THE ANTI-BIOFOULING EFFECT OF PIPER BETLE EXTRACT ON MEMBRANE BIOFOULING IN BIOREACTOR FOR BATIK WASTEWATER TREATMENT

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ABSTRACT

Navigating novel biological route to mitigate biofouling is of great worth in order to allow sustainable performance of MBRs in wastewater treatment technology. Recently, it was confirmed that a number of natural compounds in plants have an anti-biofouling effect, reducing the formation of biofilm. The main objectives of this study were to investigate the anti-biofouling effects of *Piper betle* extract (PBE) on membrane biofouling and how PBE mitigates biofouling based on quorum sensing (QS). Membrane biofouling propensity was investigated for a bacterial consortium and bacterial strains of batik wastewater. During MBR operation with bacterial consortium, a significant relationship (R^2= 0.9916) between extracellular polymeric substances (EPS) and transmembrane pressure (TMP) was revealed. MBR showed increased removal performance for dye and chemical oxygen demand (COD) removal with operation time. Fourier transform infrared spectroscopy (FTIR) showed the presence of EPS in membrane foulants. Furthermore, scanning electron microscopy (SEM) confirmed the occurrence of biofouling. The microtiter plat assay suggested that strain FS5 to be the major biofilm contributor. Batch tests of the production of EPS indicated that the *Bacillus* strain (FS5) produced a large amount of EPS compared to the bacterial consortium. This study addressed the feasibility of *Piper betle* extract (PBE) as anti-biofouling agent against the model organism *Pseudomonas aeruginosa* PAO1 and bacterial consortium. The anti-biofouling effects of PBE were evaluated via a microtiter plate assay; changes in the growth rate (μ) and EPS production. SEM was employed to qualitatively illustrate the biofilm formation. The anti-biofouling effects of PBE revealed ≥80 % reduction in biofilm formation, growth rate (87%) and reduced the EPS production. Furthermore, it decreased the soluble EPS concentration, reduced the cake resistance, and a two-fold increase in time required to reach 33 kPa of TMP. The PBE indicated a negligible effect on endogenous decay rate and biomass yield. SEM of sludge particles in PBE bioreactor showed the presence of a mixture of bacteria on its surface with a clear spherical shaped boundary. Besides that PBE indicated negligible effects on biological treatment performance. Response surface methodology (RSM) has been employed to mitigate EPS, TMP rise-up control, and dye removal in ultrafiltration MBR. The optimum conditions found to be biofouling reducer (BFR) of 0.23 mg/mg MLSS, HRT of 30.16 h and air flow rate of 0.60 l/min, with predicted values as 28.28 mg/l of EPS, 24.16 kPa of TMP and 95.65% dye removal, respectively. Validatory tests were closely agreed with the predicted values. The autoinducers production in bioreactor was confirmed using an indicator strain *Agrobacterium tumefaciens*. Moreover, three different AHLs were found in biocake using thin layer chromatographic analysis. An increase in EPS and TMP was observed with AHL activity of the biocake during continuous MBR operation, which shows that membrane biofouling was in close relationship with QS activity. PBE was verified to mitigate membrane biofouling via inhibiting AHLs production. These results exhibited that PBE could be a novel agent to target AHLs for mitigation of membrane biofouling based QS.

