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Bioflocculant 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 bioflocculants secreted from Bacillus subtilis (B. subtilis), Aspergillus oryzae (A. oryzae) and Rhizopus oligosporus (R. oligosporus). For precise investigation of bioflocculants, fourier transform infrared (FTIR) spectroscopy was performed, and pH and temperature stability, and decolorization efficiency were evaluated. In addition, the effects of bioflocculants 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. microsporus var. oligosporus were able to produce 2.51, 2.24, and 2.15 g/l of bioflocculants, respectively. The produced bioflocculants 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 bioflocculants and the involvement of N-H bands, C-O group, and carboxylic acids and their derivatives in these bioflocculant structures. Thermostability analysis of bioflocculants indicated that R. oligosporus produced more stable bioflocculants than others. It was observed that the increasing of pH caused an increase in the flocculating activity of bioflocculants produced by B. subtilis and A. oryzae. In contrast, bioflocculants from R. oligosporus showed better flocculation performance in acidic conditions. In the case of dairy wastewater, the addition of all tested bioflocculants caused a significant decrease in COD, TSS, and dyes and the best results belonged to bioflocculants from R. oligosporus.

KEYWORDS: Bioflocculant, Bacteria, Fungi, Wastewater

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Introduction

Flocculants 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 flocculants is the risk of these components for human health such as dementia (Alzheimer's disease), cancer, and neurotoxicity.[2] Bioflocculants (flocculants from microbial origin) do not have this disadvantage.[3] Another reason that bioflocculants are an interesting subject for researchers is their biodegradability. These components are advantageous over inorganic flocculants and chemically synthesized flocculants 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, bioflocculants 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 bioflocculants 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 bioflocculants produced by Bacillus subtilis PTCC 1254, Aspergillus oryzae PTCC 5163, and Rhizopus microsporus var. oligosporus PTCC 5173, the bioflocculant 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 bioflocculants 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 bioflocculant 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 × g, at 4°C for 30 minutes, and the cell free supernatant was used as the test bioflocculant. 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 bioflocculants 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 bioflocculants. Extraction of crude bioflocculants 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 supernant 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 supernant 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

bioflocculant 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 bioflocculant 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 bioflocculants. 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 bioflocculants. The bioflocculants 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 $CaCl_2$ (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 bioflocculants. This finding is in agreement with other researches that showed the bioflocculant 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.

Bioflocculant yield

Flocculating efficiency is still a limiting factor with regard to the application of bioflocculants, and strains with higher bioflocculant production are more likely to find industrial application. After 5 days of fermentation, 2.51, 2.24, and 2.15 g/l purified bioflocculants were achieved from Bacillus subtilis, Aspergillus oryzae, and Rhizopus microsporus var. oligosporus, respectively. In other studies, various bioflocculant production rates from different microorganisms have been reported such as 1.47 g/l for Bacillus sp. Strain F19[20] and 2.3-2.27 g/l for Vagococcus sp. Strain W31.[21]

Fourier transform infrared spectroscopy

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

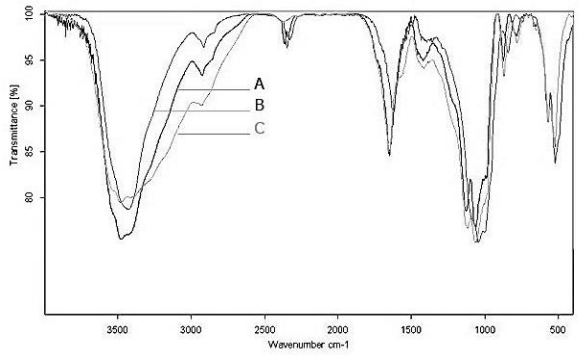

Figure 1. The effect of different loading rates of surfactant on the sorption
Contact time = 24 hours; Bromocresol green (BCG) concentration = 100 mg/l; Adsorbent dosage = 1 g/l; pH = 7

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 bioflocculant molecule. The spectrum peaks at 1000-1300 cm^{-1} suggested the presence of carboxylic acids and their derivatives.

