Identification of the effect of extracellular polymeric substances on bacterial adhesion to the membrane surface in a membrane bioreactor using *Pseudomonas fluorescens*

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Abstract In a membrane bioreactor (MBR) process containing a variety of bacteria, the bacterial adhesion to the membrane surface, prior to cake formation, causes an increased filtration resistance. In this study, *Pseudomonas fluorescens,* commonly found in the municipal wastewater treatment process with activated sludge, was used to show the effects of extracellular polymeric substances (EPS) on bacterial adhesion to the membrane surface in the MBR. Of the various roles of EPS in promoting membrane fouling, the adhesion of bacteria to the membrane surface was calculated using the specific cake resistance (α, m/kg). Although the amount of EPS binding with bacteria was increased by the addition of Ca^{2+}, there was no significant effect on the bacterial growth. The results of the particle size distribution showed that the addition of Ca^{2+} increased flocculation, allowing the formation of a complex with the bacteria and EPS. In order to identify the effects of the addition of Ca^{2+} on the hydrophobicity, the contact angle was also measured. The result showed that the addition of Ca^{2+} showed no significant differences in the hydrophobicity, even though there was an increase in flocculation. With the bacteria containing a higher EPS concentration, a higher specific cake resistance was observed. From the results of the adhesion experiment, which was conducted with various EPS levels, displayed as the COD and TOC concentration, an increased EPS concentration was shown to promote bacterial adhesion to the membrane surface.

Keywords Bacterial adhesion; extracellular polymeric substances; fouling; membrane bioreactor

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Introduction

There is currently a limitation of available water resources, which will eventually require the discovery of alternative resources, such as wastewater and rainfall. Of the alternative resources, wastewater, which should be treated for the reduction of pollution to the water system, is gaining in interest as a newly emerging water resource.

Especially, the membrane bioreactor (MBR), which is a combination of conventional wastewater treatment and a membrane filtration process, has acquired considerable attention in the development of alternative water resources as a result of the following advantages: complete solids removal, significant physical disinfection capability, superior organic and nutrient removals and small footprint. However, membrane fouling in the MBR, which increases the operational cost, limits their usage (Jang *et al.*, 2004). Membrane fouling is a ubiquitous phenomenon in MBRs, which is mainly caused by microbial substances, such as extracellular polymeric substances (EPS) and soluble microbial substances (SMP).

Of the foulants, EPS are defined as of biological origin that participate in the formation of microbial aggregates (Laspidou and Rittmann, 2002), and are known to be

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the dominant foulants in the MBR process (Nagaoka *et al.*, 1998; Chang *et al.*, 2002). The role of EPS on the fouling phenomenon is largely described as either direct (pore blocking; the accumulation of foulants within the membrane, and cake formation; the accumulation of retained solids on the membrane) or indirect (agents promoting microbial aggregate formation, increasing the adhesion of microorganisms to membrane surface).

Before cake formation on the membrane surface, bacterial adhesion to the membrane surface can cause an increase of membrane fouling in the MBR process. In this study, *Pseudomonas fluorescens,* commonly found in the municipal wastewater treatment process with activated sludge, was used to investigate the effects of EPS on bacterial adhesion to the membrane surface in the MBR. To artificially control the production of EPS, calcium was added to the culture solution for *Pseudomonas fluorescens*. Divalent ions such as calcium and magnesium are known to play a critical role in the formation of the EPS complex in the bioflocculation (Sobeck and Higgins, 2002).

Filtration theory

Carman (1938) provided a development of the standard model for incompressible cakes. The development is based on a modified form of Darcy's law for flow through porous media. The model was applied to compare the adhesion tendency with specific cake resistance (α, m/kg) in equation (5).

Flow through the cake:

$$J = \frac{1}{A}\frac{dV}{dt} = \frac{P_c}{\mu r^* L}. \tag{1}$$

Flow through the support media:

$$J = \frac{1}{A}\frac{dV}{dt} = \frac{P_m}{\mu R_m} \tag{2}$$

where J = filtrate velocity (m/s); L = cake thickness (m); P_m = pressure differences across the support medium (Pa); P_c = pressure differences across the cake (Pa); r^* = inverse of the permeability coefficient (L/m^2); V = filtrate volume (m^3); A = filtration area (m^2); μ = absolute viscosity (Pa·s); R_m = support medium resistance (L/m).

