IDENTIFIKASI SPESIES *SULFAT REDUCING BACTERIA* (SBR) DENGAN *POLIMERASE CHAIN REACTION* (PCR) PADA MEMBRANE BIOREACTOR (MBR)

IDENTIFICATION OF SULFATE REDUCING BACTERIA (SRB) SPECIES BY POLIMERASE CHAIN REACTION (PCR) IN MEMBRANE BIOREACTOR (MBR)

Dewi Dwirianti Hadiwinoto[1)], Razman Salim[2)] dan Zaini Ujang[2)]
[1)] Jurusan Teknik Lingkungan FTSP-ITS
[2)]Institute of Environmental and Water Resources Management
Universiti Teknologi Malaysia

Abstrak

Air limbah konsentrasi sulfat yang tinggi dijumpai pada air limbah domestik, efluen minyak kelapa sawit dan industri karet. Masalah yang ditimbulkannya adalah bau dan korosi dari reduksi sulfat menjadi hidrogen sulfida. Reduksi ini terjadi pada kondisi anaerobik oleh aktifitas SBR. Tujuan dari penelitian ini mengidentifikasi SBR dengan metode PCR pada MBR. MBR yang digunakan terdiri dari 2 kompartemen. Kompartemen pertama dijaga pada kondisi anaerobik sedangkan kompartemen kedua diletakkan membran mikrofiltrasi dengan ukuran pori 0,2 μm. Hasilnya adalah SRB 385 dijumpai sebagai komunitas mikroba yang dapat mereduksi sulfat pada air limbah.

Kata kunci : konsentrasi sulfat, PCR, removal sulfat, SBR

Abstract

High sulfate wastewater was mainly found in municipal, palm oil mill effluent (POME) and rubber industry. The problems were odor and corrosion which resulting from reduction of sulfate to hydrogen sulfide. This reduction occurred under anaerobic condition by sulfate reducing bacteria (SRB) activity. The objectives of this study were to identify SRB species by Polymerase Chain Reaction (PCR) method, which responsible to reduced high sulfate wastewater in immersed membrane bioreactor (MBR). The MBR consists of two compartments. Compartment 1 was maintained in anaerobic condition. Microfiltration membrane module with an average pore size of 0,2 μm was immersed in Compartment 2. The results show that characterization of SRB in MBR using molecular technique by using PCR method identified SRB (SRB385) as the microbial community, which can reduce high sulfate in wastewater.

Keywords: sulfate wastewater, PCR, sulfate removal, SRB

1. INTRODUCTION

In Malaysia and Indonesia, sulfur compounds are mainly found in municipal wastewater, palm oil mill effluent (POME) and rubber industry. The typical concentrations of sulfur compounds are 1.570 ± 1010 ppmH_2S for POME (Khoon, 1991) and 56,5 ± 21,5 mgSO_4^{2-}/L for rubber industry (Zaid Isa, 1992), respectively. It is an intention of this study to start with municipal wastewater in order to understand the mechanism and treatability of high sulfate wastewater using MBR, before further studies will be conducted on both POME and rubber wastes. Under oxygen depleted condition, sulfate is utilized as an electron acceptor and reduced into hydrogen sulfide by SRB. As a result of sulate reduction, a large amount of hydrogen sulfide is produced. It is well known that hydrogen sulfide causes significant damage to treatment facilities. Therefore, SO_4 must be removed from wastewater before disposing to the environment.

Wastewater, which is difficult to treat and requires long sludge ages, and wastewater operations where settling and clarification problems are regularly encountered, are potential applications of the MBR. MBRs are composed of two primary units; the biological unit responsible for the biodegradation of the waste compounds, and the membrane module for the physical separation of the treated water and the mixed liquor.

Biological sulfate reduction was achieved by Sulfate Reducing Bacteria (SRB) activity. And hydrogen sulfide was formed by SRB activity from sulfate reduction. Hydrogen sulfide can be converted into elemental sulfur via the biological sulfur cycle. SRB are present in anaerobic and even aerobic wastewater treatment (Lens e*t al.*, 1995a,b).

Sulfates are of considerable concern because they are indirectly responsible for two serious problem often associated with the handling and treatment of wastewater. These are odor and corrosion problem resulting from the reduction of sulfates to hydrogen sulfide under anaerobic condition (Hvitved Jacobsen *et al.*, 1998). Malaysia's Environment Act 1974 state limited that concentration of hydrogen sulfide was 5 ppm in sewage effluent. The development of treatment processes using their capacity to degrade a wide range of organic compounds (Widdel, 1988) opens promising perspectives for environmental biotechnology.

