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Technical Communication

Use of waste fermenting liquor to produce bioflocculants with isolated strains

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ABSTRACT

Dark fermentation from biomass can produce bio-hydrogen, which is a clean energy source and feedstock used in numerous industries. However, the hydrogen fermentation process produces strong waste fermenting liquor that requires polishing before disposal. This work utilized the waste fermenting liquors to produce bioflocculant from the residual organic matters. Three bacterial strains were noted to have high potential to produce bioflocculants from waste fermenting liquor. In particular, the strain BF-6, identified as *Bacillus subtilis* by 16S rRNA sequencing, was noted to effectively produce bioflocculant from ethanol-rich mixtures. The BF-6 produced carbohydrate bioflocculants from waste fermenting liquors at a yield of 2.1 $g\,l^{-1}$.

1. Introduction

Hydrogen is a clean energy source and feedstock used in many industries. Among numerous alternative methods for hydrogen production, dark fermentation of biomass to produce hydrogen has been demonstrated to be effective. The waste fermenting liquor following dark fermentation of biomass is a strong liquor containing high levels of organic substances that require further treatment before disposal.

Bioflocculant is a biodegradable polymer secreted by microorganisms with high particle flocculating capability. Bioflocculants receive academic and practical attention because they are environmentally safe to use. Salehizadeh and Shojaosadati [1] summarized relevant studies conducted up until 2000. Table 1 lists updated studies other than those listed in Ref. [1]. New bacterial strains capable of producing effective biofloccuant were identified. Most recently identified bioflocculants were polysaccharide-like substances, including poly-glutamic acids with molecular weight of 20–3000 kDa. The presence of di- or trivalent ions such as Ca^{2+} or Fe^{3+} stimulated the flocculating capability of the studied bioflocculants [2].

No studies have examined the feasibility of utilizing the waste fermenting liquors from dark fermentation processes to produce bioflocculant with isolated strains. In this study, 21 bacterial strains from a full-scale bio-hydrogen fermenter were isolated and were tested on their capability to produce bioflocculant of high efficiency from waste fermenting liquors. The strain that effectively generated bioflocculant from ethanol-rich mixtures was further examined.

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Table 1 – Literature works considering bioflocculant production from isolated microorganisms in the year 2000–2006

