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Received 22 May 2018; revised accepted 8 May 2019

doi: 10.18520/cs/v117/i1/114-121

Production of novel bio-flocculants from *Klebsiella variicola* BF1 using cassava starch wastewater and its application

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In this study, *Klebsiella variicola* BF1 that uses cassava starch wastewater to produce flocculants was identified using 16S rDNA gene sequencing. The pure flocculants of strain BF1 could be easily extracted by ethanol precipitation with a high yield of 7.5 g/l. It was mainly composed of 83.1% carbohydrates and 10.6% proteins. The flocculating activity revealed 97.6 ± 0.6% for kaolin suspension at 12.8 mg/l extracted flocculants from strain BF1 and 2.5 g/l $CaCl_2$. Interestingly, the flocculating activity was 78% without the addition of metal ions. Furthermore, flocculants of strain BF1 can be effectively applied in the treatment of cassava starch wastewater and municipal wastewater.

Keywords: Cassava starch, exopolysaccharide, flocculants, *Klebsiella variicola*, wastewater treatment.

FLOCCULANTS are widely used in industrial processes, including wastewater treatment, textiles, detergents, adhesives and oil recovery[1,2]. Flocculants consist of two main classes: (1) chemical flocculants such as polyacrylamide, polyelectrolytes, polyethyleneimine, polyaluminium chloride and aluminium sulphate, and (2) natural flocculants such as cellulose, microbial flocculants, gelatin, chitosan, gum and mucilage, sodium alginate and tannin[1,2]. The chemical flocculants have been widely used in various applications due to their effectiveness and low cost. However, chemical flocculants can negatively affect ecosystems. Therefore, it is important to replace chemical flocculants by biodegradable flocculants.

Microbial flocculants have attracted research interest[3–14]. Bacteria, fungi and algae are known to be responsible for the production of flocculants[4–11,13,14]. The large-scale production and recovery of bio-flocculants has been studied[4–11,13,14]. Therefore, they are widely applied in many industrial sectors. For the aim of commercialization, a considerable effort has gone into reducing the production cost through using some wastes rich in organic matter,

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Table 1. Properties of cassava starch wastewater and municipal wastewater before and after treatment with BP-1

Parameters	Cassava starch wastewater			Municipal wastewater		
	Before treatment	After treatment[a]	Removal rate (%)	Before treatment	After treatment[a]	Removal rate (%)
Chemical oxygen demand (COD) (mg/l)	12,475	7,353	41.1	114	46	59.6
Biological oxygen demand $(BOD)_5$ (mg/l)	8,634	4,956	42.6	87	42	51.7
Total suspended solids (TSS) (mg/l)	4,286	2,966	30.8	40	26	35
Total nitrogen (mg/l)	180	125	30.5	17	9	47.1
Total phosphorous (mg/l)	34	30	11.8	2	1.6	20
OD_{550}	0.74	0.24	67.5	0.81	0.32	61.3
pH	3.5	3.6	–	6.8	6.8	–

[a]500 ml of wastewater is poured into a 1 litre beaker, and 12.8 mg/l BP-1 is added to it. Then the solution is stirred for 20 s and left to stand without shaking for 30 min.

nitrogen and phosphorus. The ability to produce bioflocculants using dairy wastewater, excess sludge, swine wastes, rice stover and potato starch waste has also been reported[4,7–9,11–14].

Cassava (*Manihot esculenta* Crantz), also called tapioca, is one of the major staple food crops grown in more than 80 tropical countries. In Vietnam, cassava has quickly changed from a food crop to an industrial crop. The increase in global cassava processing industry has resulted in heavy water pollution, because large amounts of wastewater with extremely high concentrations of organic pollutants are released. These organic pollutants can be used to culture microorganisms for flocculants production. To the best of our knowledge, there has been no report on the flocculants-producing capabilities of microbes using cassava starch wastewater. In this study, bacteria were isolated from municipal wastewater, identified and assessed for their flocculants production using cassava starch wastewater. Utilization of cassava starch wastewater can lower the cost of flocculants production, and further reduce the pollution caused by uncontrolled emission of this type of wastewater. The composition, properties, activity and mechanism of the flocculants were also determined for a better understanding of their potential applications in various industrial processes.

In this study, cassava starch wastewater was taken from household factories in Ho Chi Minh City, Vietnam. Table 1 lists the properties of wastewater.

Bacteria were isolated from samples of Logom canal, Ho Chi Minh City and screened for flocculants production as described below. Sampling sites (10°44′N, 106°38′E) that are representative of locations receiving water discharged from human activities were selected. In the dry season of 2017 (April), samples (30 cm depth) were collected at three sites. Next, 1 ml of sample was serially diluted with distilled water (10^1–10^5 fold), and subsequently, 0.1 ml solution of each dilution was spread on the enrichment medium. The composition of the enrichment medium agar plates is as follows (per liter): beef extract 3 g, peptone 10 g, NaCl 5 g, agar 15 g, pH 7.0. The plates were inverted and incubated at 30°C for two days. A total 39 morphologically different isolates were obtained and individually inoculated for 24 h in 5 ml cassava starch wastewater medium. This wastewater medium consists of KH_2PO_4 2 g, K_2HPO_4 4 g, $MgSO_4$ 0.2 g, NaCl 0.1 g, urea 2 g in 1 litre cassava starch wastewater with the pH value adjusted to 7.0. Strains were incubated at 200 rpm on a rotary shaker at 30°C, unless otherwise stated.

