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Sources for isolation of extracellular polymeric substances (EPS) producing bacterial strains which are capable using wastewater sludge as solo substrate

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Abstract: Isolation of extracellular polymeric substances (EPS) producing bacterial strains capable of using sludge as low-cost growth substrate was carried out in this study. A total of 110 EPS producing strains were isolated from different sources, which include sludge of beer and winery wastewater treatment plant (WWTP); young, 2-month old and 10-year old leachate. Thirty seven isolated strains showed good growth in sludge medium with cell count varying from 10^6 to 10^{10} MPN/mL and total EPS concentration from 2737 to 6639 mg/L. Twenty one strains produced EPS with high flocculation activity (FA_{max} varied from 72.0% to 80.2%). The highest FA_{max} (80.2%) was observed with EPS produced by strain BES 19, which was isolated from sludge of beer WWTP. Sludge of beer WWTP, young leachate and 10-year old leachate were good sources for isolation of EPS producing bacteria.

Key words: EPS producing bacteria; Isolation; Wastewater sludge; Sludge recycle; Flocculation activity.

Introduction

Extracellular polymeric substances (EPS), synthesized and secreted by microorganism, was composed of polymeric substances with long chain, high molecular weight and contained various types of functional groups such as amino, carboxyl, carbonyl [1, 2]. These characteristics of EPS were similar to those of synthetic flocculants. Therefore, EPS could be named as bio-flocculants.

Flocculation activity (FA) is one of the key parameters to select EPS producing bacterial strain. In general, flocculation activity of EPS depends on EPS producing bacterial strains, EPS composition and ratio of protein and carbohydrate. Flocculation

activity of EPS can be explained by adsorption and bridging mechanism [3]. So far, many studies have reported the flocculation activity of EPS produced by bacteria strains. In synthetic medium, FA of EPS varies from 78 % to 99 % [4]. In case of using sludge as solo substrate, FA of EPS produced by microorganism was from 50 % to 82 % [5]. Most of EPS need divalent (Ca^{2+}, Fe^{2+}, Mg^{2+}) or trivalent (Al^{3+}, Fe^{3+}) cation to process the flocculation. Optimum concentration of EPS used in flocculation varied from 1 to 40 mg/L.

The interest in EPS has been increased remarkably in recent years as EPS can replace or reduce the amount of chemical polymer in flocculation of wastewater and sludge conditioning. Many authors reported that EPS could be applied in treating various types of wastewater such as starch wastewater [6], river water, brewery wastewater, meat processing wastewater, soy sauce brewing wastewater [7] and enhances dewaterability of wasted sludge [8, 9]. EPS showed good ability in removing various kinds of toxic contaminants such as humic acids in leachate [10] and dyes [11, 12].

The production of EPS reported till date mostly is by certain bacteria in synthetic media. It leads to a high production cost of bio-flocculants due to the high cost of growth medium (approximately 30% of total fermentation cost [13]). Therefore, much effort has been devoted to reduce the production cost by using non-expensive material and by-products of industrial process.

The wastewater sludge is a potentially economical culture medium (substrate) as it is a rich source of carbon, nitrogen, phosphorus and other nutrients. Moreover, the use of wastewater sludge will be advantageous for sludge isolated microorganisms cultivation, which is already well adapted to it. Several strains are capable to synthesize EPS as effective flocculants when using sludge as sole substrate [4, 5]. However, the number

of bacterial strains which are capable of using sludge as raw material to produce effective EPS is limited. On the contrary, thousands of EPS producing bacterial strains have been examined in synthetic and other low-cost media. Moreover, a little is known about the sources for isolation of bacterial strains which could use sludge as medium to produce EPS, apart from the study of More et al. [5] who isolated 13 strains from bio-filter of municipal wastewater treatment plant. Lack of bacterial strains and information on the source for isolation will limit the application ability of low-cost bio-flocculation. Therefore, the purpose of this study is (1) to isolate EPS producing bacterial strains from different samples, (2) to determine the characteristics and kaolin flocculation activity of EPS produced in sludge medium and (3) to investigate the relation between diversity of EPS producing bacteria and the characteristics of environment.

Materials and methods

Sample collection and isolation of EPS producing microorganism

The samples were collected from following sources

- Leachate: Different types of leachate were collected from municipal solid waste landfill, including:
    - Leachate which was generated daily from the operating cells (termed as young leachate).
    - Leachate generated from new cell and stored in pond for 2 months (termed as 2-month old leachate).
    - Leachate collected directly from the cell, which was closed since 10 years ago (termed as10-year old leachate).

- Activated sludge: Activated sludge were collected from aeration tank of wastewater treatment plants of beer and wine production.

Isolation of strains was carried out on Tryptone Soya Agar (TSA) medium using serial dilution technique. Plate was incubated at 30^{o}C for 24-48 h. After incubation, colonies with sticky and viscous growth were taken and streaked them individually on TSA plate. Pure culture of isolated strains was grown in TSB (Tryptic Soy Broth) for 24 h.

Production and harvest of EPS

In step 1, 150 mL of sludge (SS ~ 20 g/L) was first adjusted to pH 7.5 using 1 N NaOH, sterilized at 121^{o}C for 30 min. Sterilized sludge was inoculated with 3% inoculum from pure cultures prepared beforehand and incubated at 220 rpm and 30^{o}C for 24 h. It was used as the pre-culture.