Recent studies have been conducted to remove nutrients such as ammonia and phosphate. Where in MBR has been successfully used for organic and nutrient removal (Cote *et al*., 1997, Fan *et al*., 1996, Zhang *et al*., 1997, Ueda and Hata, 1999, Ujang *et al.*, 2001, Ujang *et al.*, 2002). However, there is still a vacuum in research related to treatment of high sulfate wastewater, using MBR so far. Mizuno *et al.* (1998) has done a research biological treatment of high sulfate wastewater in an acidogenic bioreactor with an external membrane system. However, no research specifically on treatment of high sulfate wastewater in immersed MBR has been done.

In order to achieve the objective for treatment of high sulfate wastewater in MBR, molecular technique has been utilized in this study to detection of the SRB in intermittent aeration immersed MBR.

Normally characterization of microbial community in wastewater is conventionally conducted using culture-based techniques (Amann *et al.*, 1998). Culture-based bacterial community analyses, however, are biased toward those organisms that can be isolated on microbial media. For wastewater treatment reactors, this cultivable fraction is typically only 15-20% of the cells that can be counted directly (Amann *et al.*, 1995).

Since cultured cteria isolates do not represent those abundant and active *in situ* it is difficult to obtain meaningful data on growth rates, substrates affinities and population size for specific groups of organisms important in MBR processes. Therefore, obtaining relevant data in an activated sludge plant is problematic. Molecular techniques offer the possibility that information useful to the operation and optimization of MBR plants can be obtained. The ability to identify and enumerate specific microorganisms *in situ*, in this case Sulfate Reducing Bacteria (SRB), in order to treatment of high sulfate wastewater related activities in the reactor is obviously of great value.

2. METHODOLOGY

A lab-scale immersed MBR was installed at the Environmental Engineering Laboratory, Universiti Teknologi Malaysia. A rectangular tank made of transparent perspex was used as MBR tank, divided into two compartments with working volume of 24 liter. The first compartment of MBR (7,5 liter) was designed for anaerobic; no diffuser put in the compartment. And the second compartment of MBR (16,5 liter) in which a membrane module was directly immersed and intermittently aerated using bubble diffusers. Aeration was supplied to immersed MBR through diffusers at 15 L/min. Membrane module used was a flat sheet type microfiltration (MF) membrane of polyolefin made by Yuasa Corporation (Japan) with an average pore size of 0,2 μm. A membrane sheet was adhered and sealed to each other along the top and the bottom edges with a PVC mesh-type spacers sandwiched between them. Both sides were connected to PVC pipes for the effluent waterway. A membrane sheet was set in plate and frame configuration, providing the total membrane area of 0,088 m^2.

Air diffuser was set below the membrane module just in the second compartment of MBR, to allow air bubbles to rise and provide the surface of MF membrane module with enough shear stress. The setting was purposely designed to create enough shear stress to remove attached biofoulant from the surface of MF membrane module during suction pump worked.

Aeration and non-aeration modes were applied in the second compartment. A small propeller with 2 cm radius was located at the bottom part of the first compartment of the MBR for the circulation of the mixed liquor and preventing the sludge from piling. Anaerob condition was applied in first compartment. Effluent from the membrane module on

the second compartment of the MBR was withdrawn using suction pump during aeration time.

The bacteria of SRB were cultivated for a period of 16 weeks in Sequencing Batch Reactor (SBR). Municipal wastewater was taken from inlet of pond in UTM campus. After the seeding period the SBR was fed with synthetic wastewater. The composition of synthetic wastewater used in this study were: Acetic Acid (600 mg/l), NH_4Cl (25 mg/l), KH_2PO_4 (12,5 mg/l), $NaHCO_3$ (125 mg/l), $MgSO_4.7H_2O$ (17 mg/l), $FeCl_3.6H_2O$ (2 mg/l), NaCl (4 mg/l), $CaCl_2.2H_2O$ (5,5 mg/l), KCl (4 mg/l), $NaSO_4$ as sulfate source (30 to 100 mg/l).