Cell morphology and Gram staining were observed by phase-contrast microscopy and light microscopy respectively. The 16S rDNA gene of the isolates was amplified by PCR using the primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′). The PCR products were sequenced and consensus sequences were obtained using Bioedit (version 7.2.6). The sequence comparisons using BLAST tool from GenBank were done for identification. The nucleotide sequences of the 16S rDNA genes from the isolated strain and the published strains were aligned using Clustal X (version 2.0.3). Using Bootstrap analysis with a default setting of 1000 trials and a seed value of 111, the phylogenetic tree was constructed.

The overnight culture of strain was diluted 1 : 50 in 500 ml fresh cassava starch wastewater medium. The flasks were then incubated for 48 h. The culture broth was collected and centrifuged at 8000 *g* for 30 min. The supernatant was subsequently collected and extracted using a modified extraction method as follows. The mixture, including pre-cooled (–20°C) absolute ethanol and supernatant in the ratio of 2 : 1 was stabilized at –20°C for 24 h. The resultant sediment was centrifuged and dried to produce the crude flocculants. These were dissolved in water, and then Sevage solution (chloroform : *n*-butanol :: 5 : 1) was added to an equal volume[3]. The mixture was then centrifuged at 8000 *g* for 30 min and the pellet was dried, yielding purified flocculants. The total sugar content and protein concentration were determined according to a previous study[1]. A FTIR spectrometer (Bruker's Vertex 79 series FT-IR, Germany) was used to examine the functional groups of flocculants.

Flocculating efficiency was studied by measuring the turbidity of a kaolin suspension. For this, 9 ml of kaolin suspension (5 g/l), 1 ml $CaCl_2$ solution (10 g/l) and 0.1 ml culture broth or 0.1 mg/l pure flocculants were mixed vigorously for 20 s and left to stand without shaking for 5 min. The turbidities of the sample supernatant and a control experiment without flocculants were measured at 550 nm. The flocculating activity can be expressed as follows

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

where a and b are the OD_{550} values of the control and sample respectively[15].

To determine the effect of temperature on flocculating activity, flocculants solutions were subjected to different temperature treatments for 30 min. The flocculating activities against kaolin suspension of these treated flocculant solutions were measured. The decrease in the total flocculating activity after the different treatments was used to evaluate the relative contribution of the protein components in the flocculants to their flocculating activity. Metal compounds (KCl, NaCl, $MgSO_4$, Fe_2SO_4, $FeCl_3$ and $AlCl_3$) were added to the mixture instead of $CaCl_2$ in order to determine their effects on the flocculating activity.

The three-level–two-factor central composite design (CCD) was applied to evaluate the most important operating variables ($CaCl_2$ (X_1) and the extracted flocculants (X_2)) in the flocculating process[5]. The ranges of the variables were chosen according to the results of a preliminary experiment as follows: flocculants dosage, 8–18 mg/l and $CaCl_2$, 1.5–3.5 g/l. Thirteen trials were performed with the independent variables at three different levels.

The response variable (Y) was calculated and fitted to a second-order model which contains the independent variables as below

$$Y = \beta_0 + \sum \beta_i X_i + \beta_{ij} X_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2, \quad (1)$$

where β_0, β_i and β_{ii} are the intercept, linear coefficient and quadratic coefficient respectively. β_{ij} is a regression coefficient of interaction between the X_i and X_j, whereas X_i and X_j are input variables that influence the response variable Y. For the experimental design, Minitab (version 16.2.4) was applied. The interaction between process variables and responses was performed using the analysis of variance (ANOVA).

To analyse the flocculating efficiency of BP-1 to municipal wastewater and cassava starch wastewater, 500 ml of wastewater was poured into a 1 litre beaker, and 12.8 mg/l BP-1 was added to it. Then the solution was stirred at 60 rpm for 20 s and left to stand without shaking for 30 min. The turbidities of the sample supernatant and a control experiment without flocculants were measured at 550 nm. Total suspended solids (TSS), chemical oxygen demand (COD), biological oxygen demand (BOD), total nitrogen and total phosphorus in wastewater and the supernatant of treated wastewater were measured according to the Association of Official Analytical Chemists[16].

In this study, the 16S rDNA gene sequence has been assigned the DDBJ/EMBL/GenBank accession number MH458937.

