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Mitigation of Biofouling by Reducing Biofilm Formation and Extracellular Polymeric Substances

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Abstract: Biofouling is a process of surface colonization by microorganisms via cell adhesion and production of Extracellular Polymeric Substances (EPS). It generally leads to severe problems in membrane bioreactor systems. This study addressed the feasibility of *Piper betle* Extract (PBE) as anti-biofouling agent against the *Pseudomonas aeruginosa*. The Minimum Inhibitory Concentration (MIC) (3000 μg mL^{-1}) of PBE was selected and was used in all experiments. The anti-biofouling effects of PBE were evaluated via a microtiter plate assay and EPS production. PBE reduced biofilm formation and EPS production.

Key words: Biofouling, extracellular polymeric substances, biofilm, microtiter plate assay, inhibition

INTRODUCTION

Membrane bioreactors (MBRs) have emerged as one of the innovative technologies in wastewater treatment (Yun *et al*., 2006). However, high-quality purification systems have faced a major problem due to biofilm formation on the membrane surface or biofouling. It reduces permeate flux, shortens the membrane life, increases the membrane cost and eventually adds additional capital cost for membrane replacement. The control of membrane irreversible fouling resulting from strongly bound fouling materials is a difficult and challenging task (Yu *et al*., 2010).

So far, extensive research has been pursued to investigate the possible methods to prevent or reduce 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. Sometimes it is hard to reach all the areas that are contaminated with biofilm. Acidic and alkaline solutions are sometimes 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).

Many natural products of plants are well known for antimicrobial activities (Kubo *et al*., 2006) and in this study, it was hypothesized that these may help to reduce biofilm formation (Sendamangalam *et al*., 2011). Consequently, the present study was undertaken to evaluate the effects of *Piper betle* extract (PBE) on biofilm formation and Extracellular Polymeric Substances (EPS) production by *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Materials: The materials used in this study were nutrient broth for cell culture; crystal violet dye (MERCK, 101408-0025) was used to stain the biofilm cells. Methanol (PROLABO) was used to fix the attached bacteria. Glacial acetic acid (DMF, Fisher scientific) was used to resolubalize the dye bound to the adherent cells. A sterile 96 well clear flat bottom tissue culture microtiter plate (Corning, SIGMA) with a lid was used for biofilm assay. All materials were autoclaved for 20 min at 120°C before use.

Model bacterial strain: Natural environment bacterial variance leads to an extremely complex biofilm system that is poorly known. Therefore, for this work, a single representative bacterium, namely *Pseudomonas aeruginosa* PAO1 was used as a model

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bacterium because of its ability to foul surfaces rapidly, its rapid reproduction rate and its significance as a pathogen (Lee *et al*., 2010). This species is gram negative; rod shaped (1.5-2 μm long and 0.3-0.6 μm wide) and can be cultured from almost all natural waters.

Preparation of bacterial suspension: The stock of the *Pseudomonas aeruginosa* PA 01 was kept in glycerol at -70°C for further use. The bacterial specie was revived in nutrient broth at 37°C overnight. The cells were collected via centrifugation at 10000 rpm for 10 min and cells were resuspended in nutrient broth. Cells concentration was standardized at 10^6 cells mL^{-1} by a spectrophotometer (Shimadzu) at 570 nm (Nalina and Rahim, 2006).

Preparation of plant extract: *Piper betle* L. leaves were obtained from Mentakab, Pahang, Malaysia. Fresh healthy leaves were washed with distilled water and air dried. Dried leaves were shredded into small pieces. 100 g of pieces were boiled in 1 liter of deionized distilled water for many hours until the final volume was 100 mL. Further, the extract was centrifuged at 10,000 rpm to remove sediments. The supernatant was divided, into 1 mL aliquots, in micro fuge tubes. It was concentrated using a speed-vacuum concentrator. The extracts were weighed into sterile micro fuge vials and prepared into stocks of 20 mg mL^{-1} using sterile distilled water as diluents and sterilized by a 0.2 μm membrane filter. The PBE (pH 6.5) was completely soluble in water. The extracts were dissolved by sonicating the microfuge vials in a sonicator (DAIHAN) and stored at 4°C until use.

Determination of MIC of PBE: The Minimum Inhibitory Concentration (MIC) of PBE was assessed using a broth dilution method (Smullen *et al*., 2007). Briefly, *Pseudomonas aeruginosa* PAO1 was grown overnight in nutrient broth medium. A 0.1 mL sample was taken from the culture when the stationary growth phase was reached after 16 h. The sample was transferred to culture tubes that contained 15 mL of the culture medium. The PBE solutions were prepared as follows: The PBE was dissolved in deionized distilled water at different concentrations (1000, 2000, 3000, 4000 and 5000 μg mL^{-1}). The PBE of various concentrations were tested for their effects on the growth of *Pseudomonas aeruginosa*. 0.1 mL of the PBE solutions of different concentrations were taken and added to the culture tubes. At a regular interval, 0.1 mL of the solution from each culture tube was serial-diluted and plated on the nutrient agar plates. The plates were incubated at 37°C and colonies were counted after 24 h. Control without PBE was also run. The MIC based on this study was further used in other experiments. The MIC assay was done in triplicates and the averages of the results were taken.

