ORIGINAL ARTICLE

Effect of sodium chloride on cell surface hydrophobicity and formation of biofilm in membrane bioreactor

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Abstract

Kuntiya, A., Nicolella, C., Pyle, L. and Poosaran, N.
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Songklanakarin J. Sci. Technol., 2005, 27(5) : 1073-1082

This work presents an experimental study aimed at assessing the relationship between cell surface hydrophobicity (CSH) and the formation of a membrane-attached biofilm. In particular, the investigation focused on the effects of growth conditions on the hydrophobicity of bacterial cells grown in suspension. Identical growth conditions were then used in a lab-scale extractive membrane bioreactor fed on phenol as the sole carbon source to assess their effect on biofilm formation. CSH was found to decrease with increasing cellular age and in the presence of sodium chloride (0.5% w/v) in the growth medium. Pictures taken by a digital camera clearly showed the differences in biofilm morphology resulting from the different growth conditions: biofilms formed in the membrane bioreactor under low CSH (2%) condition proved to be thinner and more fragile than those formed under high CSH condition (44%).

Key words : membrane- attached biofilms, membrane bioreactor, cell surface hydrophobicity

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Received, 18 October 2004 Accepted, 13 January 2005

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ผลของโซเดียมคลอไรด์ต่อไฮโดรโฟบิซิตี้ของผิวเซลล์และการเกิดของฟิล์มชีวภาพในถังปฏิกรณ์ชนิดใช้เมมเบรน

ว. สงขลานครินทร์ วทท. 2548 27(5) : 1073-1082

การทดลองนี้เป็นการศึกษาความสัมพันธ์ระหว่างไฮโดรโฟบิซิตี้ของผิวเซลล์และการเกิดของฟิล์มชีวภาพที่ยึดติดกับเมมเบรน การทดลองมุ่งเน้นไปที่ผลของสภาวะการเจริญที่มีต่อไฮโดรโฟบิซิตี้ของเซลล์แบคทีเรียที่เลี้ยงในสภาพแขวนลอย จากนั้นใช้สภาวะการเจริญอย่างเดียวกันในการเลี้ยงแบคทีเรียในถังปฏิกรณ์ชนิดใช้เมมเบรนในระดับห้องปฏิบัติการโดยมีฟีนอลเป็นแหล่งคาร์บอนเพื่อศึกษาผลที่มีต่อการเกิดฟิลม์ชีวภาพ ผลการทดลองพบว่าไฮโดรโฟบิซิตี้ของผิวเซลล์ลดลงเมื่อเซลล์มีอายุเพิ่มขึ้นและมีการเติมโซเดียมคลอไรด์ (0.5 % น้ำหนัก/ปริมาตร) ในอาหารที่ใช้เลี้ยง ภาพถ่ายจากกล้องดิจิตอลแสดงลักษณะของฟิล์มชีวภาพที่แตกต่างกันอย่างชัดเจน ฟิล์มชีวภาพที่เกิดขึ้นภายใต้สภาวะที่ไฮโดรโฟบิซิตี้ของผิวเซลล์มีค่าต่ำ (2%) มีลักษณะบางกว่าและฉีกขาดง่ายกว่าฟิล์มชีวภาพที่เกิดขึ้นภายใต้สภาวะที่ไฮโดรโฟบิซิตี้ของผิวเซลล์มีค่าสูง (44%)