Thirty-nine morphologically different isolates were selected and checked for their ability to produce high flocculants. However, only nine strains displayed high flocculating efficiency (Table 2). The growth pattern and yield of flocculants of those isolated strains were determined for four days. Among them, a Gram-negative bacterium, namely BF1 produced high flocculants and was selected for further study. The morphological characteristics of the strain include a rod-shaped bacterium without flagella, and size of approximately 0.5×2.5 μm. The colony of strain BF1 is circular, milky white, smooth and papillary, moist on the surface and not easy to pick up with loops. In order to identify the isolate, 16S rDNA gene sequencing was performed. It is closely related to *Klebsiella variicola* strain DX120E (99%), originally isolated from sugarcane roots[17]. A phylogenetic neighbour-joining tree based on the nucleotide sequences of 16S rDNA genes of strain BF1 and reported flocculants-producing bacteria was constructed, which revealed that strain BF1 was closely related to flocculants-producing *K. pneumonia* group with 92% bootstrap support (Figure 1). Based on the BLAST results, morphological and microscopic characteristics, the pure isolate was classified as *K. variicola* strain BF1.

To optimize the culture conditions for production of flocculants from strain BF1, the effect of temperature, pH, phosphate salts, extra nitrogen source and carbon source was examined. For temperature and pH, the results revealed maximum growth and flocculating rate at 30°C and 7.0 respectively. Phosphate salts (at different dosages) were found to be beneficial for cell density and flocculating efficiency in strain BF1 (Figure 2 *a*). Especially, the flocculating activity improved to 71.4% when the total added phosphate salts was 6 g/l (4 g/l K_2HPO_4 and 2 g/l KH_2PO_4). To study the effect of nitrogen sources, beef extract, peptone, yeast extract, urea and $(NH_4)_2SO_4$ were used in the same concentration (2 g/l) (Figure 2 *b*). The flocculating efficiency of five different nitrogen sources ranged from 78.8% ± 1.7% to 92.7% ± 0.7%. Specifically, peptone, beef extract and urea produced flocculants with the more 90% efficiency after 24 h of cultivation. For extra carbon sources (including 2 g/l of glucose, maltose, fructose and sucrose and 2 ml/l of 95% ethanol), the results were slightly different compared with the culture liquor from cassava starch wastewater medium (Figure 2 *c*). From these

Table 2. Screening of flocculants-producing bacterium and its flocculating ratio for kaolin suspension

Strain	Character morphology	Gram	Flocculating efficiency (%)
2	Irregular, entire, moist and white	–	31.4 ± 4.82
5 (BF1)	Circular, entire, moist and milk white	–	90.0 ± 2.46
11	Irregular, lobate, moist and white	+	73.8 ± 5.56
14	Irregular, serrate, moist and white	–	80.7 ± 3.10
17	Circular, lobate, moist and white	–	86.7 ± 3.50
21	Irregular, entire, moist and milk white	–	76.4 ± 3.19
24	Circular, serrate, dry and white	+	85.1 ± 3.43
31	Irregular, entire, moist and white	–	62.5 ± 4.26
38	Circular, serrate, moist and milk white	–	72.7 ± 9.22

Each value represents mean ± SD (n = 3).

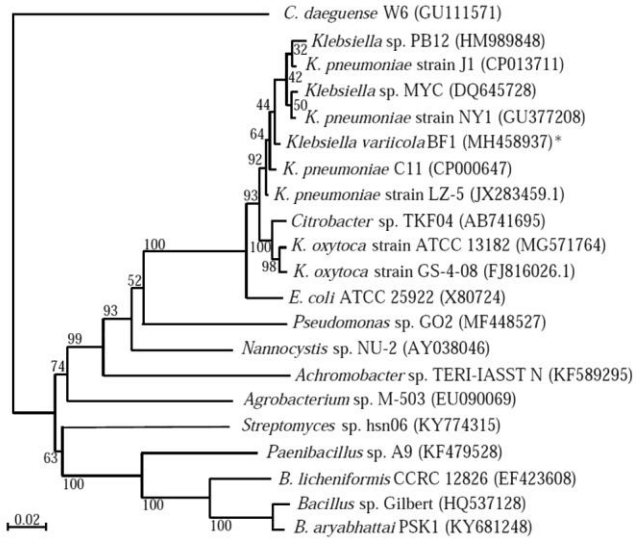

Figure 1. Phylogenetic neighbour-joining trees based on the nucleotide sequences of 16S rDNA genes of isolated (indicated by star) and the reported flocculants-producing bacteria. The strains are indicated by their EMBL/GenBank/DDBJ accession numbers after species names. Bootstrap values, indicated at the nodes, are obtained from 1000 bootstrap replicates and are reported as percentages. Bar indicates 2% sequence divergence.

results, 6 g/l phosphate salts and 2 g/l urea were chosen for BF1 to produce high flocculating activity at low cost.

Under optimal conditions, the yield of purified BP-1 could reach 7.5 g/l. The major content of BP-1 was found to be 83.1% total sugar and 10.6% protein. The functional group of BP-1 was then analysed using FTIR spectroscopy (Supplementary Figure 1). The infrared spectra of BP-1 showed characteristic functional groups that mainly included carbonyl, amino, and hydroxyl groups and amides.