PCR method was used to amplify the DNA of microorganisms from activated sludge in the immersed MBR. The purified DNA was used as template DNA for PCR reactions. Template DNA was produced from the following steps: Sample was extracted from activated sludge in the immersed MBR to isolate the DNA. The isolated DNA was further purified by cell lyses to produce the template. The template DNA was amplified with PCR mixture consists of the template, PCR buffer, dNTPs, primers, *Taq* DNA polymerase by PCR amplification. PCR products were run in electrophoresis gel. The probes were used in this study is shown in Table 1.

Table 1. The Probes Used in This Study

Probe	Specificity	Sequence (5'⟶3')
EUB338	Domain bacteria	GTCGCCTCCCGTAGGAGT
NON338	None (negative control)	ACTCCTACGGGAGGCAGC
SRB385	General SRB	CGGCGTCGCTGCGTCAGG
SRB660	Desulfobulbus spp	GAATTCCACTTTCCCCTCTG
907R	Universal probe	CCGTCAATTCTTTTGAGTTT

DNA extraction was necessary because the DNA is part of the cell. The extraction processes were conducted based on the procedures described by Mo-Bio®, USA.

Initial denaturation temperature was set at 95°C for 3 minutes. Amplification was carried out for 30 cycles with each cycle consisting of three temperatures as follows: strand denaturation at 95°C for 30 seconds, primer annealing at 64°C for 30 seconds, primer extension at 72°C for 60 seconds. The amplification was completed after final extension at 72°C for 60 seconds.

PCR products were loaded into an agarose gel and later electrophoresed to detect the amplified products. PCR products were loaded together with a 100bp DNA molecular size marker (MBI Fermentas, USA) on a 1 % agarose. TBE was used as buffer and 5 mg/mL ethidium bromide (EtBr) was used to stain gel. Electrophoresis of agarose gel was run at 40 volts for 1 hour. The ultraviolet light-box was used to observe the DNA pattern.

3. RESULTS AND DISCUSSION

The overall of high sulfate wastewater removal in MBR reached 90±1,0% to 94,2±1,0%. Sulfate can be reduced by SRB activities in anaerobic condition. The removal of sulfate is attributed to two major factors, i.e. biological transformation by SRB in MBR, and the physical filtration by the membrane module. Biological removal is a biotransformation process as stated in Equations 1 to 5, the action of the membrane is merely a filtration process.

$$SO^{2-}_4 + \text{organic matters} \xrightarrow{anaerobic} S^{2-} + H^2O + CO_2 \quad (1)$$

$$S^2 + H^+ \longrightarrow H_2S \quad (2)$$

$$HS^- + H^- \longrightarrow H_2S \quad (3)$$

$$2\,H_2S + 3\,O_2 \longrightarrow 2\,SO_2 + 2\,H_2O \quad (4)$$

$$2\,H_2S + SO_2 \longleftrightarrow 3\,S^o + 2\,H_2O \quad (5)$$

In the sulfate-reducing stage, a complete reduction of sulfate to sulfide was desired. In the second step, sulfide was removed from the liquid, by biological sulfide oxidation of sulfide to the solid elemental sulfur (S^o) (Buisman *et.al.*, 1990). Under oxygen concentration below 0.1 mg/l, S^o was the major end product of the sulfide oxidation. S^o formation required one quarter of the oxygen compared with complete oxidation and, consequently, a lower energy consumption for aeration (Janssen *et.al.*, 1997). In this study, the second step of sulfate reducing was achieved in anaerobic condition. Hence, S^o particles, which had mean diameter 3 mm (Janssen *et.al.*, 1997) was retained by membrane module which had average pore size of 0,2 μm.

In the second compartment, the sulfate concentrating was lower than the first compartment. In addition, the sulfate concentration in anaerobic condition was lower than aerobic condition. This condition shows that some portions of the sulfate that were removed in the first compartment occurred in

anaerobic condition. In this compartment, SRB was dominant since it was an anaerobic condition. Although sulfate concentration in the second compartment was observed above 50 mgS/L, the sulfate concentration in the effluent was detected below 11,5 mgS/L. This result indicates that the membrane module has successfully treatment of sulfate wastewater, coupled with bioprocess conversion by SRB.

