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Screening of Bacteria Capable of Producing Bioflocculants from Acetic and Propionic Acids

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Abstract

In order to reduce the production cost of bioflocculants we proposed to utilize acetic and/or propionic acids, which can be produced from organic wastes by anaerobic digestion, as the substrates for the bioproduction. To realize this strategy, bacterial strains which can produce kaolin-flocculating agents (bioflocculants) from acetate and/or propionate were screened from various environmental sources. Four bacterial strains which showed especially effective flocculating activity were named TKF01, TKF02, TKF03 and TKF04 and identified as *Enterobacter* sp., *Acinetobacter* sp., *Haemophilus* sp. and *Citrobacter* sp., respectively, according to their morphological and physiological properties. The optimum temperature and pH for the biofocculant production were approximately 30 ℃ and 7.0-8.0, respectively, for all the strains. They could utilize some organic acids, sugars and/or alcohols for their growth, however, acetate was the most effective substrates for bioflocculant production. The bioflocculants were found to be effective for the flocculation of a kaolin suspension in wide range of pHs (2.0-10.0) and temperatures (ca. 10- 80 ℃), while the co-presence of cations (Na^+, K^+, Ca^{2+}, Mg^{2+} etc.) did not enhance the flocculating activity. Two of the bioflocculants could flocculate a variety of suspended particles including kaolin, diatomite, bentonite, activated carbon, soil and activated sludge, while the other 2 were effective for limited ranges of particles, only kaolin and diatomite or soil. The results suggested that various types of bioflocculants can be produced from acetate and/or propionate, although no report has described bioflocculant production from these substrates so far.

Key words : bioflocculant, *Enterobacter* sp., *Acinetobacter* sp., *Haemophilus* sp., acetic acid, propionic acid

INTRODUCTION

At present, various kinds of flocculants, typically inorganic aluminum salts and organic synthetic high polymers, have been used in wastewater treatment, tap water production and dredging/ downstream processing techniques in a wide range of industrial fields[1-4]. These flocculants have been used for various purposes according to their chemical properties and toxicity and/or environmental impacts. Of these flocculants, polyaluminum chloride (PAC) which represents aluminum salts has been most widely used in water and wastewater treatment. However, health-related problems such as Alzheimer's disease have been reported recently[5-8]. Also, huge volume of sludge production is another problem of inorganic flocculants[9]. Organic synthetic polymers such as polyacrylamide (PAA) derivatives are also used frequently because

of their cost-effectiveness and superior flocculating activity, however, acrylamide monomer contained in the commercial PAA flocculants as a inpurity is a strong human carcinogen[10-11]. Although some of other synthetic organic polymers have no or little toxicity, they tend to give rise to bad influences on the environment due to low biodegradability. Therefore, it is required to develop a new type of flocculant which can be easily biodegraded and is safe for human and natural environment as an alternative product in place of the existing synthetic flocculants.

From this view point, several workers have been focusing on flocculating agents produced by microorganisms. Such flocculating agents are called bioflocculants and have a possibility to be easily decomposed and harmless for human and nature. Several types of bioflocculants have been reported up to date and it became apparent that they have efficient activity to flocculate not only inorganic but also organic suspended particles[12-15]. In spite of their high potentials to replace existing synthetic flocculants, bioflocculants have common problems of high production costs, because flocculant-producing microorganisms generally required expensive substrate such as glucose, fructose, sucrose or glutamate as carbon sources for their growth and bioflocculant production[2, 4, 16-17].

In order to reduce the production costs, we attempt here to utilize lower-molecular fatty acids (VFAs) such as acetic and propionic acids, which can be easily obtained from anaerobic digestion or thermal treatment of organic wastes including excess sewage sludge[18-19], as the substrates for bioflocculant production. This strategy seems to contribute not only to the cost-effective production of bioflocculants but also to the promotion of reduction, utilization and/or recycling of organic wastes as a secondary merit. In order to realize this strategy, a microbe which can produce a bioflocculant from VFAs is required, however, screening of such bioflocculant-producing microbes has never been attempted so far. In this study, VFA-utilizing bioflocculant-producing bacteria were screened from various environmental sources. For screened 4 bacterial strains, basic investigations were also performed on the bioflocculant production and functional properties of the produced flocculants.