	Strain	Bioflocculant	Remarks
Deng et al. [3]	*Aspergillus parasiticus*	76.3% Sugars, 21.6% protein, 320 kDa	Good in flocculating kaolin and anionic dyes
Huang et al. [4]	*Aspergillus* spp. *Penicillium* spp.	Not specified	Screening strains by NTG mutagenesis
Suh HH et al. [5]	*Bacillus* spp. A56	Polysaccharide, 7000 kDa	Effective at high ionic strength
Salehizadeh and Shojaosadati [6]	*Bacillus firmus*	Acidic polysaccharide, 2000 kDa	Enhanced flocculation with Ca^{2+}, Mg^{2+}, Fe^{2+}
Salehizadeh and Shojaosadati [7]	*Bacillus firmus*	Polysaccharide, 38% uronic acid, 6.3% pyruvic acid	Adsorption of Pb, Cu, Zn
Vijayalakshmi and Raichur [8]	*Bacillus subtilis*	Cells are the flocculants	Conditioning fine coals
Shih et al. [9]	*Bacillus licheniformis*	Poly-glutamic acid, 3000 kPa	Enhanced flocculation with Ca^{2+}, Fe^{3+} and Al^{3+}
Deng et al. [10]	*Bacillus mucilaginosus*	Polysaccharide, 47.7% neutral sugar, 19.1% uronic acid, 2.7% amino acid	Enhanced flocculation with Ca^{2+}
Fujita et al. [11]	*Citrobacter* spp. TKF04	Glucosamine, 232–440 kDa	Structure similar to chitin or chitosan
Jang et al. [12]	*Citrobacter* spp.	Glucosamine/N-acetyl-glucosamine	High C/N ratio favors flocculant production
Fujita et al. [13]	*Citrobacter* spp. TKF04	Not specified	Grows in sludge digestion liquor
Kim et al. [14]	*Citrobacter* spp. BL-4	97.3% Glucosamine, 2.7% rhamnose, 20 kDa	Structure similar to chitosan
He et al. [15]	*Corynebacterium glutamicum*	Polygalacturonic acid, 100 kDa	80% Flocculating activity in culture broth
Li et al. [16]	*Corynebacterium glutamicum*	Polygalacturonic acid	Pathway for bioflocculant synthesis
He et al. [17]	*Corynebacterium glutamicum*	Polygalacturonic acid	Effects of C/N, medium, DO on cell growth
He et al. [18]	*Corynebacterium glutamicum*	Polygalacturonic acid	Effects of pH on cell growth
Yokoi et al. [19]	*Enterobacter* spp. BY-29	Not specified	Co-production of flocculant and H_2
Lu et al. [20]	*Enterobacter aerogenes*	Acidic polysaccharide 13.2% uronic acid, 7.4% pyruvic acid, 1.6% acetic acid	Best: 45 °C with Zn^{2+}
Son et al. [21]	*Enterobacter* spp. BL-2	Cationic polyglucosamine, 106 kDa, 86.4% glucosamine, 1.6% rhamnose, 1% galactose	Structure close to chitson from crab shell
Prasertsan et al. [22]	*Enterobacter cloacae* WD7	Heteropolysaccharide (neutral sugars 29.4%, uronic acids 14.2%, amino sugars 0.93%)	Flocculate kaolin suspension of pH 2–8 and temperature 4–50 °C with $CaCl_2$
Kobayashi et al. [23]	*Klebsiella pneumoniae*	Extracellular polysaccharide	Structure of flocculant explored
Sheng et al. [24]	*Klebsiella* spp.	Not specified	Most effective at pH 6
Zhang et al. [25]	*Nannocystis* spp. NU-2	40.3% Proteins, 56.5% polysaccharides	Grows in 7% NaCl solutions
Oh et al. [26]	*Paenibacillus* spp. AM49	Not specified	Harvest *Chlorella vulgaris*
Gong et al. [27]	*Paenibacillus polymyxa* BY-28	78% Polysaccharide	Enhanced flocculation with Ca^{2+}
Cheng et al. [28]	*Saccharomycete* STSM-1	Not specified	Discussing effects of cations and bioflocculant concentrations
Zhang et al. [29]	*Sorangium cellulosum*	Exopolysaccharide, 38.3% protein, 58.5% carbohydrates	

Those not included in [1].

2. Methods

2.1. *Isolation of bioflocculant producing strains*

Liquid samples were collected from a full-scale biohydrogen fermenter. Sediment collected at the fermenter bottom was collected and was utilized to isolate strains. The composition of the screening medium (per liter) at pH 7.2 is as follows: beef extract 5.0 g, peptone 10.0 g, NaCl 5.0 g, and agar 20 g. One millilitre of the liquor sample was placed in aseptically mixed sterilized tubes containing 9 ml of the above screening medium to break up the biological aggregates. The supernatant was serially diluted with medium (10^1–10^9 fold

dilutions), and 1 ml of each 10^5–10^9 dilution was spread onto 1.2% agar plates containing the following medium at pH 7.2 (per liter): glucose 10.0 g, KH_2PO_4 2.0 g, K_2HPO_4 5.0 g, $MgSO_4$ $7H_2O$ 0.2 g, NaCl 0.1 g, urea 0.5 g, and yeast extract 0.5 g. The plates were inverted and incubated at 30 °C. Visible colonies appeared following one day of incubation. Morphologically distinguished visible colonies of strains were isolated via several cycles of replating onto the screening medium. A total of 21 morphologically different isolates were chosen from the screening medium. The isolates were individually suspended in 100 ml of shaken basel medium at 30 °C for 24 h, which were designated as "inoculum liquors" in this work.