In step 2, 150 mL of sludge (SS ~ 20 g/L) pH was adjusted to 7.5 before sterilization, and the sterilized sludge was inoculated with 3%v/v of the pre-culture from step 1, and incubated at 220 rpm and 30^{o}C for 48 h. Samples were aseptically withdrawn at 48 h for the analysis of EPS concentration, protein, carbohydrate content of EPS and flocculation activity of broth EPS.

Kaolin flocculation activity test

Flocculation activity of broth culture (or B-EPS) was tested with kaolin (5 g/L) in Jar-test. $CaCl_2$ was added to the kaolin suspension to get final concentration of 150 mg Ca^{2+}/L and pH was adjusted to 7.5. Different volumes of B-EPS was added to kaolin suspension and rapidly mixed at 100 rpm for an initial 5 min then slowly mixed at 70 rpm for an additional 30 min. Thereafter, samples were transferred to a 500 mL cylinder for settling in 30 min. The supernatant was then collected to measure the turbidity using

116 turbidity meter (Model HI 93703C, Hana). The turbidity was measured of triplicate
117 samples to take the average.

118 Flocculation activity was calculated by the following formula: FA (%) = [100*(T_o-
119 T)/T_o] where 'T_o' is the turbidity of the control (without addition of EPS) and 'T' is the
120 turbidity of the sample.

121 ***Analytical methods***

122 *Cell count*

123 All the samples were serially diluted in saline solution and used for measurement of cell
124 count by the most probable number (MPN) technique [17]. The presented results were
125 average of the triplicate samples.

126 *Extraction and dry weight (M) of the extracted EPS*

127 Fermented broth was centrifuged at 4000 x g, 4^{o}C for 15 min to obtain supernatant
128 (containing loosely bound EPS and termed as LB-EPS). Pellet was re-suspended in
129 0.5% NaCl to initial volume and heated at 60^{o}C in water bath for 30 min to release
130 tightly bound EPS (termed as TB-EPS) to liquid phase. Supernatant containing TB-EPS
131 was collected by centrifugation at 4000 x g, 4^{o}C for 15 min.

132 Supernatant containing EPS (LB and TB) was precipitated overnight in cold (-20^{o}C)
133 with 2.2:1.0 ratio of ethanol (95%) and sample, respectively. Precipitated EPS was
134 collected by centrifugation at 4000 x g, 4^{o}C for 15 min, dried at 80^{o}C and measured the
135 weight (M). Total EPS (B-EPS) was calculated by sum of LB-EPS and TB-EPS weight.
136 The presented results were average of the triplicate samples.

Protein and total carbohydrate content in the extracted EPSs

After precipitation in cold ethanol, known amount of extracted TB-EPS and LB-EPS was dissolved in deionized water to measure the protein and carbohydrate concentration. Soluble protein was determined according to the Lowry method with bovine serum albumin as the standard [18]. Carbohydrate was determined by phenol-sulfuric acid method with glucose as standard [19]. The presented results were average of the triplicates.

Result and discussion

Isolation and selection of EPS producing strains

Strains which grew well in sludge medium (cell count higher than 10^6 MPN/mL) were selected for further studies. Among 110 isolated strains (named as BES 1 to BES 110), 37 strains grew well with cell count 10^6 to 10^{10} MPN/mL in sterilized sludge. These 37 included 16 strains from sludge of Beer and Wine production WWTPs of and 21 strains from leachate.

Cell count of isolated strains from wastewater sludge was quite similar and in the range of 10^7 to 10^8 MPN/mL. Cell count (10^7 to 10^{10} MPN/mL) of strains isolated from leachate was higher than that of strains isolated from other sources. BES 38 exhibited very high cell count (10^{12} MPN/mL) while growing in sludge. The results suggested that wastewater sludge was one of the good substrates for growth of many EPS producing bacteria.

157 ***EPS production and chemical characterization of produced EPS***

158 *Indigenous EPS content in sludge*

159 Sludge (suspended solids or SS ~ 20 g/L) from beer wastewater treatment plant was
160 used as solo material to grow EPS producing bacterial strains. Five samples of sludge
161 taken from the same tank on different dates had indigenous EPS concentration ranging
162 from 3615 to 4496 mg/L or 181 to 225 mg EPS/g SS (Table 1). Purified indigenous EPS
163 from sludge contains 0.05 to 0.10 g carbohydrate/g of LB-EPS, 0.04 – 0.14 g of
164 carbohydrate/g of TB-EPS and 0.15 – 0.25 g of protein/g of LB-EPS, 0.05 – 0.15 g of
165 protein/g of TB-EPS (Fig. 1). Aggregation of microorganism in sludge to form bio-flocs
166 is well known due to polymer excreted by the microbial cells [1].

167 *Strains isolated from activated sludge of beer WWTP*

168 EPS, protein and carbohydrate content of fermented and control samples was presented
169 in Fig. 1. BES 19 revealed higher EPS concentration (5018 mg/L) than control sample
170 (3857 mg/L).

171 Carbohydrate content of purified LB-EPS (produced in sludge) varied from 0.07 to 0.11
172 g/g and was similar to carbohydrate content of LB-EPS of the control sample.
173 Carbohydrate content of purified TB-EPS varied between 0.03 - 0.10 g/g and was 2 - 3
174 times lower than that of control. Low carbohydrate content of TB-EPS produced in
175 sludge indicated that large amount of carbohydrate present in indigenous TB-EPS was
176 consumed by the inoculated strains for their growth and synthesis of new EPS.