Figure 3 shows the growth curve of strain BF1 and flocculating activity of its cassava starch wastewater medium. The flocculating rate from early stationary cultures was the highest (93.5% at 21 h). At late stationary phase, the flocculating rate started decreasing; this may be due to the de-flocculation enzyme activities. The flocculating activity of untreated BP-1 against kaolin suspension was 96.18% ± 0.81%. When BP-1 was treated at 100°C and 121°C for 30 min, flocculating activities were decreased to 75.35% ± 2.66% and 51.1% ± 4.7% respectively (Figure 4 *a*).

Figure 4 shows the effect of BP-1 dosage, $CaCl_2$ concentration and metal ions on the flocculating activity. Flocculating efficiency was more than 80% for a range of BP-1 dosages (5–50 mg/l); the maximum flocculating activity was observed at an optimal dosage of 13 mg/l (Figure 4 *b*). The addition of K^+, Na^+ or trivalent cations could not evidently enhance the flocculating activity of BP-1. The flocculating activity of BP-1 was slightly enhanced by the addition of bivalent cations, including Mg^{2+}, Ca^{2+} and Fe^{2+} (Figure 4 *c*). One common trait between BF1 and the other reported microorganisms is the positive influence of Ca^{2+} ions in aiding flocculation. Interestingly, the flocculating activity of BP-1 was 78% without the addition of any cation. As shown in Figure 4 *d*, all the flocculating activities were above 89.9% in the presence of 0.5%–4% $CaCl_2$, in which the optimal dosage was determined to be 2.5% for flocculation of kaolin solution. BP-1 showed high flocculating efficiency within a wide pH range. More than 90% removal rate was observed at either strong acidic or basic pH range. Flocculating activity of BP-1 was slightly higher in acidic (pH below 7) than in basic solution.

The interaction between the dosage of BP-1 and $CaCl_2$ content was studied by three-level–two-factor CCD analysis and response surface methodology (RSM). The fitting polynomial (eq. (2)) was obtained after data fitting. Table 3 shows the predicted and observed flocculating activities (%).

$$Y = 97.45 - 0.41X_1 + 1.46X_2 + 0.23X_1X_2 - 1.76X_1^2 - 2.14X_2^2. \quad (2)$$

ANOVA revealed that the fitted model was statistically valid with high model F-values and low P values ($P < 0.0001$). Figure 5 shows the three-dimensional response surface plot. The $CaCl_2$ dosage ($P < 0.0001$)