Let $P_t = P_c + P_m$:

$$J = \frac{1}{A}\frac{dV}{dt} = \frac{P_t}{\mu(r^* L + R_m)}. \tag{3}$$

Because the cake thickness is difficult to measure in dynamic filtration tests, the expression r^* is replaced by $\alpha\omega$, giving:

$$\frac{dV}{dt} = \frac{P_t A}{\mu\left(\alpha\omega\frac{V}{A} + R_m\right)} = \frac{P_t A^2}{\mu(\alpha\omega V + R_m A)} \tag{4}$$

where α = specific cake resistance (m/kg); ω = mass of cake deposited per unit volume of filtrate (kg/m^3).

The integrated form of equation (4) is

$$\frac{t}{V} = \frac{\mu\alpha\omega}{2P_t A^2} V + \frac{\mu R_m}{P_t A}. \tag{5}$$

Materials and methods

Cultivation of *Pseudomonas fluorescens*

Pseudomonas fluorescens, with a mean size of 2 μm, was used for the adhesion test of bacteria to the membrane surface following continuous cultivation in a master culture, at 27°C and 150 rpm, in Nutrient Broth (8 g/L DI-water), which provided consistent and repeatable cultures for various purposes. In order to maintain the growth phase and mass of the bacteria, the culture medium was periodically discarded and replenished to maintain a constant solid retention time (SRT); this being 2 days during these experiments. Before discarding and replenishing the medium, the *Pseudomonas fluorescens* used for the filtration and adhesion test was inoculated from the master culture. The culture inoculated from the master culture was cultivated at 27°C and 150 rpm in the Nutrient Broth for 24 h, and then sampled.

Separation of cultivated bacteria and extraction of EPS

In order to remove the culture solution and soluble microbial products (SMP), a washing step, including centrifugation at 7,000 rpm for 10 min, was performed three times on the cultivated *Pseudomonas fluorescens,* using pH 7 phosphate buffer containing 10 mM Na_2HPO_4 and 10 mM NaH_2PO_4 in order to emphatically remove the remaining medium and SMP.

After the washing step, EPS extraction was performed using a cation exchange resin (CER) (Dowex® Marathon® C, Na^+ form, Sigma-Aldrich, USA) extraction method (Frølund *et al.*, 1996). The CER was washed for 10 min in phosphate buffer prior to use. A 200-mL sample was transferred to a beaker, with the addition of the exchange resin (75 g of CER/g VSS) to facilitate the extraction. The CER and sample mixture was stirred at 600 rpm, using a paddle for 2 h at 4°C. After the reaction between CER and sample was complete, the mixture was centrifuged for 15 min at 12,000 g in order to remove the bacteria and CER.

Bacteria adhesion test

The experiment set-ups used for investigating the adhesion can be classified as either dynamic or static. The static and the dynamic types are defined by whether the membrane is either fixed or not fixed in a stream of fluid, respectively (Figure 1). In this study, the experiment to investigate the effects of EPS on the bacterial adhesion to the membrane surface was performed using a bench-scale cross-flow filtration system, with a flat sheet-type membrane (Figure 2). Prior to the adhesion test, the resistance of the membrane was measured using DI-water with a conductivity of 18.2 MΩ. The cross-flow velocity and pressure used in all the experiments were 126.3 cm/sec and 0.03 MPa, respectively.

