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Complete List of Authors:	Liu, Hai-Bo; Guangxi University, Chemistry & Chemical Engineering Li, Tan; Guangxi University, Chemistry & Chemical Engineering Wang, Jing; Guangxi University, Chemistry & Chemical Engineering Wei, Xiaoling; Guangxi University, Chemistry & Chemical Engineering Zhao, Hong-Yi; Guangxi University, Chemistry & Chemical Engineering Zhao, Zhong-xing; Guangxi University, Chemistry & Chemical Engineering
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PAPER

Identification and characterization of a novel Bacillus methylotrophicus with high flocculating activity

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Tan Li[#], Jing Wang[#], Xiaoling Wei, Hong-Yi Zhao, Zhong-xing Zhao, Hai-Bo Liu*

A novel bioflocculant-producing strain C412 was derived *Bacillus methylotrophicus*. The bioflocculant secreted by strain C412 is composed of 57.3% polysaccharides and 1.7% protein. The results of GPC indicated that the average molecular weight of the bioflocculant was 3.47×10^5. Maximum flocculating activity of 98.27% were achieved using an optimized culture medium by response surface methodology containing (per liter) soluble starch (19.73 g), beef extract (1.35 g), $(NH_4)_2SO_4$ (3.05 g), NaCl (1 g), KH_2PO_4 (2 g), K_2HPO_4 (5 g), and $MgSO_4$ (0.05 g) at pH 7.63. The bioflocculant is thermostable over a broad temperature range (4 °C to 60 °C) and has wide pH receptivity (4 to 13). Batch fermentation of strain C412 in high-cell density cultures showed shorter generation times in a 1.5 L fermentor (2–6 h) than in a shake-flask (13–17 h). The relationship between the expression levels of katE, pepT, and scrR and flocculating activity was established. Furthermore, a two-step flocculation mechanism (i.e., charge neutralization and bridging mechanism) was proposed based on zeta potential analysis.

Keywords: *Bacillus methylotrophicus,* Bioflocculant, Response surface methodology, Genetic expression, Flocculation mechanism

1 Introduction

The use of flocculating agents is a promising alternative approach for removal of contaminants with different surface charges. Such agents have attracted considerable attention in industrial application, such as treatment of wastewaters containing toxic compounds, freshwater treatment containing toxic suspended colloids and microalgae, dyeing pigments from textile industry, and heavy metal ions.[1-5] Flocculants are generally classified into inorganic, organic synthetic, and natural (bioflocculants). The first two conventional synthetic flocculants are widely used because of their high performance in forming flocs of colloids and low cost. However, residual aluminum from poly (aluminum chloride) and excess polyacrylamide pose neurotoxic and carcinogenic hazards

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toward human health and ecosystems; therefore, their usage should be restricted.[6]

Unlike synthetic flocculants, bioflocculants are environmentally friendly (e.g., they do not cause secondary pollution) and biocompatible. Bioflocculants secreted by microorganisms are biomacromolecules that mainly contain polysaccharides, glycoproteins, lipids, and nucleic acids. Much research on bioflocculants has focused on the development of new efficient bioflocculants and optimization of culture conditions, bioflocculant properties, and constituents. However, the mechanisms of action of the active ingredients of bioflocculants in the flocculation process are unclear. In particular, the key gene that regulates flocculation in Bacillus species remains unknown. Moreover, bioflocculant production has some disadvantages, including low yield and high cost, which limit the development of cost-effective processes for industrial application. To compensate for the disadvantages, some researchers have developed new efficient bioflocculant-producing mutants and genetic-engineered strains by flocculation-gene cloning and have used composite bioflocculant and low-cost culture substrates to enable large-scale application and reduce cost of industrial wastewater treatments.[7-11]

In the current study, a novel bioflocculant-producing strain C421 was screened on the basis of its capacity to facilitate sedimentation of kaolin suspension. The strain was identified as *Bacillus methylotrophicus* by 16S rRNA gene, gyrase B (GyrB) gene sequencing, as well as physiological and biochemical analyses. In addition, a series of experiments were systematically conducted to provide insights into a scaled-up continuous cultivation of strain C412 in a high-cell density fermentor. Relative flocculent genes, including sacA (defined as one of key enzymes in starch and sucrose metabolism pathways), scrR (defined as a substrate-binding protein in sugar transport system), pepT and katE (encoded transcriptional activator proteins in response to anaerobiosis) in different levels of flocculation were assayed by real-time PCR in different growth phases of *B. methylotrophicus*. Many previous reports about the relations between gene expression and flocculating activity were focused on yeasts.[12] A strong correlation between the expression of *FLO5* gene and the flocculation capacity of wine flocculent Saccharomyces cerevisiae strains was established.[13] This paper is the first to report about the relative flocculent genes in the strain *B. methylotrophicus* C412. Furthermore, zeta potential analysis was employed to determine the flocculation mechanisms of the novel bioflocculant (i.e., MBF412) in kaolin suspension and wastewater from synthetic ammonia industry.

2 Materials and methods

2.1 Isolation, cultivation and identification of bioflocculant-producing strain

The bacterial strain C412 was originally isolated from soil samples by enrichment–isolation-screening method based on its ability to form flocs of suspended particles in kaolin suspension. Strain C412 was conserved on agar plate at 4 °C, regenerated every 15–20 days for short-term storage, and eventually stored in −80 °C with glycerol (20% v/v final concentration). Medium A for seed preculture and bacterial screening was prepared using Luria–Bertani (LB) medium (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl). The pH of

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culture broth was adjusted to 7.0–7.2 by using 0.1 M HCl and NaOH in 1 L of deionized water, and 2% (w/v) agar was added to prepare the solid LB agar plates. The culture medium B (per liter) was composed of soluble starch (19.73 g), beef extract (1.35 g), $(NH_4)_2SO_4$ (3.05 g), NaCl (1 g), KH_2PO_4 (2 g), K_2HPO_4 (5 g), and $MgSO_4$ (0.05 g) at pH 7.63. All media were prepared with distilled water and autoclaved at 121 °C for 20 min. Biomass was determined by measuring the optical density at 600 nm (OD_{600}) by using a spectrophotometer (722 G, INESA CO., Ltd., China).