2.2. Strain identification

The DNA from the isolated strain was extracted via enzymatic lysis using extraction buffer (0.5 M 40 ml EDTA at pH 8.0, 5 M 6 ml NaCl and 154 ml ddH_2O) containing proteinase K (0.5–1 mg ml^{-1}). The samples were incubated at 37 °C for 30 min and shaken at 150 rpm. The 20% sodium dodecyl sulfate (SDS) was added to samples and incubated at 60 °C for 20 min. The samples were then subjected to two cycles of freeze-thawing. The supernatant was collected following centrifugation at 10 000 × *g* for 5 min, and the aqueous phase was extracted using chloroform:isoamyl alcohol (24:1, v:v). The DNA was precipitated utilizing two folders of pure ethanol, pelleted via centrifugation (10 000 rpm) for 10 min, and then resuspended in 1 ml of TE (10 mM Tris–HCl, 1 mM EDTA at pH 8.0).

The DNA amplification reaction was performed using primers BSF8/20:5′-AG AGTTTGATCCTGGCTCAG -3′ and PLA:5′-GGTACTTAGATGTTTCA GTTC-3′ (Wuyts, 2002; Van Camp, 1993). The PCR mixture consisted of 10 μl of 10 × buffer, 3 μl (0.6 μM) of BSF 8/20, 3 μl (0.6 μM) of PLA, 8 μl (200 μM) of dNTP, 0.5 μl (5 U μl^{-1}) of Taq enzyme, 1 μl (0.05–1 μg μl^{-1}) of template, and 74.5 μl ddH_2O. The DNA was amplified using a PTC-100 mastercycler (MJ Research, USA) by denaturation at 94 °C, 4 min, 30 cycles consisting of 94 °C for 90 s, 55 °C for 60 s, 72 °C for 90 s, and final extension at 72 °C for 10 min. The PCR-amplified 16S rRNA was sequenced using the ABI Prism model 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

2.3. Analytical analysis

The samples were taken for chemical oxygen demand (COD) determination according to the Standard Methods. A gas chromatography (GC112, Perkin Elmer, USA) with nitrogen as the carrier gas characterized the volatile fatty acids (VFA) and alcohol contents in the samples. The hydrogen flame detector with hydrogen and air flow rates of 50 and 490 ml min^{-1}, respectively, and a stainless steel column (2 m) packed with GDX-103 (60–80 meshes), were employed at 190 °C.

The carbohydrates and proteins of samples were extracted with high-speed centrifugation (12 000 × *g*) for 20 min. The resulting supernatant was added with three volumes of pure ethanol and maintained at 4 °C for 24 h to precipitate the soluble substances. The extraction procedures were repeated for a few times until no further precipitate was noted following extraction. The collected precipitated was freeze dried (EZ585Q, FTS Systems, NY, USA) and was then purified using AKTA explorer 100 (GE Healthcare, NJ, USA) with a Superdex 2000 column and ultrapure water as mobile phase. The carbohydrate content in the samples was determined by the phenol–sulfuric acid method with glucose as the standards. The protein content in the samples was measured by the modified Lowry method using bovine serum albumin as the standards.

2.4. Bioflocculant production and strain screening

The waste fermenting liquor from the full-scale biohydrogen fermenter was used to produce bioflocculant. Compositions of the waste fermenting liquors changed over time. Typical COD of the waste fermenting liquor was measured as 4490 mg l^{-1}, and the suspension had a pH of 4.02. The concentrations of ethanol, acetate, propionate, butyrate, and valerate were 689, 613, 340, 102, and 47 mg l^{-1}, respectively.

To prevent the interference of existing strains in the waste fermenting liquor, the liquor was centrifugated at 12 000 × *g* for 20 min and the resulting supernatant was filtered with 0.22 μm membrane. The 21 isolated strains were then individually added to the filtrate at 30 °C and at various mix ratios (waste fermenting liquor: inoculum liquor) for 12–36 h. The fermented broth was herein designated as "bioflocculant liquor".

In screening tests, the substrate was the waste fermenting liquor fermented with individual strains for 12 h. To prevent possible substrate inhibition, the original waste fermenting liquor was diluted by adding Milli-Q water to around 2000 mg l^{-1} COD before test.