MATERIALS AND METHODS

Media and culture conditions The basal medium (BM) contained $FeCl_3$ (0.01 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), NaCl (0.05 g), $(NH_4)_2SO_4$ (1.0 g), $CaCl_2$ (1.0 g), K_2HPO_4 (1.0 g) and yeast extract (0.1 g) in 1*l* of deionized water. Acetate-propinate (AP) medium containing 7.0 g of sodium acetate and 3.0 g of sodium propionate in 1*l* of the BM (pH 7.2) was used for screening bioflocculant-producing bacteria and also for production of bioflocculants by screened bacterial strains. The acetate and propionate concentrations in AP medium were determined so as to represent the actual composition of the anaerobic digestion liquor of sewage sludge[18]. The effects of carbon source on the bioflocculant production were investigated using BM supplemented with 1 % (w/v) sodium acetate (Ac. medium), sodium propionate (Pr. medium), sodium lactate, sodium oleate, sodium butyrate, hexadecane, methanol, ethanol, glucose or lactose. The pH of all the media was adjusted to 7.2 using 2M NaOH or HCl and the liquid culture was incubated in 10 m*l* of the medium in a test tube or 200 m*l* of the medium in a 500-m*l* Erlenmeyer flask at 30 ℃ on a reciprocal or rotary shaker at 120 rpm unless otherwise stated. For monitoring the growth, the optical density at 660 nm (OD_{660}) was measured.

Screening for bioflocculant-producing bacteria and their identification The bacterial strains were isolated from a wide variety of environment sources ; 9 biofilms from domestic kitchen drains, 9 activated sludges including scumming ones, 14 soils from agricultural fields, forests and gardens, 4 river waters, 4 sediments from domestic drainages and so on using soild AP medium (AP medium solidified with 1.5 % agar). The colonies were picked up and cultivated in liquid AP medium for 3 to 10 days at 30 ℃. The fully-grown cultures were examined for their flocculating activity against kaolin clay suspension (see below). Bacterial colonies which have considerable flocculating activity (the formation of visible aggregates and clear supernatant phases were observed: flocculating activity

was more than 10 %) were regarded as the bioflocculant-producing bacteria.

For taxonomical studies of the isolates, morphological and physiological tests were performed based on Hasegawa's procedure[20] and identification was done according to Begey's Manual of Systematic Bacteriology[21]. The API20E bacterial identification kit (BioMerieux S.A., Marcyl l' Etoile, France) was also used for ensuring the identification.

Assays of flocculating activity The flocculating activity was measured as described by Kurane *et al.*[1] with minor modifications using a suspension of kaolin clay as a test material. Kaolin clay (300 mesh, Kishida Chemical, Osaka) was suspended in deionized water at a concentration of 5,000 mg/*l* (kaolin suspension). 1 m*l* of the sample (culture broth etc.) was added to 10 m*l* of the kaolin suspension in a test tube, and the reaction mixture was shaken for 30 sec using a vortex mixer. It was allowed to stand for 5 min, and the optical density of the upper clear phase (ca. 4 m*l*) at 550 nm (OD_{550}) was measured with a spectrophotometer (A). A control experiment in which 1 m*l* of the deionized water instead of the sample was added to the kaolin suspension was also performed in the same manner, and then the OD_{550} of the upper 4 m*l* was measured (B). The flocculating activity was defined as $\{(B-A)/B\} \times 100$ (%). The activity was shown by the mean value from triplicate determination.