177 Protein content of purified EPS varied much with type of bacterial strain and was
178 between 0.17 to 0.32 g/g of LB-EPS and 0.15 to 0.23 g/g of TB-EPS. Protein content in
179 TB-EPS of fermented samples was 2 - 4 times higher than that of the control samples.

Strains isolated from activated sludge of winery WWTP

EPS concentration, EPS protein and carbohydrate content of fermented and control samples are presented in Fig. 1. EPS produced by BES 1, 2, 6 and 7 was higher than the control and EPS produced by BES 3 and 4 was lower than the control. The change of total EPS (LB-EPS plus TB-EPS) concentration was mostly due to the change of LB-EPS. The high EPS production was observed with BES 7 (5018 mg/L as compared to 3615 mg/L of the control).The trend of carbohydrate and protein content of LB-EPS and TB-EPS of isolated strains from wine manufacturing WWTP was similar to those of isolated strains from beer WWTP.

Strains isolated from leachate

EPS concentration of samples inoculated in sludge medium with strains isolated from young leachate and 10-year old leachate was between 4190 to 6639 mg/L, which was appreciably higher than the control samples (Fig. 1). On the contrary, for the strain isolated from 2-month old leachate, EPS concentration in fermented broths was lower than that of the control.

The highest EPS production was observed with BES 72 strain, which was isolated from 10-year old leachate. BES 72 produced 6639 mg EPS/L and it was 2625 mg/L higher as compared to EPS of the control. It is well known that old leachate is composed of complex organic substances with low biodegradability. Bacterial strains, which can survive in old leachate might have the ability to consume complex organic material by secreting specific enzymes. When using sludge as medium, BES 72 (isolated from 10 year old leachate) could easily use complex organic substances in sludge and produce higher concentration of EPS than the other strains.

Carbohydrate content in LB-EPS and TB-EPS of fermented broths was not much different than the control samples and was in the range from 0.10 to 0.12 (for LB-EPS) and from 0.05 to 0.15 (for TB-EPS) (Fig. 1). Protein content in LB-EPS and TB-EPS of fermented samples was much different from that of the control samples (Fig. 1). Protein content in LB-EPS and TB-EPS showed a significant difference from strain to strain and varied from 0.09 to 0.35 g/g LB-EPS and from 0.05 to 0.17 g/g TB-EPS. BES 61 had highest protein content in TB-EPS (0.42 g/g) (Fig. 1).

The results indicated that the concentration and composition of EPS (with respect to carbohydrates and protein content) of the original sludge remarkably changed after inoculation with EPS producing bacteria. The protein and carbohydrate content of the purified EPS was different for different bacterial strains with ratio of carbohydrate and protein varied from 0.29 to 1.06 for LB-EPS and from 0.04 to 2.84 for TB-EPS. More et al. (2012) reported that the ratio of carbohydrate and protein in EPS produced in sludge medium by 13 different bacterial strains varied from 0.58 to 7.76 g/g. Sludge was a complex medium and contained wide spectrum of substrates [20]. The composition of EPS would be changed depending on the type of substrate that bacterial strain can use, [21].

EPS concentration in fermented broths indicates that depending on the strains, EPS concentration can be higher or lower than sludge indigenous EPS. In sludge medium, substrate for EPS producing bacterial strains can be extracellular substances (such as natural/indigenous EPS) and/or intracellular substances (composed of nucleic acid, NAD etc.). As in sludge medium, we cannot distinguish between EPS produced by inoculated strains and sludge indigenous EPS by measuring dry weight. Broth EPS was the sum of new EPS produced by inoculated strain and sludge indigenous EPS. Because EPS production yield (mg EPS produced per mg substrate consumed) is always less

than 1, concentration of EPS after fermentation will decrease if the inoculated strain used only sludge indigenous EPS as substrate to grow and synthesize EPS. In other cases, strains prefer using intracellular substances as substrate, the total EPS will obviously increase after fermentation (provided the indigenous EPS is not simultaneously degraded).

More et al. [5] reported varied concentration of EPS 0.7 - 1.7 g/L produced by different strains isolated from municipal wastewater sludge while cultivated in sterile sludge as a growth medium. Bezawada et al. [4] reported that *Serratia* sp.1 produced maximum 3.44 g /L of EPS in 48 h fermentation using activated as production medium. Therefore, the total EPS concentration produced in this study was higher.

Flocculation activity of broth EPS

Flocculation activity of control samples

Sludge itself contained indigenous EPS and exhibited flocculation activity [22] or FA. EPS concentration found in various sludge was high (3615 - 4496 mg/L); however, flocculation activity was low (Table 1) and varied between 60.4 to 72.4%. The low FA might be due to the change in the composition of EPS, which depends on many factor such as sludge nature which in turn depends on operating conditions of WWTP, variation of influent characteristics and weather [2]. Due to low flocculation activity of indigenous EPS, it was necessary to produce EPS by isolated strains to be able to control the quality to obtain high FA and quantity of EPS.