2.5. Flocculation test

Flocculating capability of the bioflocculant liquors was determined using methods modified from Shimofuruya et al. [30]. Restated, 5 g of kaolin powers were mixed intensively with 1000 ml of water and 1.5 ml of 10% $CaCl_2$ at pH 7.0. Ten millilitre of bioflocculant liquor was then injected to the kaolin suspension, which was mixed in the mixing vessel with 160 rpm of stirring for 40 s followed by 35 rpm for a further 270 s. The supernatants for the coagulated suspension were then collected following 20 min settling, including a control without bioflocculant, with absorbance at 550 nm for the two samples being measured and denoted as OD_{550} and OD_{blank}, respectively, using a spectrophotometer (Model 721 UV/VIS spectrophotometer, Micro Photonics Inc., Allentown, PA, USA). The flocculating capability was defined as $((OD_{blank}-OD_{550})/(OD_{blank}) \times 100)$. Higher flocculating capability indicated greater removal of fine particles on settling.

3. Results and discussion

3.1. Strain screening

Following screening, ten strains among the 21 isolated strains, BF-1, BF-2, BF-4, BF-6, BF-7, BF-9, YX0 31, YX0 32, YX6 22, YX6 3, yielded effective bioflocculants from waste fermenting liquors (Table 2). The physiological and biochemical tests revealed that

the YX strains and BF-9 were closely affiliated with strain BF-4 (details not shown for brevity). In addition, the strains BF-1 and BF-2 were noted to be associated with *Achromobacter xylosoxidans* and *Kluyvera ascorbata*, respectively, which are potential human pathogens and are disregarded in tests. The strains BF-4, BF-6 and BF-7 were discussed further in this work. The partial genome of BF-4, BF-6 and BF-7 was aligned using NCBI blast with 99% sequence similarity with *Bacillus fusiformis*, *Bacillus subtilis*, and *Bacillus flexus*, deposited in GeneBank under accession number AY548950.1, AB110598.1, and AB021185.1, respectively. Fig. 1 shows their SEM images.

3.2. *Fermentation test*

The fermenting waste liquors collected at the full-scale hydrogen fermenter were fermented at 30 °C using strain BF-4, BF-6 or BF-7 at different pH, mix ratios, and fermenting times. Fresh waste fermenting liquors were collected just before the tests. To account for the effects of COD of different batches of waste fermenting liquors, the normalized flocculating capabilities were reported (Table 3). All three strains effectively produce bioflocculants at pH 4–5 and mix ratio >1:0.1. Moreover, the flocculating capability of bioflocculant liquors increased and reached a

Table 2 – Results of strain screening tests

No.	Strain	Flocculating capability	
		Inoculum liquors	Fermented liquors
1	BF-1	38.7	66.8
2	BF-2	2.8	71.0
3	YX0 31	−2.7	70.1
4	YX0 32	−28.1	72.4
5	BF-4	−4.2	71.0
6	BF-6	45.6	70.5
7	YX6 22	60.4	70.9
8	YX6 3	74.2	70.1
9	BF-9	63.6	68.7
10	BF-7	13.4	69.1
11	YX8 2	28.6	39.2
12	YX9 2	4.6	46.1
13	YX9 1	44.7	50.2
14	YT18 1	42.9	47.0
15	YT18 2	64.5	45.6
16	B7X 1	−28.1	41.5
17	B7X 2	54.8	34.6
18	B7X 3	60.4	1.4
19	B8X	66.8	42.4
20	B10X	−212	16.6
21	B10X 1	−110	27.7

Mix ratio = 1:0.1 v/v 30 °C. Fermenting waste liquor at a COD of 2000 mg l^{-1}. Fermenting time = 12 h.

Table 3 – Normalized flocculating capability of bioflocculant liquors using pure strains

Strain	PH[a]			
	3	4	5	6
BF-4	14.4	14.8	15.0	14.2
BF-6	10.2	14.2	14.6	6.6
BF-7	12.0	13.2	13.0	13.2
Strain	Mix ratio[b]			
	1:0.1	1:0.15	1:0.2	1:0.3
BF-4	7.4	8.3	9.8	14.9
BF-6	8.9	9.5	9.4	11.2
BF-7	5.8	10.2	8.6	13.8
Strain	Fermenting time (h)[c]			
	0	12	24	36
BF-4	−1.1	3.3	16.8	9.3
BF-6	3.4	13.9	15.4	1.2
BF-7	1.1	3.6	14.2	0.6

Unit in flocculating capability/COD (mg l^{-1}) * 1000.
[a] Initial COD 6780 mg l^{-1}, fermenting time = 24 h, mix ratio = 1:0.20.
[b] Initial COD 8410 mg l^{-1}, fermenting time = 24 h, pH 4.22.
[c] Initial COD 4150 mg l^{-1}, pH 4.24, mix ratio = 1:0.20.

