Research Article

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Application of *Bdellovibrio bacteriovorus* for reducing fouling of membranes used for wastewater treatment

Atıksu arıtımında kullanılan membranların kirlenme probleminin azaltılması için *Bdellovibrio bacteriovorus* uygulaması

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Abstract

**Background:** Membrane bioreactor (MBR) systems used for wastewater treatment (WWT) processes are regarded as clean technologies. Degradation capacity of the predator bacterium, *Bdellovibrio bacteriovorus*, was used as a cleaning strategy for reducing membrane fouling.

**Method:** Wastewater with different sludge age and hydraulic retention time were filtered through Poly(ether)sulphone (PES) membranes using dead end reactor. Change in filtration performance after cleaning of membrane surface by *B. bacteriovorus* was measured by comparison of flux values. Bacterial community of the sludge was determined by 16SrRNA sequence analysis. Community profile of membrane surface was analyzed by fluorescent in situ hybridization technique.

**Results:** After cleaning of MP005 and UP150 membranes with predator bacteria, 4.8 L/m²·h and 2.04 L/m²·h increase in stable flux at steady state condition was obtained as compared to the control, respectively. *Aeromonas*, *Proteus*, and *Alcaligenes* species were found to be dominant members of the sludge. *Bdellovibrio bacteriovorus* lysed pure cultures of the isolated sludge bacteria successfully. FISH analysis of the membrane surface showed that Alfa-proteobacteria are the most numerous bacteria among the biofilm community on the membrane surface.

**Conclusion:** Results suggested that cleaning of MBR membranes with *B. bacteriovorus* has a potential to be used as a biological cleaning method.

**Keywords:** *Bdellovibrio bacteriovorus*; Biofilm; Membrane fouling; Wastewater treatment; Membrane bioreactor (MBR) systems.

Özet

**Genel bilgi:** Atıksu arıtımı için kullanılan membran biyoreaktör (MBR) sistemleri temiz teknolojiler olarak bilinmektedir. Membran yüzeyinde biriken mikroorganizmalar nedeniyle membranın kirlenmesi MBR sistemlerin en önemli problemidir ve membranın kullanım ömrünün kısıtlamasına neden olmaktadır. *Bdellovibrio bacteriovorus* membranın tıkanmasının temel nedeni olan biyofilm komunitelerinin Gram negatif bakterileri yiyerek beslenen avcı bakteridir.

**Amaç:** Bu çalışmada avcı bakteri *B. bacteriovorus* 'un membranın tıkanma probleminin azaltılması amacıyla kullanım potansiyeli araştırılmıştır.

**Yöntem:** Farklı çamur yaşları ve hidrolik bekleme sürelerine sahip atıksular Ölü Üç Reaktör hücresi kullanılarak Polietersülfon (PES) membranlardan filtre edilmişlerdir. *Bdellovibrio bacteriovorus* ile temizleme işleminden sonra filtrasyon performansındaki değişim membran aklarının kryaslanması ile ölçülmüştür. Aktif çamurun bakteri profilini 16SrRNA dizi analizi ile belirlenmiştir. Membran yüzeyindeki biyofilm komunitesi Floresan in Situ Hibridizasyonu tekniği ile analiz edilmiştir.
**Bulgular:** MP005 ve UP150 membranlarının avcı bakteri ile temizlenmesi sonucunda kontrol membranına kıyasla kararlı akıda sırasıyla 4,8 L/m²·s ve 2,04 L/m²·s ortalama artış tespit edilmiştir. Aeromonas, Proteus, ve Alcaligenes türlerinin çamurun baskın üyeleri olduğu görülmüştür. Bdellovibrio bacteriovorus’un izole edilen saf bakteri kültürlerini başarrière bir şekilde parçaladığı gözlemlemiştir. Membran yüzeyinin FISH analizi Alfa-proteobakterilerin membrandaki çoğunlukta olduğunu göstermiştir. Membran yüzeyinin FISH analizi Alfa-proteobakterilerin kültürlerini başarılı bir şekilde parçaladığı gözlemlemiştir. Membran yüzeyinde çoğunlukta olduğunu göstermiştir. Membran yüzeyinin FISH analizi Alfa-proteobakterilerin kültürleri başarılı bir şekilde parçaladığı gözlemlemiştir.