In this experiment, the membrane module functions as a physical barrier to retain the SRB in the reactor and filter out the biologically treated wastewater. Lens and Hulshoff Pol (2000) observed that typical diameter for SRB is within the range of 0,5 to 2 width/μm and 1,5 to 2,8 length/μm. These indicated that the size of SRB is typically bigger than 0,2 μm. Therefore, the microfiltraltion membrane module that was used in this study has successfully retained the SRB in MBR. Hence, higher reduction of high sulfate wastewater was achieved in Stage 2. Sulfate removal was occurred by SRB activity. From equation 1, it was clear that complete reduction of sulfate occurred in anaerobic condition; when non-aeration mode was applied. The SRB formed were single scattered cells to clustered cells, in bulkwater and biofilm (Okabe *et al*, 1999). Thus sulfate reduction was obtained by transformation of sulfate by SRB in MBR and in biofilm.

Conventional method to detect the activity of biological reaction in the wastewater treatment process has heavily relied on the consumption rate of substrates. However, little attention has been directed to the number or spatial distribution of specific bacteria in microbial community in the reactor. This is partly because difficulty of characterization of bacterial population in engineered system. The traditional culture dependent monitoring technique do not allow exact number and localization of specific bacteria because bias of cultivation. In the other words, a reactor or a treatment system has been so far regarded as a "black-box" with respect to microbial community.

Among molecular techniques, PCR is a powerful tool to analyze undefined microbial communities. PCR technique has been used to detect bacteria in a variety of environmental samples at very low previously considered undetectable. One of PCR step that the DNA was extracted from biomass sample (sludge). The biomass samples were extracted from 5 different module situation: 1st compartment, anaerobic condition; 2nd compartment, anaerobic condition; 2nd compartment, aerobic condition; 1st compartment (batch reactor); 2nd compartment (batch reactor)

Extracted DNA product was detected to confirm that extraction of DNA was successfully obtained. The extracted DNA was electrophoresis in 1 % of agarose gel. The photograph of the extracted DNA product is shown in Figure 1.

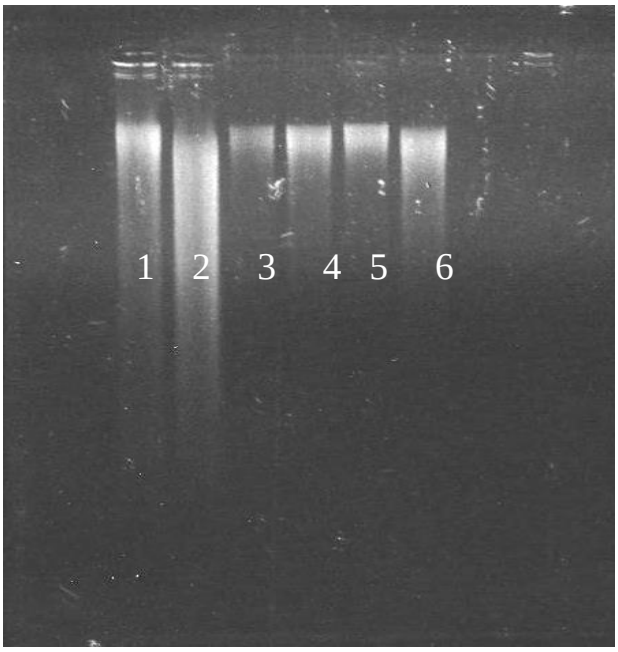

Figure 1. Photograph of The Extracted DNA
Lane 1: 1st compartment under anaerobic condition*
Lane 2: 1st compartment under anaerobic condition
Lane 3: 2nd compartment under aerobic condition
Lane 4: 2nd compartment under anaerobic condition
Lane 5: 1st compartment (batch reactor)
Lane 6: 2nd compartment (batch reactor)

The DNA extraction protocol from Mobio® (DNA water extraction kit) was applied successfully for the isolation of DNA from sludge in all compartments. It can be seem that active bacterial cells in MBR will be deposited in the sludge. The extracted DNA is termed as template DNA.

The template DNA was mixed with PCR mixture, as described previously. The PCR mixture was amplified in Cetus Cycler 9700. Amplification was carried out for 30 cycle with each cycle consisting of three temperatures as described previously. The PCR products were loaded into 1% of agarose gel and electrophoresed at 40 volts for 1 hour. Although, the experiments were carried out for many times, the result only showed the best photograph.

The photograph of PCR product is shown in Figure 2.