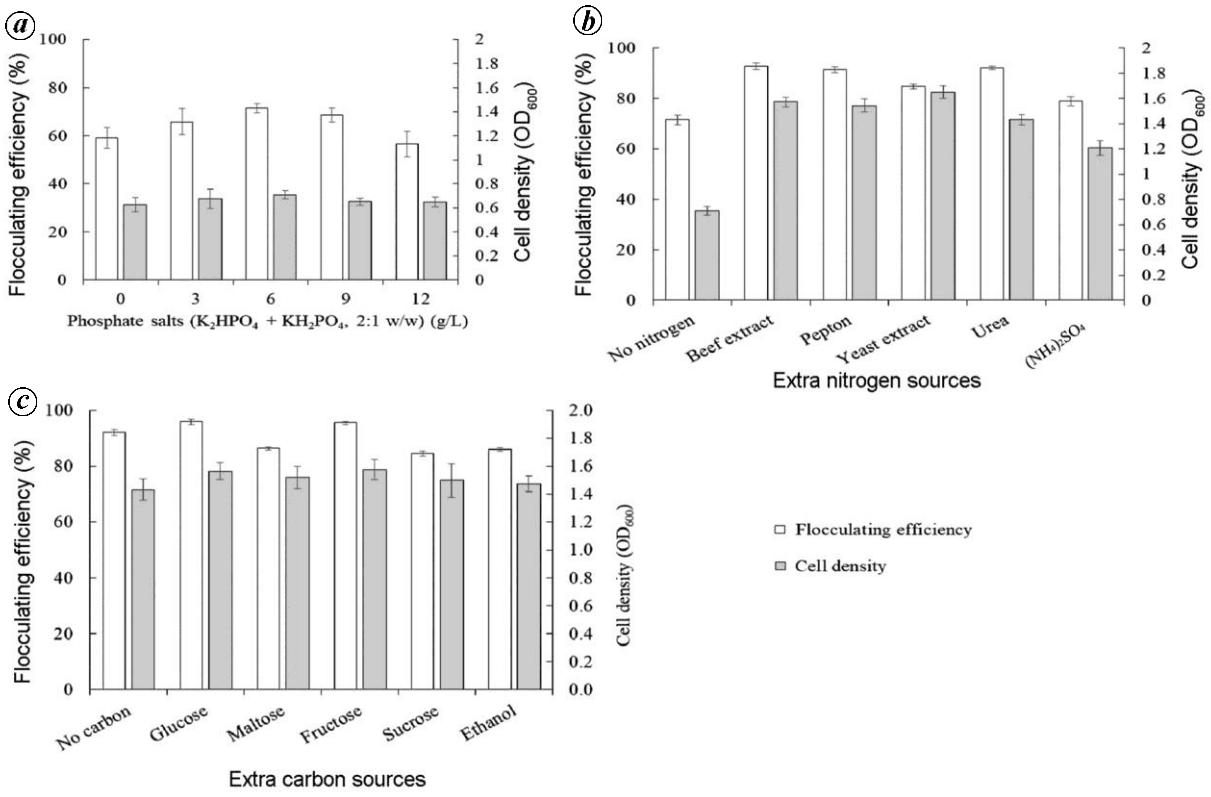

Figure 2. Effects of (***a***) phosphate salts, (***b***) extra nitrogen sources and (***c***) extra carbon sources on flocculating efficiency and cell density of strain BF1. The medium consists of (***a***) $MgSO_4$ 0.2 g, NaCl 0.1 g and different concentrations of phosphate salts; (***b***) K_2HPO_4 4 g, KH_2PO_4 2 g, $MgSO_4$ 0.2 g, NaCl 0.1 g and 2 g of different extra nitrogen sources and (***c***) K_2HPO_4 4 g, KH_2PO_4 2 g, $MgSO_4$ 0.2 g, NaCl 0.1 g, urea 2 g, and 2 g of different extra carbon sources in 1 litre cassava starch wastewater with pH value adjusted to 7.0. Error bars indicate standard deviation of triplicate experiments.

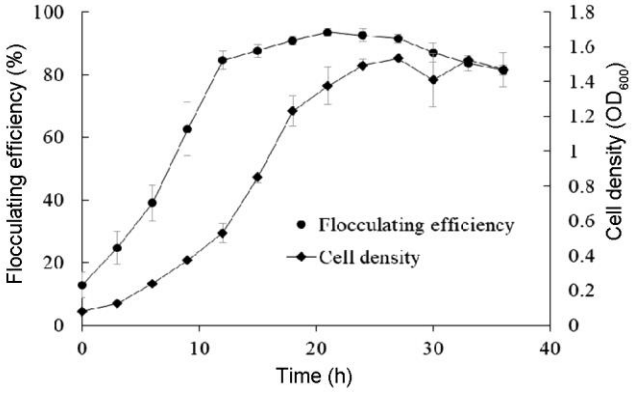

Figure 3. Growth curve of strain BF1 and flocculating activity of its cassava starch wastewater medium. The medium consists of K_2HPO_4 4 g, KH_2PO_4 2 g, $MgSO_4$ 0.2 g, NaCl 0.1 g, urea 2 g in 1 litre cassava starch wastewater with pH value adjusted to 7.0. Error bars indicate standard deviation of triplicate experiments.

exhibited a higher influence than the BP-1 dosage ($P = 0.037$) on the flocculating activity. According to the regression model, the maximum flocculating activity of 97.71% was obtained under the following conditions: $X_1 = 12.8$ mg/l and $X_2 = 2.67$ g/l. Under optimized condition, the observed flocculating activity was 97.6% ± 0.6%. The result closely agrees with the model prediction. Thus, the model is considered to be reliable for describing the effects of BP-1 and $CaCl_2$ dosages on flocculating activity.

In this study, the bonding types in kaolin–Ca^{2+}–BP-1, kaolin–Mg^{2+}–BP-1 and kaolin–BP-1 systems were tested by EDTA, EGTA, HCl and urea treatment. After addition of 3 M HCl, 1 M EDTA or 1 M EGTA, the flocculation in three systems did not occur in 30 min observation. No significant de-flocculation phenomenon was observed in the urea (3 M) added group.

Finally, we determined whether BP-1 flocculants could be used to improve the efficiency of municipal wastewater and cassava starch wastewater treatment. After treatment with 12.8 mg/l BP-1, the residual TSS, COD, BOD_5, total nitrogen and total phosphorus of cassava starch wastewater and municipal wastewater, were found to be 2996, 7353, 4956, 124 and 30 mg/l and 26, 46, 42, 9 and 1.6 mg/l respectively, which were lower than the initial concentrations (Table 1), indicating that the bio-flocculants can be used as an effective pretreatment for cassava starch wastewater and municipal wastewater.