Thermostability of the purified bioflocculants

The effect of temperature on flocculating rate was determined and the results are presented in figure 2. In all tested bioflocculants, 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, bioflocculants 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 bioflocculant produced by Aeromonas sp. after being heated at 100°C for 60 minutes.[22] Gong et al. observed that the bioflocculant 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 bioflocculant 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 bioflocculant CBF-F26 was observed when the purified bioflocculant was heated over 100°C for 30 minutes. The residual flocculating activity of this bioflocculant was more than 90%.[9]

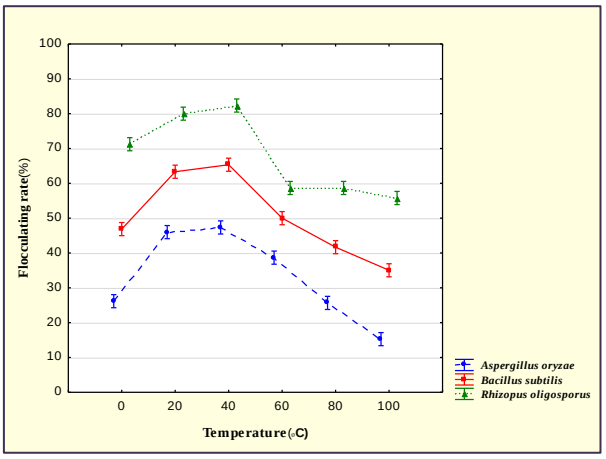

Figure 2. Thermostability of bioflocculants

The presence of different functional groups, which are noticeable in FTIR spectrum, could explain different performances of the tested bioflocculants (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 bioflocculants 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, bioflocculants from R. oligosporos 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 bioflocculants

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

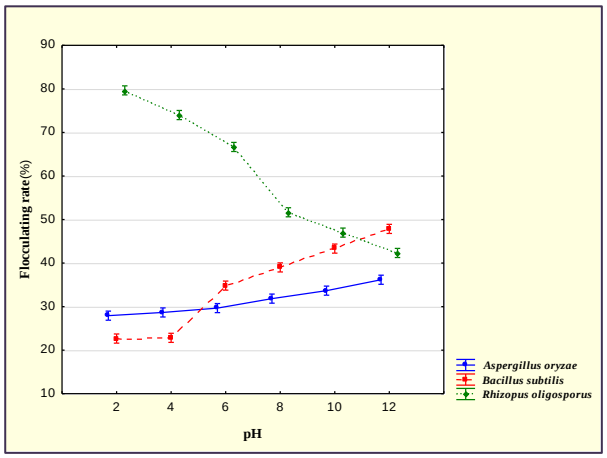

Figure 3. The effect of pH on flocculating activity in 5 g/l kaolin suspension

The increasing of pH caused an increase in the flocculanting activity of B. subtilis and A. Oryzae, but not that of R. oligosporos. Indeed, the three studied bioflocculants 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 bioflocculants observed different flocculating efficiencies at different pH values. Yokoi et al. stated that pH is a significant factor affecting the efficiency of bioflocculants.[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 bioflocculants from Gyrodinium impudicum KG03 occured 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, bioflocculants 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 bioflocculant (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 bioflocculant 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 bioflocculants on COD content of dairy wastewater. Addition of bioflocculants has significant effect on lowering COD content.

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

Adsorbate	Control	Bacillus subtilis	Aspergillus oryzae	Rhizopus oligosporus
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.