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The problem normally associated with membrane bioreactor operation is excessive biofilm growth in process equipment, resulting in reduction in heat and mass transfer fluxes, increased frictional resistance to fluid flow across the membrane, corrosion inside pipes, and problems in medical applications (Flemming *et al*., 1996). In membrane processes the surface properties of membranes may promote bacterial adhesion and the formation of membrane attached biofilms (MABs) is documented in membrane processes such as extractive membrane bioreactors (EMBs) (Livingston, 1993a,b) and bubbleless membrane bioreactors (Brindle and Stephenson, 1996). In these processes, MABs prove to be detrimental for process performance because of the build up of severe mass transfer limitation (Nicolella *et al*., 2000a). Flemming *et al*. (1996) reported techniques such as coatings to alter the surface energy of the substratum and mechanical treatment (e.g. brushing) to detach biofilm from the substratum. None of these techniques can be applied to avoid formation and accumulation of MABs in EMBs since coatings would modify transport properties of membranes and mechanical treatment is unfeasible in membrane modules. A novel extractive liquid membrane biphasic bioreactor, where the benefits of high shear forces and the use of a biphasic biomedium are effectively combined, has been reported to be operated without any biofilm formation or reduction in organic substrate flux across the membrane (Nicolella *et al*., 2000b; Splendiani *et al*., 2003). However, this bioreactor presents a very sophisticated process design.

Among the factors affecting biofilm formation, cell surface hydrophobicity (CSH) is an important property ruling the mechanism of bacterial attachment (van Loosdrecht *et al*., 1987). Experimental evidence suggests that CSH is influenced by factors such as the presence of cell appendages containing protein (Zita and Hermansson, 1997; Korber *et al*., 1989; Stenstrom and Kjellberg, 1985) and extracellular polymeric substances (Caccavo *et al*., 1997; Rosenberg *et al*., 1983). Results from many investigators have shown that CSH depends on growth conditions (Dabrowski *et al*.,2001, 2002; Kjelleberg and Hermansson, 1984; Wrangstadh *et al*., 1986;

Waligora *et al*., 1999). The influence of growth rate (Allison *et al*., 1990; van Loosdretch *et al*., 1987; Waligora *et al*., 1999) and cellular age (Fattom and Shilo, 1984; Rosenberg *et al*., 1980) on CSH have been reported. However, no correlation between attachment and CSH was reported. Caccavo *et al*. (1997) have reported that cells of bacterial strains with their surfaces composed predominantly of exopolysaccharide are significantly less hydrophobic than those composed of protein. So far, no specific information on the effect of exopolysaccharide on CSH is available even though it has been known that the exopolysaccharides are composed of hydrophobic and hydrophilic components and that the measured hydrophobicity reflects the average of the hydrophobicity of the two components (Daffochio *et al*., 1995).

In this work, the effects of growth conditions such as mineral medium composition and cellular age on CSH of *Pseudomonas* sp. grown in suspension were investigated. Identical growth conditions were then applied in a lab-scale extractive membrane bioreactor to assess their effect on MABs formation. The aim was to assess the relationship between the CSH and the formation of MABs in order to control the operating conditions to minimize biofilm formation in a membrane process.

Materials and Methods

Bacterial strain and inoculum preparation

Pseudomonas sp. isolated from a soil sample was used in this work. The bacterium, which was grown on phenol as carbon source, was maintained at 4ºC on nutrient agar slopes supplemented with 0.2 g/l phenol, in order to prevent possible plasmid loss. To prepare an inoculum, the bacterium was grown in nutrient broth supplemented with 0.2 g/l phenol at ambient temperature (30-35ºC) with orbital shaking (100-110 rpm) for 18-20 hours. Bacterial biomass was then harvested (16,000 g, 10 min, 4ºC), washed once with and suspended in sterile distilled water giving an OD of 0.9 at 600 nm when determined spectrophotometrically before being used.

Phenol solution and mineral salt medium

A stock solution of phenol was prepared by dissolving phenol pellets in distilled water (70 g/l). The stock solution was added to the feed flask to give/maintain its concentration at 0.75 g/l. The mineral salt medium used was that reported by Ralston and Vela (1974), but bromothymol blue was not included.

Phenol assay

Phenol was estimated by a direct photometric method (Greenberg *et al.,* 1992), which was based on a rapid condensation with 4-aminopyrine followed by oxidation with potassium ferricyanide under alkaline condition to give a red-coloured compound.