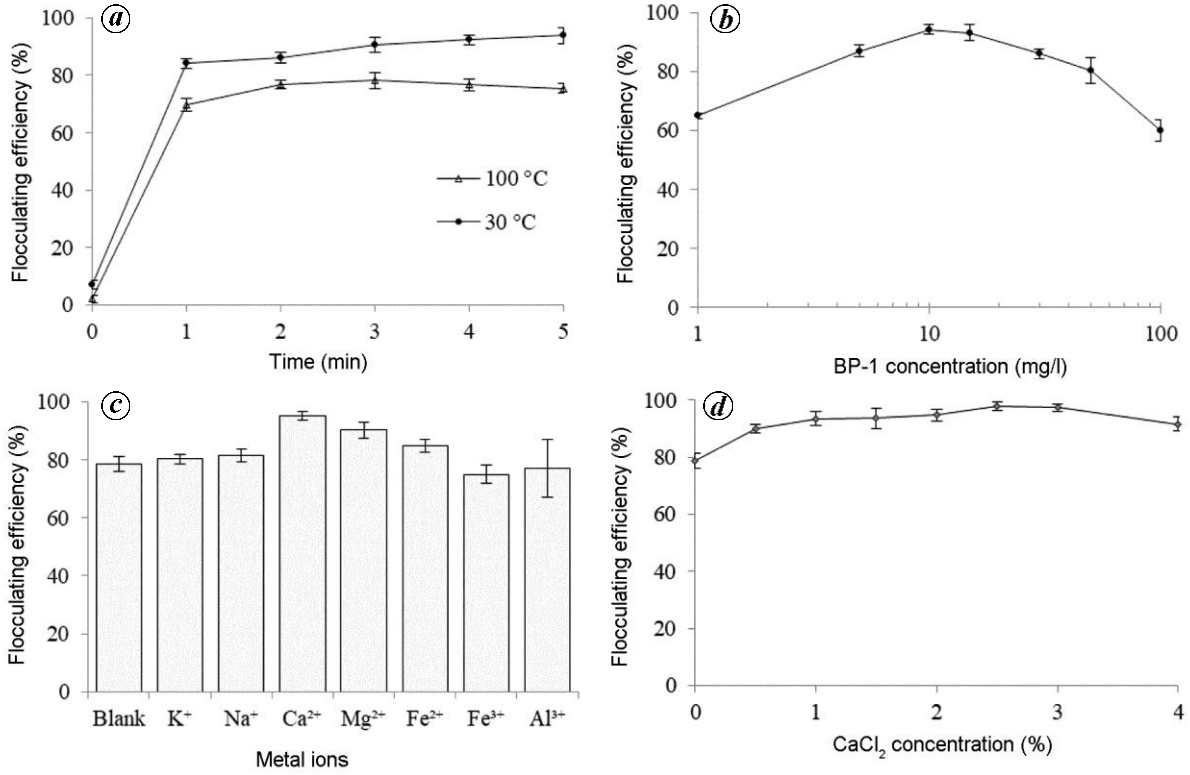

Figure 4. Effects of temperature, BP-1 dosage, pH and metal ions on flocculating efficiency. ***a***, Temperature treatment for 30 min, 30 mg/l BP-1, pH 6.5; ***b***, BP-1 conc. pH 6.5, 30°C; ***c***, 10 mg/l BP-1, pH 6.5 and metal ions: 0.1 M; ***d***, 10 mg/l BP-1 and pH 6.5. Error bars indicate standard deviation of triplicate experiments.

Table 3. Central composite design for optimization of the flocculation parameters of kaolin suspension with BP-1

	Factor					
	BP-1		$CaCl_2$		Flocculating efficiency (%)	
Run	X_1	A (mg/l)	X_2	B (g/l)	Actual value	Predicted value
1	–1	8	0	2.5	95.88 ± 1.91	96.10
2	–1	8	–1	1.5	92.90 ± 0.95	92.73
3	0	13	–1	1.5	93.88 ± 1.16	93.85
4	0	13	1	3.5	97.12 ± 1.24	96.77
5	0	13	0	2.5	97.25 ± 0.42	97.45
6	0	13	0	2.5	97.25 ± 1.22	97.45
7	–1	8	1	3.5	95.20 ± 1.28	95.19
8	1	18	–1	1.5	91.19 ± 2.02	91.45
9	1	18	1	3.5	94.43 ± 1.51	94.83
10	1	18	0	2.5	95.88 ± 1.84	95.28
11	0	13	0	2.5	97.25 ± 1.43	97.45
12	0	13	0	2.5	97.84 ± 1.21	97.45
13	0	13	0	2.5	97.25 ± 0.64	97.45

The low production and high cost of bio-flocculants have greatly affected their practical uses; therefore it is necessary to select microorganisms capable of producing high yields of desirable flocculants from low-cost material. Our interests in the characterization of *K. variicola* BF1 stemmed from its ability to produce a high yield of BP-1 using cassava starch wastewater. Table 4 summarizes the bio-flocculants producing microorganisms and their properties, including flocculating efficiency, optimum condition for flocculation and characterization of bio-flocculants[4–14]. In general, the genus *Klebsiella* produces very low yields of its flocculants products, including *K. oxytoca* GS-4-08 (0.2 g/l), *Klebsiella* sp. S11 (0.9 g/l), *Klebsiella* sp. PB12 (1.3 g/l), *K. pneumoniae*

