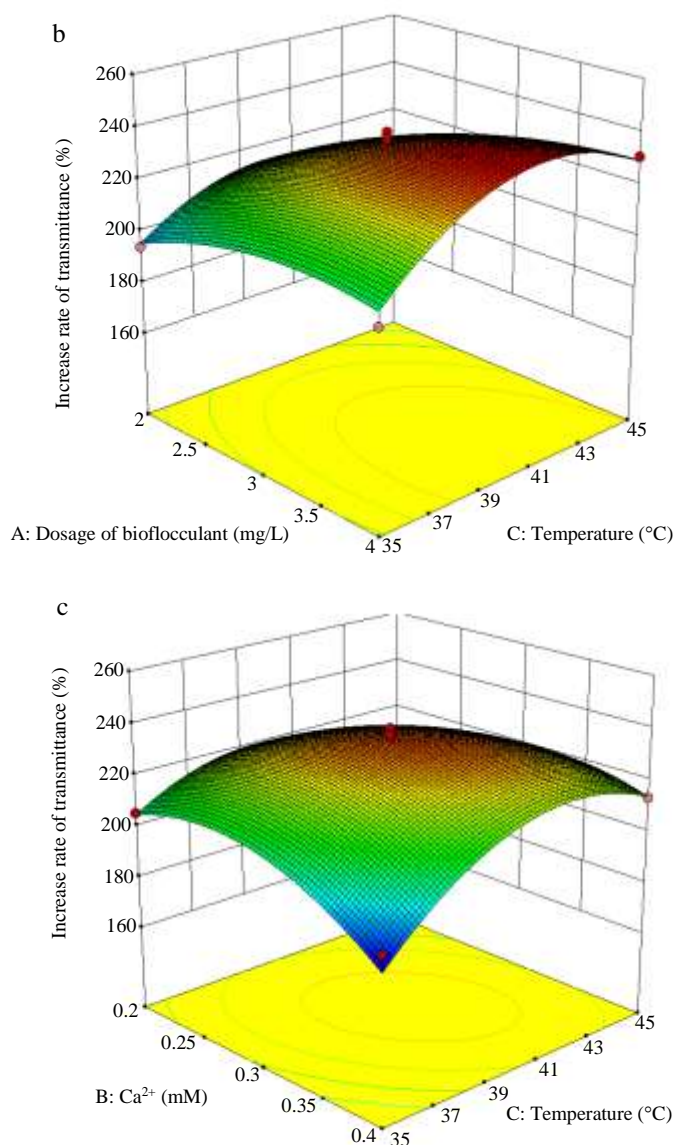
Fig. 12: (a,b,c) Showed the three-dimensional surface response plot and their interactions between the variables

Figure 12 Response surface plots representing the effects of variables on increase rate of transmittance (Y, in percent). A dosage of bioflocculant (mg/L) and $CaCl_2$ (mM); b dosage of bioflocculant (mg/L) and temperature(°C); c $CaCl_2$ (mM) and temperature (°C).

These surface plots, therefore, allow for visualization of the optimum levels of each variable to maximize the flocculating efficiency. The optimal conditions for the bioflocculant were obtained using the "numerical optimization" function within the Design-Expert 10.0. With 3.94 mg/L bioflocculant, 0.32 mM $CaCl_2$ and flocculation system temperature of 41.5°C, the highest increase rate of transmittance was predicted to be 241.04%. To verify the reproducibility of the model, all experiments were performed under the optimal

conditions. The results showed that the increase rate of transmittance was 240.63, 242.51 and 243.64%. This suggested that the model can reasonably optimize the flocculation conditions and predict the flocculation efficiency for rice vinegar clarification.

Conclusion

The bacterium S-3#, isolated from the fresh sand bones, was identified as *Paenibacillus* sp. It has shown to be able to synthesize bioflocculants effectively. Moreover, the bioflocculant possessed wide range of adaptations at pH 2.0-9.0. It was found that extracellular polysaccharide was the main constituent of the bioflocculant. The Box-Behnken design showed that

the optimal flocculation conditions used in rice vinegar clarification were 3.94 mg/L bioflocculant, 0.32 mM $CaCl_2$ and flocculation system temperature of 41.5°C, respectively. The increase rate of transmittance was over 240.63% under these conditions. Suggesting that the bioflocculant may have promising applications in clarification of brewing food items.

Although bioflocculant has many advantages over other flocculants, the bioflocculant is much expensive due to the high cost of the productive process. To overcome this impediment, future emphasis should be placed on finding out a low-cost medium. The application of bioflocculant in the production of beer will also be studied in the future.

Acknowledgment

Author's Contributions

Dehui Dai: Has contributed in the experiment, paper writing and publication.

Weilian Hu: Has contributed in the experiment design and revised the manuscript.

Ethics

There are no ethical issues to declare that could arise upon the publication of this manuscript.

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