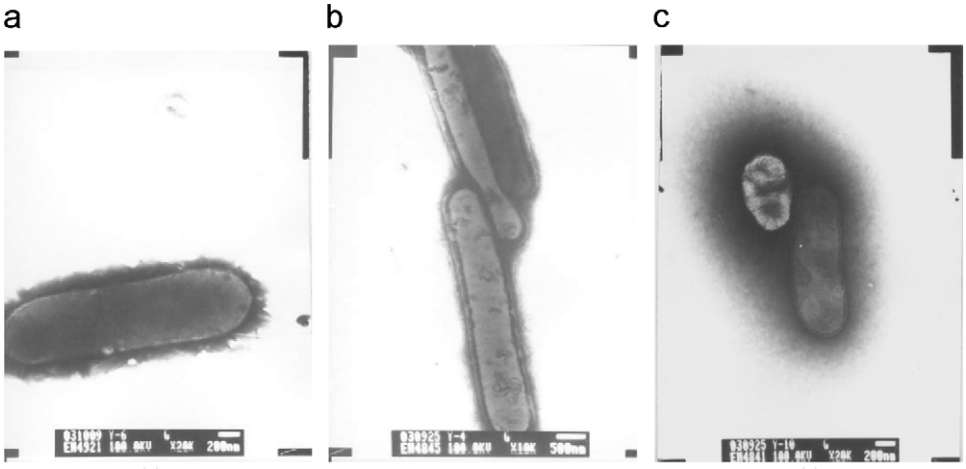

Fig. 1 – SEM images of the isolated strains: (a) BF-4, (b) BF-6, and (c) BF-7.

maximum at 24 h of fermentation. Prolonged fermentation to 36 h significantly reduced the yielded flocculating capability.

Numerous impurities were present in the waste fermenting liquors. To demonstrate the capability of bioflocculant without possible inhibition by the impurities, synthetic medium with mixtures of ethanol and volatile fatty acids as carbon source were utilized to produce bioflocculants (Table 4). The strain BF-6 yielded the highest flocculating capability (>99) at 1000–1100 mg l^{-1} ethanol, 700–900 mg l^{-1} acetate, 150–250 mg l^{-1} propionate, and 100–300 mg l^{-1} butyrate. Ren et al. [31] revealed that ethanol is one of the main metabolites in their highly efficient biohydrogen fermentation system. Hence, in the next section, the strain BF-6, which effectively produced bioflocculant from the ethanol-rich medium, was further examined.

3.3. *Bioflocculant production using strain BF-6*

The growth curve of the strain BF-6 was obtained in the waste fermenting liquor (COD of 4490 mg l^{-1}, ethanol, acetate, propionate, butyrate, and valerate of 689, 613, 340, 102, and 47 mg l^{-1}, respectively) at 30 °C. Using the growth data (Fig. 2), the logarithmic growth phase for the strain BF-6 lasted approximately for 15 h with a generation time of 3.6 h. The flocculating capabilities of broth fermented for 12 h were 76% of those of broth fermented for 24 h (Fig. 2). Consequently, the bioflocculant was mainly produced during the logarithmic growth phase of BF-6.