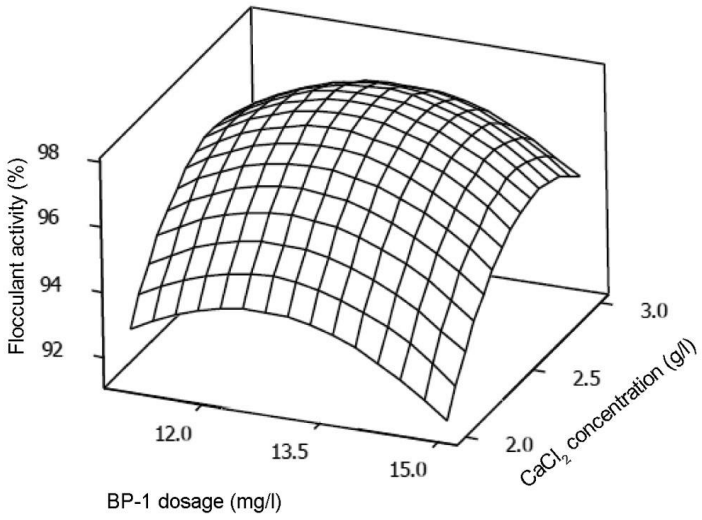

Figure 5. Response surface plots for optimization of flocculating activity.

C11 (1.6 g/l), *K. mobilis* (2.5 g/l), *K. pneumoniae* LZ-5 (2.8 g/l), *K. pneumoniae* H12 (3 g/l) and *K. variicola* B16 (3.08 g/l)[4–14]. Interestingly, *K. pneumoniae* NY1 was reported to show good flocculants production (14.9 g/l)[18]. Few strains from other genera reported high yields of flocculants, such as *Agrobacterium* sp. M-503 (14.9 g/l)[19], *Nannocystis* sp. NU-2 (14.8 g/l)[20], *Bacillus licheniformis* CCRC 12826 (14 g/l)[21] and *Achromobacter* sp. TERI-IASST N (ref. 22). In this study, the pure flocculants (BP-1) could be easily extracted from cassava starch wastewater medium by ethanol precipitation with a yield of 7.5 g/l. Compared to strains which were isolated from river water or wastewater, strain BF1 revealed 5.8- and 36-fold higher yield than *Klebsiella* sp. PB12 (ref. 7) and *Citrobacter* sp. TKF04 (ref. 23) respectively, in producing pure flocculants.

The flocculating activity was found to be 97.6% ± 0.6% for kaolin suspension with 12.8 mg/l BP-1 and 2.5 g/l $CaCl_2$. The flocculating activity of BP-1 was 78%, without the addition of metal ions. Mandal *et al.*[7] reported that flocculating activity of the bio-flocculants produced by *Klebsiella* sp. PB12 could reach 98% for kaolin suspension with 17 mg/l EPS and 4 mM $CaCl_2$. However, in the absence of Ca^{2+} ions, no effective flocculation was observed which indicates the requirement of $CaCl_2$ for effective flocculation by forming Ca^{2+}-mediated complexes of EPS and kaolin. There are only few studies reporting high flocculating activity with or without Ca^{2+} ions, in which flocculants produced by *K. pneumoniae* strain YZ-6 and LZ-5 showed 96.5% and 98% activity with Ca^{2+} ions for kaolin suspension at 50 and 54.3 mg/l EPS respectively[6,8]. In the absence of Ca^{2+} ions, flocculants produced by both strains still reached 80% efficiency. This is in agreement with our results, but the required dose of BP-1 is much lower, suggesting its potential industrial use.

Compositions of bio-flocculants are often reported to be glycoprotein-like substances, where the conjugates play an important role in exhibiting flocculating activities[24–26]. Interestingly, some studies report the absence of proteins in bio-flocculants, that indicates the important role of polysaccharides in flocculation[15]. In this study, FTIR analysis of BP-1 revealed the presence of –OH or –NH groups, in which the broad spectra appeared to be similar to that of a sugar–protein complex. In addition, the major contents of BP-1 were found to be 83.1% total sugar and 10.6% protein. These results are in disagreement with previous studies (Table 4).

The coordination of flocculants with kaolin and metal ions was examined for the flocculation mechanism of BP-1. The positive influence of Ca^{2+} and Mg^{2+} ions on flocculating efficiency was recognized. Therefore, the bonding types in kaolin–Ca^{2+}–BP-1, kaolin–Mg^{2+}–BP-1 and kaolin–BP-1 systems were tested by EDTA, EGTA, HCl and urea treatment. Urea is known to disrupt hydrogen bonds, while HCl destroys the ionic bonds[27]. No significant de-flocculation phenomenon after addition of 3 M urea was observed, suggesting that hydrogen bonds do not exist predominantly in BP-1. After the addition of 3 M HCl, the cloudy kaolin–Ca^{2+}–BP-1, kaolin–Mg^{2+}–BP-1 and kaolin–BP-1 systems did not occur in 30 min observation, suggesting the role of ionic bonds in these systems. To further study the role of Ca^{2+} and Mg^{2+} ions in the systems, 1 M EDTA and 1 M EGTA were added. EDTA and EGTA are well known as chelating agents, in which EGTA has a higher affinity for Ca^{2+} but a lower affinity for Mg^{2+} compared to EDTA[27]. Interestingly, our results revealed that all systems are sensitive to EDTA or EGTA; this might be because the chelating agents have high affinity to Ca^{2+} and Mg^{2+} ions, and also to BP-1 flocculants. This can be explained by the fact that high concentration of EDTA, EGTA and HCl affects the