The 16S rDNA nucleotide sequence applied for species identification was amplified using universal oligonucleotide primers.[14] The oligonucleotide primersused for the GyrB gene amplification were UP-1S (GAAGTCATCATGACCGTTCTGCA) and UP-2SR (AGCAGGGTACGGATG TGCAGCC).[15] The resulting 16S rDNA and GyrB sequence sequenced by Sangon Corp (Beijing, China) were submitted to NCBI to determine potentially homologous sequences by a web-based tool Blast. Multiple reference alignment was then performed using the Clustral X2 software, and a neighbor-joining phylogenetic tree was constructed using MEGA software (version 6.06).[16] To characterize the strain C412, various morphological features of the strain were demonstrated by LB agar culture and light microscopy (ML32, China). The physiological and biochemical characteristics of the isolated strain C412 were analyzed using routine methods referred to Bergey's Manual of Systematic Bacteriology.[17]

2.2 Determination of flocculating activity

The flocculating activity of bioflocculant-producing stain C412 was detected by the kaolin suspension method previously described with slight modification.[18] The fermentation broth was centrifuged (7000×g at 4 °C for 15 min) to obtain supernatants regarded as liquid MBF412. Subsequently, 0.1 mL of 10% (w/v) $CaCl_2$, which was used as the conditioning agent, and 0.2 mL of cell-free-supernatant liquid MBF412 were dispersed into 20 mL of kaolin clay solution (5 g/L) in a 30 mL bottle. The mixture was oscillated using a vortex mixer for 30 s and then left to stand for 10 min. The decrease in turbidity of upper phase (2 mL) was measured using a spectrophotometer at 550 nm (OD_{550}). In the control experiment, the fermentation broth was replaced with fresh sterile culture medium. All assays were conducted in triplicates, and the flocculating activity was calculated as follows:

$$\text{Flocculating activity} = (B - A)/B \times 100\%,$$

where A and B are the OD_{550} of the supernatant and control samples, respectively.

2.3 The optimization of the preliminary culture medium by single-factors

The preliminary culture medium B used for optimizing the cultivation broth was composed of 30 g of glucose, 3 g of peptone, 1 g of NaCl, 5 g of K_2HPO_4, and 2 g of KH_2PO_4 in 1 L of deionized water at pH 7. The effects of five carbon sources (i.e., sucrose, glucose, lactose, soluble starch, and ethanol, all at 30 g/L), seven nitrogen sources (i.e., peptone, yeast extract, $(NH_4)_2SO_4$, beef extract, urea, peptone, $(NH_4)_2SO_4$, beef extract, and $(NH_4)_2SO_4$, all at 3 g/L), and eight metal ion containing monovalent, divalent, and trivalent salts (i.e., NaCl, KCl, $MgSO_4 \cdot 6H_2O$, $CuSO_4$, $MnSO_4 \cdot H_2O$, $CaCl_2$, $Al_2(SO_4)_3 \cdot 18H_2O$, and $FeCl_3 \cdot 6H_2O$, all at 1 g/L) in the basal-culture medium on cell growth and flocculating activity were investigated.

2.4 Plackett–Burman (PB) design

A two-level PB design with eight independent parameters, namely, soluble starch (A), beef extract (B), $(NH_4)_2SO_4$ (C), peptone (D), NaCl (E), KH_2PO_4(F), $MgSO_4 \bullet 6H_2O$ (G), and pH (H) was applied in a 12-run trial. The levels of every significant variable are selected according to a preliminary literature review[5] and shown in Table S1. Each variable was set on two levels, i.e., high and low, and was designated as +1 and −1, respectively. A medium constituent was considered to have a significant effect at 95% level ($p < 0.05$) from the regression analysis. The PB design was based on the following first-order model:

$$Y = a_0 + \sum^{k} a_i x_i$$

Where Y, a_0, a_i, x_i and i are the response (flocculation activity), model intercept, linear coefficient, the level of the independent variable, and number of involved variables, respectively. Critical factors of media components were determined by PB design, and then the path of steepest ascent (Table S2) was applied to adjust the factors to best experimental region.

2.5 Optimization of critical media by central composite design

The central composite design (CCD) is used for the optimization of the amount of key variables and elucidates the interactions among the variables.[19] In this part, a four-factor five-level (−2, −1, 0, 1, 2) CCD model was implemented to determine the optimal cultivation parameters for strain C412 growth. The experiments were conducted in 30 runs with low and high value, including six replications at the center point to evaluate the pure error. The design matrix and experimental data are presented in Table S3. The model was expounded by a second-order polynomial:

$$Y = a_0 + \sum^{k} a_i x_i + \sum^{k} \sum^{k} a_{ij} x_i x_j + \sum^{n} a_{ij} x_i^2, i \neq j$$

Where Y is the predicted response, a_0, a_i, a_j and a_{ij} are the regression coefficients for offset term, liner, squared, and interaction, respectively, and are the independent variables. Data of the linear relationships and interactions within variables in quadratic model are represented as elliptical contours and 3D surface plots generated from Design-expert® software (State-Ease version 8.06 Inc., US state). Fisher distribution of the regression, p-value, lack of fit, pure error, and coefficients of determination R^2 were used to assess the significance of the regression coefficient.

2.6 Batch fermentation of strain C412

Strain C412 was inoculated into 100 mL of optimized culture solution in a 250 mL Erlenmeyer flask at 37 °C and 120 rpm to obtain high-cell density culture. A 500 mL shake flask with 250 mL fermented liquid was cultivated for 72 h, and the biomass, pH, and extra- and inner-cell flocculating activities were monitored at different time intervals to obtain the growth curve of C412. The other 250 mL bottles with 100 ml fermented liquid (each OD_{600} was 2.94 for 10 h) were centrifuged to obtain wet cells and resuspended in one flask to get 2.3-fold enrichment cell culture. The wet cells were dispersed into 1 mL of distilled water with 100 µl of lysis buffer for microorganism to direct PCR (TaKaRa). The liquid was prepared at 80 °C for 30 min in a water bath to release intracellular polysubstances.

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In addition, a series of scaled-up batch fermentation were conducted in a laboratory-scale 1.5 L fermentor (ZYBIO-6001-miniQ-4, ZIYU BIOTECHNOLOGY, China). The working volume was 800 mL based on the fermentation results for the shaking flask experiments. The strains were cultured at constant rotation speed (150 rpm), temperature (37 °C), and aeration rate (6.5 ft^3/h) were optimized mainly for the continuous operation of high-cell density strain C412 cultures in a fermentor. When one cycle fermentation was completed, the cells were centrifuged and inoculated in same volume of fresh culture medium.