Flocculation activity of broth EPS

Effect of volume and concentration of broth EPS (B-EPS) produced by various strains isolated from wine WWTP, beer WWTP and leachate on flocculation activity was

shown in Figs. 2, 3 and 4, respectively. Flocculation activities of EPS of control samples were depicted by the continuous line. The results indicated that flocculation activity was first increased sharply with the increase of EPS volume and reached maximal when using EPS in the range from 2 to 3 mL. Further increase of EPS volume did not increase the flocculation activity, it remained constant. Several previous studies showed that the flocculation activity of EPS produced in sludge medium first increased to a maximum value and after that decreased [4, 5]. A different trend of flocculation activity of this study than those reported previously might be due to the difference in colloidal content of sludge medium. In previous studies [4, 5], sludge used for growing EPS producing bacterial strains was from backwash of bio-filter operation of the municipal wastewater treatment plant, which contained high concentration of colloidal particles. On the contrary, the sludge used in this research was from activated sludge tank, which had good settleability and low colloidal particles in the supernatant (< 20 mg/L). Lower colloidal particles concentration lead to an improvement of flocculation activity [4]. Thus, flocculation activity did not decrease or decreased less when using EPS in high volume.

The EPS produced by the strains isolated from winery WWTP sludge did not bring significant enhancement in flocculation activity compared to EPS of the control sample (Figs. 2 and 5). FA_{max} (maximum flocculation activity) of samples varied from 67.9% to 71.9% and FA_{max} of the control sample was 70.9%. Winery wastewater was rich in nitrogen (TN ~ 354.6 mg N/L) and poor in organic compounds (COD = 216 mg O_2/L, BOD_5 = 111 mg O_2/L) (Table 2). On the contrary, the BOD:TN ratio of sludge used as growth medium was between 5 to 10. A significant change in BOD:TN ratio of growth medium might lead to poor production and probably change of protein and carbohydrate moieties of EPS in such a way that modified the flocculation activity of the EPS of the

276 strains isolated from the sludge of winery WWTP.

277 EPS produced by the strains isolated from sludge of beer WWTP showed a remarkable
278 enhancement in flocculation activity compared to the control EPS samples (Figs. 3 and
279 5), except for EPS produced by strains BES 16 and BES 28, which revealed flocculation
280 activities close to the control. Maximum flocculation activities (FA_{max}) of samples
281 varied from 71.9 to 80.2% (Fig. 5.b) and they were 11.5 - 19.8% higher than the
282 corresponding control sample (Fig. 5.a). The highest maximum flocculation activity
283 (80.2%) was obtained with EPS produced by strain BES 19. Among 3 sources (beer
284 WWTP sludge, winery WWTP sludge and leachate), beer WWTP sludge was the best
285 source for isolation of EPS producing bacteria (Fig. 5). These finding were also in line
286 with the high indigenous EPS concentration in beer WWTP sludge (3615 - 4496 mg
287 EPS/L), which suggested that microbial community of beer WWTP was rich in strains
288 that are capable to produce EPS. EPS producing microorganisms are well known for
289 having a subtle role in floc formation in activated sludge system [1]. Many researchers
290 argued that isolated strains from activated sludge have good ability to produce EPS as
291 bio-flocculants [7, 20, 23]. In this study, beer wastewater treatment plant sludge was
292 selected for isolating microorganisms based on the hypothesis that wastewater of beer
293 production activity is rich in organic compounds and high BOD:TN ratio (as shown in
294 Table 2), which can be a favourable condition for EPS producing microorganisms [24].

295 Similar to strain isolated from beer WWTP sludge, many strains isolated from young
296 and 10-year old leachate produced EPS with flocculation activity higher than that of the
297 control samples (Figs. 4a, 4c, 4d, 4f and 5). BES 45 exhibited flocculation activity
298 lower than that of the control and other three strains (BES 58, 60 and 65) had little
299 higher flocculation activity (about 3% higher) as compared to the control. Remaining

300 strains have shown the increase of flocculation activity from 4.9% - 6.9% as compared
301 to the corresponding control samples. The highest flocculation activity (79.3%) was
302 attained with EPS from strain BES 56.

303 Although EPS produced by the strains isolated from 2-month old leachate showed
304 significant enhancement in flocculation activity (ΔFA_{max} = 5.7 - 16.6%), maximum
305 flocculation activities of these strains were low (FA_{max} = 65.5 - 72.4%). High increase
306 in flocculation activity was due to the low flocculation activity (59.8%) of the control
307 sample

308 The results revealed that young and 10-year old leachate were potential sources for
309 isolating EPS producing bacteria. It could be due to better surviving conditions for
310 microorganisms. Young leachate had high COD and BOD concentration (Table 2),
311 which was a favourable condition for EPS producing bacteria [24]. Old leachate
312 contained low BOD concentration (due to degradation of organic compounds) but
313 comprised of high concentration of toxic compounds such as heavy metals (due to
314 mineralization) and phenolic and aromatic compounds. Therefore, the isolated bacterial
315 strains from 10 year old leachate developed a capacity to produce high amount of EPS
316 to protect themselves against adverse conditions created by the toxic elements [1, 25].
317 Conversely, 2-month old leachate has low BOD content and heavy metals in organic
318 form. It is not ideal condition for the growth of EPS producing bacteria.