Cell density assay

Suspended biomass concentration was determined by a spectrophotometer (PYE UNICAM PU 8600 UV/VIS, Philips). The absorbance of samples was measured at 600 nm and the biomass concentration was calculated from a standard calibration curve.

Cell surface hydrophobicity (CSH) assay

Cell surface hydrophobicity was determined by the "Bacterial Adherence To Hydrocarbon" (BATH) method, which was based on the distribution of cells between water and an organic phase after a brief period of mixing. Results were expressed as the percentage of cells which were excluded from the aqueous phase, viz. (Ai-Af)/Ai x 100, where Ai and Af are the initial and net final optical densities of the aqueous phase, respectively. In this research, cells were harvested, washed and resuspended in a PUM buffer (Rosenberg *et al*., 1980) to a specified concentration before conducting the test as described by Mozes and Rouxhet (1987).

Shake flask experiments

Shake flask experiments were carried out in order to investigate the extent to which 0.5 g/l phenol could be used as a sole source of carbon and energy for the bacterium under aerobic growth

conditions. A range of growth conditions was used in these experiments. An inoculum and mineral medium were first prepared as described above; for those experiments in which the effect of sodium chloride on growth was to be studied, the salt was added to the medium at a concentration of 0.5% (w/v). One ml of the inoculum was inoculated into each of twenty 250 ml Erlenmeyer flasks containing 100 ml mineral salt medium containing 0.5 g/l phenol, initial pH 8.0. The flasks were then placed on the same shaker used for inoculum preparation for several days at ambient temperature (30-35ºC). Flasks were taken in turn at predetermined times and assayed for residual phenol (Greenberg *et al*., 1992), cell density, and cell surface hydrophobicity (Rosenberg *et al*., 1980; Mozes and Rouxhet, 1987).

Membrane bioreactor (MBR)

The membrane bioreactor used in this study, schematically represented in Figure 1, consisted of a single silicone rubber tube (3 mm i.d; 0.3 mm wall thickness and 200-240 mm length) held in a glass column (15 mm i.d., 300 mm length). The annular region outside the membrane was connected to a jacketed stirred tank bioreactor (1L, Lh fermentation, UK). The feed solution containing the phenol was circulated through the tube side of the membrane. A pH controller (Anglicon Solo2) held the pH on the fermenter side at 7.8-8.2 via addition of acid or alkali as necessary. The fermenter loop temperature was held constant at 30ºC by running warm water from a water bath (Grant) through the jacket. Agitation was at 150 rpm (Lh fermentation agitator 502D) with aeration sufficient to maintain 6-7 mg/l dissolved oxygen (DO) in the bioreactor.

MBR batch experiment

In a batch experiment, 15 ml of the inoculum was added to the bioreactor, which had been filled with 800 ml of mineral salt medium. The medium was recirculated by a gear pump (Micropump, Mod. GJ -N25FF1SAB1) from the bioreactor around the shell side of the membrane module at a flow rate of 900 ml/min. Aqueous phenol solution was recirculated through the membrane tube by an identical gear pump at a flow rate of 1,440 ml/min. Fifteen ml and 5 ml samples per day were withdrawn from the bioreactor and the feed flask, respectively, and assayed for phenol concentration (Greenberg *et al*., 1992). Samples from the bioreactor were additionally analyzed for suspended biomass concentration and cell surface hydro-

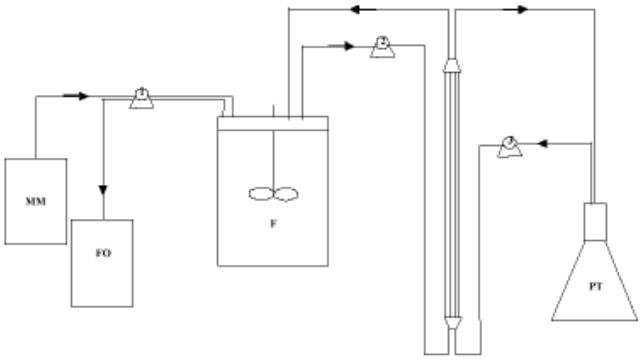

Figure 1. Simple schematic representation of the single tube extractive membrane bioreactor system: MM is the mineral medium tank; FO the feed outlet; F the bioreactor; PT the phenol tank. For continuous mode of operation, a multichannel pump (1) was used to connect the mineral medium tank and the feed outlet to the bioreactor. Mineral medium was pumped through the shell side of the membrane module by gear pump (2), and phenol pumped from the phenol tank through the membrane tube by gear pump (3).

phobicity (Mozes and Rouxhet, 1997).