2.7 Real-time PCR

The relative expression levels of target genes encoding certain enzymes that produce extracellular bioflocculants were assayed using quantitative reverse transcription PCR (qRT-PCR). The assay was conducted for four different levels of flocculating capability samples from different growth phases (A: flocculating activity = 0 (time = 4 h), B: 35.9% (6 h), C: 66.7% (8 h), D: 89.2% (10 h)) containing pepT [GenBank: ABS75938.1], sacA [GenBank: ABS75857.1], scrR [GenBank: CCP23111.1], and katE [GenBank: ABS75946.1], sacA predicted cytoplasmic protein of 497 amino acids located in the cytoplasmic membrane with a size of 56.4 KDa, which was defined as a probable beta-fructosidase, and pepT (defined as a tripeptide aminopeptidase), scrR (defined as a probable transcriptional regulator) and katE (defined as a probable catalase 2) were all located in the cytoplasm.[8] The two-step PCR program on the AnalytikJena instrument (qTOWERE2.2, Germany) consisted of an initial denaturation for 10 min at 95 °C and amplification using 50 cycles of 10 s at 95 °C and 1 min at 60 °C. The primer sequences used for qRT-PCR analysis (5'–3') and their product size are given in Table S4. gatB was used as housekeeping gene for all qRT-PCR analyses.[20] Gene expression levels were all normalized with respect to the relative expression value of the housekeeping gene gatB. The relative transcript levels were calculated using $2^{-(\Delta\Delta Ct)}$ as previously described.[21]

2.8 Extraction and characteristics of bioflocculant

Pure bioflocculant was obtained from the ethanol-insoluble pellet after removing the cells by centrifugation at low temperature.[5,22] The viscous fermentation broth was centrifuged at 7000×g for 20 min at 4 °C to removal bacterial cell precipitates. The supernatant was dissolved into two volumes of pre-cooled anhydrous ethanol to resuspend the precipitate and then left overnight at 4 °C. The extracellular polymers precipitate was derived by centrifugation, washed with 75% ethanol, redissolved in deionized water for dialysis overnight at 4 °C, and then lyophilized in a vacuum freeze-drier (Xianou-18N, China) to yield the purified bioflocculant (i.e., MBF412).

The total polysaccharide and protein content of MBF412 from lyophilized material were measured by previous methods.[2,23] Fourier transform infrared spectrophotometry (Nicolet is50, USA) was employed over a wavenumber range of 400–4000 cm^{-1} to determine the functional groups. Molecular weights (MWs) of MBF412 were evaluated by Gel-Permeation Chroma-tography (GPC) using a Waters e2695 GPC system equipped with a Waters 2489 Refractive Index (RI) detector (Shimadzu, Japan) and a WAT011530 column operated at 35 ℃. Dextran-polysaccharide standards of known molecular

weight (Mw: 5.2, 11.6, 23.8, 48.6, 148, 273, 277, 401 kDa) were applied for constructing a calibration curve. The ultrapure water used as the mobile phase at a flow rate of 1 ml/min. The sample was filtrated though 0.45 um filter before injection, and then a 30 ul sample was injected in each run. Based on these experiments, a standard curve between elution volume and the logarithm of molecular weight could be acquired.

2.9 Thermostability and pH stability of purified MBF412

To evaluate the thermal stability of MBF412, six samples were prepared in deionized water (800 mg/L) to achieve an initial flocculating activity of over 90%. The pH of aqueous solution was adjusted to a pH range of 4–13 (i.e., a total of eight solutions) by using 1 M NaOH or HCl and then stored at 4 °C overnight.[24] Kaolin suspension was dispersed into the eight solutions to adjust the concentration to 5 g/L. Afterward eight aliquots of bioflocculant solutions were prepared at 4, 20, 40, 60, 90, and 100 °C for 30 min in a water bath.[24] The aliquots were then dispersed into 5 g/L kaolin suspensions at different pH values with $CaCl_2$ (as coagulant aid) to detect the flocculating rates at room temperature.

2.10 Zeta potential analysis

After adding Na^+, Ca^{2+}, Al^{3+}, and MBF412, the zeta potential of kaolin suspensions at different pH values without additives were measured using a NanoBrookOmni zeta potential analyzer (Brookhaven, USA) to elucidate the flocculation mechanism.

2.11 Sedimentation treatment of industry wastewater

MBF412 solution (200 µl, 800 mg/L) and $CaCl_2$ (100 µl, 10%) were dispersed into 20 mL of wastewater from synthetic ammonia industry in Nanning, China. After 5 min of sedimentation, the supernatant was obtained for further analysis. Turbidity was measured at 860 nm wavelength[5]. The turbidity of the pollutants was calculated by the following equation: removal efficiency = $(A_0 - A_r)/ A_0 \times 100\%$, where A_0 and A_r are the initial and final values, respectively.

3 Results and discussions

3.1 Isolation and identification of strain C412

Over 130 bacterial strains with flocculating activity were isolated from soil samples. Among these strains, the strain C412 showed a high flocculating activity with high-cell growth and stable generations. The key indicators of physiological and biochemical experiments are shown in Table S5. The 16S rDNA (1464 bp) and GyrB (1154 bp) sequences of the isolated strain C412 were submitted to GenBank database with accession numbers of KR045745 and KR150369, respectively. 16S rDNA gene shared excessive similarity (99%) with Bacillus species and species relationships were obscure because of a high genetic similarity.[25,26] Additionally, the GyrB sequence amplification of the strain C412 was further carried out. The neighbor-joining phylogenetic analysis of similar sequences to GyrB sequences obtained by the Blast tool is illustrated in Fig.1.

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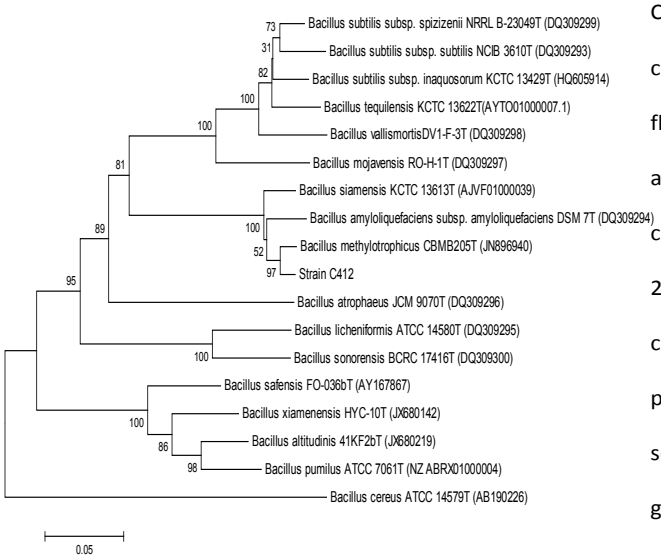

Fig. 1. Neighbor-joining phylogenetic tree of strain C412 based on GyrB sequences.

