



Biofloculant production by different microbial species and their potential application in dairy wastewater treatment

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Original Article

Abstract

The aim of this study was to characterize the biofloculants secreted from *Bacillus subtilis* (*B. subtilis*), *Aspergillus oryzae* (*A. oryzae*) and *Rhizopus oligosporus* (*R. oligosporus*). For precise investigation of biofloculants, fourier transform infrared (FTIR) spectroscopy was performed, and pH and temperature stability, and decolorization efficiency were evaluated. In addition, the effects of biofloculants use on dairy wastewater properties, including chemical oxygen demand (COD) and total suspended solids (TSS), were assessed. The experiments showed that 5-day fermented *B. subtilis*, *A. oryzae*, and *R. oligosporus* var. *oligosporus* were able to produce 2.51, 2.24, and 2.15 g/l of biofloculants, respectively. The produced biofloculants differed in terms of performance rate. The order of performance rate at 20-40°C was *R. oligosporus* > *B. subtilis* > *A. oryzae*. FTIR analysis revealed differences between the chemical structures of the three biofloculants and the involvement of N-H bands, C-O group, and carboxylic acids and their derivatives in these biofloculant structures. Thermostability analysis of biofloculants indicated that *R. oligosporus* produced more stable biofloculants than others. It was observed that the increasing of pH caused an increase in the flocculating activity of biofloculants produced by *B. subtilis* and *A. oryzae*. In contrast, biofloculants from *R. oligosporus* showed better flocculation performance in acidic conditions. In the case of dairy wastewater, the addition of all tested biofloculants caused a significant decrease in COD, TSS, and dyes and the best results belonged to biofloculants from *R. oligosporus*.

KEYWORDS: Biofloculant, Bacteria, Fungi, Wastewater

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Introduction

Floculants are components with a wide range of application in different industries like textile manufacturing, pharmacology, cosmetology, as well as wastewater treatment processes.¹ The main disadvantage of chemical floculants is the risk of these components for human health such as dementia (Alzheimer's disease), cancer, and neurotoxicity.² Biofloculants (floculants from microbial origin) do not have this disadvantage.³ Another reason that biofloculants are an interesting subject for researchers is their

biodegradability. These components are advantageous over inorganic floculants and chemically synthesized floculants in numerous applications including wastewater treatment, and downstream processing for food and fermentation industries, due to their nontoxicity, harmlessness, and biodegradability.⁴ In wastewater treatment, biofloculants have been used to treat dye solutions, inorganic solid suspensions, downstream processing, food and industry wastewater, heavy metals, among others.⁵

Literature reports indicate that a number of biofloculants have been produced from different microorganisms such as *Klebsiella*,⁶ and *Bacillus agaradhaerens*.⁷ The aim of this

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study was to isolate biofloculants produced by *Bacillus subtilis* PTCC 1254, *Aspergillus oryzae* PTCC 5163, and *Rhizopus microsporus* var. *oligosporus* PTCC 5173, the biofloculant production property of which, to our knowledge, has not been assessed thus far. Fourier transform infrared (FTIR) spectroscopy was performed, and pH and temperature stability, and decolorization efficiency were evaluated. Moreover, the effects of using biofloculants on dairy wastewater properties, including chemical oxygen demand (COD) and total suspended solids (TSS) were assessed.

Materials and Methods

Bacillus subtilis PTCC 1254 (*B. subtilis*), *Aspergillus oryzae* PTCC 5163 (*A. oryzae*), and *Rhizopus microsporus* var. *oligosporus* PTCC 5173 (*R. oligosporus*) were purchased from the Iranian Research Organization for Science and Technology in lyophilized form. Dairy wastewater was collected from a local dairy factory. All chemicals were purchased from Merck Company.