ABSTRAK

Penerokaan novel penggunaan biologi untuk mengurangkan kotoran adalah amat signifikan bagi prestasi MBR yang mapan dalam teknologi rawatan air sisa. Kini, telah dibuktikan terdapat beberapa sebatian semulajadi dalam tumbuhan boleh bertindak sebagai kesan anti sumbat bio yang boleh mengurangkan pembentukan biofilem. Objektif utama kajian ini adalah untuk mengenalpasti kesan anti sumbat bio daripada ekstrak daun sireh (PBE) terhadap membran yang tersumbat dan bagaimana PBE menyingkirkan kotoran berdasarkan kuorum penderiaan (QS). Kecenderungan membran tersumbat telah dikenalpasti bagi konsortium bakteria dan strain bakteria dalam air sisa batik. Semasa operasi MBR dengan konsortium bakteria, hubungan yang signifikan (R2 = 0.9916) antara bahan polimer luar sel (EPS) dan tekanan transmembran (TMP) yang telah ditunjukkan. MBR menunjukkan penyingkiran prestasi yang meningkat untuk pewarna dan penyingkiran permintaan oksigen secara kimia (COD) dengan masa operasi. Spektroskopi jelmaaan Fourier inframerah (FTIR) menunjukkan kehadiran EPS dalam kotoran membran. Tambahan pula, mikroskopi imbasan elektron (SEM) mengesahkan berlakunya kesumbatan. Plat mikrotiter asai mencadangkan bahawa strain FS5 menjadi penyumbang lapisan yang utama. Ujian kelompok pengeluaran EPS menunjukkan bahawa EPS yang dihasilkan oleh strain *Bacillus* (FS5) adalah dalam jumlah yang besar berbanding dengan konsortium bakteria. Kajian ini memberi tumpuan kepada kemungkinan ekstrak daun sireh (PBE) sebagai agen anti-sumbat bio terhadap organisma model *Pseudomonas aeruginosa* PAO1 dan konsortium bakteria. Kesan-kesan anti-sumbat bio terhadap PBE dinilai melalui plat mikrotiter asai; perubahan dalam kadar pertumbuhan (μ) dan pengeluaran EPS. SEM telah digunakan untuk menggambarkan pembentukan biofilem secara kualitatif. Kesan anti-sumbat bio terhadap PBE menunjukkan pengurangan ≥ 80% dalam pembentukan biofilem, kadar pertumbuhan (87%) dan mengurangkan pengeluaran EPS. Tambahan pula, ia menurun kepekatan EPS larut, mengurangkan rintangan kek, dan meningkat dua kali ganda dalam masa yang diperlukan untuk mencapai 33 kPa bagi TMP. PBE menunjukkan kesan yang tidak siknifikan pada kadar pereputan dalaman dan hasil biojisim. SEM zarah enapcemar di dalam bioreaktor PBE menunjukkan kehadiran campuran bakteria pada permukaannya dengan sempadan berbentuk sfera yang jelas. Selain itu, PBE menunjukkan kesan yang tidak signifikan mengenai prestasi rawatan biologi. Kaedah gerak balas permukaan (RSM) telah digunakan untuk mengurangkan EPS, maningkatkan kawalan TMP, dan penyingkiran pewarna dalam MBR ultraturasan. Keadaan optimum didapati pada pengurang kotoran (BFR) sebanyak 0.23 mg/mg MLSS, HRT pada 30.16 jam dan kadar aliran udara pada 0.60 l/min, dengan nilai-nilai yang diramalkan sebagai 28.28 mg/l EPS, 24.16 kPa bagi TMP dan 95.65% penyingkiran pewarna. Ujian pengesahan menunjukkan nilai yang sangat hampir dengan nilai-nilai yang diramalkan. Pengeluaran auto-pencetus di dalam bioreaktor telah disahkan dengan menggunakan penunjuk strain *Agrobacterium tumefaciens*. Selain itu, tiga AHLs yang berbeza telah didapati di kek menggunakan analisis kromatografi lapisan nipis. Peningkatan dalam EPS dan TMP diperhatikan dengan aktiviti AHL daripada kek semasa operasi MBR secara berterusan, menunjukkan bahawa membran yang tersumbat mempunyai hubungan yang rapat dengan aktiviti QS. PBE telah disahkan boleh mengurangkan membran yang tersumbat dengan menghalang pengeluaran AHLs. Keputusan ini menunjukkan bahawa pihak PBE boleh menjadi ejen novel untuk mensasarkan AHLs bagi mengurangkan membran yang tersumbat berdasarkan QS.