The strain C412 is the closest phylogenic neighbor of *B. methylotrophicus*, with 98% similarity. The 16S rRNA and GyrB gene analyses, as well as the morphological, physiological, and biochemical characterization showed that the isolated strain C412 was derived *B. methylotrophicus.*

3.2 Optimization of culture condition for strain C412

3.2.1 Variables influencing the flocculating activity

Preliminary studies of the composition of culture medium and cultivation condition were conducted to determine the critical factors influencing the flocculating activity during the fermentation period. As shown in Fig. 2 (a, b, c, d), the strain C412 could utilize sucrose, glucose, and soluble starch as sole carbon and energy source for cell growth. The highest flocculating activity was achieved when the soluble starch acted as the carbon source and then reached to 90% at concentrations exceeding 15 g/L after 24 h of cultivation (Fig. 2e). In terms of cost, the soluble starch was chosen as the sole carbon source in the following experiments because of it produced more satisfactory results than the other carbon sources. All single and complex nitrogen sources supported good cell growth or efficient flocculation of kaolin suspension, except for the urea source. A mixed-nitrogen source containing 1.5 g/L ammonium sulfate and 1.5 g/L beef extract was more suitable for strain C412 production than the other nitrogen sources. The beef extract concentration was varied from 1–1.5 g/L (Fig. 2f). The flocculating activity reached the maximum when the ammonium sulfate concentration varied from 2.5–3.5 g/L (Fig. 2g). High concentrations of ammonium sulfate inhibited the extracellular flocculating activity and biomass, and ammonium sulfate can possibly precipitate protein.[27] Fig. 2h shows that the flocculating activity of strain C412 was stimulated in the presence of K^+, Na^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, and Al^{3+} in the fermentation process, whereas Cu^{2+} and Fe^{3+} had no notable effect on stain C412 production (Fig. 2h); Similarly, the PM-5 produced by Aspergillus Niger has the same phenomenon.[28] Mg^{2+} could significantly stimulate cell growth. The initial pH of culture medium can affect the

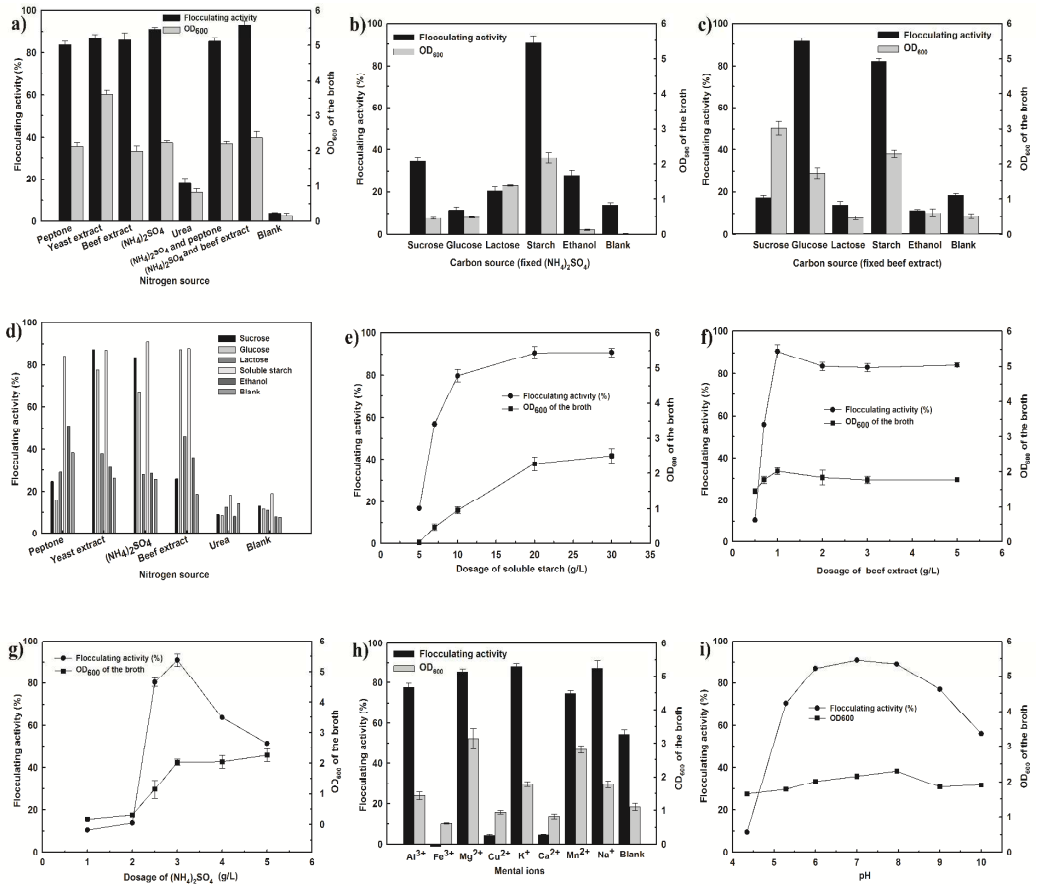

Fig. 2. The influence of cultivating broth component and condition of single factors on flocculating activity: **a):** Nitrogen sources (fixed soluble starch); **b) and c):** Carbon source **(**b) fixed beef extract; **c):** fixed $(NH_4)_2SO_4$); **d):** Nitrogen and carbon sources; **e), f) and g):** Dosage of soluble starch, beef extract and $(NH_4)_2SO_4$, respectively; **h):** Mental ions; **i):** pH of the cultivation broth.

nutrient absorption and enzymatic activity of bioflocculant-producing strains.[29] The optimal initial pH of strain C412 was in the range of 7 to 8 (Fig. 2i).

3.2.2 Screening of critical factors by PB and the steepest ascent experiment

Experimental results of the PB design for eight critical medium components are presented in Table S1. The fitted first-order model equation for predicted flocculating activity regardless of their significance could be written as: Y=87.94 + 2.24A + 0.87B + 0.42C − 0.048D + 0.19E + 8.892E-003F − 0.047G + 0.79H. Statistical analysis showed that the p-values and determinant coefficient R^2 of the first-order model were 0.0177 and 0.99, respectively. Among the variables (Table S6), soluble starch (A), beef extract (B), $(NH_4)_2SO_4$ (C), and pH (H) had p-values below the significance level of 0.05, indicating positive effects on flocculating activity. Thus, these four variables (i.e., A, B, C, and H) were chosen for the steepest ascent experiment (Table S2). The steepest ascent experiment, supported by PB design, increased from the center point of the PB design to close the

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optimal experimental space of maximum response. Maximum flocculating activity was obtained in the steepest ascent experiment with 17.8 g/L soluble starch, 1.30 g/L beef extract, and 3.05 g/L $(NH_4)_2SO_4$ at pH 7.60, which were then used as the center point of CCD.