319 EPS produced by 21 isolated bacterial strains showed a good flocculation activity (from
320 72.0% to 80.2%), whereas EPS of BES 19 and BES 56 had the highest FA_{max} of 80.2%
321 and 79.3%, respectively. Optimum EPS concentration was 4 - 10 mg EPS/L for
322 flocculation. Table 3 summarized FA_{max} and concentration of EPS used for flocculation
323 activity of bioflocculants produced by different strains reported in the literature. FA_{max}

obtained in this study was similar to FA_{max} reported using similar growth medium (sludge) and the same protocol to determine the flocculation activity. FA_{max} reported in this study was somewhat lower than others employing synthetic medium and dairy wastewater as production medium. It might be due to the difference in settling condition. In this study, samples were transferred to 500 mL cylinder for stetting instead of settling in 500 mL flask used in other studies. It is well-known that higher settling depth and smaller settling area of cylinder reduced settling efficiency. Moreover, some studies used optical density at 550 nm instead of turbidity (measured at 860 nm); therefore, it is difficult to compare those flocculation activities.

The flocs, which were big and visible in kaolin solution were observed when using EPS produced by strain BES 19 and 72. These strains produced high EPS concentration. It can be concluded from these results that strains which produce high concentration of EPS will have good flocculation activity. However, it cannot be generalised that the strains which produce less EPS have low flocculation activity. Several fermented samples, which had lower EPS concentration than their corresponding control had higher flocculation activity such as BES 38 (isolated from 2-month old leachate) and 50 (isolated from young leachate).

Conclusion

Thirty seven EPS producing bacterial strains capable to grow in sludge medium were isolated from various sources. Twenty one strains could produce EPS with maximum flocculation activity of 72.0% to 80.2%. Highest EPS production of 6639 mg/L and 5018 mg/L was observed with strains BES 72 and BES 19, respectively. The highest flocculation activity of 80.2% was obtained using EPS of strain BES 19, which was isolated from beer wastewater treatment plant. Diversity of strains which produced

effective EPS in sludge medium was rich in places that received rich organic wastewater or wastewater containing toxic components.

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Nomenclature

ΔFA: Difference between maximum flocculation activity of B-EPS after fermentation and B-EPS of control (sludge indigenous EPS) (%)

B-EPS: Broth EPS

EPS: Extracellular polymeric substances

FA: Flocculation activity (%)

FA_{max}: Maximum flocculation activity (%)

LB-EPS: Loosely bound EPS

M: EPS dry weight (mg/L)

T: Turbidity (NTU)

TB-EPS: Tightly bound EPS

WWTP: Wastewater treatment plant

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442

443

444 Table 1. Flocculation activity and EPS dry weight of control samples

Samples	Control 1	Control 2	Control 3	Control 4	Control 5
FA_{max} (%)	60.4±0.7	70.9±0.3	67.6±0.6	59.8±0.8	72.4±0.3
$M_{B\text{-}EPS}$ (mg/L)	3857±35	3615±56	4015±47	3948±71	4496±43
$M_{LB\text{-}EPS}$ (mg/L)	3120±22	3178±24	3456±21	2731±22	3586±14
$M_{TB\text{-}EPS}$ (mg/L)	738±18	755±26	559±16	1217±24	910±19

445 Note: $M_{B\text{-}EPS}$, $M_{LB\text{-}EPS}$ and $M_{TB\text{-}EPS}$: dry weight of B-EPS, LB-EPS and TB-EPS, respectively;
446 Control 1: Control sample for strains isolated from sludge of beer WWTP; Control 2: control
447 sample for strains isolated from sludge of winery WWTP; Control 3: control sample for
448 strains isolated from young leachate; Control 4: control sample for strains isolated from 2-
449 month old leachate; Control 5: control sample for strains isolated from 10-year old leachate;

450

451 Table 2. Characterization of influent of beer and winery WWTP and leachate

Parameters	Beer WWTP	Winery WWTP	Young leachate	2-month old leachate	10-year old leachate
pH	5.58 ±0.01(*)	10.23±0.01 (*)	7.75±0.01	8.23±0.01	8.00±0.01
COD (mg O_2/L)	2570±30	216±2	4619±60	947±7	360±5
BOD_5 (mg O_2/L)	2127±35	111±3	3120±52	382±4	43±1
TN (mg N/L)	15.3±0.2(**)	354±2	782±5	864±6	717±4
TP (mg P/L)	2.51±0.01	5.50±0.02	14.73±0.08	13.09±0.06	18.90±0.05
BOD:TN	130	0.3	4.0	0.4	0.06

452 (*) Before pumped in biological process of WWTP, pH of wastewater was neutralized to 7.5

453 (**) In aeration unit, ammonium chloride was added to adjust ratio of BOD:TN to 100:5

454

455

456 Table 3. Comparison of different bio-flocculants concentrations used and their
457 flocculation activity

EPS producing bacterium	Growth medium	Cation added	EPS conc. (mg/L)	FA_{max} (%)	References
Serratia ficaria	Synthetic	Ca^{2+}	0.4 (*)	96.1	[7]
Klebsiella sp. N10	Synthetic	Ca^{2+}	34	86.5	[9]
K. mobilis	Dairy wastewater	Ca^{2+}	5.2	94.6	[16]
Serratia sp.1	Wastewater sludge	Ca^{2+}	3.5	79.1	[3]
Serratia sp.1	Waste water sludge	Ca^{2+}	6.9	70.4	[4]
21 selected bacterial strains	Waste water sludge	Ca^{2+}	~ 10	72.0 – 80.2	This study