MBR semi-batch experiments

The two semi-batch experiments were set up in exactly the same manner as that of the batch experiment, except that each time a sample was withdrawn from the bioreactor or the feed flask the volume of the biological growth medium was restored with fresh medium. The liquid volume in the feed flask was also restored with phenol stock solution in such a concentration as to restore the phenol concentration in the feed to the desired value of 0.75 g/l. Mineral salt concentration was restored to its original concentration once phenol accumulation was detected in the bioreactor. In these experiments, the formation of biofilm and the changes in its morphology were observed and registered in pictures taken by a digital camera.

Images

Pictures of the biofilm were taken using a digital camera (Camedia, Olympus C-2500L).

Results and Discussions

Shake flask experiments

Figure 2 (a, b) shows the change in phenol concentration, biomass concentration and cell surface hydrophobicity (CSH) during growth in the absence and in the presence of sodium chloride, respectively. Figure 2b shows that phenol degradation and cell growth were slightly faster in the presence of sodium chloride; phenol consumption was virtually complete in the presence of salt and the growth yield coefficient was lower in this case. This agrees with the result of Strachan *et al*. (1996) who reported that salt led to decreased growth as a consequence of the increased maintenance requirements. As can be seen from both sets of results cell hydrophobicity decreased as the cell concentration increased over time. Since the average cellular age in shake flask experiments increases with time (in this experiment it did so while the culture time was less than about 10/μ, data not shown), it was inferred from the results presented in Figure 2a and Figure 2b that CSH decreased with increasing cellular age: note that cell concentrations were adjusted to a constant value before conducting hydrophobicity tests, so the results were not the effect of increasing concentration. The hydrophobicity of cells grown in the medium with the addition of sodium chloride fell to zero faster than that of cells grown in the absence of the salt. By comparing Figure 2a and Figure 2b, it could be concluded that the addition of sodium chloride resulted in a faster decrease in

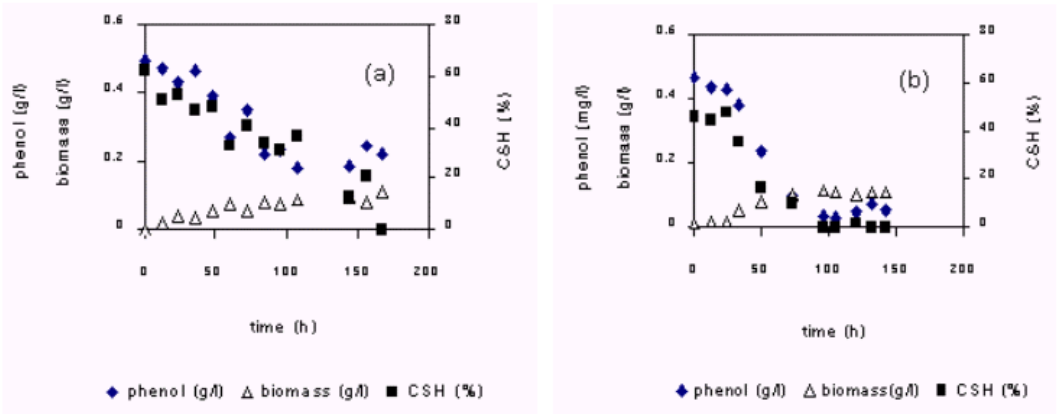

Figure 2. Shake flask experiments. Changes over time of phenol concentration, biomass concentration and cell surface hydrophobicity (CSH).
(a) Results from growth in the absence of NaCl.
(b) Results from growth in the presence of NaCl.