Biofilm reduction assay: The *Pseudomonas aeruginosa* PAO1 was grown over night in nutrient broth, at 37°C. Aliquots of 100 μL were inoculated in 9 parallel wells of a microtiter plate. Two microliter of PBE was added to each well. The final concentration of PBE in a well was 3000 μg mL^{-1}. Control (without PBE) was also run in the study. Then the plate was incubated at 37°C for 12, 24, 36, 48, 60 and 72 h. Then the content of each well was aspirated. The wells were rinsed three times after incubation period with 150 μL of physiological saline. The plate was vigorously shaken so that non-adherent bacteria removed and fixed the remaining bacteria with 100 μL of 99.99% ethanol for 10 min. The liquid was poured off from the plate and the plate was dried in the air. The adhered bacterial material was stained adding 100 μL of crystal violet (2%) for 15 min. The tape water was used to rinse off excess violet and was air dried. The dye bound to the adherent cells was re-dissolved with 100 μL of 33% (v/v) glacial acetic acid per well (Burton *et al*., 2007) and the adhered cells were quantified via an ELISA reader (TECAN) at 570 nm. The tests were done in triplicates and the averages were taken.

Effect of PBE on EPS production: To produce EPS, two experiments, control and PBE treated were run in parallel. The bacterium was grown as pure culture in a reported mineral medium. The composition of mineral medium utilized for the biopolymer production was as follows; 25 g L^{-1} glucose, 0.2 g L^{-1} $MgSO_4.7H_2O$, 2 g L^{-1} K_2HPO_4, 1 g L^{-1} KH_2PO_4, 1 g L^{-1} NH_4Cl and 0.01 g L^{-1} yeast extract. Bacterial strain was inoculated in the medium from the slants and it was incubated in an orbital shaker at 250 rpm for 6 days at 25°C. After 6 days, the broth changed into highly viscous. A control without PBE was also run in parallel. After each day, the medium was centrifuged at 6000 g for 15 min at 4°C to obtain slime EPS (in centrifuged supernatant) and capsular EPS (in bacterial pellet or microorganisms) (Zhang *et al*., 2002). The supernatant was precipitated with chilled ethanol (2.2 volumes) and the mixture was incubated at -20°C for 1 h. The precipitates of EPS were centrifuged at 6000 g for 15 min at 4°C and the pellet containing slime EPS was collected and dried at room temperature. Dry weight of the capsular EPS, crude slime EPS and bacterial broth (combined slime and capsular EPS) were quantified by drying at 105°C to a constant weight (APHA, 2005).

Statistical analysis: All experiments were conducted in triplicates and results obtained in experiments were expressed in terms of means (average) and standard deviation (±) using SPSS 10.0 software.

RESULTS AND DISCUSSION

Various concentrations (1000-5000 μg mL^{-1}) of PBE were used and the least effective concentration (3000 μg mL^{-1}) of PBE was selected as the MIC. Figure 1 shows the log (CFU mL^{-1}) results for the cells measured soon after the addition of PBE. The control in Fig. 1 shows the number of cells in the growth medium without the PBE. These results demonstrate that the CFU, i.e., cell viability in the presence of the PBE was not significantly affected, when the PBE concentration was at the MIC. The concentration of PBE was selected at 3000 μg mL^{-1}. This would make sure that the augmentation of extract would not kill the bacteria cells but instead would allow the growth of cells to be its minimum. As seen in Fig. 1, it can be clearly observed that the bacterial culture grew exponentially until the beginning of stationary phase and started to die once the PBE were added. The PBE were added after 16 h of inoculation.

The results of microtiter plate are shown in Fig. 2 and the percentage reduction from the control is shown in Table 1. In Fig. 2, each bar graph represents the absorbance of crystal violet dye bound to biofilm cells. Therefore, a large absorbance indicates more biofilm formation. PBE showed an inhibitory effect on the biofilm formation by *Pseudomonas aeruginosa*, with a 79% minimum biofilm reduction at concentration of 3000 μg mL^{-1}. Biofouling causing bacteria are protected by formation of biofilm and hence, are less prone to antimicrobial agents than their planktonic counterparts (Wilson, 1996). Unlike the effects of PBE on planktonic cells, as determined by MIC, PBE exhibit 80% reduction of biofilm even at 72 h biofilm. However, in terms of 79% minimum biofilm reduction, we revealed that PBE have prominent effect on the formation of biofilm by *Pseudomonas aeruginosa*.