**Tartışma ve Sonuç:** Bdellovibrio bacteriovorus MBR sistemlerin temizilenmesinde biyolojik temizleme ajani olarak kullanım potansiyeline sahiptir. Arıtımların düzenli temizlenmesi sonucunda kontrol membrana kıyasla MP005 ve UP150 membranlarının avcı bakteri ile temizlenmesi sonucunda kontrol membranına kıyasla kararlı akıda sırasıyla 4,8 L/m²·s ve 2,04 L/m²·s ortalama artış tespit edilmiştir. Aeromonas, Proteus, ve Alcaligenes türlerinin çamurun baskın üyeleri olduğu görülmüştür. Bdellovibrio bacteriovorus’un izole edilen saf bakteri kültürlerini başarrière bir şekilde parçaladığı gözlemlemiştir. Membran yüzeyinin FISH analizi Alfa-proteobakterilerin membrandaki çoğunlukta olduğunu göstermiştir. Membran yüzeyinin FISH analizi Alfa-proteobakterilerin kültürleri başarılı bir şekilde parçaladığı gözlemlemiştir.

**Anahtar kelimeler:** Atıksu arıtımı; Bdellovibrio bacteriovorus; Biyofilm; Membran kirilenmesi; Membran Bioreaktör (MBR) Sistemleri.

**Introduction**

Domestic and industrial wastewater discharges and the contaminated rainwater run-off cause significant unfavorable impacts on the environment. There are many different technologies for wastewater treatment (WWT) including physico-chemical and/or microbial ones [1]. Membrane bioreactors (MBRs) are now broadly favorable treatment technologies that combines membrane processes and suspended growth bioreactors [2]. MBR application in WWT is an activated sludge process serving as a replacement for secondary settling tanks. Because of more stringent discharge regulations, water reuse initiatives and decrease in membrane cost, MBRs have gained increasing popularity.

In MBR systems, membrane fouling is one of the most important problems during operation which leads to a frequent cleaning and/or replacement of membranes, and increases operation cost [3]. As soon as the membrane surface contact with wastewater, deposition of particles and bacterial cells occurs onto membrane surface. The microorganism and their products contribute to membrane fouling. As a dynamic process, biofouling occurs as a result of two main mechanisms: colonization of membrane surfaces with microorganisms and production of membrane foulants by microorganisms in the waste water [2]. Although this fouling and biofilm formation on membrane surface enhances the removal of microbes by acting as a secondary barrier, it also reduces the flux and permeability of the membranes [4]. Thus, fouling needs to be kept under control in order to increase the membrane lifetime and decrease the operational costs.

In order to decrease the fouling problem, physical and chemical cleaning methods are frequently used. Back flushing (backwashing), a physical cleaning method, has been applied on fouled membranes in many cases. This method successfully removes most of the reversible fouling due to pore blocking, and partially dislodges loosely attached sludge cake from surface [5]. Respectively, newer treatment method, air flushing (air sparging), is another physical cleaning concept where air is used as a back flushing medium. During the filtration process, deposition of particles and bacterial cells causes forming a slimy gel layer on the membrane surface that cannot be removed easily by physical cleaning methods. Different types of chemical cleaning methods may be recommended on a weekly/monthly/yearly basis to increase the membrane life by removing the irreversible fouling which cannot be removed by physical cleaning methods [6] Chemicals like HCl, HNO3, NaOCl, H2O2, citric acid have been employed on membranes to remove organic and inorganic fouling [7]. Biological control strategies are still at research and development stage. Inhibition of quorum sensing system, nitric oxide-induced biofilm dispersal, enzymatic disruption of extracellular polysaccharides, proteins, and DNA, inhibition of microbial attachment by energy uncoupling, use of cell wall hydrolases, and disruption of biofilm by bacteriophages can be listed under biological ways of microbial attachment control strategies [8].