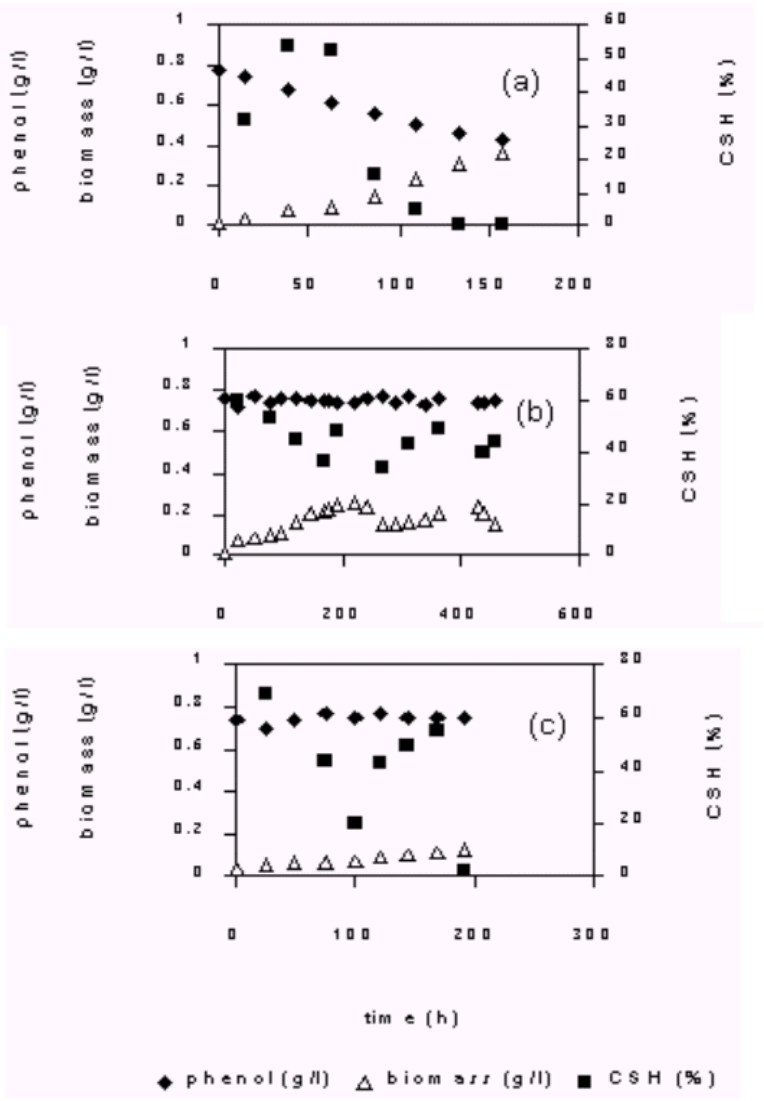

Figure 3. Change over time of phenol concentration, biomass concentration and cell surface hydrophobicity (CSH).
(a) Result from a batch experiment.
(b) Result from a semi-batch experiment in the absence of NaCl.
(c) Result from a semi-batch experiment in the presence of NaCl.

hydrophobicity. There appear to be at least three possible reasons for the observed changes in hydrophobicity. First, the presence of salts has been reported to increase exopolysaccharide production (Raihan *et al*., 1992; Singh and Fett, 1995) although the mechanism is not completely understood; this may, however, account for a drop in hydrophobicity (Caccavo *et al*., 1997), if the exopolysaccharides are predominantly neutral or hydrophilic. Second, it has been reported that the production of exopolysaccharides is higher with aged cells (Fattom and Shilo, 1984). Third, nutrient starvation in the batch culture may be another reason for lowering the hydrophobicity since this also triggers the production of exopolysaccharides (Wrangstadh *et al*., 1986).