The growth medium for biofloculant production was composed of glucose (20 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $(NH_4)_2SO_4$ (0.2 g), K_2HPO_4 (5 g), urea (0.5 g), yeast extract (0.5 g), and KH_2PO_4 (2 g) in a liter at pH of 6.5 and sterilized through autoclaving at 121–124°C for 15 minutes.⁸ The strain was pre-cultured in 50 ml of growth medium in a 250 ml flask on the rotary shaker (160 rpm) at 28°C for inoculation preparation. After 18 hours of cultivation, 4% (v/v) culture broth of *B. subtilis* was inoculated into 200 ml of the production medium in a 500 ml flask.⁹ Batch fermentation was carried out under the same cultivation conditions as those for pre-cultivation. To determine flocculating activity, 2 ml of culture broth was centrifuged at $4,000 \times g$, at 4°C for 30 minutes, and the cell free supernatant was used as the test biofloculant. Bacterial count was determined through standard spread plate technique using nutrient agar and all plates were incubated at 35°C for 36 hours.

R. oligosporus and *A. oryzae* were obtained from the Iranian Research Organization for Science and Technology (with spore count of 10^6 – 10^7 spores/ml). The culture was rehydrated and revived in yeast-malt (YM) nutrient broth at 24°C. Then, the culture was transferred onto potato dextrose agar. Incubation was conducted at 24°C, for 7 days. After harvesting of fungal sporangiospores, the culture was transferred into steril distilled water containing 0.85% (w/v) saline solution (NaCl) and 0.5% (v/v) Tween 80, and more dilution performed to reach spore count of 10^6 – 10^7 spores/ml.¹⁰

To separate bacterial cell and biofloculants from culture broth, centrifugation was performed at 10000 rpm, at 5°C, for 15 minutes. After the separation process, 2 clear layers appeared and the supernatant layer was removed to extract crude biofloculants. Extraction of crude biofloculants from supernatant involved a precipitation process using 4 volumes of cold ethanol (95%). The mixture was later left for 24 hours before it was centrifuged again at 10000 rpm for 15 minutes (5°C). Subsequently, the supernatant was diluted using 4-fold volume of cold ethanol and stored at 4°C for 24 hours, and the precipitated layer was lyophilized for further investigation.¹¹

Flocculation activity was determined by preparing kaolin clay suspension with the concentration of 4 mg/l, then, pouring 100 ml of suspension along with 3 ml $CaCl_2$ (1% w/w) and 2 ml culture supernatant into a 250-ml flask, mixing for 1 minute using a magnetic stirrer VELP model, and transferring it to a 100-ml measuring cylinder. Sedimentation occurred after 5 minutes. Optical density of supernatant was determined using a UV spectrophotometer (model Jenway6305) at 550 nm. Flocculation activity was determined using the following formula:

$$\text{Flocculating Activity (\%)} = [(A - B/A)] \times 100$$

Where A and B are optical densities of the control and sample measured at 550 nm, respectively.

The concentration of 0.1 mg/ml of the

biofloculant solution was prepared. The justified pH of individual kaolin solutions in separate flasks ranged from 2–12 prior to the determination of flocculating activity at each of these pH values.¹²

The biofloculant was dissolved in distilled water to reach the concentration of 0.1 mg/ml. The given amount of it (10 ml) was kept at 0–100°C for 30 minutes in order to measure the temperature stability of biofloculants. For this reason, kaolin suspension method was used.⁹

In order to obtain insight into the molecular structure of biofloculants, FTIR spectroscopy was used to determine the functional groups of the biofloculants. The biofloculants were ground with KBr salt at 25°C and pressed into a pellet for FTIR analysis over a wave number range of 4,000 to 400 cm⁻¹.¹³

In decolorization experiments, 1 ml of culture broth and 1 ml of CaCl₂ (1% wt) were added to 30 ml of dye solutions (100 mg/l basic fuchsine), then, the pH of the suspension was adjusted to 8 with NaOH solutions (10% wt). The mixture was stirred for 1 minute, held for 10 minutes, and then, the supernatant was obtained for analysis. The absorbance of each sample was measured using a spectrophotometer at 543 nm wavelength. The residual concentration of the dye in the samples was then calculated and the decolorization efficiency was calculated based on the initial and final (after treatment) dye concentrations.⁵

In this study, it was confirmed that the 3 tested flocculants possessed adequate flocculating activity in kaolin suspension method, so their flocculatin activity in dairy wastewater was assessed. Dairy wastewater was collected from a local dairy factory using sterile containers. The parameters evaluated for each of the collected wastewater samples were TSS and COD. The COD analyses were used as a measurement of the oxygen demand equivalent of the organic constituents in the sample. The method outlined by Gong et al. was used to perform COD tests.¹⁴