TABLES OF CONTENTS

CHAPTER 4 RESULTS AND DISCUSSION

LIST OF TABLES

LIST OF FIGURES

A. tumefaciens bioassay results. Bioassay was carried out after 4 days operation, (d) quantitative analysis of EPSs in biocake in the MBR under various operating conditions

LIST OF ABBREVIATIONS/SYMBOLS

AFM	Atomic Force Microscopy
AHL	Acyl homoserine lactones
AI	Autoinducer
ATP	Adenosine triphosphate
BOD	Biological oxygen demand
CCD	Central composite design
CLSM	Confocal laser scanning microscopy
COD	Chemical oxygen demand
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DOE	Department of Environment
DOTM	Direct observation through the membrane
EPS	Extracellular polymeric substances
F/M	Food to microorganism ratio
FISH	Fluorescence *in situ* hybridization
FTIR	Fourier transform infrared spectroscopy
GCMS	Gas chromatography-mass spectrometry
HHL	Hexanoyl homoserine lactone
HPLC	High pressure liquid chromatography
HRT	Hydraulic retention time
MEC	Magnetic enzyme carrier
MLSS	Mixed liquor suspended solids

OD	Optical Density
OFAT	One-factor-at-a-time
OHL	Octanoyl homoserine lactone
OLR	Organic loading rate
PBE	*Piper betle extract*
PCR	Polymerase Chain reaction
QS	Quorum Sensing
R_c	Cake resistance
RSM	Response surface methodology
SEM	Scanning electron microscopy
SMBRs	Submerged membrane bioreactors
SMP	Soluble microbial product
SNP	S-nitroso-N-acetylpenicillamine
SRT	Sludge retention Time
TLC	Think layer chromatography
TMP	Transmembrane pressure
UF	Ultrafiltration membrane
$^{\circ}C$	Degree Celsius
min	Minute
cm^2	Square centimeter
g	Gram
H	Height
h	Hour

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

This chapter describes the background of batik dye wastewater, treatment methods, membrane biofouling and biological mitigation of membrane biofouling. The first part of chapter explains about batik wastewater; nature and hazards of dye wastewater. Meanwhile, the second part explains a brief background of the treatment methods for dye wastewater. The third part explains about membrane biofouling. The fourth part discusses mitigation of membrane biofouling. Finally, for the last part of this chapter, problem statement, objectives and the scopes of study were described.

1.2 BACKGROUND

1.2.1 BATIK WASTEWATER, NATURE AND HAZARDS OF DYES

In Malaysia, the textile sector is the third largest foreign exchange earner after the palm oil and electronic industries (Malaysian Textile Manufacturers Association (MTMA), 2008). Approximately 1500 textile industries operate in Malaysia, most of which are backyard industries making the local ‘batik’. These homemade textile industries are well known in Malaysia. These industries are traditionally inherited from generation to generation. The batik making process can generally be categorized into four processes, cloth preparation, application of wax, dyeing of cloth and removal of wax in boiling water (Ahmad et al., 2002).

These textile facilities discharge a large amount of wastewater, which contains many types of dyes, solvents, salts and detergents (Marcucci et al., 2003, and Ali et al., 2009). Wastewater dye affects water transparency, gas solubility, and aesthetics of aquatic systems, and can be toxic to aquatic organisms (Vandevivere et al., 1998, and Siddiqui et al., 2010). Moreover, most synthetic azo dyes are noxious, mutagenic and carcinogenic, causing danger to organisms (Nilsson et al., 1993, and Siddiqui et al., 2009). The limits for the discharge of color effluents are 100 and 200 Platinum–Cobalt units according to standards A and B, respectively (Department of Environment (DOE), 2010).

1.2.2 TREATMENT TECHNIQUES

Textile wastewater is often treated with physio-chemical methods, but these methods are generally very expensive (Robinson et al., 2001). Moreover, the complex molecular structure of dyes makes them more resistant to degradation via biological methods. Thus, there is an urgent need to develop a suitable technology for treatment of dyes in textile effluent.

In recent years, submerged membrane bioreactors (SMBRs) have received significant interest, because they eliminate the need of a secondary settling tank. SMBRs also having small reactor space and it usually produce very less amount of sludge (Yun et al., 2006). However, the membrane fouling prevents its large scale application, as it is a major hitch (Meng et al., 2009). Fouling is of various types, e.g. organic, inorganic, and physical and biofouling (Kramer and Tracey, 1995). Of these, “biofouling” resulting from extracellular polymeric substances and microbial cells is a difficult task (Yu et al., 2010). It reduces the membrane life span; decreases the permeate flux, and ultimately add an extra capital cost for the replacement of a membrane (Yu et al., 2010).

