Membrane Water Treatment, *Vol. 9, No. 3 (2018) 195-203*
DOI: https://doi.org/10.12989/mwt.2018.9.3.195

Effects of N-acetylcysteine on biofilm formation by MBR sludge

WonJung Song, Harshad Lade, YoungJae Yu and JiHyang Kweon*

Department of Environmental Engineering, Konkuk University, 120 Neungdong-ro, Gwangjin-gu, Seoul 05029, Republic of Korea

(Received January 9, 2018, Revised April 29, 2018, Accepted May 2, 2018)

Abstract. N-acetylcysteine (NAC) has been widely used as an initial mucolytic agent and is generally used as an antioxidant to help alleviate various inflammatory symptoms. NAC reduces bacterial extracellular polymeric substances (EPS) production, bacterial adhesion to the surface and strength of mature biofilm. The efficacy has been shown to inhibit proliferation of gram-positive and gram-negative bacteria. In membrane bioreactor (MBR) processes, which contain a variety of gram negative bacteria, biofilm formation has become a serious problem in stable operation. In this study, use of NAC as an inhibitor of biofilm contamination was investigated using the center for disease control (CDC) reactors with MBR sludge. Biomass reduction was confirmed with CLSM images of membrane surfaces by addition of NAC, which was more efficient as the concentration of NAC was increased to 1.5 mg/mL. NAC addition also showed decreases in EPS concentrations of the preformed biofilm, indicating that NAC was able to degrade EPS in the mature biofilm. NAC addition was also effective to inhibit biofilm formation by MBR sludge, which consisted of various microorganisms in consortia.

Keywords: Extracellular polymeric substance; N-acetyl cysteine; MBR sludge; CDC reactor; biofilm formation

1. Introduction

Membrane bioreactor (MBR) has been applied to various fields such as water treatment, sewage treatment, water reuse and seawater desalination due to high biomass concentration, small site area and high water quality. However, decreases in filtration permeability due to membrane contamination and frequent shutdowns due to membrane cleaning and replacement have become problems in stable MBR operation (Wang *et al*. 2009). As filtration continues, unwanted biofilm occurs on the membrane surface by microorganisms, which are attracted by convective flow through membrane pores. When microorganisms reach the membrane surface and their population increases, microorganisms secrete extracellular polymeric substances (EPS) and form a biofilm. EPS produced by bacteria enhance the attachment of bacteria on the membrane surface and create biofilms, of which the removal becomes more difficult.

The formation of biofilm is related to several mechanisms including quorum sensing between microorganisms. Microbial signal transduction in quorum sensing of gram-negative bacteria occurs when microorganisms produce N-acyl homoserine lactones (AHLs), signaling substances between them, and when the concentration of AHLs increases above a certain level in the reactor, EPS are generated and biofouling occurs on the membrane surface. The EPS are high molecular weight substances secreted by microorganisms and consist of various organic substances such as polysaccharides, proteins, nucleic acids and lipids (Zeng *et al*. 2016). The EPS produced on the solid surface promote the attachment of microorganisms by altering the physicochemical properties of the surface such as charge, hydrophobicity, and roughness. Cell adhesion to the solid surface is inhibited by electrostatic interaction at low EPS concentrations and it is enhanced by polymer interactions at high EPS concentrations (Tsuneda *et al*. 2003). It causes severe reduction in the operating efficiency of the membrane process.

A variety of methods including backwash and chemical cleaning have been studied to seek solutions to solve biofouling problem. Optimization of membrane backwashing including frequency and duration improves cleaning efficiency. The efficiency of backwash depends on the operating factors such as permeate flux and temperature, characteristic of the contaminants, and the backwash flux (Smith *et al*. 2006). Vargas *et al*. (2008) studied the control method of backwashing in the MBR through monitoring of transmembrane pressure (TMP) and flux; backwash was performed either when the flux was reduced below the lowest allowable flux or TMP reached the maximum allowable TMP. The results suggested that the both TMP and flux should be kept below the thresholds during most of the filtration time. However, backwash causes an interruption of the process operation and loss of water production, and additional valves and tanks. Also, the frequency and duration of backwash are often implemented by pre-determined scenarios based on experience or experimentation, which were hard to change between runs of the process while adequate backwash frequency and duration should be determined based on the variations of feed and operational conditions.

Another common anti-fouling strategy is to inject continuously chemical agents or antimicrobial substances into feed water. The efficacy of biofouling control by these

*Corresponding author, Professor
E-mail: jhkweon@konkuk.ac.kr

Copyright © 2018 Techno-Press, Ltd.

ISSN: 2005-8624 (Print), 2092-7037 (Online)

substances is influenced by activity of microorganisms in the system, types and concentrations of the substances. The operational conditions such as frequency of dosing and contact time and characteristics of raw water such as pH and the organic concentration also affect greatly on the efficiencies of the control method (Gogate *et al*. 2007). Chlorine is a widely used chemical cleaning agent to reduce biofouling in MBR. However, most of the polymeric membranes are susceptible to be degraded by chlorine. Chlorine dioxide is an alternative to chlorine because of its germicidal effect, the generation of less harmful by-products and the relatively weak effect on the polymer membrane. However, the main disadvantage is high costs and handling issues because chlorine and chlorine dioxide gas are produced on-site in the field (Saad 1992). Quorum-quenching enzymes and bacteria are also investigated extensively in the past decade to reduce biofouling in MBR. Oh *et al*. (2013) studied the MBR performance by encapsulating quorum quenching bacteria isolated from actual MBR plants into microbial vessels. The increase of TMP was delayed in MBR with the vessel containing the strain. However, they used a single strain and the strains contained in the vessel might be deteriorated due to contamination by microorganisms which could pass through the vessel or death due to the external environment. The inhibitory effects of quorum quenching were probably effective for early stage of biofilm and might be less efficient to mature biofilm.