The TSS test was performed according to Method 2540 D of the Standard Methods. After filtration, the solids retained on a 0.45 m pore size filter paper were taken as the TSS. Samples were homogenized by vigorous mixing.¹⁵

All data were treated in replicates and the mean values were calculated. Data were subjected to one-way analysis of variance (ANOVA) using Minitab statistical package (release 12, Minitab, Inc., USA). All P values of less than 0.01 were considered as significant. All experiments were performed in triplicates.

Results and Discussion

The experiments confirmed the ability of all tested microorganisms in producing biofloculants. This finding is in agreement with other researches that showed the biofloculant activity of other strains from similar families such as *Bacillus licheniformis* X14,¹⁶ *Aspergillus niger*,¹⁷ *Aspergillus flavus*,¹⁸ and *Rhizopus* sp. M9 and M17.¹⁹

Effect of inoculum size

In all cases, the flocculating activity initially increased with increasing of the inoculum size. At the inoculum size of 1%, the maximum flocculating activity was obtained (details not shown). As a result, an inoculum size of 1% was used for all subsequent cultures.

Biofloculant yield

Flocculating efficiency is still a limiting factor with regard to the application of biofloculants, and strains with higher biofloculant production are more likely to find industrial application. After 5 days of fermentation, 2.51, 2.24, and 2.15 g/l purified biofloculants were achieved from *Bacillus subtilis*, *Aspergillus oryzae*, and *Rhizopus microsporus* var. *oligosporus*, respectively. In other studies, various biofloculant production rates from different microorganisms have been reported such as 1.47 g/l for *Bacillus* sp. Strain F19²⁰ and 2.3–2.27 g/l for *Vagococcus* sp. Strain W31.²¹

Fourier transform infrared spectroscopy

Analysis of functional groups in the purified biofloculants was carried out and results are shown in figure 1.

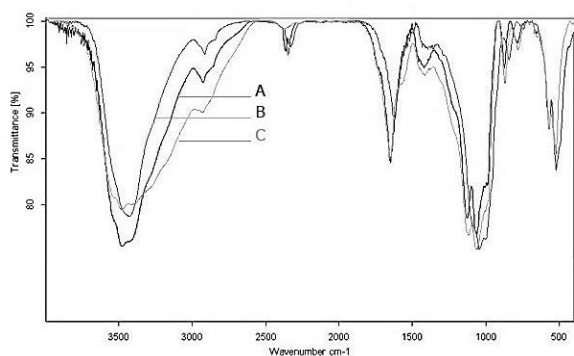


Figure 1. The effect of different loading rates of surfactant on the sorption
 Contact time = 24 hours; Bromocresol green (BCG) concentration = 100 mg/l; Adsorbent dosage = 1 g/l; pH = 7

FTIR analysis revealed the presence of different functional groups in the molecule. In figures 1-3, the spectrum peak at the range of 3400-3500 cm^{-1} showed the presence of N-H bands in the molecule. The weak band noticed at 2500-3300 cm^{-1} indicated the presence of carboxylic acids and their derivatives. The spectrum peak at 1630-1695 cm^{-1} was an indication of the presence of an amide group. The vibration peak at 1300-1450 cm^{-1} corresponded to the C-O group in the biofloculant molecule. The spectrum peaks at 1000-1300 cm^{-1} suggested the presence of carboxylic acids and their derivatives.

Thermostability of the purified biofloculants

The effect of temperature on flocculating rate was determined and the results are presented in figure 2. In all tested biofloculants, the best performance was in the range of 20-40°C. Higher temperatures caused a greater decrease in flocculant rate, and the lowest magnitude was reached at 100°C. Among the three microorganisms, biofloculants extracted from *R. oligosporous* showed more stability against temperature changes. The results indicated that the flocculation performance was in the order of *R. oligosporous* > *B. subtilis* > *A. oryzae* (Figure 2).