WWT with MBRs can be regarded as clean technology since it replaces secondary settlement tank. However, harmful chemical agents are introduced to the environment during membrane cleaning process. Therefore, potential of biological ways of membrane cleaning methods should be introduced for the aim of clean environment. Biofilms causing membrane fouling consist of multiple bacterial species including Gram-negative ones. Some previous researches have revealed that Bdellovibrio can consume Escherichia coli prey in simple biofilms, in some cases destroying the biofilms altogether [9]. Bdellovibrio bacteriovorus is a Gram-negative, obligately aerobic bacterium that preys upon a wide variety of other Gram-negative bacteria [9]. Bdellovibrio life cycle consists of an attack phase cell that attaches to other Gram-negative bacteria, penetrates their periplasm, multiplies in the periplasmic space and finally bursts the cell envelope to start the new cycle. Depending on the prey and environment, this life cycle takes roughly 3–4 h. Dashiff et al. [10] showed lysis activity of B. bacteriovorus on biofilms formed by different bacterial species, especially pathogenic ones. Although the main role of B. bacteriovorus in nature is not completely known, there is evidence that these bacteria play a role in the microbial ecology of natural environments, controlling the population size of bacterial ecosystems. It is known that B. bacteriovorus efficiently degrades biofilms formed...
by many Gram-negative bacteria including *Aeromonas*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Proteus*, *Pseudomonas*, *Salmonella* etc. [10]. Most of those bacteria are members of wastewater sludge which contribute plugging of the membrane in MBR system. It is also reported that most of the bacteria colonizing membrane surfaces belong to different Proteobacteria group, which are known to be Gram-negative [11]. Being a Gram-negative predator feeding on other Gram-negative bacteria, *B. bacteriovorus* has great potential to aid in a variety of fields where biofilm formation creates problem.

Up to our knowledge, activity of this predator on biofilm formed by wastewater sludge has not been investigated yet. In this study, potential of the predator bacterium *Bdellovibrio* as a biological cleaning agent for mitigation of membrane fouling and for increasing membrane lifetime was investigated.

### Materials and methods

#### Bacteria and culturing

*Bdellovibrio bacteriovorus* HD100 and *E. coli* S17 were kindly provided by Dr. Liz Sockett from the University of Nottingham (UK). *Bdellovibrio bacteriovorus* were produced in HM buffer which contains *E. coli* cells as prey [12]. HM buffer was prepared as 10 mM HEPES buffer containing 1 mM CaCl₂ and 0.1 mM MgCl₂ as the final concentration and the buffer pH was fixed at 7.2. *Bdellovibrio bacteriovorus* were grown in suspensions of prey at 29°C plates. Wastewater from bench scale activated sludge reactor fed in laboratory was used for biofilm formation. Large solid particles of wastewater sludge was eliminated by precipitation and supernatant part was used as bacterial suspension for biofilm formation. Two hundred and thirty microliter of *E. coli* or wastewater suspension with an OD₆₀₀ of 0.7 was added to the wells and incubated at 30°C for 3 h for biofilm formation. After washing of the wells *B. bacteriovorus* culture was added to the wells and incubated for 3 h at 30°C or 37°C. Wells were washed with tap water and remained biofilms in the wells were stained with 1% crystal violet for 10 min. Crystal violet was removed from the wells, washed twice and biofilm remained in the wells was dissolved in ethanol:acetone solution (70:30). Intensity of violet color was measured at 570 nm wavelength by using plate reader spectrophotometer for determination of biofilm thickness.

#### Degradation of suspended pure cultures of sludge bacteria

*Escherichia coli* and isolated sludge bacteria were streaked on NA and grown for o/n period. They were collected from agar surface and dissolved in HM buffer to obtain a solution of OD₆₀₀ of 0.3–0.4 absorbance. *Bdellovibrio bacteriovorus* was inoculated as 1/10 volume of the bacterial cell culture medium and incubated 30°C at 180 rpm for 3 days. A control medium without *B. bacteriovorus* were also prepared for calculation of % lysis activity of *B. bacteriovorus* on bacterial isolates. % lysis activity was calculated by using equation 1.

\[
\% \text{ lysis by } B. \text{ bacteriovorus} = \frac{(\text{biomass of control})t - (\text{biomass of culture with } B. \text{ bacteriovorus})t}{(\text{biomass of control})t} \times 100
\]

at 180 rpm. As number of prey cells decreased, absorbance at OD₆₀₀ decreased from 0.3 (6.6 × 10⁴ cfu/mL) to 0.08 (3 × 10⁵ cfu/mL) at the end of 3 days of incubation. The co-culture was filtered for removal of prey cell debris and then used for biofilm removal.