N-acetylcysteine (NAC) has been widely used initially as a mucolytic agent in the treatment of chronic bronchitis and acetaminophen overdose (Stey *et al*. 2000). NAC is generally used as an antioxidant and helps relieve symptoms caused by various diseases that are aggravated by reactive oxygen species. NAC is a derivative of cysteine with an acetyl group attached to the amino group of cysteine. This compound crosses the cell membrane and replenishes intracellular glutathione (GSH) and protects cells from oxidative stress. NAC breaks disulfide bonds in biofilm and inhibits bacterial biofilm formation. This reduces the bacterial EPS production, which in turn reduces bacterial adhesion to the surface and breaks the mature biofilm (Demonech *et al*. 2017, Perez-Giraldo *et al*. 1997, Marchese *et al*. 2003, Silveira *et al*. 2013). NAC is classified as a non-antibiotic drug and does not have anti-bacterial properties. Parry *et al.* (1977) reported that NAC inhibited the growth of gram-positive and gram-negative bacteria. *P. aeruginosa*, which was more susceptible to infection than other tested microorganisms, were inhibited by NAC. The minimum inhibitory concentration of NAC was in the range of 2-20 μg/mL. Roberts *et al.* (1981) found that 2 to 5 percent of NAC had antibacterial effects against *P. aeruginosa*. The antibacterial effect of NAC was due to the inhibition of use of amino acids or the reaction with bacterial cell proteins by retaining a sulfhydryl group. Zhao *et al*. (2010) investigated the biofilm characteristics using *P. aeruginosa* strains with increasing concentrations of NAC. As the NAC concentration was increased from 0.5 to 5 mg / mL, the total biomass was decreased from 2.79 to 0.23 mg / mL. In addition, the mean thickness and the substratum coverage were also decreased. The surface to volume ratio was increased from 1.39 to 4.47 $\mu m^2/ \mu m^3$. The increased ratio reflected that the environment around microbes needed more surface to obtain nutrients from surroundings. These results suggested that the application of NAC may be effective as a method to reduce biofilm and the EPS generated by microorganisms.

The purpose of this study was to better understand how NAC affects biofilm formation process of microorganisms in consortia. More specifically, we investigated effects of NAC concentrations on biofilm inhibition using MBR sludge. The CDC reactors with polyvinylidene fuloride (PVDF) membranes were operated at three different NAC concentrations. To understand inhibitory effects of NAC on thick and mature biofilm, biofilm was pre-formed on the membranes and NAC was applied in the reactor. The biofilm characteristics were examined with EPS production and image analyses using a confocal laser scanning microscopy. In addition, influence of biomass concentration on inhibition response of MBR sludge was examined with an NAC dose of 1.5 mg/mL.

2. Materials and method

2.1 NAC reagent

N-Acetylcysteine (≥99% purity) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). The molecular weight of NAC was 162.21 g/mol. A powder form of NAC was dissolved in distilled water to make a stock solution with a concentration of 100 mg/mL.

2.2 Operation of CDC reactors with different NAC concentrations

The effect of NAC concentrations on biofilm formation was investigated in a CDC biofilm reactor study (BioSurface Technologies Corp., Bozeman, MT, U.S.A.) using MBR sludge. A microfiltration flat membrane (Merck Millipore, Darmstadt, Germany) with a pore size of 0.22 μm were cut into 1.5 cm x 1.5 cm pieces and affixed to one side of the rod using a double-sided cellophane tape. Then, the rods were placed in the CDC reactor in such a way that the fixed membrane faced inside the reactor, rendering a total surface area of 2.25 cm^2. Six rods were used and two PVDF membrane specimens were attached to one rod.

The MBR sludge was collected from a wastewater treatment plant (Guri, Gyunggi-do, Korea). Four CDC reactors were prepared. At the beginning of the experiments, 500 mL of the sludge was filled in each CDC biofilm reactor. The mixed liquor suspended solid concentrations in the reactor were 5200 mg/L. The quorum sensing signal compounds, i.e., AHLs, were added to the reactor to stimulate biofilm formation. Four types of AHL were used: N-butanoyl-L-homoserine lactone (C4-HSL), N-hexanoyl-L-homoserine lactone (C6-HSL), N-Octanoyl-L-homoserine lactone (C8-HSL) and N-decanoyl-L-homoserine (C10-HSL). The dosage of AHLs was 80 μg/L. The signal compounds were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

A synthetic wastewater was fed to the CDC reactor, which simulated the aquatic condition of the MBR plants in practice. The chemical oxygen demand of the feed water

was 500 mg/L. The feed water was made of glucose, yeast extract, bactopeptone, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4$, $FeCl_3$, $CaCl_2$, $MnSO_4$ and $NaHCO_3$ (Nam *et al*. 2015). The temperature was maintained at 30 ± 0.5°C and a stirrer with a speed of 150 rpm was continuously operated. The synthetic wastewater with AHLs was dosed once a day for 3 days to form biofilm. After 3 days of biofilm formation, NAC was added to the CDC reactors with concentrations of 0.5, 1.0 and 1.5 mg/mL, respectively. The concentrations were determined below the maximum inhibitory concentration found in the previous study (Kappachery *et al*. 2012). A control CDC reactor was run in parallel under the same condition. The characteristics of biofilm were analysed after 24 hours of the NAC addition. The six membrane specimens were used to extract EPS and two membranes were used for the CLSM analysis.

2.3 Operation of CDC reactors with different biomass concentrations

The effect of NAC on different MLSS concentrations was investigated using the CDC reactor followed by the similar way explained in the previous section except the incubation period for the pre-formed biofilm. The 3 days of incubation time for pre-formed biofilm were not applied in these experiments. The MBR sludge from the wastewater treatment plant was collected on the experimental day. The sludge was filled up to the CDC reactor. The synthetic wastewater was fed to make a COD of 500 mg/L. The MLSS concentration of the CDC reactor was 5200 mg/L, which is in the range of the typical MLSS concentration of the MBR operation in Korea. The sludge was diluted with tap water to lower MLSS concentration, which yielded an MLSS concentration of 2500 mg/L. The sludge after two hours of settling and withdrawing supernatant was filled up to the CDC reactor, in which the MLSS concentration was 8200 mg/L. The MLSS concentration of 2500 mg/L was found in the MBR treating reuse wastewater. The MLSS concentration of 8200 mg/L was often found in treatment plants with high strength wastewater. A CDC reactor as a control was also operated in parallel under the same condition. Therefore, three pairs of experiments, i.e., three biomass concentrations and with/without NAC addition were performed separately. The dose of NAC was 1.5 mg/mL for these experiments. After 24 hours of inoculation, biofilm of the membrane surface was analysed.