To find out whether the bioflocculants were secreted outside or accumulated inside or surface of the bacterial cells, the flocculating activity was measured with the whole culture, cell-free supernatant, cell suspension and sonicated cell suspension. The fully-grown bacterial cells in AP medium were centrifuged (20,000 × g, 10 min, 4 ℃) to obtain the cell-free supernatant. The remaining cell pellet was suspended to the original volume of the BM after washing twice with deionized water. The cells were disrupted by sonication using an ultrasonicator UD-210 (Tomy, Tokyo, Japan) for 5 min at the output 5 under 0 ℃.

For the investigation of pH effect on the flocculating activity, the pH of the kaolin suspension was adjusted to various values using HCl or NaOH. The effect of the temperature was studied using the kaolin suspension and sample, the temperature of which were adjusted to a definite value. To investigate the effect of co-presence of various cations, kaolin suspensions were prepared in 50 mM Tris-HCl (pH 7.0), and 0.1 m*l* of a cation solution was added to the reaction mixture; NaCl, KCl, $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$, $FeCl_2$, $AlCl_3 \cdot 6H_2O$ and $FeCl_3 \cdot 6H_2O$ solutions as the sources of Na^+, K^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, Al^{3+} or Fe^{3+}, respectively. For investigating the flocculating activity against various suspended particles, suspensions of diatomite (Kishida Chemical, Oaska), cellulose powder (> 300 mesh, Advantec Toyo Ltd. Co., Tokyo), activated carbon (powder, Kishida Chemical, Osaka), bentonite (300 mesh, Kishida Chemical, Osaka), dry yeast (Asahi Brewery Ltd. Co., Tokyo), soil and activated sludge were also examined in the same manner as kaolin. To prepare the soil suspension, a garden soil sample was stirred in deionized water, allowed to stand for 5 min, and the upper phase was obtained. For the assay using activated sludge, the measurement of OD_{550} of the reaction mixture was performed after allowing the mixture to stand for 1 min.

RESULTS

Screening and identification of the flocculant-producing bacteria Out of 1564 bacterial colonies obtained from various environmental sources, 102 exhibited flocculating activity by forming visible aggregates and clear supernatant phases in the flocculating tests when using 1 m*l* of fully-grown culture broth in AP medium. Although most colonies regarded as the bioflocculant-producing bacteria showed 20 to 80 % of the flocculating activity against the kaolin suspension, 4 strains exhibited specially strong flocculating activity of more than 90 %. Four bioflocculant-producing bacteria were purified from these colonies (one strain from each colony) and designated strains TKF01, TKF02, TKF03 and TKF04. The strains TKF01, TKF02 and TKF04 were isolated from biofilms inside kitchen drains, while TKF03 from a soil sample. These showed strong flocculating activity even in the pure culture grown in AP medium.

Table 1 shows morphological and physiological features of the isolates. Based on the results, strains TKF01, TKF02, TKF03 and TKF04 were tentatively identified as belonging genera *Enterobacter*, *Acinetobacter*, *Haemophilus* and *Citrobacter*, respectively.

Properties of the bioflocculant production

Fig. 1 shows the cell growth and flocculating activity of the four strains during cultivation on AP medium. Here the flocculating activity was assayed using 1 m*l* of the culture broth including whole cells. For all strains selected, similar trend of the

Table 1 Morphological and physiological properties and identification of flocculant producing bacteria

Characteristics	TKF01	TKF02	TKF03	TKF04
Morphology	Rods	Rods	Rods	Rods
Gram stain	−	−	−	−
Motility	+	+	+	+
Catalase	+	+	+	+
Oxidase	−	−	−	−
OF test	F	O	−	F
Argininedehydrogenase	+	−	+	+
Urease	N.D.	−	−	−
β-glucosidase	N.D.	−	+	N.D.
Protease	N.D.	−	+	N.D.
β-galactosidase	N.D.	−	−	+
Fermentation of				
d-glucose	+	+	+	+
d-manitol	+	−	+	+
l-arabinose	+	+	−	+
Identification*	*Enterobacter* sp.	*Acinetobacter* sp.	*Haemophilus* sp.	*Citrobacter* sp.
Identification code no.	(no. 3305573)	(no. 0043473)	(no. 1554751)	(no. 3204572)

Symbols : +, positive ; −, negative ; O, oxidative ; F, fermentative ; N.D., not determined.
*,identification by API 20 NE kit. Code numbers are shown in parenthesis.