Following the above fermentation test with BF-6, the COD of the supernatant declined from 4490 to 3450 mg l^{-1}, with the

Table 4 – Bioflocculant liquors from synthetic medium using pure cultures

Strain	Ethanol (mg l^{-1})	Acetate (mg l^{-1})	Propionate (mg l^{-1})	Butyrate (mg l^{-1})	Flocculating capability
BF-4	800	700	150	150	85.4
	900	900	300	100	99.5
	1000	600	200	300	98.7
	1100	800	100	250	93.9
	1200	1000	250	200	94.3
BF-6	800	800	200	200	92.5
	900	1000	100	150	97.3
	1000	700	250	100	99.0
	1100	900	150	300	99.7
	1200	600	300	250	96.5
BF-7	800	900	250	250	87.3
	900	600	150	200	88.8
	1000	800	300	150	91.0
	1100	1000	200	100	99.4
	1200	700	100	300	97.8

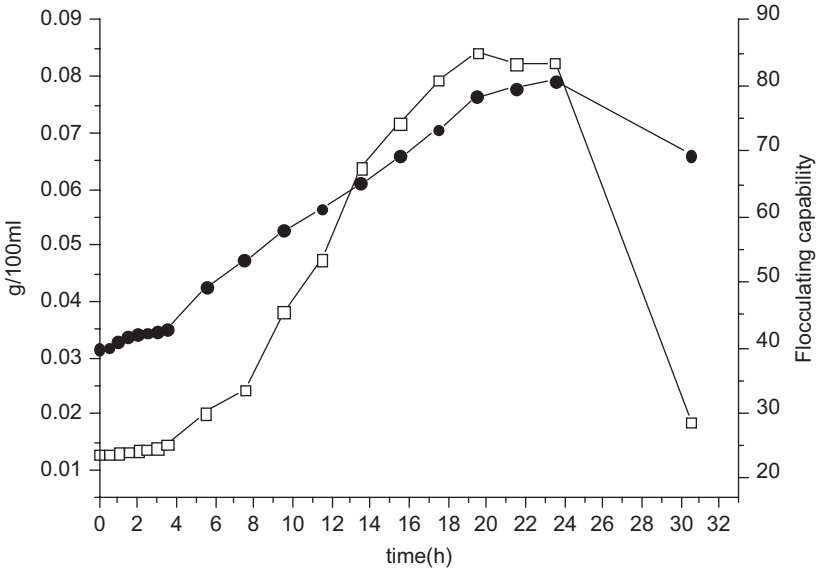

Fig. 2 – Growth curves of BF-6 and the flocculating capabilities of bioflocculant liquors. Mix ratio = 1:0.1 v/v 30 °C. Fermenting waste liquor at a pH of 4.02, COD of 4490 mg l^{-1}, ethanol, acetate, propionate, butyrate, and valerate of 689, 613, 340, 102, and 47 mg l^{-1}, respectively. Open symbols: cell weight; close symbols: flocculating capability.

corresponding concentrations of ethanol, acetate, propionate, butyrate, and valerate being 10.5, 474, 184, 63.8, and 10.4 mg l^{-1}, respectively. The strain BF-6 hence consumed more than 98% of ethanol, and part of acetate (23.4%), propionate (45.9%), and butyrate (37.5%) in producing bioflocculant. Restated, the bioflocculant was mainly produced with the consumption of ethanol in waste fermenting liquor.

The freeze-dried and purified products from bioflocculant liquor with strain BF-6 weighted 2.1 g l^{-1}, consisting of 91% (w/w) carbohydrates and 5.1% (w/w) proteins. This observation correlates with the recent findings that the most effective bioflocculants were polysaccharide-like substances [2,3].

4. Conclusions

The feasibility of using waste fermenting liquors to produce bioflocculants was examined using a few isolated strains from a full-scale biohydrogen fermenter. Following screening, three strains, BF-4, BF-6 and BF-7, identified as *B. fusiformis*, *B. subtilis*, and *B. flexus*, respectively, effectively produced bioflocculants from the waste fermenting liquor at pH 4–5, mix ratio >1:0.1, and fermenting time of 24 h. Using synthetic medium enriched with ethanol, the strain BF-6 yielded the highest flocculating capability (>99) among the tested strains. The strain BF-6 produced bioflocculant during its logarithmic growth phase, by mainly consuming ethanol to produce bioflocculant. The bioflocculant mainly comprised carbohydrates (91% w/w) and was produced by BF-6 at a yield of 2.1 g l^{-1}.

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