2.4 Extraction of EPS

The thermal extraction method was applied to extract EPS from the biofilm formed on the membrane surface. The membrane was carefully removed from the CDC rod and the residue was washed with 20 mL of 0.9% NaCl solution. After washing, the membrane was transferred to a conical tube containing 15 mL of 0.9% NaCl solution and vortexed for 5 minutes and sonicated for 60 minutes (B5510, Branson Ultrasonics, U.S.A.). The membrane was then removed from the solution and the solution was again centrifuged (5,000 xg, 20 min, 4°C) to collect the detached biofilm from the membrane surface. After centrifugation, the supernatant was discarded and the same amount of 0.9% NaCl solution was added to re-suspend the biofilm. The re-suspended biofilm solution was heated in a drying oven at 100°C for 60 minutes and was cooled to a room temperature. The centrifugation (5,000 xg, 20 minutes, 4°C) was applied and the supernatant was filtered with a 0.45 μm syringe filter to analyze concentrations of protein and polysaccharide, which are the main composition of EPS.

Proteins were measured by the Bradford method. Since the quantification of protein was measured by absorbance, a standard curve was made using several concentrations of bovine serum albumin (BSA) standards, which were prepared by diluting a stock solution with 2 mg/mL of BSA concentration. Dilution was in steps of 0-10 μg/mL. Each sample and 1 ml of protein dye supplied by The Protein Assay Kit (BR500, Bio-rad, U.S.A.) were placed in a micro cuvette and allowed to stand for 10 minutes. Absorbance was measured at 595 nm. The protein of the sample was calculated from the absorbance data of the standard solutions. A Genesys 10 UV/vis spectrophotometer (Thermo Scientific, U.S.A.) was used for absorbance measurement. The amounts of polysaccharide were measured by a total organic carbon (TOC) analyser (SIEVERS 5310C, GE, Australia) and polysaccharide was quantified by dissolved organic carbon (DOC).

2.5 Confocal laser scanning microscopy (CLSM) observation

The CLSM analysis was performed to observe biofilm on membrane surfaces. After incubation, the membranes were detached from the CDC rod and dried in a cooler (HB-603C, JSR, Korea). The 5mM SYTO9 nucleic acid stain in dimethyl sulfoxide was purchased from Invitrogen (Molecular Probes, Eugene, OR, U.S.A.). The SYTO9 green fluorescent nucleic acid stain has been shown to stain live and dead gram-positive and gram-negative bacteria. After drying, the membranes were placed in small petri dish and 50μL of diluted SYTO 9 dye were put on the membrane surface for 4 times using micropipette (i.e., total 200 μL). The lids of petri dishes were closed and the petri dishes were wrapped with aluminium foil. The membranes were sat for 30 min at room temperature under the dark. The excess stain was carefully washed with deionized sterile water and the membranes were mounted on glass slides (covered with a coverslip). Microscopic observation and image acquisition were performed on the stained membranes using a confocal laser scanning microscopy (LSM 710, ZEISS, Germany).

The biofilm structure was quantified from the confocal stack using an image analysis software COMSTAT2. Five parameters obtained from the software were total biomass, surface to biovolume ratio, average thickness, maximum thickness and roughness coefficient. The biomass is determined by the volume of all voxels containing biomass divided by the area covered by biomass, which gives a value independent of the observed area size. The unit of the biomass is thus $\mu m^3\ \mu m^{-2}$. The voxel is the smallest three-dimensional picture element in the system. The surface to biovolume ratio (SBR) reflects what fraction of the biofilm is in fact exposed to nutrient flow. The surface area in this parameter accounts any enclosed surfaces inside