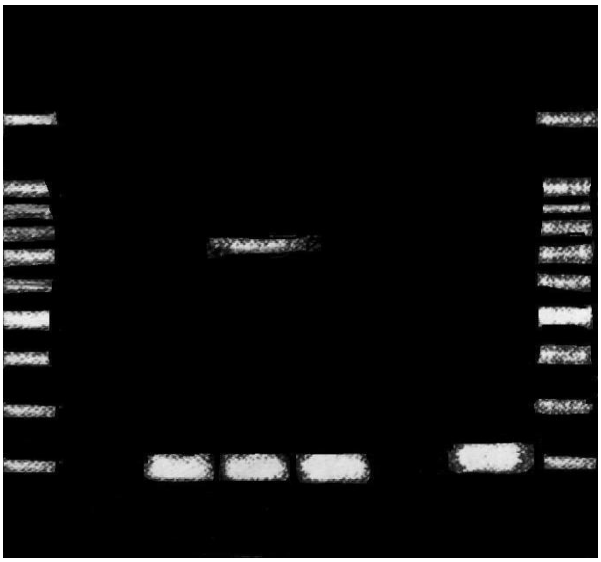

Figure 2. PCR Amplification
Lane 1: molecular weight marker
Lane 2: negative control (distilled water)
Lane 3: DNA template from 2nd compartment under aerobic condition
Lane 4: DNA template from 1st compartment under anaerobic condition
Lane 5: DNA template from 2nd compartment under anaerobic condition
Lane 6: negative-control (no DNA template)
Lane 7: DNA template from 1st compartment (batch reactor)
Lane 8: molecular weight marker

Figure 2 represents a series of PCR products. This results indicate the presence of general SRB (SRB385) in all compartment under all conditions. The bands appeared after PCR products were analyzed by 1% agarose gel electrophoresis. A primer set of primer 385 and 907R appeared in all series. The result indicated the SRB 385 was found in all compartments, even under anaerobic and aerobic conditions. This result was confirmed by previous study (Okabe *et al*., 1998, Okabe *et al*., 1999) that found of SRB even in aerobic condition.

By using the PCR technique described in this study, the community development could be observed. Detection of different SRB groups was also observed. This would be difficult to achieve with conventional cultivation technique. However, PCR technique also has bias and limitation. First, PCR amplification is not quantitative, therefore band intensities cannot be extrapolated to indicate the abundance of particular bacterial population. Second, the oligonucliotides, only SRB385 was used to exclude most non-SRB and to enrich the SRB populations in this study. These biases demonstrate the importance of combining different molecular methods and comparing them with the activity measurement.

From this research it was found that SRB exists in MBR. The source of wastewater from WSP was already containing SRB and various types of microorganisms. In mixed cultures communities of bacteria work together in harmony, in which that product of one are consumed by the other. In the other hand it is difficult to maintain pure culture environment in wastewater. In wastewater SRB385 were formed single scattered cells to clustered cells.

4. CONCLUSION

The removal of sulfate is attributed to two major factors, i.e. biological transformation by SRB in MBR, and the physical filtration by the membrane module. Characterization of SRB in MBR was successfully conducted using PCR. The microbial community in MBR that responsible for high sulfate waste-water removal was characterized as general of SRB (using SRB385 probe). The SRB community was highest in anaerobic condition than in anaerobic condition.

REFERENCES

Amann, R.I., Ludwig, W. and Schleifer, K.H. (1995). **Phylogenetic Identification and *in situ* Deyection of Individual Microbial Cells without Cultivation**. *Microbiol. Rev.* **Vol. 59** (1). pp. 143 - 169.

Amann, R., Lemmer, H. and Wagner, R. (1998). **Monitoring the Community Structure of Wastewater Treatment Plants: A Comparison of Old and New Techniques**. *FEMS Microbiol. Ecol.* **Vol. 25** (3). pp. 205 -215.

Buisman, C.J., Lettinga, G. and Wit, B. (1990). **Biothechnological Sulphide Removal in Three Polyurethane Carrier Reactor: Stirred Reactor, Biorotor Reactor and Upflow Reactor**. *Water Research.* **Vol. 24**. pp. 245.

Cote, P., Buisson, H., Pound, C. and Arakaki,G. (1997). **Immersed Membrane Activated**

Sludge For The Reuse Of Municipal Wastewater. *Desalination.* **Vol. 113**. pp. 189 -196.