Table 4. Properties of reported flocculants-producing bacteria

Microorganisms	Source	Pure flocculants in culture broth (g/l)	Flocculating rate (%)	Condition for flocculating activity				Flocculants composition (%)		Accession number	Reference
				Dose (mg/l)	pH	Temperature (°C)	Time (min)	Carbo-hydrate	Proteins		
Klebsiella variicola BF1	River water	7.5	97.6 (77)	12.8	6.5	30	5	83.1	10.6	MH458937	The present study
Klebsiella variicola B16	Soil	3.08	94.1	12.5 ml	7.0	RT	5	81.8	15.9	–	14
Streptomyces sp. hsn06	Sludge	–	92.7	20	5.0	25	5	+	0	KY774315	29
Pseudomonas sp. GO2	Sludge	–	94.7 algae	12.5	7.0	25	1	59	32.1	MF448527	25
Bacillus aryabhattai PSK1	Soil	6	90 (71.6)	–	6.0	25	3	+	+	KY681248	24
Pseudomonas veronii L918	Soil	3.39	92.5	2.8	7.0	28	1	77.1	4.8	–	27
Klebsiella oxytoca GS-4-08	Midgut	0.2	25 (0)	40	4.0	25	10	+	0	FJ816026	4
Klebsiella pneumoniae C11	Sludge	1.6	92.3	25 ml	7.0	RT	5	91.2	4.6	CP000647	5
Klebsiella pneumoniae YZ-6	Human saliva	–	96.5 (80)	50	7.0	30	5	95.1	3.4	–	6
Achromobacter sp. TERI-IASST N	Sludge	10.5	90	–	6.0	37	3	57	13	KF589295	22
Klebsiella sp. PB12	River water	1.3	98 (5)	17	7.0	RT	5	76.4	0	HM989848	7
Klebsiella pneumoniae LZ-5	Sputum	2.8	98 (80)	54.3	3.3	26	5	96.8	2.1	JX283459	8
Klebsiella pneumoniae J1	Sludge	–	67.8	5	5.0	30	20	–	–	CP013711	9
Bacillus sp. Gilbert	Sediment	–	91	2 ml	3.0	28	5	~100	0	HQ537128	30
Klebsiella pneumoniae NY1	Sediment	14.9	97	41.6	3.0	30	5	66	26	GU377208	18
Agrobacterium sp. M-503	Sludge	14.9	+	5 ml	7.0	RT	1	97	3	EU090069	19
Chryseobacterium daeguense W6	Sludge	–	(96.9)	1.2	5.6	15	1	13.1	32.4	GU111571	26
Klebsiella terrigena	Wastewater	–	62.3	2	7.2	30	5	66.8	2.45	–	10
Bacillus sp. F19	Soil	3.9	(97)	2	2.0	RT	5	66.4	16.4	–	31
Klebsiella mobilis	Soil	2.5	95.4	2 ml	6.0	30	10	+	0	–	12
Klebsiella sp. MYC	Sludge	–	93.3	5 ml	7.0	RT	5	–	–	DQ645728	13
Paenibacillus sp. A9	Soil	0.7	(99.6)	0.1 ml	7.0	30	5	93	0	KF479528	16
Nannocystis sp. NU-2	Soil	14.8	90	30	7.0	RT	5	56.5	40.3	AY038046	20
Bacillus licheniformis CCRC 12826	–	14.2	–	3.7	7.0	RT	5	–	–	EF423608	21
Citrobacter sp. TKF04	Kitchen drain	0.2	(90)	1	4.0	RT	5	10	0	AB741695	23
Klebsiella sp. S11	Sludge	0.9	69.1	15	7.0	RT	5	72	+	–	2
Klebsiella pneumoniae H12	Soil	3	+	–	–	RT	5	+	–	–	13
Klebsiella oxytoca ATCC13182	Crud Soil	3.3	+	–	–	RT	5	–	–	MG571764	13

[a]Flocculating efficiency is evaluated by measuring the turbidity of kaolin suspension, unless otherwise stated. Values in parenthesis indicate the absence of $CaCl_2$. RT, Room temperature; –, Not determined.

stability of the protein components, or disrupts the interaction between the proteins and polysaccharides. This reconfirms that the protein is one of the major flocculating components in BP-1. In previous studies, some proteins or polysaccharides with high molecular weight have been reported as adhesions, which can promote the cells initial attachment of the cells on the solid surface to form biofilms[28]. Therefore, the flocculation of BP-1 may be achieved by adhesion mechanism, in which the proteins or polysaccharides can attach on the surface directly and bridge the kaolin clay particles, and thus promote their flocculation.

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Received 2 December 2018; revised accepted 29 April 2019

doi: 10.18520/cs/v117/i1/121-129