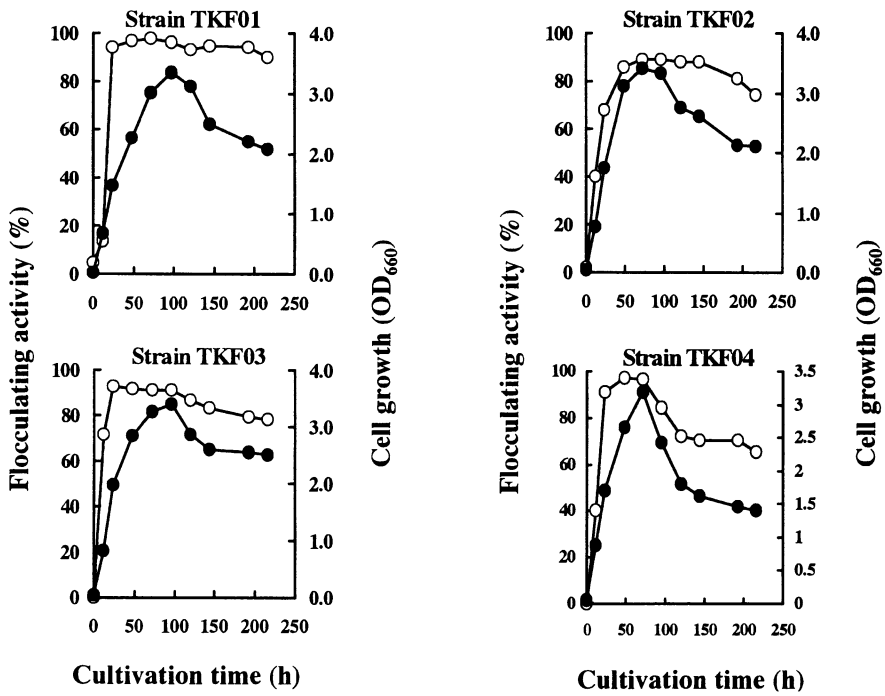

Fig. 1 Cell growth and flocculant production by TKF01, TKF02, TKF03 and TKF04 in AP medium. Symbols : cell growth (●) ; flocculating activity (○)

bioflocculant production was observed. The flocculating activity of the culture broth, i.e. bioflocculant production, increased during the logarithmic growth and reached a maximum value (more than 90 %) after about 3 days. Thereafter the flocculating activity continued to be stable for 3-4 days, followed by a slight decrease.

Whether the bioflocculants were secreted outside or accumulated inside or surface of the bacterial cells was investigated by the flocculation tests as shown in Table 2. The kaolin-flocculating activity of the culture broth was almost same as that of its cell-free supernatant, while the cell suspension and the cell extract showed no or little activity for all strains. These indicates that the most or major portion of the bioflocculants produced by these strains were secreted into the medium, though a little portion seems to exist on the cell surface for strains TKF02 and TKF03. Based on the results, cell-free supernatant samples were used for the flocculating activity assays in the following studies.

In order to optimize the cultivations conditions for the bioflocculant production by strains TKF01 to TKF04, the effect of temperature and initial pH of the AP medium on the cell growth and the flocculating activity were investigated. The results are shown in Figs. 2 and 3 after 3-day incubation under different temperatures and pH. The bioflocculant production of each strain considerably depended on the cultivations temperature, and the optimum temperature for the bioflocculant was found to be 30 ℃, although that for the cell growth was 37 ℃ (Fig. 2). On the other hand, the initial pH of the medium showed slight effect on the bioflocculant production within the pH range of 7.0-10.0 where the bacterial strains could grow, while the optimum pH for strain TKF02 was 7.0 and bioflocculant productivity was a little lower at higher pH (Fig. 3).