Membrane bioreactor experiments

Cell surface properties vs MAB formation and growth. The evolution over time of cell surface hydrophobicity (CSH) in the EMB system (Figure 3) was similar to that observed in shake flask experiments (see Figure 2). The results of two very different experiments are shown in Figure 3. Figure 3a shows the results of an experiment carried out in pure batch mode, whereas Figures 3b and 3c show the results of experiments carried out in semi-batch mode, in which the phenol and mineral concentrations were replenished from time to time. When the experiment was operated in a pure batch mode, that is without adding minerals and without replenishing the phenol in the feed, with proper control of the growth medium pH, CSH also decreased with time and reached zero in 134 hours (Figure 3a). This suggests that the hydrophobicity depended on cellular age, at least in the pH range used here. The conditions for the cells in this experiment were rather similar to those in pure batch culture (shake flask experiments), since they were exposed to a fall in nutrient concentration and also to a decline in phenol flux because of the fall in feed concentration. This combination of conditions would lead inevitably to starvation and, ultimately, death of the cell population. Thus, it was concluded that increasing domination of the culture by old cells accounted for the low hydrophobicity. In contrast, when the experiment was operated in a semi-batch mode, i.e., minerals were added to the bioreactor from time to time to compensate for their decline as indicated by accumulation of phenol, and the phenol concentration was kept more or less constant by regular addition of phenol on the feed side, CSH decreased much more slowly over time (Figure 3b, 3c). In the absence of sodium chloride in the medium, even though the experiment had run for 455 hours, the CSH still remained high (44%) (Figure 3b). In contrast, in the presence of sodium chloride CSH decreased faster and was almost zero (2%) in only 243 hours (Figure 3c). This mirrorred the effect of sodium chloride on hydrophobicity in pure semi-batch culture.

The results could also be interpreted in the light of observations on the biofilm. In the experiment using mineral salt medium (Figure 3b), MAB formation started within 24 hours after an inoculum was added to the bioreactor. Cells initially formed small colonies on the membrane surface and over time joined together and grew leading to the formation of a biofilm (Figure 4a). The biofilm covered the whole surface of the membrane tube with a thickness varying along the membrane length. It was initially homogeneous in structure and lightly yellow in color with a fragile 'waving' appearance. Biofilm detachment was observed as early as a few days after its formation (Figure 4b), followed by re-formation of the active biofilm. The most likely reason for the intermittent biofilm instability was nutrient starvation and anaerobosis close to the membrane in particular. Shirtliff *et al*. (2002) have also argued that the nature of the biofilm itself once it gains maturation should also be taken into account. Biofilm detachment was possibly affected by stress due to the hydrodynamic conditions outside the film and/or enzymatic degradation (Boyd and Chakrabarty, 1994; Allison *et al*., 1998). Random detachment and attachment were observed throughout the experiment and a thick but fluffy, cotton-like biofilm was obtained after 20 days (Figure 4c). The increase in biomass concentration in the bioreactor over time (Figure 3b) was accompanied by visibly thicker biofilm which was in contrast to some other researchers' work (Livingston *et al*., 1993a; Zhang *et al*., 1998) which reported a steady decrease in biomass concentration when initial attachment was started. This was probably due to the dynamic nature of the biofilm formed in this experiment.

· · · On the other hand, when sodium chloride was present in the biomedium the start of biofilm formation was similar to that observed in the absence of the salt, but a thinner and more fragile biofilm was formed (Figure 5a). This biofilm was also homogeneous and initially covered the whole surface of the membrane tube. Detachment also occurred but not on such a large scale as observed in the absence of sodium chloride in the biomedium. Moreover, after the first biofilm had been detached there was no substantial biofilm reformation even