Bioflocculants from R. oligosporous 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]

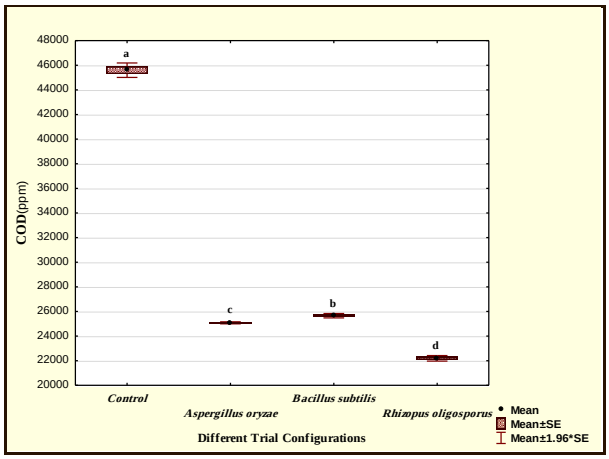

Figure. 4. The effect of bioflocculant addition on COD content of dairy wastewater
The same letter illustrates lack of significant difference at the 0.01 probability level.

Conclusion

According to this study, bioflocculants 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 bioflocculant property in the structure of the tested bioflocculants. Due to their higher bioflocculating rate and thermal stability, bioflocculants from R. oligosporous 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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References

1. Salehizadeh H, Yan N. Recent advances in extracellular biopolymer flocculants. Biotechnol Adv 2014; 32(8): 1506-22.
2. Banks WA, Niehoff ML, Drago D, Zatta P. Aluminum complexing enhances amyloid beta protein penetration of blood-brain barrier. Brain Res 2006; 1116(1): 215-21.
3. Yang Z, Yang H, Jiang Z, Cai T, Li H, Li H, et al. Flocculation of both anionic and cationic dyes in aqueous solutions by the amphoteric grafting flocculant carboxymethyl chitosan-graft-polyacrylamide. J Hazard Mater 2013; 254-255: 36-45.
4. Wang L, Ma F, Lee DJ, Wang A, Ren N. Bioflocculants from hydrolysates of corn stover using isolated strain Ochrobactium ciceri W2. Bioresour Technol 2013; 145: 259-63.
5. Zhang CL, Cui YN, Wang Y. Bioflocculant produced from bacteria for decolorization, Cr removal and swine wastewater application. Sustain Environ Res 2012; 22(2): 129-34.
6. Yin YJ, Tian ZM, Tang W, Li L, Song LY, McElmurry SP. Production and characterization of high efficiency bioflocculant isolated from Klebsiella sp. ZZ-3. Bioresour Technol 2014; 171: 336-42.
7. Liu C, Wang K, Jiang JH, Liu WJ, Wang JY. A novel bioflocculant produced by a salt-tolerant, alkaliphilic and biofilm-forming strain Bacillus agaradhaerens C9 and its application in harvesting Chlorella minutissima UTEX2341. Biochem Eng J 2015; 93: 166-72.
8. Zhang ZQ, Lin B, Xia SQ, Wang XJ, Yang AM. Production and application of a novel bioflocculant by multiple-microorganism consortia using brewery wastewater as carbon source. J Environ Sci (China) 2007; 19(6): 667-73.
9. Wang L, Ma F, Sun D, Li A, Guo J, Yu B. Characterization of a compound bioflocculant produced by mixed culture of Rhizobium radiobacter F2 and Bacillus sphaeicus F6. WORLD J MICROB BIOT 2011; 27(11): 2559-65.
10. Ozsoy HD, Kumbur H, Saha B, van Leeuwen JH. Use of Rhizopus oligosporus produced from food processing wastewater as a biosorbent for Cu(II) ions removal from the aqueous solutions. Bioresour Technol 2008; 99(11): 4943-8.
11. Azmi MA, Norli I, Farehah ZA, Ishak SA, Siti Norfariha MN, Azieda AT. Crude and pure bioflocculants produced from bacillus subtillis for low concentration of copper (Cu2+) removal. Iran J Energy Environ 2015; 6(2): 103-10.
12. Xiong Y, Wang Y, Yu Y, Li Q, Wang H, Chen R, et al. Production and characterization of a novel bioflocculant from Bacillus licheniformis. Appl Environ Microbiol 2010; 76(9): 2778-82.