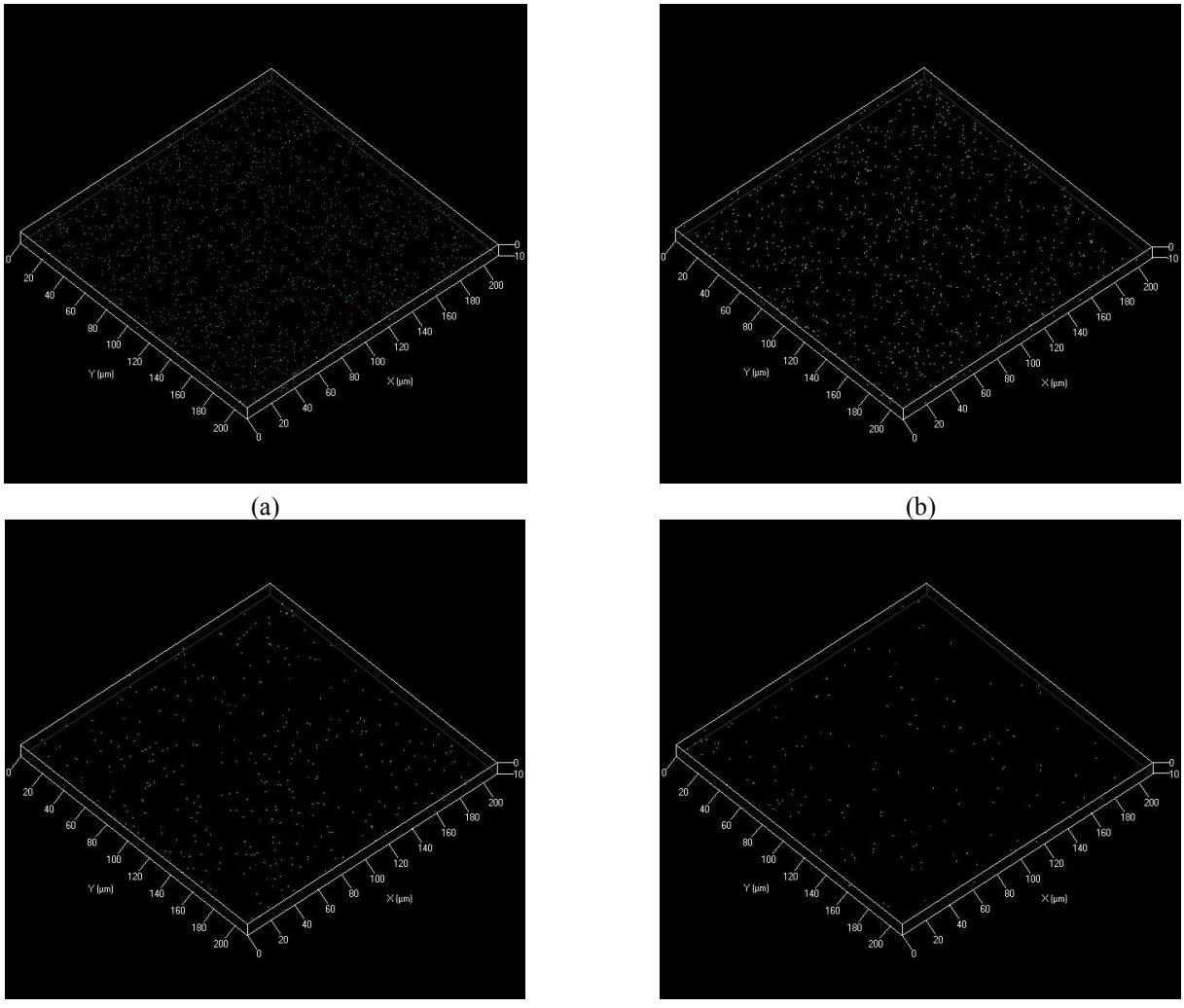


Fig. 1 The CLSM images of membrane surface in contact with different concentrations of NAC. (a) Control, (b) NAC 0.5 mg/mL, (c) NAC 1.0 mg/mL and (d) NAC 1.5 mg/mL. The bacterial cells were stained with SYTO 9, expressed in green, and magnified at 40×.

microcolonies.

For instance, a low nutrient condition leads to an increase in the ratio to improve contact to the limited supply of nutrients. The unit of SBR is μm^2 μm^{-3}. The average and maximum thickness are calculated from thickness distribution of the top image of the biofilm. The roughness, a measure for the variability in the height of the biofilm, was analyzed with individual thickness measurement and average thickness of the measured values.

3. Results and discussion

3.1 Effect of NAC on mature biofilm: CLSM observation

Three different concentrations of NAC, (i.e., 0.5, 1.0, and 1.5 mg/mL) was added to the CDC reactor, in which thick and mature biofilm was formed on PVDF membranes by three days of incubation with MBR sludge. After one day of operation with the designated concentrations of NAC, the PVDF membranes were taken out for CLSM observation. The SYTO 9 staining was performed on the PVDF membrane samples. The biofilm formed on the membrane surface was viewed using the image analysis software COMSTAT2. The three-dimension images of the membrane surfaces are shown in Fig. 1.

The biofilm on the membrane surface was decreased as the concentration of NAC was increased. The CLSM image of the membrane from the control reactor, in which NAC was not added, showed a considerable amount of green fluorescence throughout the membrane surface, which reflected a substantial amount of biofilm formation by the MBR sludge on the PVDF membrane surface (Fig. 1a). However, when NAC was contained, it greatly inhibited biofilm formation on the membrane surface as evidenced by the reduction in intensity of green fluorescence (Fig. 1b, 1c and 1d). The reactor containing 1.5 mg/mL of NAC substantial inhibited biofilm formation, which revealed the little green fluorescence color on the image (Fig. 1d). These CLSM observations indicated that addition of NAC caused strong inhibition of microbial biofilm formation on the PVDF membrane.

The CLSM image of the biofilm formed on the membrane surface was quantified using the COMSTAT2 program. The quantified results of biofilm measurement are shown in Table. 1. The values of total biomass were decreased with increasing NAC doses while other parameters such as average thickness and SBR were insignificantly varied with the increasing NAC doses. The total biomass of biofilm from the membranes of the control reactor was 0.035 $\mu m^3/\mu m^2$. The total biomass was decreased to 0.027, 0.017 and 0.008 $\mu m^3/\mu m^2$, respectively corresponding to the NAC doses of 0.5, 1.0, and 1.5 mg/mL although the average thickness of the biofilm was observed in relatively narrow ranges of 1.148-1.230 μm. The SBR was detected in the range of 18.944 and 19.977 μm. Other parameters such as the maximum thickness and roughness were also showed small changes in the calculated values. Since the dosing of NAC in our study was occurred after three days of inoculation of the MBR sludge, some extents of biofilm formation were already established before the addition of NAC, which probably yielded the similar values of thickness and other parameters. In the COMSTAT2 program, the voxels containing biomass were detected and the heights of the voxels were used to calculate the thickness distribution. In the image of the membrane treated with 1.5 mg/mL of NAC in Fig. 1, there were numerous empty spots and the sparse sites with biomass accumulation. It seems that biofilm at the high dose of NAC was formed with the heights similar to the biofilm with other conditions.

Quah *et al*. (2012) examined inhibitory effects of NAC on biofilm formed by a single strain, *Enterococcus faecalis*. The CLSM analyses of the biofilm cultured on dentin disk by *E. faecalis* revealed that exposure of the strain to 50 mg/mL of NAC noticeably inhibited the biofilm formation, similar to the results from this research, and thus scarcely detected in the CLSM analysis. NAC addition was supposed to destruct biofilm, which was probably due to blocking effects on exopolysaccharide production. Several mechanisms were proposed for NAC to interfere production of EPS, which are one of the major components of biofilm. For an example, sulfhydryl groups in the NAC could destroy disulfide bonds of bacterial enzymes, which are involved in EPS production or excretion through thiol-disulfide exchange. Indirect effects on bacterial cell metabolism and EPS production with antioxidants were also proposed for the inhibitory effects on biofilm by NAC (Quah *et al*. 2012). Muranaka *et al*. (2013) used NAC to inactivate *Xylell fastidiosa*, which caused infection on plants. The infected clusters are characterized by dense mature biofilm. When NAC with a concentration of 1.0 mg/mL was inoculated, small cluster cells were visualized. As the NAC concentration was increased from 2 mg/mL to 6 mg/mL, the dispersed cells only, not clustered cells were remained on the surface. The NAC concentrations higher than 1.0 mg/mL evoked reduction of biofilm formation and total EPS. In addition, the biofilm reduction was occurred because cells attachment was hindered (Muranaka *et al*. 2013). Since the SYTO9 used in our study were stained both live and dead cells, the limited information on the activity of biomass capable of biofilm formation was given by CLSM images. Therefore, another analysis such as EPS production should be investigated for better understanding of the NAC effects on biofilm formation.