3.2.3 Optimization of culture component by CCD

A standard RSM (five-level four-factor CCD) was adopted to optimize the dosage of each variable (Table S3). The interactions among the key variables on flocculating activity and their optimal levels were determined by the fitted second-order response surface model (in terms of coded factors): $Y = 97.52 + 2.25\ A + 0.66\ B + 0.66\ C + 0.46\ D - 1.95\ A^2 - 0.89\ B^2 - 0.96\ C^2 - 1.34\ D^2 - 0.42\ A\ B + 0.51\ A\ C + 0.48\ A\ D + 0.12\ B\ C + 0.37\ B\ D - 0.17\ C\ D$. An effective model with proportional relationships among the soluble starch (A), beef extract (B), $(NH_4)_2SO_4$ (C), and pH (D) and outstanding flocculating activity (Y) was established. ANOVA of CCD was used for statistical evaluation of the model Table S7; the corresponding F-value (456.67) and p-value (<0.001) implied that the model was highly significant. The adequacy of the model was scrutinized by the greater approach between the determination coefficient R^2 (0.9978) and the adjusted determination coefficient $adjR^2$ (0.9957). The “Lack of fit F-value” (0.45) and the “Lack of fit p-value” (0.8661) indicated no statistical significance with the pure error. A relatively low value of coefficient of variation (0.23) is an indication of a better reliability of the experimental values.

The individual F-value and p-value of linear coefficients (A, B, C, and D) and quadratic term coefficients (A^2, B^2, C^2, and D^2) showed extremely significant effects on flocculating activity ($p < 0.001$). Among the variables, soluble starch exerted the most prominent proportional relationship with flocculating activity. Fig. S1 illustrates the interactive relationship between independent and dependent variables by maintaining the third variable constant at zero level, as visualized by 3D response surface plots. All the interactions between the selected four variables were significant. The proportions of independent variable (per liter) as predicted by the model with maximal flocculating activity (98.58%) were 19.73 g of soluble starch, 1.35 g of beef extract, 3.05 g of $(NH_4)_2SO_4$, 1 g of NaCl, 2 g of KH_2PO_4, 5 g of K_2HPO_4, and 0.05 g of $MgSO_4$ at pH 7.63. The suitability of the model equation was verified under these optimal conditions.

3.3 Batch growth of strain C412

The flocculation efficiency of strain C412 in shake flask scale was shown in Fig. 3a. In general, the flocculating activity of bioflocculant secreted by strain C412 almost paralleled the cell growth. The bioflocculant-producing strain C412 underwent logarithmic growth within 3–4 h; the strain C412 reached the stationary phase after 11 h, and the maximum flocculating activity (98.3%) was obtained at 13 h. The maximum flocculating activity of the intracellular bioflocculant was 82.4% at 6 h ahead of the extracellular bioflocculant (i.e., 13 h) and a majority of flocculants were released into the fermentation broth when bacterial growth, which indicated that the bioflocculant was produced by biosynthesis rather than cell autolysis. At the last death phase (after 40 h), the flocculating activity decreased from 92.4% to 72.77% with cell growth slowly dropping because of the deflocculating enzyme effect

and cell lysis.[30] The pH showed the similar profile compared to that of flocculating activity.

Compared with the values before optimization, the time and numerical values of the maximum flocculating activity of C412 under optimized culture broth were achieved in 13 h (instead of 17 h) and enhanced to 98.3% (in close agreement with the model prediction of 98.6%). In addition, a 4.25±0.2 g/L bioflocculant production was obtained, which also verified the accuracy of the RSM model.

As shown in Fig. 3b, the flocculating activity and biomass slightly declined upon successive cultivation of up to three passages in high-cell density cultivation. Strain C412 remained over 92.4% of its flocculation efficiency (the stationary phase) when the cell density was maintained within 3.58 to 6.78. Moreover, the maximum flocculating activity was achieved within 2 h, which greatly shortened the bioflocculant production period from 10 h. The high-density cultivation broth can reduce the time and cost of the metabolism as well as growth of strain C412. Shorter generation times of strain C412 with high efficiency and stability are advantages over other bioflocculant-producing strains.

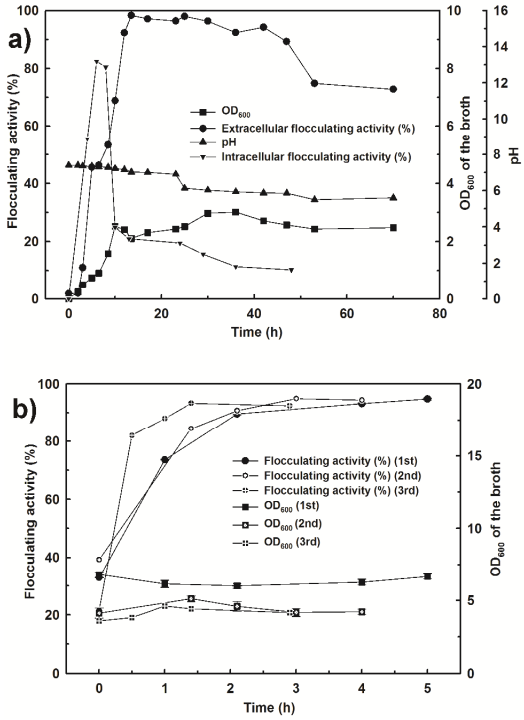

Fig. 3. a): Time course profile of flocculation efficiency of strain C412 in shake flask scale; **b):** Flocculation efficiency of strain C412 in three-cycle fermentations.

Based on the results of flask fermentation, experiments were conducted to investigate the correlated characteristics in a 1.5 L fermentor. The time course of bioflocculant produced by strain C412 was shown in Fig. 4a. The maximum flocculating activity was attained after 6–7 h under the optimized cultivation condition in the fermentor. Cell concentrations of strain C412 steadily increased until it reached the stationary phase (OD_{600}=2.588). After the 10 h cultivation of strain C412, the wet cells obtained by centrifugation were washed with distilled water, and then placed into the next fermentor with the same volume of cultivation broth under the same conditions for a batch high-cell density culture (Fig. 4b). With further progression (Fig. 4c), the time needed to reach maximum flocculating activity decreased from 4 h to 2 h,

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which could be attributed to the high cellular adaptation and acclimation of the strain. These high cellular adaptation and acclimation provide the fastest laboratory cultivation rate of high-cell density cultures of bioflocculant-producing strains reported to date.