1.2.3 MEMBRANE BIOFOULING

Membrane biofouling is a pervasive membrane system problem. Biofouling process involves adhesion and growth of microorganisms on the membrane surface (Flemming et al., 1997, and Wang et al., 2005). Biofouling is hard to control, even by reducing the number of microorganisms in the feed water, because they can multiply even if their number is strongly diminished, and they will do so if nutrients are available (Ridgway and Flemming, 1996). Since microorganisms are abundant in wastewater effluents and due to prevention methods such as disinfection or MF/UF pretreatment in technical systems neither leads to sterility nor is maintained over a long period of time. Moreover, the microorganisms will always invade and colonize the system. Thus if they removed to 99.99% there are still enough cells left which can grow at the expense of biodegradable substances in the feed stream. In membrane systems biofouling represents the Achilles heel of the process because all other fouling components such as organic and inorganic dissolved substances can be removed by pretreatment (Ridgway and Flemming, 1996). Biofouling leads to considerable technical problems and economic loss. During the past two decades, the membrane bioreactor (MBR) has emerged as one of the innovative technologies in wastewater treatment. Biofouling is still an unsolved problem (Yeon et al. 2009).

Extracellular polymeric substances (EPSs) are found to be key substances to cause membrane biofouling (Massé et al., 2002, and Rosenberger et al., 2002). These compounds are high molecular weights that are produced by bacterial cells. These compounds comprised of polysaccharides, lipoproteins, proteins, and deoxyribonucleic acid (DNA). Recently, studies on EPS in either soluble microbial product (SMP) or bound form have attained growing interest (Yeon et al., 2009). EPS shows an essential component of biofilm development and structure, particularly mechanical strength, attachment, and protection against environmental deleterious effects (Tansel et al., 2008). It is necessary to remove EPS (soluble) from the activated sludge, because they can pose internal fouling and hence decrease the membrane flux (Lee et al., 2003).

It has been known that biofilm formation in liquid–solid interfaces is controlled via quorum sensing (QS). QS refers to the density-dependent regulation of gene expression in microorganisms (Freeman and Bassler, 1999). During the bacterial quorum sensing, *N*-acyl homoserine lactones (AHL) are produced from the microorganisms. AHL has been known as quorum sensing molecules (i.e., autoinducer (AI)) transferring bacterial signals from one to another and, thus controlling the rate and the extent of biofilm formation (Hu et al., 2003).

1.2.4 MEMBRANE BIOFOULING CONTROL

So far, extensive research has been pursued to investigate the possible methods to control membrane biofouling. Many physico-chemical methods have been used, for regular physical and chemical cleaning, etc. (Ramesh et al., 2006), and they may not be effective and energy efficient. Although several biofouling control techniques have been developed through engineering (Yeon et al., 2005), material (Yu et al., 2007), and chemistry approaches (Lee et al., 2001), all these attempts have the limitation that they are essentially not able to prevent the biofilm formation because it is intrinsically a natural biological process. Sometimes it is hard to reach all the areas that are contaminated with biofilm. Acidic and alkaline solutions are occasionally used to remove biofilm from surfaces by washing, but there is an issue of adverse environmental impact. Thus, it appears that biological control of microbial attachment would be a novel promising alternative for mitigating membrane biofouling and would be a new niche that deserves further study (Xiong and Liu, 2010). It would be better to prevent biofilm formation rather than killing the cells after it forms. However, killing the cells using antibiotics, as practiced in industry, for example, does not always work, because it is not usually possible to kill all the cells completely for an extended time, and some cells still can attach onto the solid surface to form a biofilm (Costerton, 1999).

Recently, a number of QS inhibitors have been discovered and it has revealed a novel way to target QS to mitigate biofilm. Not surprisingly, the QS inhibitors of plants such as *Vanilla planifolia* (Choo et al., 2006), *Bucida buceras* (Adnonizio et al., 2006), and

Terminalia catappa (Taganna et al., 2011) inhibit bacterial QS. Results from these studies enabled us to hypothesize that, in principle; membrane biofouling originating from biofilm formation could also be alleviated through QS control, e.g., the blocking of intercellular communication to mitigate membrane biofouling.