458 (*) 0.4 mL of bio-flocculants/L of kaolin solution

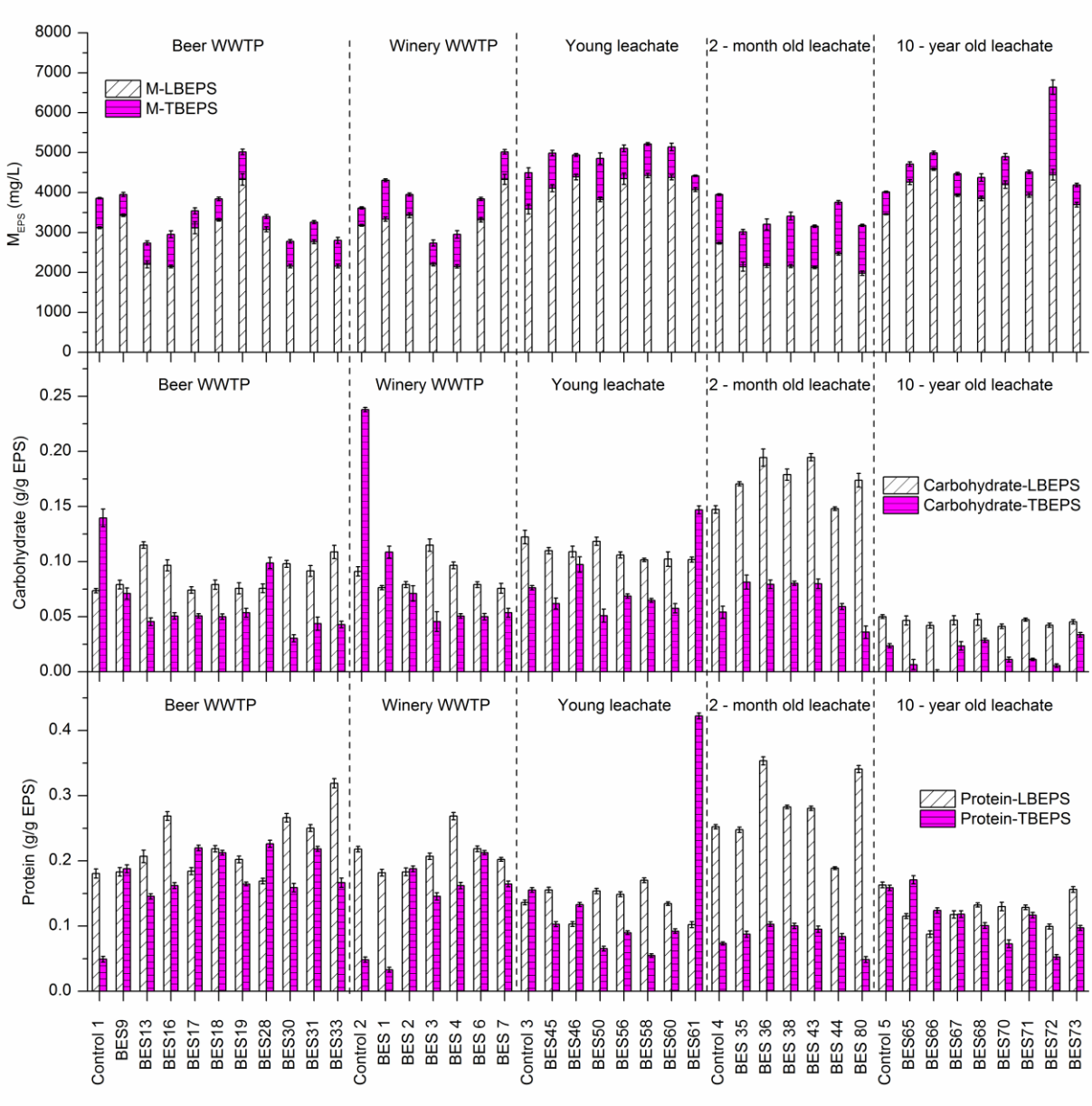
Fig. 1. Dry weight, protein and carbohydrate content of EPS produced by isolated strains