The effects of the carbon source on the bioflocculant production were also investigated as shown in Table 3. Although all the strains could grow on some of the tested carbon sources in addition to acetate and/or propionate which were used for their isolation, acetate was the most effective substrate for the bioflocculant production of all the strains. Propionate was also effective for TKF02 and TKF04, while others were not. However, the data suggested that strain TKF02 could utilize a relatively wide range of carbon sources for the bioflocculant production, though the flocculating activity was not so high.

Properties of the flocculating activity of the bioflocculants The flocculation properties of the bioflocculants produced by strains TKF01 to TKF04 were investigated using the cell-free supernatants of the culture on AP medium. The effects of temperature and pH of the reaction mixture on the kaolin-flocculating activity are shown in Figs. 4 and 5, respectively. It was found that effective flocculation of kaolin occurred in the temperature range of 10-80 ℃ for TKF01, TKF03 and TKF04, while the flocculating activity showed a considerable drop at the temperature higher than 60 ℃ for TKF02. On the other hand, the bioflocculants of all the strains exhibited relatively stable and effective flocculating activity in the pH range of 4 to 10, although there was a general trend observed that the flocculating activity was slightly decreased as pH became higher. These results suggested that the bioflocculants produced by strains TKF01 to TKF04 are relatively thermostable and their flocculating activity is hardly affected by pH. The effects of various cations on the activity

Table 2 Determination of the location of bioflocculants

	Flocculating activity (%)			
	TKF01	TKF02	TKF03	TKF04
Culture broth	92	90	92	94
Cells suspension	N.A.	14	11	N.A.
Sonicated cells suspension	N.A.	N.A.	N.A.	N.A.
Supernatant	94	92	95	97

N.A. : no activity

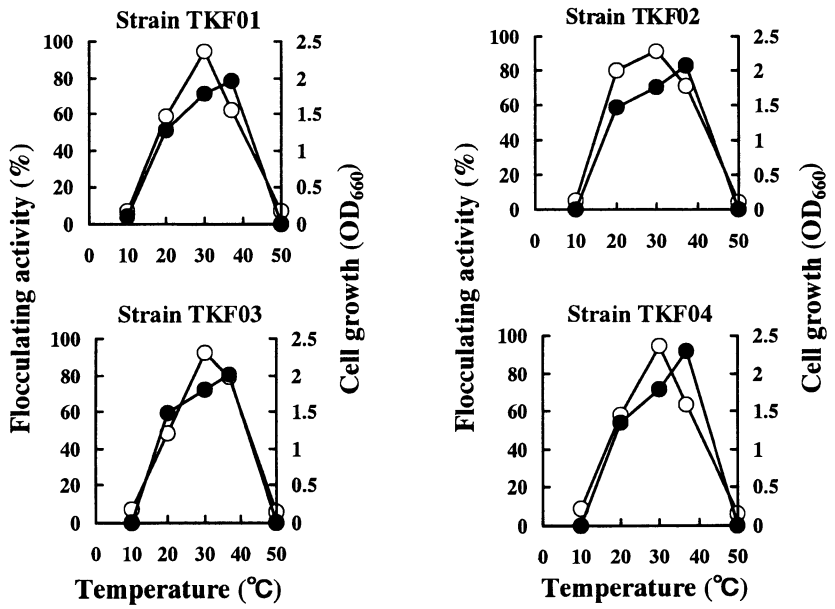

Fig. 2 Effects of cultivation temperature on the cell growth and bioflocculant production by TKF01, TKF02, TKF03 and TKF04.
Symbols : cell growth (●) ; flocculating activity (○)