#### Lysis capability of *Bdellovibrio bacteriovorus* on wastewater bacteria

#### Degradation of biofilm formed by wastewater sludge

The effect of *B. bacteriovorus* on biofilms formed by *E. coli* or wastewater bacteria was analyzed in 96-well polystyrene plates. Wastewater from bench scale activated sludge reactor fed in laboratory was used for biofilm formation. Cleaning effect of *B. bacteriovorus* on plugged membranes used in dead-end reactor was tested by using the micro filtration Poly(ether)sulphone (PES) membrane (with pore size of 0.05 mm). Secondary wastewater from a domestic WWT plant was fed every day in aerated 10 L batch reactor with synthetic wastewater (for final concentrations of ingredients in mg/L: glucose, 650; peptone, 50; urea, 100;
(NH₄)₂SO₄, 50; KH₂PO₄, 50; K₂HPO₄, 5; NaHCO₃, 100. Trace minerals were also included). This wastewater had MLSS around 3000 mg/L and particle size of 80–95 μm. Feeding period was adjusted in order to obtain activated sludge with different sludge retention time (SRT) (20 days or 10 days) and hydraulic retention time (HRT) (48 h or 96 h). Properties of wastewater used in this study are listed in Table 1.

A dead-end stirred cell setup was used to study the membrane filtration performance (Figure 1A). This system is useful for speeding up the filtration by applying pressure and resulting in shorter process period. The system consisted of a filtration cell (model HP4750 Stirred Cell, USA) with a total cell volume of 250 mL and an effective membrane area of 14.6 cm². The membrane was placed on rigid sponge part of the dead end cell. The cell was fitted with a pressure gauge. Pressurized nitrogen gas (at 1.5 bar) was used to force the wastewater through the membrane. The wastewater in the cell was stirred at the rate of 500 rpm. The permeation flux was determined from the amount of permeate collected through the membranes per unit time using equation 2.

\[ J = \frac{Q}{A \times \Delta t} \]  

where Q is the quantity (L) of the permeate sample collected over a period of time (Δt, h); A is the effective membrane area (m²).

In each set of experiment, two membranes were used. One of the membranes was used as control and treated with buffer after each filtration, and second membrane was used as experiment membrane and treated with \( B. \) bacteriovorus culture for 3 h at 180 rpm at 30°C by insertion of the membrane into 20 mL \( B. \) bacteriovorus cell solution. Then, membranes were placed in dead end reactor cell again for the next filtration. Membranes were used for five to seven filtrations and after each filtration experiment membrane was cleaned with \( B. \) bacteriovorus. Operational steps are seen in Figure 1B. Improvement in stable flux through membranes were compared by calculating \( \Delta J_s \) as shown in equation 3.

\[ \Delta J_s = J_{n-1} - J_{n} \]  

Stable flux value (\( J_s \)) is mean value of the flux values obtained at last 30 min of the filtration where the flux through membrane is stabilized. \( \Delta J_s \) = difference between stable flux obtained at 1st filtration and nth filtration of an individual membrane. More positive (or less negative) value for \( \Delta J_s \) was regarded as plugging of the membrane at lower level. \( \Delta J_s \) vs. \( V \) (total volume of filtrate) for each filtration was plotted for treated and non-treated (control) membranes and slopes were analyzed.

### Table 1: Properties of wastewaters used for dead-end filtration experiments.

| Wastewater from | MLSS (mg/L) | Particle size (μm) | HRT (h) | SRT (days) | Hydrophobicity (%) and EPS protein (mg/L) |
|----------------|-------------|-------------------|--------|-----------|-----------------------------------|
| 1-Aerated batch reactor | 3000 | 80–95 | 48 | 20 | – |
| 2-Aerated batch reactor | 2900 | 80–95 | 48 | 10 | 33 ± 6.3% 15.73 mg/L |
| 3-Aerated batch reactor | 1600 | 80–95 | 96 | 20 | 48 ± 8.1% 25.68 mg/L |

MLSS, mixed liquor suspended solids; HRT, hydraulic retention time; SRT, solid retention time; EPS, extracellular polymeric substances.

