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

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

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 ($F_{\text{max}}$ varied from 72.0% to 80.2%). The highest $F_{\text{max}}$ (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 as 10-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°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°C for 30 min. Sterilized sludge was inoculated with 3% inoculum from pure cultures prepared beforehand and incubated at 220 rpm and 30°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°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
turbidity meter (Model HI 93703C, Hana). The turbidity was measured of triplicate samples to take the average.

Flocculation activity was calculated by the following formula: $\text{FA (\%)} = \left[ \frac{100 \times (T_o - T)}{T_o} \right]$ where ‘$T_o$’ is the turbidity of the control (without addition of EPS) and ‘$T$’ is the turbidity of the sample.

**Analytical methods**

**Cell count**

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

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

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

Supernatant containing EPS (LB and TB) was precipitated overnight in cold (-20°C) with 2.2:1.0 ratio of ethanol (95%) and sample, respectively. Precipitated EPS was collected by centrifugation at 4000 x g, 4°C for 15 min, dried at 80°C and measured the weight ($M$). Total EPS (B-EPS) was calculated by sum of LB-EPS and TB-EPS weight. 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 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.
Indigenous EPS content in sludge

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

Strains isolated from activated sludge of beer WWTP

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

Carbohydrate content of purified LB-EPS (produced in sludge) varied from 0.07 to 0.11 g/g and was similar to carbohydrate content of LB-EPS of the control sample.

Carbohydrate content of purified TB-EPS varied between 0.03 - 0.10 g/g and was 2 - 3 times lower than that of control. Low carbohydrate content of TB-EPS produced in sludge indicated that large amount of carbohydrate present in indigenous TB-EPS was consumed by the inoculated strains for their growth and synthesis of new EPS.

Protein content of purified EPS varied much with type of bacterial strain and was 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 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). $F_{\text{A}\max}$ (maximum flocculation activity) of samples varied from 67.9% to 71.9% and $F_{\text{A}\max}$ 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\textsubscript{2}/L, $\text{BOD}_5$ = 111 mg O\textsubscript{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
strains isolated from the sludge of winery WWTP.

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

Similar to strain isolated from beer WWTP sludge, many strains isolated from young and 10-year old leachate produced EPS with flocculation activity higher than that of the control samples (Figs. 4a, 4c, 4d, 4f and 5). BES 45 exhibited flocculation activity lower than that of the control and other three strains (BES 58, 60 and 65) had little higher flocculation activity (about 3% higher) as compared to the control. Remaining
strains have shown the increase of flocculation activity from 4.9% - 6.9% as compared to the corresponding control samples. The highest flocculation activity (79.3%) was attained with EPS from strain BES 56.

Although EPS produced by the strains isolated from 2-month old leachate showed significant enhancement in flocculation activity ($\Delta F_{A_{\text{max}}} = 5.7 - 16.6\%$), maximum flocculation activities of these strains were low ($F_{A_{\text{max}}} = 65.5 - 72.4\%$). High increase in flocculation activity was due to the low flocculation activity (59.8%) of the control sample.

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

EPS produced by 21 isolated bacterial strains showed a good flocculation activity (from 72.0% to 80.2%), whereas EPS of BES 19 and BES 56 had the highest $F_{A_{\text{max}}}$ of 80.2% and 79.3%, respectively. Optimum EPS concentration was 4 - 10 mg EPS/L for flocculation. Table 3 summarized $F_{A_{\text{max}}}$ and concentration of EPS used for flocculation activity of bioflocculants produced by different strains reported in the literature. $F_{A_{\text{max}}}$
obtained in this study was similar to $FA_{\text{max}}$ reported using similar growth medium (sludge) and the same protocol to determine the flocculation activity. $FA_{\text{max}}$ 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 settling 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.

Acknowledgements
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Nomenclature
\[ \Delta FA: \ \text{Difference between maximum flocculation activity of B-EPS after fermentation and B-EPS of control (sludge indigenous EPS)} \ (%)]
\[ \text{B-EPS: Broth EPS} \]
\[ \text{EPS: Extracellular polymeric substances} \]
\[ \text{FA: Flocculation activity} \ (%)]
\[ \text{FA}_{\text{max}}: \ \text{Maximum flocculation activity} \ (%)]
\[ \text{LB-EPS: Loosely bound EPS} \]
\[ \text{M: EPS dry weight (mg/L)} \]
\[ \text{T: Turbidity (NTU)} \]
\[ \text{TB-EPS: Tightly bound EPS} \]
\[ \text{WWTP: Wastewater treatment plant} \]

References


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

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

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

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Beer WWTP</th>
<th>Winery WWTP</th>
<th>Young leachate</th>
<th>2-month old leachate</th>
<th>10-year old leachate</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.58 ±0.01 (*)</td>
<td>10.23±0.01 (†)</td>
<td>7.75±0.01</td>
<td>8.23±0.01</td>
<td>8.00±0.01</td>
</tr>
<tr>
<td>COD (mg O₂/L)</td>
<td>2570±30</td>
<td>216±2</td>
<td>4619±60</td>
<td>947±7</td>
<td>360±5</td>
</tr>
<tr>
<td>BOD₅ (mg O₂/L)</td>
<td>2127±35</td>
<td>111±3</td>
<td>3120±52</td>
<td>382±4</td>
<td>43±1</td>
</tr>
<tr>
<td>TN (mg N/L)</td>
<td>15.3±0.2 (** )</td>
<td>354±2</td>
<td>782±5</td>
<td>864±6</td>
<td>717±4</td>
</tr>
<tr>
<td>TP (mg P/L)</td>
<td>2.51±0.01</td>
<td>5.50±0.02</td>
<td>14.73±0.08</td>
<td>13.09±0.06</td>
<td>18.90±0.05</td>
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<tr>
<td>BOD:TN</td>
<td>130</td>
<td>0.3</td>
<td>4.0</td>
<td>0.4</td>
<td>0.06</td>
</tr>
</tbody>
</table>

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

(**) In aeration unit, ammonium chloride was added to adjust ratio of BOD:TN to 100:5
Table 3. Comparison of different bio-flocculants concentrations used and their flocculation activity

<table>
<thead>
<tr>
<th>EPS producing bacterium</th>
<th>Growth medium</th>
<th>Cation added</th>
<th>EPS conc. (mg/L)</th>
<th>FA&lt;sub&gt;max&lt;/sub&gt; (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Serratia ficaria</em></td>
<td>Synthetic</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>0.4 (*)</td>
<td>96.1</td>
<td>[7]</td>
</tr>
<tr>
<td><em>Klebsiella</em> sp. N10</td>
<td>Synthetic</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>34</td>
<td>86.5</td>
<td>[9]</td>
</tr>
<tr>
<td><em>K. mobilis</em></td>
<td>Dairy wastewater</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>5.2</td>
<td>94.6</td>
<td>[16]</td>
</tr>
<tr>
<td><em>Serratia</em> sp.1</td>
<td>Wastewater sludge</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>3.5</td>
<td>79.1</td>
<td>[3]</td>
</tr>
<tr>
<td><em>Serratia</em> sp.1</td>
<td>Waste water sludge</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>6.9</td>
<td>70.4</td>
<td>[4]</td>
</tr>
<tr>
<td><em>21 selected</em></td>
<td>Waste water sludge</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>~10</td>
<td>72.0 – 80.2</td>
<td>This study</td>
</tr>
</tbody>
</table>

*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 ($\Delta F_{A_{\text{max}}}$); (b) Maximum flocculation activity ($F_{A_{\text{max}}}$)