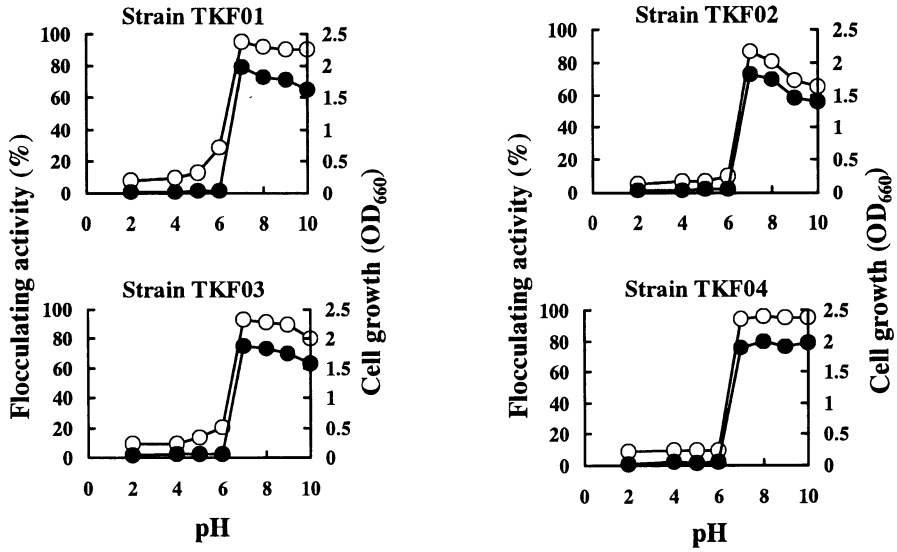

Fig. 3 Effects of initial pH of the medium on the bioflocculant production by TKF01, TKF02, TKF03 and TKF04.
Symbols : Cell growth (●) ; Flocculating activity (○).

Table 3 Effects of various carbon source on the bioflocculant production

Carbon sources	Strain TKF01		Strain TKF02		Strain TKF03		Strain TKF04	
	Cell growth	F.A.(%)	Cell growth	F.A.(%)	Cell growth	F.A.(%)	Cell growth	F.A.(%)
Sodium acetate	+ +	93	+ +	87	+ +	98	+ +	96
Sodium propionate	−	21	+ +	73	+	22	+	98
Sodium butyrate	+	29	+	51	+	36	+	N.A.
Sodium lactate	+ +	17	+ +	55	+	N.A.	+	14
Sodium oleate+Al	+ +	30	+ +	26	+ +	43	+ +	19
Hexadecan	+	38	+ +	59	+ +	38	+	N.A.
Methanol	+	16	+	18	+ +	27	−	N.A.
Ethanol	−	N.A.	+	59	+ +	76	−	N.A.
Glucose	+ +	36	+	34	+	15	+ +	13
Lactose	+	N.A.	+	N.A.	+	N.A.	+ +	37

F.A., Flocculating activity (%) ; −, No growth ; +, OD_{660} = 0.1−0.5 ; + +, OD_{660} = 0.5−1.5 ; N.A., No activity (<10%)

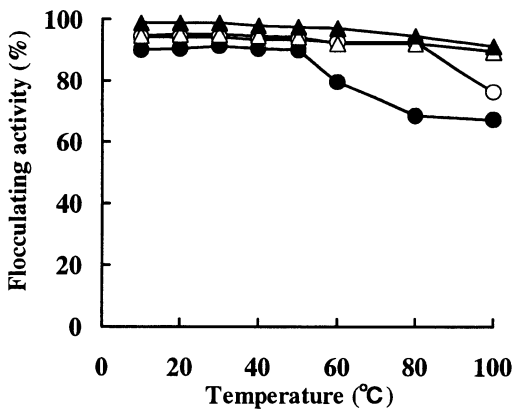

Fig. 4 Effects of reaction temperature on the flocculating activity.
Symbols : TKF01 (○), TKF02 (●), TKF03 (△), TKF04 (▲).