13. Cosa S, Okoh A. Bioflocculant production by a consortium of two bacterial species and its potential application in industrial wastewater and river water treatment. Pol J Environ Stud 2014; 23(3): 689-96.
14. Gong WX, Wang SG, Sun XF, Liu XW, Yue QY, Gao BY. Bioflocculant production by culture of Serratia ficaria and its application in wastewater treatment. Bioresour Technol 2008; 99(11): 4668-74.
15. Boltz J, La Motta E, Madrigal J. The role of bioflocculation on suspended solids and particulate COD removal in the trickling filter process. J Environ Eng 2006; 132(5): 506-13.
16. Li Z, Zhong S, Lei HY, Chen RW, Yu Q, Li HL. Production of a novel bioflocculant by Bacillus licheniformis X14 and its application to low temperature drinking water treatment. Bioresour Technol 2009; 100(14): 3650-6.
17. Aljuboori AH, Uemura Y, Osman NB, Yusup S. Production of a bioflocculant from Aspergillus niger using palm oil mill effluent as carbon source. Bioresour Technol 2014; 171: 66-70.
18. Aljuboori AH, Idris A, Al-joubory HH, Uemura Y, Ibn Abubakar BS. Flocculation behavior and mechanism of bioflocculant produced by Aspergillus flavus. J Environ Manage 2015; 150: 466-71.
19. Pu SY, Qin LL, Che JP, Zhang BR, Xu M. Preparation and application of a novel bioflocculant by two strains of Rhizopus sp. using potato starch wastewater as nutrilite. Bioresour Technol 2014; 162: 184-91.
20. Zheng Y, Ye ZL, Fang XL, Li YH, Cai WM. Production and characteristics of a bioflocculant produced by Bacillus sp. F19. Bioresour Technol 2008; 99(16): 7686-91.
21. Gao J, Bao HY, Xin MX, Liu YX, Li Q, Zhang YF. Characterization of a bioflocculant from a newly isolated Vagococcus sp. W31. J Zhejiang Univ Sci B 2006; 7(3): 186-92.
22. Li XM, Yang Q, Huang K, Zeng GM, Liao DX, Liu JJ, et al. Screening and characterization of a bioflocculant produced by Aeromonas sp. Biomed Environ Sci 2007; 20(4): 274-8.
23. Li Q, Liu HL, Qi QS, Wang FS, Zhang YZ. Isolation and characterization of temperature and alkaline stable bioflocculant from Agrobacterium sp. M-503. N Biotechnol 2010; 27(6): 789-94.
24. Salehizadeh H, Shojaosadati SA. Extracellular biopolymeric flocculants. Recent trends and biotechnological importance. Biotechnol Adv 2001; 19(5): 371-85.
25. Dermlim W, Prasertsan P, Doelle H. Screening and characterization of bioflocculant produced by isolated Klebsiella sp. Appl Microbiol Biotechnol 1999; 52(5): 698-703.
26. Yokoi H, Natsuda O, Hirose J, Hayashi S, Takasaki Y. Characteristics of a biopolymer flocculant produced by Bacillus sp. PY-90. J Ferment Bioeng 1995; 79(4): 378-80.
27. Golob V, Vinder A, Simoni M. Efficiency of the coagulation/flocculation method for the treatment of dyebath effluents. Dyes Pigments 2005; 67(2): 93-7.
28. Deng S, Yu G, Ting YP. Production of a bioflocculant by Aspergillus parasiticus and its application in dye removal. Colloids Surf B Biointerfaces 2005; 44(4): 179-86.
29. Huang X, Bo X, Zhao Y, Gao B, Wang Y, Sun S, et al. Effects of compound bioflocculant on coagulation performance and floc properties for dye removal. Bioresour Technol 2014; 165: 116-21.
30. Huang X, Gao B, Yue Q, Zhang Y, Sun S. Compound bioflocculant used as a coagulation aid in synthetic dye wastewater treatment: The effect of solution pH. SEP PURIF TECHNOL 2015; 154: 108-14.