Table 1 Effects of NAC concentrations (mg/mL) on biofilm structures on the PVDF membranes

Parameters	control	NAC 0.5	NAC 1.0	NAC 1.5
Total biomass ($\mu m^3\ \mu m^{-2}$)	0.035	0.026	0.017	0.008
Surface to biovolume ratio ($\mu m^2\ \mu m^{-3}$)	19.977	19.857	19.738	18.944
Average thickness (μm)	1.148	1.155	1.166	1.230
Maximum thickness (μm)	2.273	2.273	2.273	3.409
Roughness Coefficient	1.999	2.000	1.999	1.991

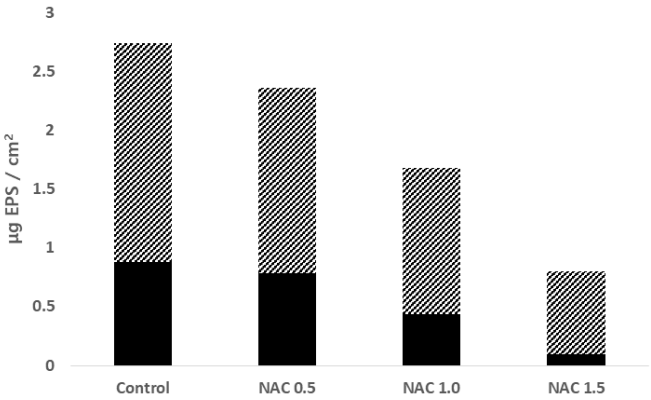


Fig. 2 Effect of NAC on EPS production ▨: polysaccharide ■: protein

3.2 Effects of NAC on mature biofilm: EPS analysis

To understand the effect of NAC on microbial biofilm formation, the MBR sludge was used for the CDC reactor operation. The EPS concentrations of the membrane surfaces were analysed. EPS are one of the main components of biofilm and play a role in establishing and maintaining the structural stability of microorganisms. EPS are a major cause of biofouling in the MBR process, and proteins and polysaccharides are known to be major components of EPS. The amounts of EPS in terms of protein and polysaccharide were monitored after 24 hours of inoculation with the NAC addition.

The results presented in Fig. 2 revealed different EPS production characteristics of the biofilm depending on the doses of NAC. Total EPS concentration of the biofilm from the control reactor was 2.74 $\mu g/cm^2$. The EPS concentrations were decreased to 2.36 $\mu g/cm^2$, 1.68 $\mu g/cm^2$ and 0.80 $\mu g/cm^2$, respectively, as the concentration of NAC was increased to 0.5 mg/mL, 1.0 mg/mL, and 1.5 mg/mL. The decrease in the EPS concentration on the membrane surface was not significant with the NAC concentration of 0.5 mg/mL. As the concentration of NAC was increased to 1.0 mg/mL, the EPS concentration was decreased to 61% of the EPS concentration from the control reactor. Only approximately 30% of the EPS was measured from the biofilm produced on the membranes of the reactor with the NAC concentration of 1.5 mg/mL. The decrease of the total EPS was attributed mostly by the decrease of polysaccharide at the NAC dose of 0.5 mg/mL. Both

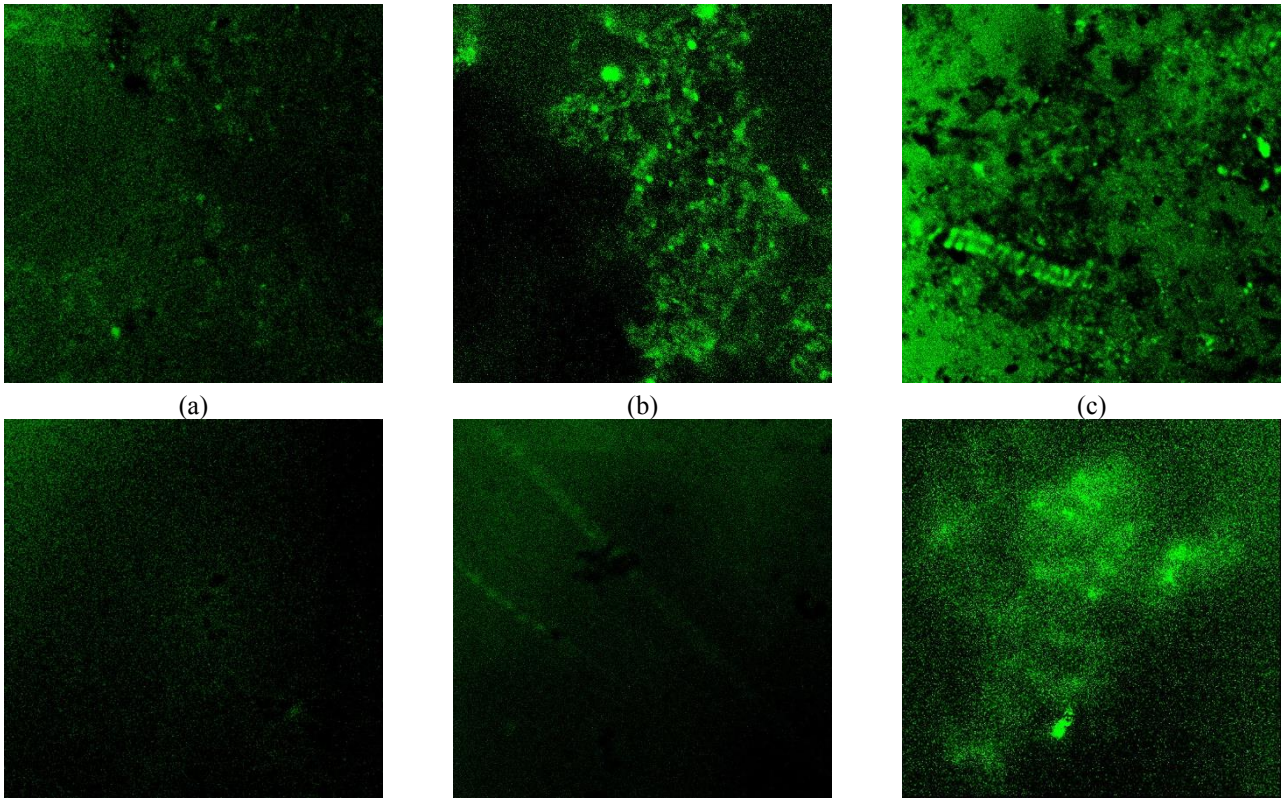


Fig. 3 The CLSM images of biofilm from the reactors w/o (above) or with (below) NAC addition at different MLSS concentrations: (a) and (d) 2500 mg/L, (b) and (e) 5200 mg/L, and (c) and (f) 8200 mg/L

Table 2 Effects of MLSS for inhibitory result of NAC (1.5 mg/mL) on biofilm structures

MLSS (mg/L)	2500		5200		8200	
parameters	Control	NAC	Control	NAC	Control	NAC
Total biomass ($\mu m^3\ \mu m^{-2}$)	0.180	0.290	0.102	0.054	6.066	0.064
Surface to biovolume ratio ($\mu m^2\ \mu m^{-3}$)	9.252	8.912	17.972	19.089	11.436	19.021
Average thickness (μm)	2.701	2.898	1.372	0.926	32.129	2.017
Maximum thickness (μm)	16.060	13.766	9.419	3.829	66.999	5.743
Roughness Coefficient (-)	1.981	1.954	1.958	1.986	0.620	1.997

polysaccharide and protein were decreased at the NAC doses of 1.0 mg/mL and 1.5 mg/mL.