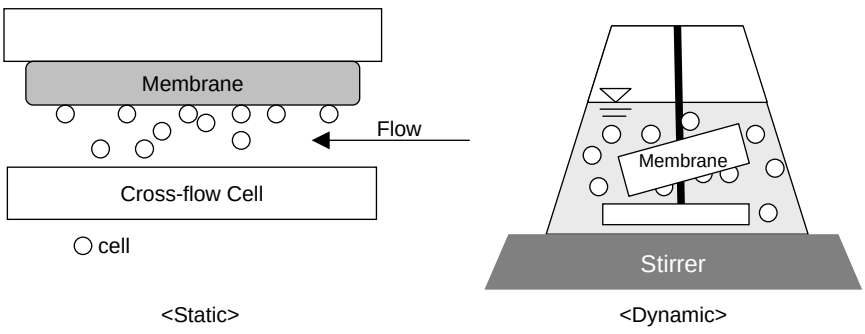

Figure 1 Type of adhesion test

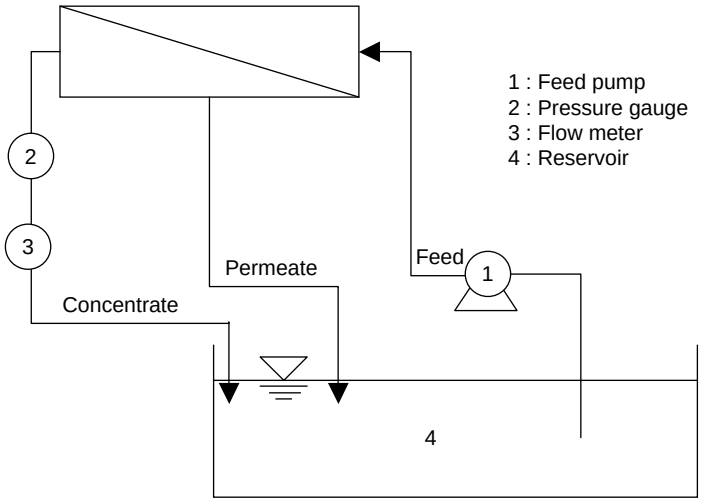

Figure 2 Cross-flow unit for the adhesion test

For adhesion of the bacteria to the membrane, the experiment was conducted without permeate during 2 h. To prevent the production of SMP and removal of the bioactivity, the feed solution was kept on an ice bed. Following adhesion to the membrane, the bacteria remaining in the filtration unit were washed using 1 L of DI-water, under the same conditions as for the adhesion period. The increased resistance due to the bacterial adhesion was measured using DI-water.

After the adhesion process, the degree of bacterial adhesion was calculated in terms of the specific cake resistance. The membrane used in this study was made of polyvinyl difluoride, with a pore size of 0.3 μm (GE Osmonics, Inc., USA).

Analysis

The mixed liquor volatile suspended solids (MLVSS) of the washed culture were measured using glass fibre filtration, following the Standard Method (APHA, 1998). The hydrophobicity of the membrane, and the bacteria on the membrane, were evaluated using water contact angle measurements (CAMs). The membrane with the deposited cake was dried and then transferred to the contact angle measurement system. A drop of DI-water was placed on the cake, using a micrometer equipped with a stainless steel needle. A video image system was used to view the sessile drop from above. The drop shape was captured after 5 sec, and the image of sessile drops used to estimate the contact angle values. The COD and TOC in the supernatant, representing the EPS concentration per cell mass, were analysed using a COD kit (Humas, Korea; Low range COD (15–150 mg/L COD): reaction digestion method) and TOC analyser (Sievers 820, USA), respectively.

Results and discussion

Effect of Ca^{2+} on EPS production

In this study, the production of EPS was artificially controlled by the addition of Ca^{2+}. In activated sludge, the presence of divalent cations tends to increase complex with EPS, which also promotes the aggregation of bacterial cells and the formation of floc and biofilms (Wingender *et al.*, 1999; Liu and Fang, 2003). Growth curves were constructed in relation to the optical density (OD) to identify the effects of Ca^{2+} on the growth of *Pseudomonas fluorescens*. Despite increasing the additional Ca^{2+} concentration, the results of the growth of *Pseudomonas fluorescens* showed similar tendencies (data not shown).

Figure 3 shows the EPS concentrations as various Ca^{2+} additions. The EPS concentration per cell mass was calculated as the TOC and COD on the addition of 10, 50 and 100 mM Ca^{2+}. On increasing the addition of Ca^{2+}, the EPS production per bacterial mass gradually increased in terms of the TOC; the COD concentration also increased with Ca^{2+} addition.

The particle size distribution was measured to identify the effects of Ca^{2+} on the flocculation. As shown in Figure 4, the particle size distribution exhibited the two maxima. The peak near 1 μm would be due to the non-aggregated bacteria, with the peak above 1 μm representing the aggregated bacteria. The volume fraction of bacteria in relation to size was divided into aggregated (larger than 3 μm) and non-aggregated bacteria (smaller than 3 μm). Because *Pseudomonas fluorescens* has a mean size of 2 μm, and due to the result obtained for the particle size distribution near 3 μm, with the particle size distribution exhibiting a minimum between the two peaks, the division of aggregated and non-aggregated bacteria was set at 3 μm. As shown in Figure 3, the volume fraction of the aggregated bacteria increased with increasing EPS production.