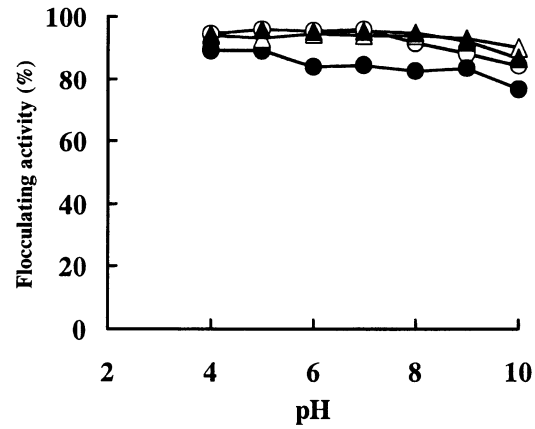

Fig. 5 Effects of pH of reaction mixture on the flocculating activity
Symbols : TKF01 (○) ; TKF02 (●) ; TKF03 (△) ; TKF04 (▲)

of the bioflocculants were also investigated as shown in Fig. 6, since some of synthetic and bio-flocculants are known to require cations like Ca^{2+} as assistants for expressing their flocculating activity. The flocculating activity of all the bioflocculants was not enhanced by the addition of any cations. Although high concentrations of some cations resulted in the decrease of the activity, the reasons are not clear.

Further, the flocculating activity of the bioflocculants against a variety of inorganic and organic suspended particles was investigated for comparison with that of PAA (1 mg/*l*) and PAC (100 mg/*l*) (Table 4). The bioflocculants by strains TKF01 and TKF02 showed significant flocculating activity against relatively limited ranges of the particles. The flocculant of TKF01 was effective only for kaolin and diatomite, while that of TKF02 only for kaolin and soil suspensions. On the other hand, the bioflocculants of TKF03 and TKF04 could efficiently flocculate wide range of the particles including kaolin, bentonite, activated carbon, soil and activated sludge, even compared with PAA and PAC. Especially the flocculant of TKF04 showed a significant flocculation against all the tested particles.

DISCUSSION

Until now, numerous studies have been reported on the flocculant production by microorganisms from different view points. Some of previously-reported microbial