Thus, it is clear that mitigation of biofilm attachment on membrane would be a new alternative to control membrane biofouling and it would be a new route which needs further research (Xiong and Liu, 2010). Several plant products are known for their antibacterial activities (Kubo et al., 2006), and in this study, it was hypothesized that these may help reduce biofilm formation (Sendamangalam et al., 2009). The extract of *Piper betle* plant (PBE) leaves has been reported to possess many biological activities that contributed its role as an antibacterial agent (Nair and Chanda, 2008). *Piper betle extract* control the growth of many gram-positive and gram- negative microbes (Nair and Chanda, 2008).

1.3 PROBLEM STATEMENT

Biofouling control is a difficult and challenging task because some microorganisms survive and grow rapidly. Biofouling control techniques have been developed, but they are not able to prevent the biofilm formation because it is intrinsically a natural biological process. Therefore, elucidating mechanisms involved in biofilm formation as well as developing the methods for controlling biofilm formation is important for the efficient application of membrane technologies. It would be better to prevent biofilm (biofouling) formation rather than killing the cells after it form biofilm because once biofilm form it's 10-1000 times more resistant. Several plant products are known for their antibacterial activities (Kubo et al., 2006, and Sendamangalam et al., 2011), and in this study, it was hypothesized that these may help reduce biofilm formation. No information, however, is available on the *Piper betle* (L.) extract to mitigate membrane biofouling. In this study, the concept of bacteriostatic effect and quorum sensing (QS) of *Piper betle* extract on biofouling control was evaluated. For this research, we hypothesized that in principal, preventing biofilm formation, targeting QS system and extracellular polymeric substances (EPS) production, membrane biofouling could also be alleviated through the use of *Piper*

betle extract (PBE), to control its growth, formation of biofilm, disruption of QS and production of EPS.

1.4 RESEARCH OBJECTIVES

a) Main objective

This study seeks to evaluate the biological control agent (*Piper betle* extract) to control membrane biofouling in a submerged membrane bioreactor.

b) Specific Objectives

i. To determine the biofouling potential of batik wastewater by indigenous bacteria.

ii. To evaluate the anti-biofouling effect of *Piper betle* extract (PBE) against model bacterium *Pseudomonas aeruginosa* and bacterial consortium.

iii. To determine the influence of PBE on extracellular polymeric substances (EPS), biokinetic parameters, sludge properties, membrane filterability and reactor treatment performance.

iv. To determine the optimized conditions for EPS removal, transmembrane pressure rise-up control and dye removal in membrane reactor using response surface methodology (RSM).

v. To determine how PBE target the Quorum Sensing (QS) (autoinducer signals) to mitigate membrane biofouling.

1.5 SCOPE OF STUDY

To accomplish the above objectives, the following tasks were undertaken:

1. The resistance-in-series model was applied to find out the main contributor to membrane fouling. The bacterial consortium in membrane bioreactor (MBR) exhibited a significant relationship (R^2= 0.9916) between EPS and transmembrane pressure (TMP).
2. Biofilm formation was qualitatively illustrated via scanning electron microscopy (SEM), to confirm the occurrence of biofouling. FTIR spectra of membrane foulants qualitatively confirmed the presence of polysaccharides and proteins as major components.
3. Microtiter plat assay was carried out to determine the biofouling activity of indigenous bacteria from batik wastewater. Batch tests of the production of extracellular polymeric substances (EPS) by *Bacillus* strain (FS5) was compared to the bacterial consortium.
4. The anti-biofouling effects of PBE against model bacterium *Pseudomonas aeruginosa* and bacterial consortium were evaluated via a microtiter plate assay; changes in the growth rate (μ) and EPS production. SEM was employed to qualitatively illustrate the biofilm formation.
5. The influence of different concentrations of *Piper betle* extract (PBE) as a biofouling reducer, on soluble and bound EPS was carried out in submerged membrane bioreactor.
6. The effect of addition of PBE on cake resistance and time to reach 33 kPa of TMP was also determined.
7. The effect of PBE on biokinetic properties of sludge, sludge volume index, biomass in reactor, chemical oxygen demand and color removal was also carried out.
8. The effects of PBE dosage (mg/mg MLSS), HRT (h), and air flow rate (l/min) on EPS mitigation, TMP rise-up control and dye removal were