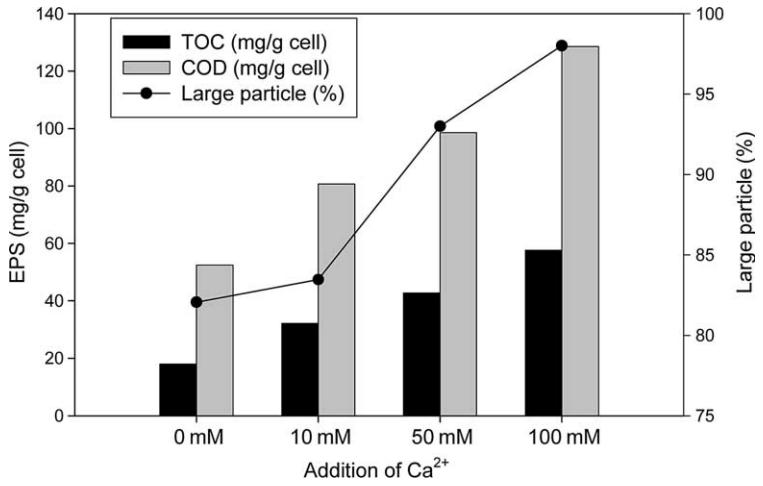

Figure 3 Variation in the EPS relation to the addition of Ca^{2+} and the fraction of large particles with a diameter greater than 3 μm

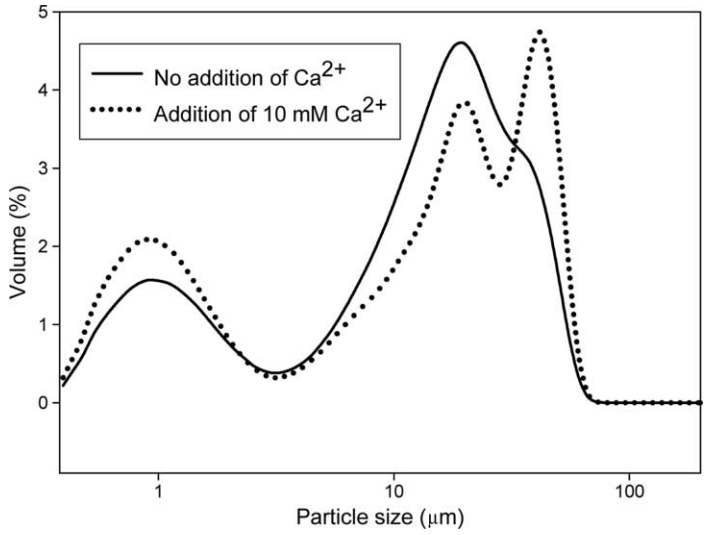

Figure 4 Particle size distributions of bacteria with and without Ca^{2+}

Sludge flocculation generally increases in relation to the hydrophobicity of cells and flocs (Liu and Fang, 2003). The addition of Ca^{2+} affected the flocculation of bacteria, as shown by the results of the particle size distribution. In order to verify whether the addition of Ca^{2+} affected the hydrophobicity of *Pseudomonas fluorescens*, the contact angle was measured. With accumulation of bacteria on the membrane, decreases in the contact angle were observed but with no significant differences between the contact angles result on the addition of Ca^{2+}, although the amounts of total EPS, as TOC and COD, were increased. Also, no significant relationship was found in this study between the hydrophobicity and flocculation of *Pseudomonas fluorescens* on the addition of Ca^{2+}. The addition of Ca^{2+} to *Pseudomonas fluorescens* was assumed to promote flocculation binding with EPS, but not affect the hydrophobicity.

Effect of EPS on bacterial adhesion to the membrane surface

The submerged MBR needs higher aeration intensity than that required for conventional activated sludge to enhance the turbulence and sweeping effects to reduce the formation of a cake layer on the membrane (Stephenson *et al.*, 2000). Prior to the formation of a cake layer on the membrane, the adhesion or adsorption of bacteria, and/or soluble matters, such as SMP and colloids, onto the membrane surface and within the membrane pore occur in MBR (Güell *et al.*, 1999).

The adhesion of bacteria to the membrane surface was affected by many factors including bacterial characteristics, the chemical and physical nature of the target material surface, and factors relating to the bacterial suspension medium including the physical conditions of the medium and the presence of the EPS and SMP. The EPS that always exists around bacteria, which promote flocculation, should be considered as the main factor for the adhesion of bacteria to the membrane surface. Following the adhesion test with a cross-flow filtration unit, the effects of EPS on the adhesion of bacteria to the membrane was investigated using the specific cake resistance.