**Figure 1:** Experimental set-up for filtration using dead end cell (A) and operation steps for treatment of membrane with Bdellovibrio cell solution (B).

Wastewater in the Dead end cell is filtered through membrane with the applied pressure. Membrane is then cleaned with \( B. \) bacteriovorus cells for 3 h between each filtration.

**Determination of bacterial community of the sludge**

Wastewater taken from domestic WWT plant was serially diluted in saline solution and 100 μL from diluted
samples were spread on nutrient agar (NA) plates. After incubation at 30°C for 24 h, colonies appeared on nutrient agar were picked and streaked on new NA. Pure cultures were obtained and they were used genomic DNA isolation. Universal primers were used for amplification of 16SrDNA. The deduced nucleotide sequence of 16SrDNA (performed by Refgen, Turkey) was compared with the National Center for Biotechnology Information (NCBI) database using the BLAST search available through the center’s website (http://www.ncbi.nlm.nih.gov/BLAST).

Fluorescent in situ hybridization for community profile analysis of membrane surface biofilm

The FISH analyses were carried out with the membranes used for wastewater filtration experiments in dead-end cell as described in Section “Fluorescent in situ hybridization for community profile analysis of membrane surface biofilm”. Two hundred and fifty microliter wastewater was filtered through the membranes at 1.5 bar pressure in dead-end cell, and the membranes were used for bacterial community profile analysis. After removing cake layer on the membranes, they were incubated in 4% paraformaldehyde solution for 30 min. Following washing with PBS solution, fixed cells on the membrane were transferred on the surface of gelatin coated slides for 3 min. Cells stuck on the slides were dehydrated with 50%, 80% and 95% of alcohol solutions for 3 min each. After drying of the dehydrated slides, cells were hybridized with dropping of 10 μL 50 μM DNA probes separately and were incubated in dark at 4°C for 16 h. Unbound oligonucleotides were removed by rinsing with washing buffer [0.9 M NaCl, 20 mM Tris/HCl (pH 7.4), 0.01% SDS]. Slides were subsequently incubated at 46°C for 30 min with washing buffer and rinsed with distilled water. Cells were analyzed with an Accu-Scope Inc. Microscope (Sea Cliff, NY, USA) and Unitron 67xSC67 camera fitted for epifluorescence microscopy. For each sample and oligonucleotide probe used, around 1000 cells obtained from five independent, randomly chosen microscopic fields were microscopically enumerated at a 20-fold magnification. Oligodeoxynucleotide probes 5’-labelled with Cy3 were purchased from Iontek (Turkey). The oligonucleotide probes, their sequences and target-organisms are listed in Table 2.

% hydrophobicity = \frac{O.D \ (Initial \ bacterial \ suspension) - O.D \ (aqueous \ phase)}{O.D \ of \ initial \ bacterial \ suspension} \times 100 \tag{4}

Scanning electron microscopy analysis of membrane surface

After the membranes were used for filtration of activated sludge in dead-end system (at least seven series of filtration was performed with 300 mL working volume dead-end reactor system), cake layer was removed from the surface of the membranes and they were washed with water and kept in 4% glutraldehyde solution at 4°C until analysis. The membranes were put into a Petri plate together with a small piece of wetted cotton to provide a humid environment during drying. Microorganisms on the surface of the membranes were examined with scanning electron microscope (TUBİTAK MAM, Turkey).

Determination of sludge parameters

MLSS concentration of wastewater was measured according to standard methods. For determination of extracellular polymeric substances (EPS) concentrations for proteins, sludge samples were fractionated in water phase and biomass by centrifugation. EPS in the biomass phase were then analyzed for proteins through photometric method according to Lowry et al. [17]. Microbial adhesion to hydrocarbon (MATH) test [18] was applied for determination of microbial hydrophobicity. The percent hydrophobicity was calculated by using equation 4.

Results and discussion

Microbial attachment-based biotechnologies have been widely applied for WWT. However, in other situations, attached microorganisms on membrane, biomaterials, ship hulls, water pipes, marine constructions, etc. are unwanted and would cause serious and costly disturbances [8]. In despite of environmental concerns, chemical cleaning strategies for biofilm removal have been widely applied. As in all other areas of technology, cleaner biological biofilm removal strategies are trying to be developed for MBRs.  