These findings are in accordance with that of other researches that reported a decrease in flocculant activity at higher temperatures. Li et al. reported a decrease of only 9.2% in flocculating activity of the biofloculant

produced by *Aeromonas* sp. after being heated at 100°C for 60 minutes.²² Gong et al. observed that the biofloculant produced by *Serratia ficaria* could retain its flocculating activity after being heated at 100°C for 15 minutes, mainly due to polysaccharide backbone.¹⁴ Li et al. reported that the biofloculant produced by *Agrobacterium* sp. M503 retained its flocculating activity up to 70°C and a further increase in temperature up to 121°C had no effect on flocculating activity.²³ High thermostability of a compound biofloculant CBF-F26 was observed when the purified biofloculant was heated over 100°C for 30 minutes. The residual flocculating activity of this biofloculant was more than 90%.⁹

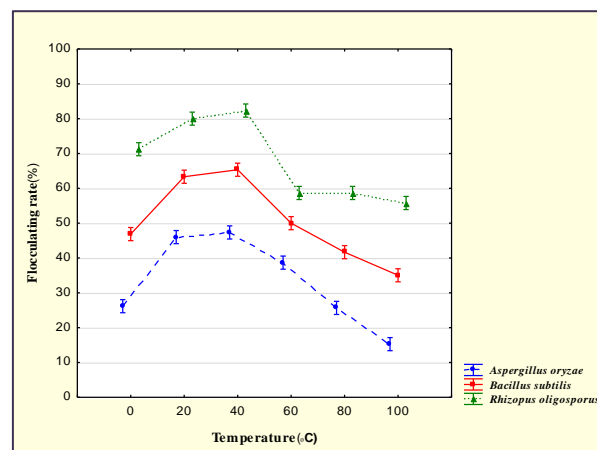


Figure 2. Thermostability of biofloculants

The presence of different functional groups, which are noticeable in FTIR spectrum, could explain different performances of the tested biofloculants (figure 1). It has been approved that some functional groups like hydroxyl and phosphate groups and ester bonds have more effect on enhancing flocculation activity.²⁴ Based on the FTIR, lack of the band related to O-H stretching vibration at 2925 cm^{-1} in biofloculants produced by *A. oryzae* might explain its lower flocculating rate because the abundance of hydroxyl groups could build up strong attraction forces between polysaccharide molecules.²⁵ Thereby, biofloculants from *R. oligosporous* and *B. subtilis*, with regular linear structure, might cause better approach-ability

between molecules resulting in their higher flocculating activity.

Effect of pH on the flocculating activity of purified biofloculants

The effect of pH on the flocculating activity of the biofloculants was investigated using biofloculant dosage of 0.1 mg/ml at different pH values ranging from 2–12 and figure 3 displays the results.

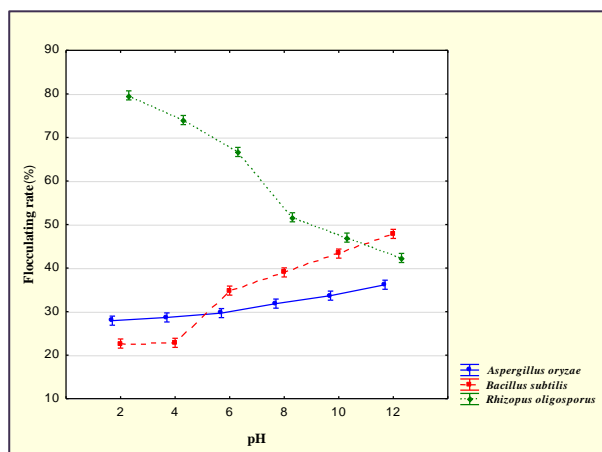


Figure 3. The effect of pH on flocculating activity in 5 g/l kaolin suspension

The increasing of pH caused an increase in the flocculating activity of *B. subtilis* and *A. Oryzae*, but not that of *R. oligosporus*. Indeed, the three studied biofloculants showed different behaviors in response to various pH values that could be due to their different chemical structures and presence of different functional groups that is apparent in their FTIR spectrum.