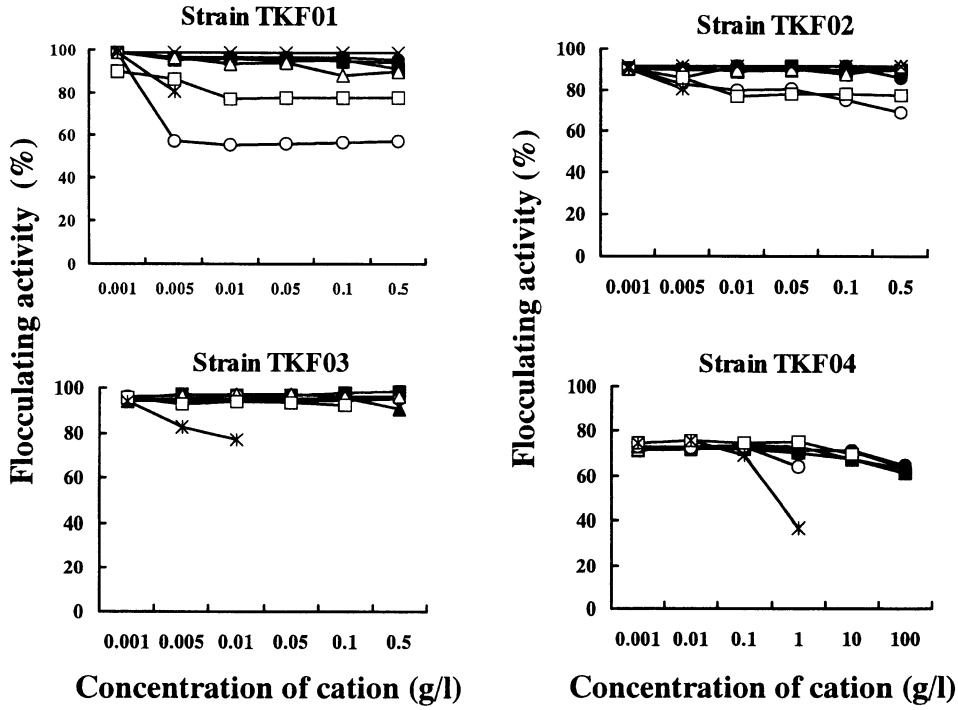

Fig. 6 Effects of cations addition on the flocculating activity. (A) TKF01 (B) TKF02 (C) TKF03 (D) TKFO4
Symbols : ● (Na^+) ; ▲ (K^+) ; ■ (Mg^+) ; ○ (Fe^{2+}) ; △ (Ca^{2+}) ; □ (Al^{3+}) ; ✳ (Fe^{3+}) ; × (Control)

Table 4 Flocculating activity of the crude bioflocculants on various suspended particles compared with those of PAC and PAA

Suspension	Flocculating activity (%)					
	TKF01	TKF02	TKF03	TKF04	PAC	PAA
Kaolin	92	89	95	96	49	73
Bentonite	–	33	94	96	–	–
Diatomite	75	25	49	78	41	83
Activated carbon powder	40	44	90	80	–	89
Cellulose powder	17	–	15	40	–	–
Dry yeast	–	–	–	19	–	12
Soil	–	92	73	94	17	83
Activated sludge	12	–	87	71	65	–

–, no activity (<10%)

flocculants were tentatively or completely identified as glycoproteins[3], proteins[22,23], oligosaccharides[16,24], glycolipids[25], cellulose[26], DNA[27] and complex hetero-polymers[28], however, the bioproduction of such known flocculants generally requires expensive substrates[2,4,16,17]. In order to reduce the production cost of bioflocculants we proposed to utilize VFAs (acetic and/or propionic acids), which can be easily produced from organic wastes, as the substrates for the bioproduction. However, screening of microbes which can produce bioflocculants from VFAs has never been attempted. Consequently, such a bioflocculant-producing microbe has never been reported so far. Our

experimental results suggested that a variety of bioflocculants can be produced from VFAs by various bacteria. Therefore, it may be possible to find new kinds of VFA-utilizing bioflocculant-producing bacteria by further screening.

All the strains produced bioflocculants simultaneously with their cell growth. This may indicate that the bioflocculants were not produced by cell autolysis but by biosynthesis[29-30]. During the cultivation of these strains on AP medium, the bioflocculants were secreted into the medium as a common property. Thus the bioflocculants of these strains were extracellular, and this is advantageous for recovery and purification of the bioflocculants. It was also observed commonly for all the strains that the flocculating activity in the medium showed a slight or considerable decrease after reached maximum values, suggested that the bioflocculants were biodegraded by the corresponding producing bacteria themselves. Therefore, it may be a little difficult to optimize the bioflocculant production in batch cultivations. On the other hand, this might demonstrate that the bioflocculants are readily biodegradable, i.e. suitable for environmental uses.

The bioflocculants produced from VFAs showed efficient kaolin-flocculation in the pH range of 4-10 and temperature range of 10-80 ℃, and they could flocculate various inorganic and organic suspended particles. These results suggest that these bioflocculants have potentials to be applied to waters or wastewaters with various physical and chemical properties. Further, since their flocculating was not affected in the presence or absence of cations like Ca^{2+}, they seem to be cost-effective flocculants in the practical application.

Because we could obtain promising candidates of the bioflocculant-producing bacteria, strains TKF01 to TKF04, there may be a possibility to realize the strategy of organic wastes (VFAs) utilization for bioflocculant production as cheap substrates. However, further studies are required to optimize the production processes, establish the recovery and purification methods, and characterize the chemical structures of the bioflocculants.

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