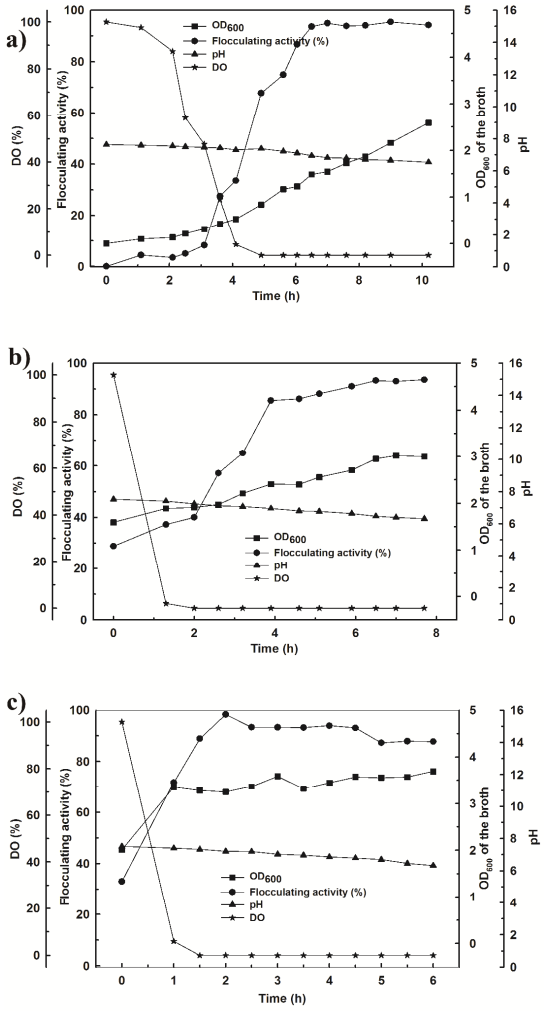

Fig. 4. Time course profile of flocculation efficiency of strain C412 in 1.5-L fermentor. **a):** The first round fermentation; (b) The second round fermentation; **c):** The third round fermentation.

Thus, the current methods extremely shortened the cultivation period. Dissolved oxygen (DO) value rapidly decreased from 100% to 0% in the early stage (normal and high-cell density fermentation for 4 and 1 h, respectively). DO participated in and promoted the bioflocculant production at the first stage in a 1.5 L fermentor. A segmented-oxygen-control strategy was proposed wherein a steady supply of oxygen was provided in the first 3–4 h, and a large demand for DO was supplied within 4–17 h to reduce the energy consumption of the fermentation, which is beneficial for the bioflocculant production. Batch cultivation of Dehalococcoides mccartyi in high-density cultures also showed a fast-rate growth and satisfied achievement.[21] Similarly, semi-continuous production of bioflocculant was carried out to prove its feasible and promising application.[31] Our findings contribute to the cost-effective and large-scale production of bioflocculant production.

3.4 Relative expression of pepT, sacA, scrR, and katE in strain C412

In this part, four samples with different levels of flocculating activity (A=0, B=35.9%, C=66.7%, and D=89.2%) from different growth phases were investigated. A sample with non-flocculating activity was chosen as a control. Fig. 5 shows the relative expression of the four flocculent genes. Among the four genes encoding the flocculent protein, the differences observed among the genes of katE, pepT, and scrR showed gradual increase in the gene expression levels of the four selected samples with the increase in flocculation capacity relative to the control. In particular, katE gene expression displayed an increasing change from 13.22-, 33.38-, and 768.56-fold, Pept gene expression increased from 3.30-, 9.09-, and 190.17-fold, and scrR gene expression increased from 1.74-, 7.49-, and 11.59-fold that of the control. By contrast, the sacA gene showed no difference. In addition, samples with higher flocculating activity showed higher relative gene expression levels. A significant increase was found in the katE,

pepT, and scrR gene expression levels, suggesting a direct relationship among the flocculating capabilities of these genes. katE, pepT, and scrR genes may have a leading function or may induce a synergistic effect with other relative flocculent genes to affect the flocculating capability of strain C412.

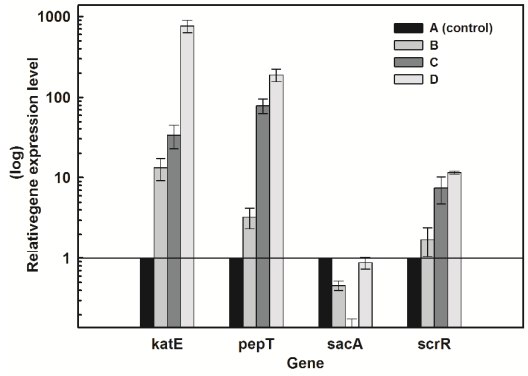

Fig. 5. Relative expression of pepT, sacA, scrR, and katE in strain C412; Four samples with different levels of flocculating activity (A=0 (control), B=35.9%, C=66.7%, and D=89.2%); Values represent the means of experiments performed in triplicate, and error bars show standard deviations.

In this study, the statistically reliable experimental data presented in the current study demonstrated a particular relationship between the expression of relative flocculent genes and the flocculation capacity of the novel *B. methylotrophicus* C412.

3.5 Characterization of MBF412

3.5.1 Composition analysis of MBF412

The UV spectra of the purified C412 presented no obvious two small peaks at around 280 and 260 nm (Data were not shown), indicating trace amount of protein at each spectrum. The total sugar and protein contents of MBF412 were 57.3% and 1.71%, respectively, which further indicate that MBF412 is mainly composed of polysaccharides. The results of GPC indicated that the average molecular weight of MBF412 was 3.47×10^5. The infrared spectrum profile of MBF412 coincided with that of the polysaccharide waveform topography (Fig. 6).[32]

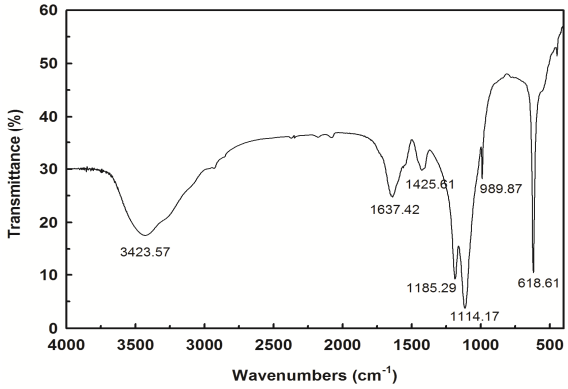

Fig. 6. The infrared spectrum profile of the purified MBF412.