The study by Marchese *et al*. (2003) also evaluated biofilm activity of *Escherichia coli* under NAC exposure. The NAC concentrations used were ranged from 0.007 to 8 mg/mL. The biofilm production by *E. coli* was decreased as the NAC concentrations were increased. The biofilm reduction was directly proportional to the increases in NAC concentration, except for the lowest dose of 0.007 mg/L. The maximal effects were observed at the doses of 4-8 mg/ml and the biofilm formation was reduced by 40-50%, compared with the biofilm without any inhibition by NAC. The authors concluded that rupture of residual biofilms was promoted, in addition to inhibiting synthesis of the biofilms (Marchese *et al*. 2003).

3.3 Application of NAC on different MLSS concentrations

The amounts of biomass in wastewater treatments are expressed as MLSS because heterogeneous microbial communities are involved in removal of organic matter. The MLSS are determined by several factors of operational condition including organic strength of feed, hydraulic retention time, sludge retention time and so on. The high MLSS concentrations indicate in general resilience properties of microbial community, which might yield less effect of NAC on inhibition of biofilm formation than the normal MLSS concentrations. Three different MLSS concentrations, i.e., 2500 mg/L, 5200 mg/L, and 8200 mg/L, were used to evaluate effects of amount and diversity of microbial populations on the inhibitory effects of NAC. The NAC concentration used for these experiments was 1.5 mg/mL. The three control reactors without NAC additions were also operated in parallel with the reactors of the three different MLSS concentrations. The PVDF membranes were exposed to the MBR sludge with or without NAC dose for one day prior to the biofilm characterization. The CLSM image analyses from the observation of the membrane surface of biofilm are shown in the Fig. 3. The images showed that the biofilm from the membranes of the control reactor without NAC addition built a substantial depth of biofilm and layers of the biofilm became thickened with increasing the MLSS concentrations. The amount of biomass presented on the membrane surface of the reactor contacted with the NAC concentrations also showed some

increases, however, the increase was not noteworthy compared to the biofilm of the control reactors.

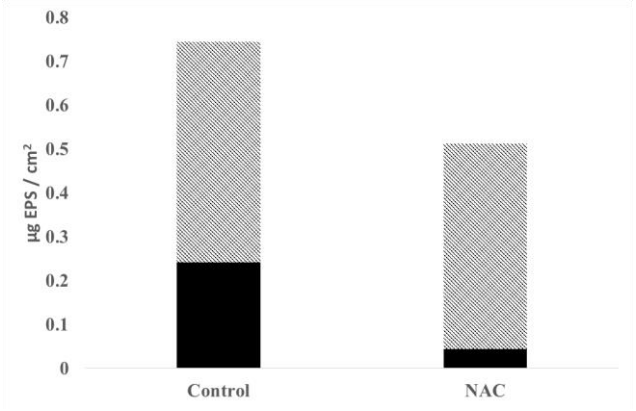
The quantified parameters from the CLSM images by the COMSTAT2 program were presented in Table 2. The significant structural differences were shown at the MLSS concentrations of 5200 mg/L and 8200 mg/L while there was little difference with the MLSS concentration of 2500 mg/L. At the two high MLSS concentrations, the total biomass, average thickness, and maximum thickness of the biomass were all decreased for the biofilms grown in the presence of NAC. The SBR and roughness coefficients showed the reverse trends at the two high MLSS concentrations. For instance, the decrease of the total biomass was substantial at the highest MLSS concentration, i.e., 8200 mg/L, at which the total biomass was decreased from 6.066 μm^3 μm^{-2} to 0.064 μm^3 μm^{-2} with the NAC dose while the total biomass was decreased from 0.102 μm^3 μm^{-2} to 0.054 μm^3 μm^{-2} with the NAC dose at the MLSS concentration of 5200 mg/L. The great structural difference shown in the two MLSS concentrations revealed that NAC would be effective to various microbes with heterogeneity. At the low MLSS concentration, the biofilm formation by overnight incubation was not sufficient enough to distinguish the effects of NAC therefore, the values of the parameters were similar regardless of presence of NAC.

The EPS concentrations were measured from the membranes exposed at the different MLSS concentrations as presented in Figure 4. The total EPS concentrations of biofilm were gradually increased with increasing MLSS concentration. In the case of the reactors without NAC addition, the total EPS concentrations were 0.74 $\mu g/cm^2$ at the MLSS of 2500 mg/L, 1.19 $\mu g/cm^2$ at the MLSS of 5200 mg/L and 1.48 $\mu g/cm^2$ at the MLSS of 8200 mg/L. The extracted EPS was measured as polysaccharide and protein. At the MLSS concentration of 2500 mg/L, the protein and polysaccharide were 0.24 $\mu g/cm^2$ and 0.50 $\mu g/cm^2$. The polysaccharide fraction was 68% of the EPS of the sample.

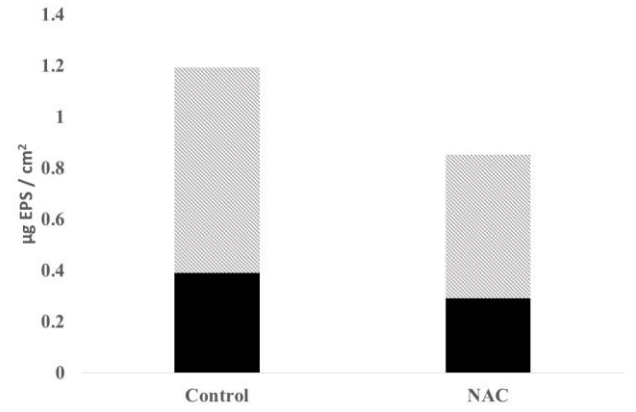
The EPS of the biofilm formed at the MLSS concentration of 5200 mg/L also showed a very similar composition like 0.39 $\mu g/cm^2$ of protein and 0.80 $\mu g/cm^2$ of polysaccharide. The experiments conducted with a MLSS concentration of 8200 mg/L showed that protein was 0.44 $\mu g/cm^2$ and polysaccharide was 1.04 $\mu g/cm^2$. The compositional fraction of polysaccharide in the EPS was in the range of 67 and 70% in this study.