Fan, X.J., Manem,J., Qian,Y. and Urbain,V. (1996). **Nitrification and Mass Balance with a Membrane Bioreactor for Municipal Wastewater Treatment**. *Wate. Science. Technology*. **Vol. 34** (1-2). pp. 129 - 136.

Hvitved-Jacobsen, T., Tanaka, N. and Vollertsen, J. (1998). **Wastewater Quality Changes during Transport in Sewers An Integrated Aerobic and Anaerobic Model Concept for Carbon and Sulfur Microbial Transformations**. *Water Qual. Int. '98.* **Vol. 8**. pp. 252 - 259.

Janssen, A.J.H., Lens, P., Lettinga, G. and Ma S.C. (1997). **Performance of a Sulphide Oxidizing Expanded Bed Reactor with a Spatially Separated Aeration Unit**. *Biotech. Bioeng.* **Vol. 53**. pp. 32 - 40.

Khoon, Q.S. (1991). **Effluent Treatment: Anaerobic Digestion and Biogas Utilization**. PORIM:Palm Oil Mill Engineers / Executive Training Course. **Vol. 18**.

Lens, P.N., Masone, A., Rozzi, A. and Verstraete, W. (1995a). **Effect of Sulfate Concentration and Scrapping on Aerobic Fixed Film Reactors**. *Wat. Res*. **Vol. 29**. pp. 857 - 870.

Lens P.N., Cronenberg C.C., De Poorter M.P. and Verstraete W.H. (1995b). **Sulfate Reducing and Methane Producing Bacteria in Aerobic Wastewater Treatment Systems**. *Wat. Res.* **Vol. 29** (3). pp. 871 - 880.

Lens, P. and Hulshoff, P.L. (2000). **Environmental Technologies to Treat Sulfur Pollution**. IWA Publishing. London.

Mizuno, O, Noike, T. and Takagi, H. (1998). **Biological Sulfate Removal in an Acidogenic Bioreactor with an Ultrafiltration Membrane System**. *Wat. Sci. Tech*. **Vol. 38** (4 - 5). pp. 513 - 520.

Okabe, S., Itoh, T., Matsuda, T., Satoh, H. and Watanabe, Y. (1998). **Sulfate Reduction and Sulfide Oxidation in Aerobic Mixed Population Biofilms**. *Wat. Sci. Technol.* **Vol. 37** (4 - 5). pp. 131 - 138.

Okabe, S., Itoh, T., Satoh, H. and Watanabe, Y. (1999). **Microbial Ecology of Sulfate Reducing Bacteria in Wastewater Biofilm Analyzed by Microelectrodes and FISH (Flourescent *In Situ* Hybridization) Technique**. *Wat. Sci. Tech*. **Vol. 39** (7). pp. 41 -47.

Ueda, T. and Hata, K. (1999). **Domestic Wastewater Treatment by a Submerged Membrane Bioreactor with Gravitational Filtration**. *Wat. Res.***Vol. 33**. pp. 2888 - 2892.

Ujang, Z., Khor, S.L. and Salim, M.R. (2001). **Instability of Phosphorous Removal in Membrane Bioreactor**. *JSPC-VCC Seminar on Water Resources*. University Malaya Sabah. October 8 - 9.

Ujang, Z., Khor, S.L. and Salim, M.R. (2002). **The Effect of Aeration and Non-aeration Time on Simultaneous Organic, Nitrogen and Phosphorous Removal Using an Intermittent Aeration Membrane Bioreactor**. *Wat. Sci. Tech.* **Vol. 46** (9). pp. 193 - 200.

Widdel, F. (1988). **Microbiology and Ecology of Sulfate and Sulfur-Reducing Bacteria**. In **Biology of Anaerobic Microorganisms**. (edited by Zehnder, A.J.B.). John Wiley. New York. pp. 469 - 585.

Zaid Isa. (1992). **Pollution Control in The Natural Rubber Industry in Malaysia**. *Kuala Lumpur: ENSEARCH*. **Vol. 6**.

Zhang, B., Ohgaki, S., Kamiko, N. and Yamamoto, K. (1997). **Floc Size Distribution and Bacterial Activities in Membrane Separation Activated Sludge Processess for Small Scale Wastewater Treatment / Reclamation**. *Wat. Sci. Technol.* **Vol. 35** (6). pp. 37 - 44.