A broad and strong stretching peak at around 3423 cm^{-1} could be assigned to the vibration of OH and NH groups, which indicated the existence of hydrogen bonds. The peak at 1637 cm^{-1} is a characteristic of OH-bending vibration, and that at 1425 cm^{-1} is a typical C-H distortion vibration–absorption peak. The peaks at 1114 and 1185 cm^{-1} could be attributed to C-O stretching vibration from methoxy group and C-OH, indicating the existence of polar groups, such as carboxyl and hydroxyl amino. These groups could provide adsorption site for contaminants and promote agglomeration of pollutants and bioflocculants. The peaks in the 1000-1200 cm^{-1} range are characteristic absorption locations of sugar derivatives. The characteristic peaks of MBF412 indicated the existence of carboxyl groups, amino groups, and hydrogen bonds from carbohydrates and amides. In conclusion, the MBF412 is a β-type heteropolysaccharide containing trace proteins, which is similar to that of the ZZ-3 from *Klebsiella*.[21]

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3.5.2 Effect of temperature and pH on flocculating activity and stability

Furthermore, the effect of pH on MBF412 was determined at various temperatures ranging from 4 °C to 100 °C and different pH from 4–13 for a comprehensive evaluation of the flocculation performance. Figure 7 shows that MBF412 was heat resistant 4–100 °C at pH 7. Thus, the bioflocculant can be extensively applied at wide temperature range. At pH 7, the flocculating activity of MBF412 began to decrease when the temperature is over 60 °C; the flocculating activity was 77.3% at 80 °C and 28.6% at 100 °C. The MBF412 maintained high flocculating activity of over 90% from pH 4–13 below 60 °C.

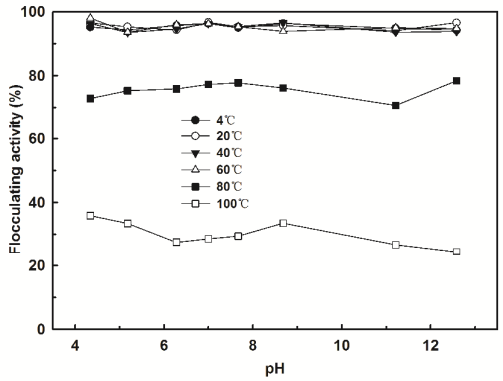

Fig. 7. The pH receptivity and thermostability of MBF412.

It is speculated that the active ingredients of polysaccharide is conducive to increase the flocculating activity and the constituent of bioflocculant containing protein was sensitive to temperature may reduce the flocculating activity, but the Macromolecular chain structure was still existed under high temperature, echoing the previous study concerning *Bacillus amyloliquefaciens*.[33] The bioflocculant MBF412 exhibited high thermostability and pH receptivity, which may be attributed to its main components, namely, polysaccharides and polar functional groups. Thus, the bioflocculant is active over a wide pH range and it can resist both acidic and alkaline conditions of sewage.

3.6 Flocculation mechanism

Zeta potentials of all tested samples were negative at pH 2.0–13.0 in kaolin suspension with $CaCl_2$. Fig. 8 shows that the zeta potential values of kaolin suspension (5 g/L) with or without Ca^{2+} and bioflocculant additions and MBF412 were negative at pH 7. The zeta potential of kaolin suspension (20 mL) vitiated from −29.78±0.3 mV to −13.69±0.27 mV and to −45.7±0.19 mV after addition of $CaCl_2$ and bioflocculant, respectively. Adding $CaCl_2$ favors the flocculating process, whereas the addition of MBF412 alone (without Ca^{2+}) has a negative effect on the flocculating activity. These effects may be due to the electrical repulsion of identically charged layers around kaolin particles. Other metal coagulant aids containing Na^+ and Al^{3+} and ammonia-contaminated wastewater were also studied. The variation of zeta potential had a strong resemblance with the flocculation process with the addition of Ca^{2+}. Bridging, charge neutralization, and a combination of the two mechanisms have been regarded as dominant mechanisms for bioflocculants.[29] In addition, the chemical composition, polar functional groups, and molecular structure of many bioflocculants could influence the effectiveness of the bridging mechanism.[18] Hypothetically, the first step of the flocculation mechanism is likely the charge neutralization on the basis of the decrease of zeta potentials of kaolin suspensions after adding $CaCl_2$. The second step was mainly the bridging mechanism induced by the addition of MBF412. The main active ingredients of bioflocculant are polysaccharides with a large number of polar functional groups

(i.e., carboxyl groups, amino groups, and hydrogen bonds), which may account for the second step of mechanism. This proposed mechanism needs further investigation.

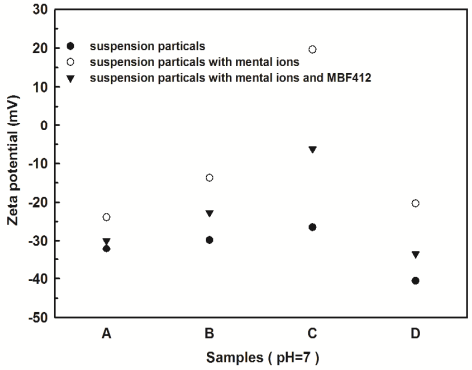

Fig. 8. The Zeta potential of the kaolin suspension (A, B and C) and wastewater from synthetic ammonia industry (D); Mental ions (**A):** Na^{+}; **B)** & **D):** Ca^{2+}; **C):** Al^{3+}); All the data are the mean of triplicates.

3.7 Sedimentation treatment of industry wastewater by MBF412

MBF412 solution and $CaCl_2$ were dispersed into 20 mL of wastewater from synthetic ammonia industry. After 5 min of sedimentation, the supernatant was obtained for further analysis. The particles from the suspension of wastewater were dried at 105 °C to a constant weight. The amount of removed suspended particles in wastewater decreased from 874 mg/L to 2 mg/L; thus, the removal efficiency in terms of turbidity was 96.1% within 5 min. The zeta potential of the pollutant particulates in the settlement process is shown in Fig. 8. The settling process was in accordance with the charge neutralization and bridging mechanism. Fig. 9 illustrates the capability of the current bioflocculant in treating wastewater from industrial plants.

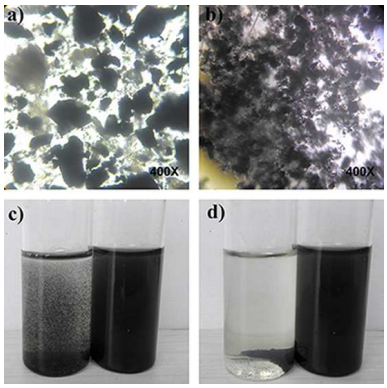

Fig. 9. Image-analysis of the wastewater: **a):** Polluted particles before sedimentation examined under a microscope; **b):** Polluted particles with CaCl2 and bioflocculant after sedimentation examined under a microscope; **c):** Polluted particles during sedimentation; **d):** Polluted particles after sedimentation.