A study on effects of sludge characteristics on MBR removal efficiency conducted by Massé *et al*. (2006) showed that the compositional ratio of EPS between polysaccharide and protein was approximately 76%. However, another study using MBR process showed that the polysaccharide fraction was 89% under the sludge retention time of 40 days (Al-Halbouni *et al*. 2008). In an MBR-treated landfill leachate treatment, the protein on the membrane surface where fouling occurred was about 5 times higher than the polysaccharide (Wang *et al*. 2016). In the study by Harshad *et al*. (2015), effect of quorum sensing inhibition on biofilm formation was investigated and proteins were present with 38-50% of total EPS. The compositional ratio of polysaccharide in the EPS varied from 50% to 89% depending on the operational conditions.

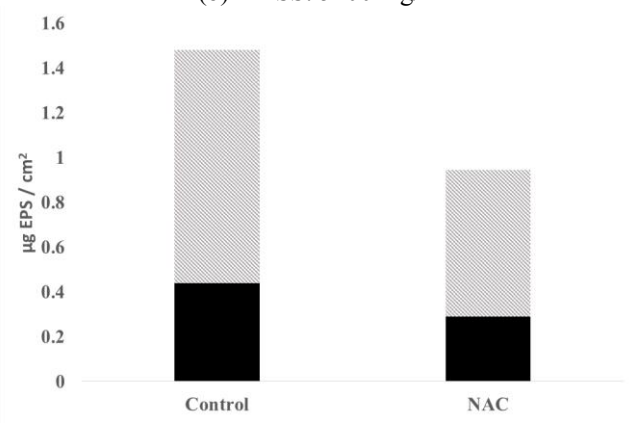
The addition of NAC definitely decreased the amounts



(a) MLSS: 2500 mg/L



(b) MLSS: 5200 mg/L



(c) MLSS: 8200 mg/L

Fig. 4 Effects of NAC addition on EPS at various MLSS concentrations ▨: polysaccharide ■: protein

of EPS regardless of MLSS concentrations. The EPS concentration with NAC addition was reduced to 64%-71% of the EPS without NAC addition. For instance, at the MLSS concentration of 8200 mg/L, the EPS was reduced to 64% of the EPS of the reactor without NAC addition. The reduction rate of protein and polysaccharide was various at the different MLSS concentration. At the low concentration of MLSS, i.e., 2500 mg/L, protein was reduced greatly while polysaccharide was relatively maintained. At the two high concentrations of MLSS, polysaccharide reduction was more substantial than the protein concentration reduction. The EPS concentration of the 8200 mg/L NAC-containing

reactor was 0.94 μg/cm^2, which was much lower than the EPS of the control reactor, i.e., 1.48 μg/cm^2. The respective fractions of the EPS with the NAC-containing reactor were reported as 0.29 μg/cm^2 for protein and 0.65 μg/cm^2 for polysaccharide while the fractions of those without NAC were 0.44 μg/cm^2 for protein and 1.04 μg/cm^2 for polysaccharide.

Similar to the results from our study, Olofsson *et al*. (2003) also demonstrated that the NAC application suppressed biofilm accumulation on the machine in the paper making process. They assessed efficacy of NAC by varying incubation times for 10 different bacterial strains isolated from the paper mill. As the incubation time was increased from 24 hours to 72 hours, numbers of adherent cells were increased without NAC addition. The NAC addition decreased adherence of microorganisms with time from the beginning of the experiments. The presence of NAC reduced number of community bacteria in attached species by more than 57%. Microbial populations were closely related to biofilm formation and decrease in the numbers of adherent microbial populations caused by NAC was equivalent to a decrease in the biofilm that occurred.

4. Conclusions

This study investigated effects of NAC addition on biofilm formation reduction using MBR sludge. Degree of biofilm formation reduction was examined with CLSM image analyses and EPS measurements. The NAC concentration was varied as 0, 0.5, 1.0 and 1.5 mg/mL and the MLSS of MBR sludge used was 2500, 5200, and 8200 mg/L. The application of NAC to the MBR sludge definitely decreased biofilm formation on the membrane surface. Also, the NAC addition reduced biofilm substantially when the MBR sludge with a MLSS concentration of 8200 mg/L was applied.

• The mature biofilm formed on the membrane surface was observed using the MBR sludge at different concentrations of NAC. The CLSM analyses showed that total biomass of the biofilm formed in the presence of NAC decreased with increasing NAC concentration. Especially, biofilm formation was substantially inhibited at a NAC concentration of 1.5 mg/mL.

• The reduction of EPS was occurred as the concentration of NAC was increased. The maximum reduction, approximately 70%, was obtained at the concentration of 1.5 mg/mL of NAC, compared to the EPS concentration without NAC addition.

• The effect of NAC on biomass concentration was investigated. The CLSM analyses showed that biofilm of the control reactors was increased with increasing MLSS concentrations. The NAC addition unquestionably decreased biofilm formation at the reactors with MLSS concentrations of 5200 and 8200 mg/L. The EPS concentrations were decreased in the reactors with NACs compared to those in the control reactors.

• The NAC addition could be a possible anti-biofouling strategy for the reduction of biofilm in MBR process. Other operational conditions such as flux and TMP and removal of NAC by MBR sludge should be further investigated.

Acknowledgments

This work was supported by National Research Foundation of Korea (NRF) Grant funded by the Korean Government (Ministry of Science, ICT and Future Planning) - NRF - 2015R1A2A2A03005392 and the Korea Institute of Energy Technology Evaluation and Planning (KETEP) and the Ministry of Trade, Industry and Energy (MOTIE, 20174010201490).