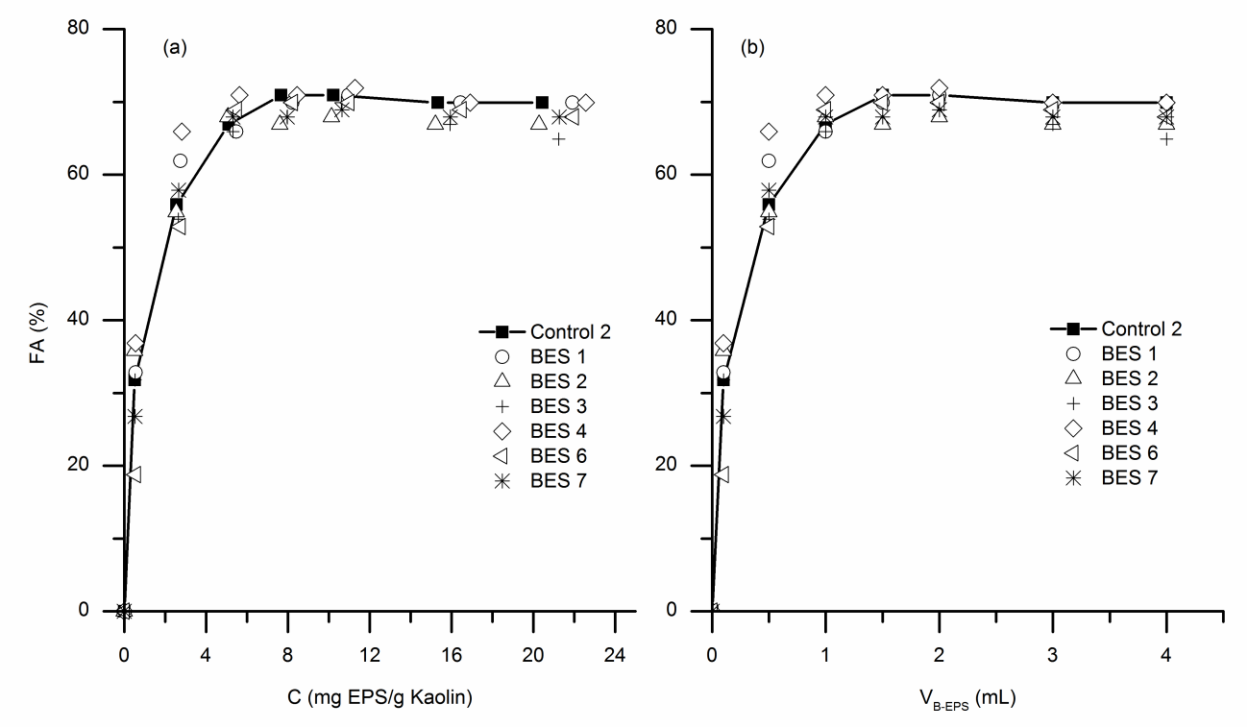
Fig. 2. Effect of broth EPS produced by isolated strains from winery WWTP sludge on flocculation activity. (a) EPS concentration vs FA; (b) EPS volume vs FA

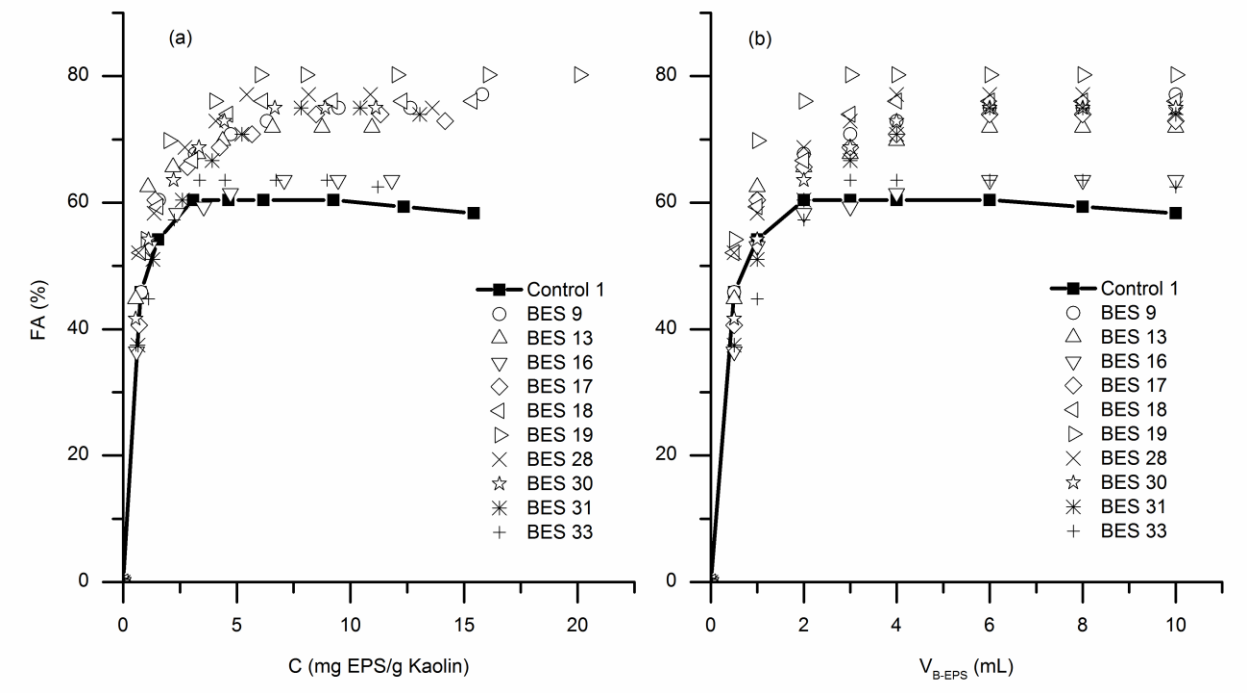
Fig. 3. Effect of broth EPS produced by isolated strains from Beer WWTP sludge on flocculation activity. (a) EPS concentration vs FA; (b) EPS volume vs FA