Conclusions

A novel bioflocculant-producing strain was identified as B. methylotrophicus C412 by 16S rDNA and gyrase B sequencing analysis, as well as morphological, physiological and biochemical characterizations. High-cell density culture of strain C412 decreased the time to reach the maximum flocculating activity from 13–17 h to 2–6 h. This significant decrease offers a cost-effective method for large-scale bioflocculant production. Significant increase of katE, pepT, and scrR gene expression levels in different growth phases showed relative relationships with flocculating activity. Furthermore, a combined mechanism of bridging and charge neutralization was proposed for the settlement process. The removal efficiency 96.1% in industry wastewater demonstrated the potential of MBF412 for treating industrial wastewater.

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References

1. S. V. Patil, C. D. Patil, B. K. Salunke, R. B. Salunkhe, G. A. Bathe and D. M. Patil, *Appl. Biochem. Biotechnol*, 2011, 163, 463-472.
2. J. Y. Guo, C. P. Yang and L. Y. Peng, *Bioresour. Technol*, 2014, 152, 490-498.
3. G. Prochazkova, P. Kastanek and T. Branyik, *Bioresour. Technol*, 2015, 177, 28-33.
4. X. Huang, X. W. Bo, Y. X. Zhao, B. Y. Gao, Y. Wang, S. L. Sun, Q. Y. Yue and Q. Li, *Bioresour. Technol*, 2014, 165, 116-121.
5. O. Li, C. Lu, A. Liu, L. Zhu, P.-M. Wang, C. D. Qian, X. H. Jiang and X. C. Wu, *Bioresour. Technol*, 2013, 134, 87-93.
6. M. Kimura, Y. Matsui, K. Kondo, T. B. Ishikawa, T. Matsushita and N. Shirasaki, *Water Res*, 2013, 47, 2075-2084.
7. J. He, Q. Zhen, N. Qiu, Z. Liu, B. Wang, Z. Shao and Z. Yu, *Bioresour. Technol*, 2009, 100, 5922-5927.
8. S. Yan, N. Wang, Z. Chen, Y. P. Wang, N. He, Y. J. Peng, Q. B. Li and X. Deng, *Funct. Integr. Genomics*, 2013, 13, 425-434.
9. C. O. Letelier-Gordo, S. L. Holdt, D. De Francisci, D. B. Karakashev and I. Angelidaki, *Bioresour. Technol*, 2014, 167, 214-218.
10. L. Y. Peng, C. P. Yang, G. M. Zeng, L. Wang, C. H. Dai, Z. Y. Long, H. Y. Liu and Y. Y. Zhong, *Appl. Microbiol. Biotechnol*, 2014, 98, 6847-6858.
11. G. Zhao, F. Ma, L. Wei and H. Chua, Bioresour. Technol, 2012, 113, 83–88.
12. P. Govender, M. Bester and F. F. Bauer, *Appl. Microbiol. Biotechnol*, 2010, 86, 931-945.
13. R. Tofalo, G. Perpetuini, P. Di Gianvito, M. Schirone, A. Corsetti and G. Suzzi, *Int. J. Food microbiol,* 2014, 191, 45-52.
14. L. Sang-Cheol, S.-H. Kim, I. H. Park, S. Y. Chung, M. S. Chandra and C. Yong-Lark, *Biotechnol. Bioprocess Eng,* 2010, 15, 246-253.
15. S. Yamamoto and S. Harayama, *Appl. Environ. Microbiol*, 1995, 61, 1104-1109.
16. J. Saegusa, H. Kurikami, R. Yasuda, K. Kurihara, S. Arai, R. Kuroki, S. Matsuhashi, T. Ozawa, H. Goto, T. Takano, H. Mitamura, T. Nagano, H. Naganawa, Z. Yoshida, H. Funaki, T. Tokizawa and S. Nakayama, *Health Phys*, 2013, 104, 243-250.
17. K. Kersters and M. Vancanneyt, 2005.
18. Y. J. Yin, Z. M. Tian, W. Tang, L. Li, L. Y. Song and S. P. McElmurry, *Bioresour. Technol*, 2014, 171, 336-342.
19. M. A. Bezerra, R. E. Santelli, E. P. Oliveira, L. S. Villar and L. A. Escaleira, Talanta, 2008, 76, 965-977.
20. L. Reiter, A. B. Kolst and A. P. Piehler, *J. Microbiol. Meth,* 2011, 86, 210-217.
21. A. G. Delgado, D. Fajardo-Williams, S. C. Popat, C. I. Torres and R. Krajmalnik-Brown, *Appl. Microbiol. Biotechnol,* 2014, 98, 2729-2737.
22. C. Zhong, A. Xu, L. Chen, X. Yang, B. Yang, W. Hong, K. Mao, B. Wang and J. Zhou, *Colloid surface B*, 2014, 122, 729-737.
23. M.M. Bradford, Analys. Biochem. , 1976, 72, 248–254.
24. N. He, Y. Li and J. Chen, Bioresource Technology, 2004, 94, 99-105.
25. W. Liu, X. Wang, L. Wu, M. Chen, C. Tu, Y. Luo and P. Christie, *Chemosphere*, 2012, 87, 1105-1110.
26. M. Yamashita and J. B. Fenn, *J. Phys. Chem*, 1984, 88, 4451-4459.
27. T. H. de Koker, M. D. Mozuch, D. Cullen, J. Gaskell and P. J. Kersten, *Appl. Microbiol. Biotechnol*, 2004, 70, 5794-5800.
28. A. H. Aljuboori, Y. Uemura, N. B. Osman and S. Yusup, *Bioresour. Technol*, 2014, 171, 66-70.
29. S. Q. Xia, Z. Q. Zhang, X. J. Wang, A. Yang, L. Chen, J. F. Zhao, D. Leonard and N. Jaffrezic-Renault, *Bioresour. Technol*, 2008, 99, 6520-6527.
30. A. H. R. Aljuboori, A. Idris, N. Abdullah and R. Mohamad, *Bioresour. Technol*, 2013, 127, 489-493.
31. J. N. Wang, A. Li, J. X. Yang, J. H. Wang, J. B. Guo, F. Ma, S. N. Shi, S. Zhang and N. Q. Ren, Rsc Adv, 2013, 3, 18414-18423.
32. M. Pathak, A. Devi, K. G. Bhattacharyya, H. K. Sarma, S. Subudhi and B. Lal, Rsc Adv, 2015.
33. Y. J. Lee, B. K. Kim, B. H. Lee, K. I. Jo, N. K. Lee, C. H. Chung, Y. C. Lee and J. W. Lee, *Bioresour. Technol*, 2008, 99, 378-386.