Up to now B. bacteriovorus biofilm degradation capacity has been analysed for many different Gram-negative bacteria. In this study, B. bacteriovorus activity was investigated to introduce its potential to be used as biological
cleaning strategy for fouled membranes. Its lysis capacity was first tested on biofilm formed by wastewater sludge in 96-well plates. Table 3 shows % lysis of sludge biofilm by *B. bacteriovorus* at 30 and 37 °C. *Bdellovibrio bacteriovorus* degraded *E. coli* biofilms by 67% (data not shown). *Bdellovibrio bacteriovorus* showed higher activity at 30 °C, and 18% decrease was observed in the thickness of biofilm in the wells after *B. bacteriovorus* treatment at that temperature. Being a member of a biofilm community brings some advantages to the bacteria, one of which is resistance to predators. Wastewater sludge contains numerous types of microbial species which would form a more difficult biofilm as compared to the biofilm formed by single bacterial species. In this study, it was showed that *B. bacteriovorus* successfully degrades the wastewater sludge biofilm, underlining its potential to be used as an agent for reducing undesired biofilm occurring in various water treatment applications. Time period for biofilm cleaning by *Bdellovibrio* cells is critical, since longer cleaning period results in formation of extra biofilm by *Bdellovibrio* itself and thickening of the biofilm (data not shown).

During WWT using MBR system, an incompressible biofilm layer form on the surface of the membrane because of the high filtration rate through the membrane. For the investigation of performance of *B. bacteriovorus* on mitigation of membrane fouling, PES membranes with pore size of 0.05 μm (MP005) and ultrafiltration membrane (UP150) were used in this study. The experiments were performed with Dead end reactor system operated by using wastewater with different characteristics listed in Table 1. Membranes were used at least for seven filtration with duration of 1–2 h each. After each filtration, the membrane was cleaned with *B. bacteriovorus* for 3 h as illustrated in Figure 1B. After cleaning step, membranes were used for next filtration. Figure 2 shows results obtained with PES M005 and PES UP150 membranes. Stable flux through these membranes cleaned with *B. bacteriovorus* after each filtration was found to be higher than the stable flux through the control membranes. Slope lines of ΔJs (Jsns–Js1) values was added to the graphs for differentiating trend of ΔJs of experiment and control membranes. Steeper slope for *Bdellovibrio* treated membrane shows improvement in membrane filtration as compared to the non-treated control membrane. Cleaning effect of predator bacteria was more distinct when sludge with SRT of 10 days and HRT of 2 days was used for filtration (Figure 2A). 4.8 L/m²·h and 2.04 L/m²·h increase in stable flux as compared to control was obtained when the MP005 and UP150 membrane was treated with predator bacteria, respectively. Results suggest that if membrane is cleaned with *B. bacteriovorus* at steady-state condition, improvement in membrane life-time can be obtained and plugging of the membrane would be delayed.

In our study, it was observed that characteristic of the wastewater affects *B. bacteriovorus* performance on cleaning of bacterial layer on the membrane surface. When SRT and HRT were increased to 20 days and 4 days, respectively, sludge hydrophobicity and proteinous EPS values increased. In this condition, sludge bacteria age because of low food/microorganism ratio. Slope difference between ΔJs values of treated and control membranes for the sludge with 20 day SRT (Figure 2B) is lower as compared to that of the sludge with 10 days SRT (Figure 2A, C and D). In other words, *Bdellovibrio* treatment is more successful for the sludge with young microorganisms.

Accumulated bacteria on the membrane surface is not the only factor causing membrane fouling. Organic

**Table 3:** Degradation of biofilm formed by wastewater sludge by *B. bacteriovorus* in 96 well plates.

|                          | Absorbance at 570 nm (biofilm thickness) 30°C | Absorbance at 570 nm (biofilm thickness) 37°C | % Activity at 30°C | % Activity at 37°C |
|--------------------------|-----------------------------------------------|-----------------------------------------------|-------------------|-------------------|
| *Bdellovibrio* treated biofilm | 0.807 ± 0.03                                 | 0.885 ± 0.01                                  | 18.23             | 6.4               |
| Control (buffer treated biofilm) | 0.987 ± 0.04                                 | 0.946 ± 0.02                                  |                   |                   |

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and inorganic residues adhering the membrane has an important effect on the formation of biofilm layer. Fouling mechanism depends on many other factors including membrane type and is affected by sludge characteristics such as floc size distribution, dissolved inorganic compounds, soluble microbial products (SMP) and extracellular polymeric substrate concentration (EPS) [2, 18–22].