The results of the adhesion test on the addition of Ca^{2+} are given in Figure 5 using four different feed solutions which have a different EPS concentration per cell mass but the same cell mass. The results revealed that the EPS promotes the adhesion of bacteria to the membrane surface. Because the SMP existing in the system had been removed by washing three times, the EPS bound to the bacteria had a dominant effect on the adhesion

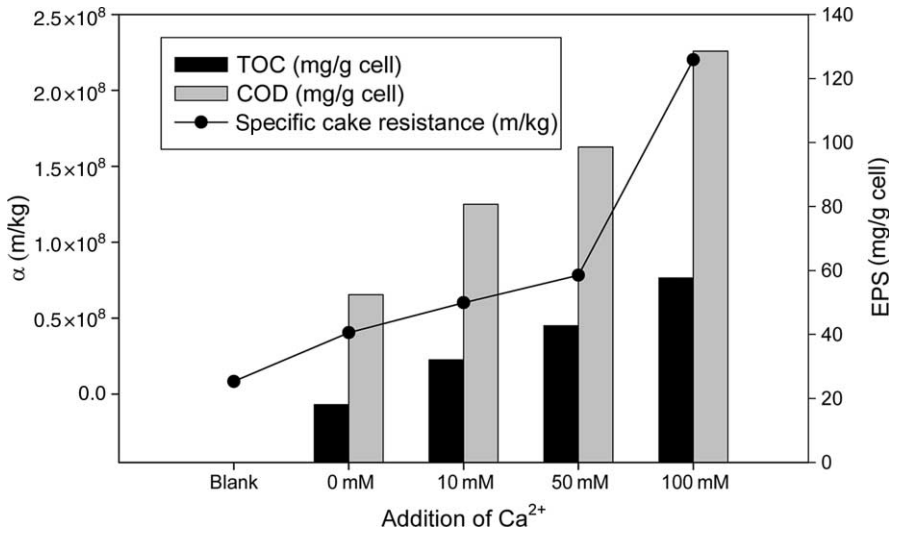

Figure 5 Results of the adhesion test

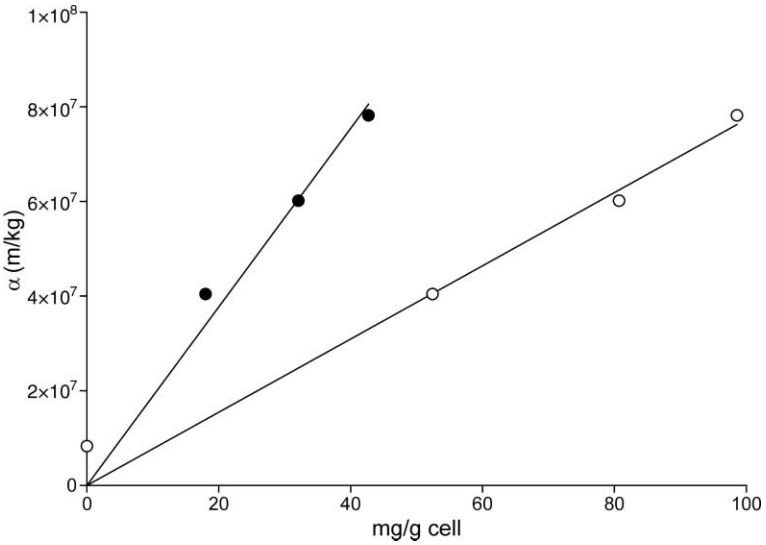

Figure 6 Relationship between the specific cake resistance and EPS concentration per cell mass

between the bacteria and membrane surface. During filtration, the EPS promoted the adhesion of the bacteria onto the membrane surface; thereby, increasing the filtration resistance. That is, increases of bacterial EPS promote the accumulation of bacteria on the membrane surface. Also, the relationship between the specific cake resistances, as calculated from equation (5), and the EPS concentration per cell mass, as the TOC and COD, is shown in Figure 6.

Conclusions

In this study, Ca^{2+} was artificially added to increase the complex of EPS with bacteria. Although the amount of EPS bound with bacteria increased on the addition of Ca^{2+}, there was no significant effect on the bacterial growth. With the particle size distribution results, the curves obtained had two peaks: aggregated and non-aggregated bacteria. The fraction of aggregated bacteria with diameters greater than 3 μm increased on the addition of Ca^{2+}. These results showed that the addition of Ca^{2+} increases flocculation allowing the formation of a complex between the bacteria and EPS. The contact angle results showed no significant differences in hydrophobicity on the addition of Ca^{2+}, even though the flocculation was enhanced. The results of the adhesion experiments, conducted with various EPS levels, indicated that increases of bacterial EPS promoted the adhesion of bacteria on the membrane surface.

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