Researches on various biofloculants observed different flocculating efficiencies at different pH values. Yokoi et al. stated that pH is a significant factor affecting the efficiency of biofloculants.²⁶ Wang et al. reported that pH influences the stability of

particles and floc formation.⁹ Yin et al. reported that the best flocculation of biofloculants from *Gyrodinium impudicum* KG03 occurred at pH of 4.⁶ The compound biopolymer CBF-F26 produced by a mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6 had flocculating activity between pH of 7–9.⁹

Color removal (%)

The presence of dyes in wastewater is one of the most serious environmental issues.³ Dyes can be removed through coagulation flocculation without decomposition, which may produce more harmful compounds.²⁷ As can be seen in table 1, biofloculants from *Rhizopus oligosporus* had stronger decolorizing ability for basic fuchsine (30.66%) than that of *Bacillus subtilis* (21.66%) and *Aspergillus oryzae* (15.33%). It appears that composition and functional groups and dyes may play an important role.²⁸ Huang et al. reported that color removal performance of compound biofloculant (CBF) with aluminum sulfate (AS) and poly aluminum chloride (PAC) was significantly improved.²⁹ This was attributed to the adsorption and bridging effect of CBF.²⁹ These findings were confirmed by Huang et al. in their study in 2015.³⁰

Real wastewater

TSS and COD content were measured as indicative of biofloculant efficiency in dairy wastewater and the results are presented in table 1.

Chemical oxygen demand and total suspended solids removal

Figure 4 and table 1 depict the effect of different biofloculants on COD content of dairy wastewater. Addition of biofloculants has significant effect on lowering COD content.

Table 1. The effect of different biofloculants on dairy wastewater properties

Adsorbate	Control	<i>Bacillus subtilis</i>	<i>Aspergillus oryzae</i>	<i>Rhizopus oligosporus</i>
Total suspended solids (mg/l)	50.5000 ^a	40.5867 ^b	40.1600 ^c	30.4933 ^d
Chemical oxygen demand (ppm)	45600.0000 ^a	25650.6667 ^b	25080.0000 ^c	22190.3333 ^d
Color removal (%)	-----	21.6667 ^b	15.3333 ^c	30.6667 ^a

Means, within each column, followed by the same letter are not significantly different at the 0.01 probability level.

Biofloculants from *R. oligosporus* showed the highest COD removal rate (44.66% removal). This finding is in accordance with other researches on this topic. Boltz et al. showed that, in a Trickling Filter Process, bioflocculation caused a reduction in the COD and TSS of wastewater by a first-order bioflocculation rate equation.¹⁵

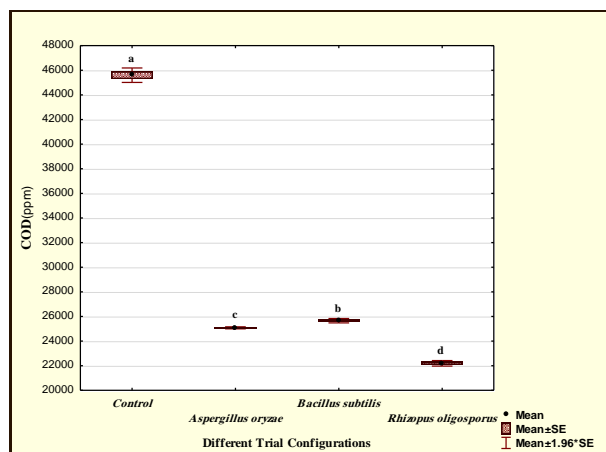


Figure 4. The effect of biofloculant addition on COD content of dairy wastewater
The same letter illustrates lack of significant difference at the 0.01 probability level.

Conclusion

According to this study, biofloculants from *Rhizopus oligosporus*, *Aspergillus oryzae*, and *Bacillus subtilis* were efficient in the removal of COD and TSS of dairy wastewater. FTIR analysis suggested the presence of functional groups, such as hydroxyl, carboxyl, and amino groups, underlying biofloculant property in the structure of the tested biofloculants. Due to their higher bioflocculating rate and thermal stability, biofloculants from *R. oligosporus* could be a good alternative for the bioflocculation process of dairy wastewaters.

Conflict of Interests

Authors have no conflict of interests.

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