Moreover, bacterial adhesion governed by Van der Waals, electrostatic and acid–base interactions is crucial in the biofilm process. These interactions depend on the physicochemical properties of substratum and the bacterial surface such as hydrophobicity, surface charge and electron donor–electron acceptor (acid–base) properties [19–22]. In the present study, we observed effect of sludge age on hydrophobicity and proteinous EPS, which are important factors determining the characteristics of biofilm.

Performance of *Bdellovibrio* as cleaning agent for membranes was previously investigated [23]. In that study, *E. coli* cell culture was used instead of real wastewater sludge, and *B. bacteriovorus* was inoculated in *E. coli* cell culture and the filtration performance in Dead end system was measured. They showed that flux through membrane was increased at high predator to prey cell ratio. In a similar study [24], bacteriophage activity as biological cleaning method for membranes was tested. The activity of bacteriophages was analysed on *Pseudomonas aeruginosa*, *Acinetobacter johnsonii* and *Bacillus subtilis* which were inoculated into the sterilized wastewater. Forty percent increase in the flow rate in the presence of bacteriophage was reported. Viruses, however, have narrow host range and can lyse only specific type of bacterial species. Advantage of *Bdellovibrio* as cleaning strategy is its ability to lyse almost all type of Gram-negative
bacterial species. Activity of a predator bacterium or a bacteriophage would be different in a solution containing a pure bacterial species and in a complex wastewater sludge. Harsh conditions in wastewater sludge may not be suitable for Bdellovibrio activity. Because of that, in this study, we cleaned the membranes with *B. bacteriovorus* out of the MBR tank. This strategy was thought to be more solution oriented because it is focusing on to the dynamic membrane, without harming the sludge community that is necessary for reducing BOD. Increase in the membrane filtration performance after treatment shows potential of these predator bacteria for increasing membrane lifetime. Better performance on membrane cleaning can be obtained by increasing the number of predator/prey ratio as suggested by [23].

Figure 3 shows SEM micrographs of microorganisms on membrane surface after filtration of wastewater. After about 10–14 h filtration in total, membrane surface was not fully occupied by a bacterial biofilm. Individual bacterial cells seen on membrane surface can be regarded as the pioneers of biofilm formation. Degradation of these pioneers by *B. bacteriovorus* may cause an improvement in the stable flux of the membrane. Number of bacterial cells present on the surface of the membrane cleaned with *B. bacteriovorus* (Figure 3A) was lower as compared to control (Figure 3B).

Bacterial community profile of wastewater used in this study was investigated by isolation and genetic identification of bacterial species. Cultured bacterial species isolated from domestic wastewater sludge are listed in Table 4. According to 16SrRNA analysis, most of the cultivable bacteria isolated from the aerated batch reactor were found to be Aeromonas species. The lysis activity of *B. bacteriovorus* on those individual species of wastewater bacteria is seen in Figure 4. *Bdellovibrio bacteriovorus* showed high lysis activity for the isolates showing 99% identity to *Aeromonas hydrophila* and *A. veronii*, *Proteus vulgaris* and *P. penerri* and *Alcaligenes feacalis* and *A. aquaticus*.