In view of the fact that, EPS gives a gel matrix in which microorganisms are embedded, they provide a considerable hindrance to permeate flux in the MBRs. Microbial biofilms play a vital role in biofouling and biodeterioration (Dunne Jr., 2002). The bacterium was screened to study its potential of EPS production. In general, slime and capsular EPS are produced by bacterial cells to protect them against unfavorable environmental conditions such as: occurrence of toxic compounds, desiccation, uptake of metal ions and low temperature or high osmotic pressures (Hirst *et al*., 2003). In this context, the effect of PBE on EPS production was studied. The EPS concentrations were smaller than those in the control.

Table 1: Absorbance of biofilm and the percentage reduction of biofilm

Time (h)	Control Ab.	PBE Ab.	Reduction (%)
12	0.85±0.13	0.11±0.06	87.06
24	1.42±0.10	0.24±0.07	83.10
36	1.85±0.08	0.35±0.06	81.08
48	2.35±0.15	0.47±0.05	80.00
60	2.74±0.27	0.57±0.07	79.10
72	3.77±0.12	0.74±0.05	80.37

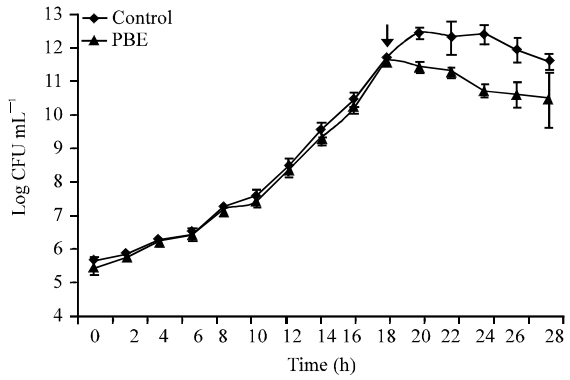

Fig. 1: The log (CFU mL^{-1}) results for the cells measured for control and PBE treated

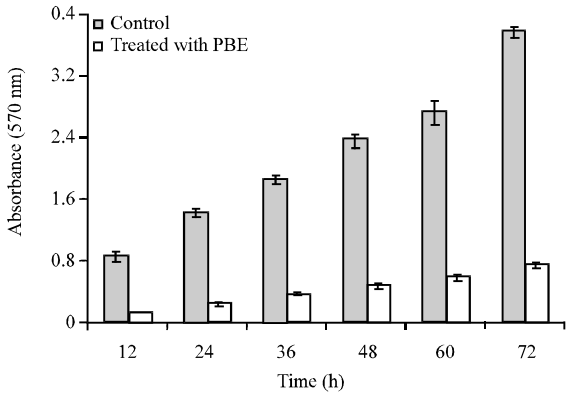

Fig. 2: Absorbance of biofilm formed at different time intervals at MIC of PBE

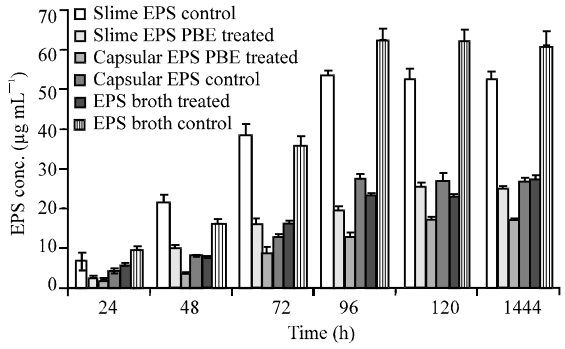

Fig. 3: Extracted EPS (slime, capsular, broth) concentrations of control and PBE treated

EPS concentrations, at the end of each day are presented in the Fig. 3. The quantity of slime EPS (52.54±3.47 μg mL^{-1}) and capsular EPS (26.61±2.50 μg mL^{-1}) produced by control were higher than PBE treated, after 6 days. Similarly the total EPS concentration (60.17±7.56 μg mL^{-1}) after 6 days was also higher than PBE treated. Subramanian *et al.* (2010) studied individual bacterial strains and they observed that individual microbial strains grow in a non-competitive environment and thus produce higher EPS concentration (0.5-34 μg mL^{-1}). It has been found that the amount of EPS is closely related to membrane permeability (Chang and Lee, 1998).

CONCLUSION

These investigations exhibit that PBE is effective in reducing biofilm formation, EPS production and growth rate caused by *Pseudomonas aeruginosa*. At MIC level, PBE used in experiments, reduced the bacterial growth rate and biofilm formation. While PBE greatly reduced EPS production compared to the control.

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