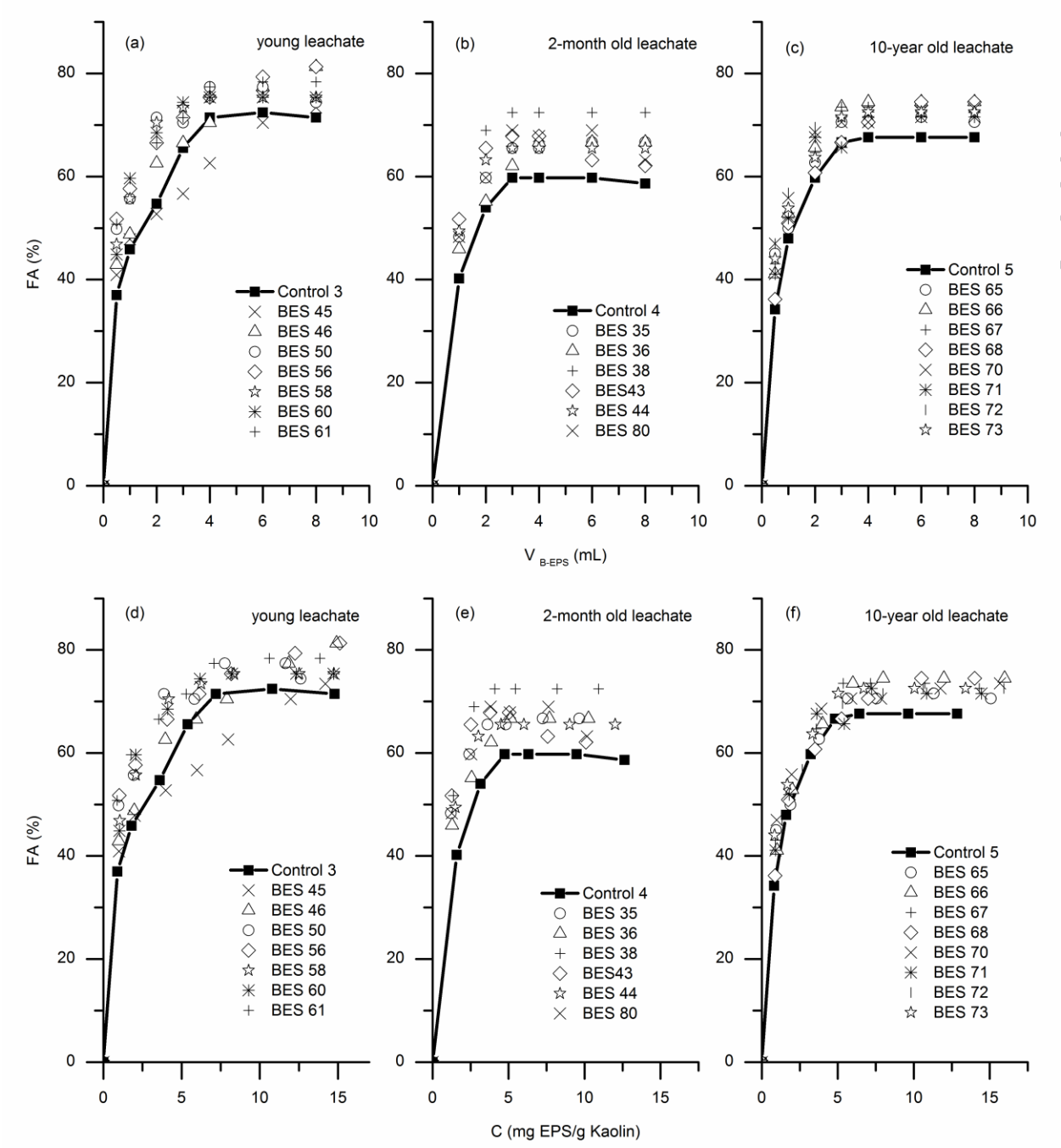
Fig. 4. Effect of broth EPS produced by isolated strains from leachate on flocculation activity. (a), (b) and (c) EPS concentration vs FA; (d), (e) and (f) EPS volume vs FA

Fig. 5. Comparison of flocculation activity of all isolated strains. (a) Difference between maximum flocculation activity of sample and correlative control (ΔFA_{max}); (b) Maximum flocculation activity (FA_{max})


Beer WWTP
Winery WWTP
Young leachate
2 - month old leachate
10 - year old leachate
M-LBEPS
M-TBEPS
M_{EPS} (mg/L)
Carbohydrate-LBEPS
Carbohydrate-TBEPS
Carbohydrate (g/g EPS)
Protein-LBEPS
Protein-TBEPS
Protein (g/g EPS)
Control 1
BES9
BES13
BES16
BES17
BES18
BES19
BES28
BES30
BES31
BES33
Control 2
BES 1
BES 2
BES 3
BES 4
BES 6
BES 7
Control 3
BES45
BES46
BES50
BES56
BES58
BES60
BES61
Control 4
BES 35
BES 36
BES 38
BES 43
BES 44
BES 80
Control 5
BES65
BES66
BES67
BES68
BES70
BES71
BES72
BES73






(a)
young leachate
(b)
2-month old leachate
(c)
10-year old leachate
FA (%)
V B-EPS (mL)
Control 3
BES 45
BES 46
BES 50
BES 56
BES 58
BES 60
BES 61
Control 4
BES 35
BES 36
BES 38
BES43
BES 44
BES 80
Control 5
BES 65
BES 66
BES 67
BES 68
BES 70
BES 71
BES 72
BES 73
(d)
young leachate
(e)
2-month old leachate
(f)
10-year old leachate
C (mg EPS/g Kaolin)