Microbial interactions and activities in wastewater and also on membrane surface should be well understood for developing novel solutions for membrane fouling problem. Microorganisms and their organic products are the main cause of membrane fouling. Early pioneers colonizing the surface of the membrane provide suitable conditions that allow for biofilm formation to progress during the continuous operation of the MBR system [9]. Type of pioneers adhering membrane surface show differences in many studies. This difference may be caused by characteristics of wastewater used in the study. In general, Gram-negative bacteria seems to play important role as being pioneers for starting biofilm formation on membrane surfaces. According to Jinhua et al. [26], Gamma-proteobacteria more selectively adhere and grow on the membrane surface than other microorganisms. Miura et al. [27] reported that the biofilm formation induced the membrane fouling and the microbial communities on membrane surfaces were quite different from those in the planktonic biomass in the mixed liquor. According to their findings, Beta-proteobacteria probably played a major role in development of the mature biofilms, which led to the severe irreversible membrane biofouling. In the study of Zhang et al. [11], it was observed that mostly Alfa and Gamma proteobacteria colonized on membrane surface. In our study, results obtained in FISH analysis of membrane surface showed that most of the bacteria sticking on the membrane surface belong to Alfa-proteobacteria. Number of bacterial clusters in the biofilm community in
decreasing order was found to be as follows: Alfa-proteobacteria > Gamma-proteobacteria > Nitrosomonas > Nitrobacter > Aeromonas. As shown in Table 5 number of Alfa-proteobacteria in the biofilm was successfully reduced by \textit{B. bacteriovorus} activity.

Results suggested that cleaning of MBR membranes with \textit{B. bacteriovorus} species has a potential to be used as a biological cleaning method. This strategy can be integrated to chemical cleaning methods in order to decrease the amount of chemical agents used for membrane cleaning.

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\begin{table}[h]
\centering
\caption{Amount of bacterial clusters/genus on Bdellovibrio treated and non treated membranes with respect to FISH.}
\label{tab:5}
\begin{tabular}{|c|c|c|}
\hline
Clusters/Genus & Bdellovibrio treated membrane surface & Untreated membrane surface (control) \\
\hline
Aeromonas & ■■ & ■■■■■\tabularnewline
Gamma-proteobacteria & ■■■■■ & ■■■■■■■\tabularnewline
Nitrosomonas & ■■■■ & ■■■■■■■\tabularnewline
Nitrobacter & ■■■■ & ■■■■•\tabularnewline
Alfa-proteobacteria & ■■■■■ & ■■■■■■■\tabularnewline
\hline
\end{tabular}
\end{table}

\textit{BAC and AC coded bacteria were isolated from aerated batch reactor and characterized in this study. NAC coded bacteria were isolated from jetloop reactor sludge operated for denitrification process [25].}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4}
\caption{Activity of \textit{B. bacteriovorus} on suspended pure cultures of bacteria isolated from wastewater sludge.}
\end{figure}

\begin{table}[h]
\centering
\caption{Microbial species isolated from sludge according to 16S rDNA sequence analysis.}
\label{tab:4}
\begin{tabular}{|c|c|}
\hline
Isolate codes & Genus or species with respect to 16SrRNA sequence analysis (% identity) & Clusters \\
\hline
BAC 1 & Aeromonas Sp. (98%) & γ-proteobacteria \\
BAC 2 & Aeromonas hydrophila, A. veronii (99%) & γ-proteobacteria \\
BAC 3 & A. veronii, A. hydrophila (99%) & γ-proteobacteria \\
BAC 4 & Corynabacterium glutamicum (99%) & Actinobacteria \\
BAC 5 & Aeromonas sp, A. jandei, (99%) & γ-proteobacteria \\
AC 3 & Aeromonas sp, A. jandei, (99%) & γ-proteobacteria \\
AC 5 & Leucobacter aridicolis (99%) & Actinobacteria \\
AC 6 & Bacillus cereus (100%) & Firmicutes \\
AC 8 & Rhodococcus erythropolis (99%) & Actinobacteria \\
AC 9 & Bordatella (99%) & β-proteobacteria \\
NAC 1 & Proteus vulgaris, P. penerri (99%) & γ-proteobacteria \\
NAC 2 & Proteus penerri, P. vulgaris (99%) & γ-proteobacteria \\
NAC 4 & Alcaligenes faecalis (99%) & β-proteobacteria \\
NAC 5 & Alcaligenes faecalis, A. aquaticus (99%) & β-proteobacteria \\
NAC 6 & Staphylococcus hominis (99%) & Firmicutes \\
NAC 7 & Staphylococcus haemolyticus, S. epidermidis (99%) & Firmicutes \\
NAC 8 & Alcaligenes faecalis, A. aquatilis (99%) & β-proteobacteria \\
NAC 9 & Corynabacterium glutamicum (98%) & Actinobacteria \\
\hline
\end{tabular}
\end{table}

\textit{Table 4: Microbial species isolated from sludge according to 16S rDNA sequence analysis.}
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