References

Al-Halbouni, D., Traber, J., Lyko, S., Wintgens, T., Melin, T., Tacke, D., Janot, A., Dott, W. and Hollender, J. (2008), "Correlation of EPS content in activated sludge at different sludge retention times with membrane fouling phenomena", *Water Res*., **42**(6), 1475-1488.

Domenech, M. and Garcia, E., (2017) "N-Acetyl-L-Cysteine and cysteamine as new strategies against mixed biofilms of nonencapsulated streptococcus pneumoniae and nontypeable haemophilus influenza", *Antimicrobial Agent. Chemotherapy*, **61**(2) 1-10

Gogate, P.R. (2007), "Application of cavitational reactors for water disinfection: Current status and path forward", *J. Environ. Manage.*, **85**(4), 801-815.

Kappachery, S., Paul, D. and Kweon, J.H. (2012), "Effect of N-acetylcysteine against biofouling of reverse osmosis membrane", *Desalination*, **285**, 184-187.

Lade, H., Paul, D. and Kweon, J.H. (2015), "Combined effects of curcumin and (-)-epigallocatechin gallate on inhibition of N-Acylhomoserine lactone-mediated biofilm formation in wastewater bacteria from membrane bioreactor", *J. Microbiol. Biotechnol*, **25**(11), 1908-1919.

Marchese, A., Bozzolasco, M., Gualco, L., Debbia, E.A., Schito, G.C. and Schito, A.M. (2003), "Effect of fosfomycin alone and in combination with N-acetylcysteine on E. coli biofilms", *Int. J. Antimicrobial Agents*, **22**, 95-100.

Massé, A., Spérandio, M. and Cabassud, C. (2006), "Comparison of sludge characteristics and performance of a submerged membrane bioreactor and an activated sludge process at high solids retention time", *Water Res.*, **40**(12), 2405-2415.

Muranaka, L.S., Giorgiano, T.E., Takita, M.A., Forim, M.R., Silva, L.F., Coletta-Filho, H.D., Machado, M.A., and de Souza, A.A. (2013), "N-Acetylcysteine in agriculture, a novel use for an old molecule: Focus on controlling the plant-pathogen xylella fastidiosa", *PloS one*, **8**(8), e72937.

Nam, A., Kweon, J., Ryu, J., Lade, H. and Lee, C. (2015), "Reduction of biofouling using vanillin as a quorum sensing inhibitory agent in membrane bioreactors for wastewater treatment", *Membr. Water Treat.*, **6**(3), 189-203.

Oh, H.S., Kim, S.R., Cheong, W.S., Lee, C.H., and Lee, J.K. (2013), "Biofouling inhibition in MBR by Rhodococcus sp. BH4 isolated from real MBR plant", *Appl.Microbiol. Biotechnol.*, **97**(23), 10223-10231.

Olofsson, A.C., Hermansson, M. and Elwing, H. (2003), "N-acetyl-L-cysteine affects growth, extracellular polysaccharide production, and bacterial biofilm formation on solid surfaces", *Appl. Environ. Microbiol.*, **69**(8), 4814-4822.

Parry, M.F. and Neu, H.C. (1977), "Effect of N-acetylcysteine on antibiotic activity and bacterial growth in vitro", *J. Clinical Microbiol.*, **5**(1), 58-61.

Perez-Giraldo, C., Rodriguez-Benito, A., Moran, F.J., Hurtado, C., Blanco, M.T. and Gomez-Garcia, A.C. (1997), "Influence of N-acetylcysteine on the formation of biofilm by Staphylococcus epidermidis", *J. Antimicrobial Chemotherapy*, **39**(5), 643-646.

Quah, S.Y., Wu, S., Lui, J.N., Sum, C.P. and Tan, K.S. (2012), "N-acetylcysteine inhibits growth and eradicates biofilm of Enterococcus faecalis", *J. Endodontics*, **38**(1), 81-85.

Roberts, D. and Cole, P. (1981), "N-acetylcysteine potentiates the anti-pseudomonas activity of carbenicillin in vitro", *J. Infection*, **3**(4), 353-359.

Saad, M.A. (1992), "Biofouling prevention in RO polymeric membrane systems", *Desalination*, **88**(1-3), 85-105.

Silveira, L.F. M., Baca, P., Arias-Moliz, M.T., Rodríguez-Archilla, A. and Ferrer-Luque, C.M. (2013), "Antimicrobial activity of alexidine alone and associated with N-acetylcysteine against Enterococcus faecalis biofilm", *J. Oral Sci.*, **5**(3), 146-149.

Smith, P.J., Vigneswaran, S., Ngo, H.H., Ben-Aim, R. and Nguyen, H. (2006), "A new approach to backwash initiation in membrane systems", *J. Membr. Sci.*, **278**(1), 381-389.

Stey, C., Steurer, J., Bachmann, S., Medici, T. C. and Tramer, M.R. (2000), "The effect of oral N-acetylcysteine in chronic bronchitis: a quantitative systematic review", *Euro. Respir. J.*, **16**(2), 253-262.

Tsuneda, S., Aikawa, H., Hayashi, H., Yuasa, A. and Hirata, A. (2003), "Extracellular polymeric substances responsible for bacterial adhesion onto solid surface", *FEMS Microbiol. Letters*, **223**(2), 287-292.

Vargas, A., Moreno-Andrade, I. and Buitrón, G. (2008), "Controlled backwashing in a membrane sequencing batch reactor used for toxic wastewater treatment", *J. Membr. Sci.*, **320**(1), 185-190.

Wang, G., Huang, L. and Xia, Z. (2016), "Study of membrane fouling properties of landfill leachate treatment by the SBAC-MBR process", *Environ. Eng. Sci.*, **33**(12), 978-985.

Wang, Z., Wu, Z. and Tang, S. (2009), "Extracellular polymeric substances (EPS) properties and their effects on membrane fouling in a submerged membrane bioreactor", *Water Res.*, **43**(9), 2504-2512.

Zeng, J., Gao, J.M., Chen, Y.P., Yan, P., Dong, Y., Shen, Y., Gue, J.S., Zeng, N. and Zhang, P. (2016), "Composition and aggregation of extracellular polymeric substances (EPS) in hyperhaline and municipal wastewater treatment plants", *Scientific Reports*, **6**.

Zhao, T., and Liu, Y. (2010), "N-acetylcysteine inhibit biofilms produced by Pseudomonas aeruginosa", *BMC Microbiol.*, **10**(1), 140.

CC