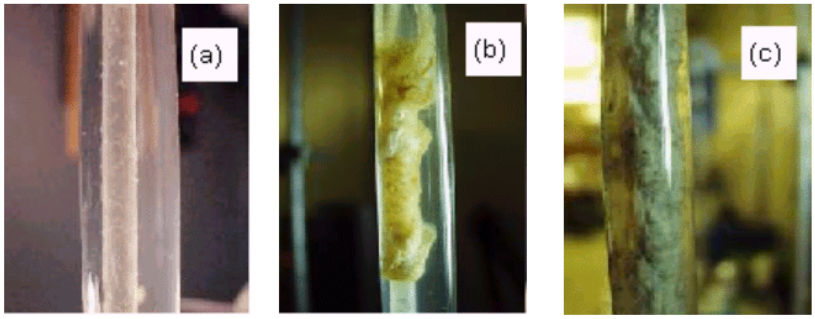

Figure 4. Different stages of MAB formation when *Pseudomonas* sp. was grown in a mineral salt medium in a semi-batch experiment.
(a) newly formed biofilm (2d);
(b) detached and rolling biofilm (7d);
(c) thick but fluffly, cotton-like biofilm (20d).

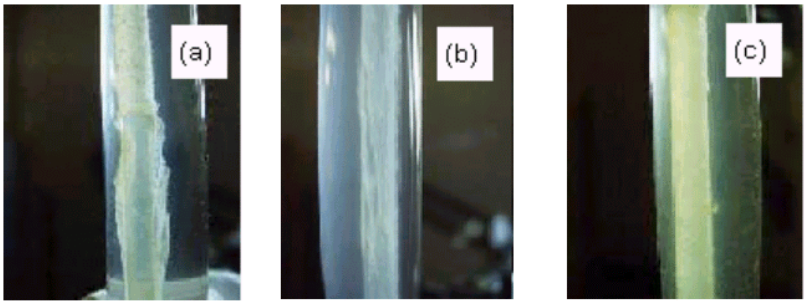

Figure 5. Different stages of MAB formation when *Pseudomonas* sp. was grown in a mineral salt medium supplemented with sodium chloride (0.5% w/v) in a semi- batch experiment.
(a) newly formed biofilm (2d);
(b) biofilm with random detachment (3d);
(c) uniformed biofilm (7d).

though scattered attachment was observed all over the membrane surface (Figure 5b). Interestingly, this happened when the cell hydrophobicity of the suspended cells was already low or zero, suggesting that non-hydrophobic cells were not attracted to hydrophobic silicone rubber membrane surface (and it was also much sooner than in the no-salt experiment). It also further suggests that interactions between cells and the membrane did, as hypothesised, play an important role in forming and controlling the biofilm in this membrane system. Since this latter effect happened when cell hydrophobicity of the suspended cells was low or zero, it is concluded that residual hydrophobicity in the absence of sodium chloride enabled the biofilm to reform and rebuild, whilst once the hydrophobicity had fallen to zero, the biofilm could not reform. It is suggested, therefore, that in the presence of sodium chloride, the cell physiology, surface properties and exopolymer production were changed; this was in line with the results reported above on the effect of sodium chloride on cell growth and maintenance requirement. The results demonstrated that the presence of sodium chloride in the medium and cellular age did affect CSH and consequently biofilm formation and growth. An increase in biomass concentration in the bioreactor over time was also detected in this

experiment (Figure 3c).

Phenol removal in the membrane bioreactor was stable throughout the experiments without any degradation problem due to biofilm formation (data not shown). Further investigation into the extraction rate of phenol through the membrane in the presence of the biofilm is needed to better understand process performance of the system used in this work.

Conclusions

The effect of growth conditions on cell surface hydrophobicity (CSH), and consequently on membrane-attached biofilm formation and growth, in an extractive membrane bioreactor was investigated in this work. CSH decreased with cellular age. Changing the medium composition by the addition of sodium chloride (0.5% w/v) resulted in a faster decrease in the CSH. The formation, growth and reformation after detachment of the membrane-attached biofilm was also slower in the presence of sodium chloride, confirming the cell growth studies. CSH plays a key role in biofilm formation and growth in membrane systems, and its control could be an important element in biofilm system performance.

Acknowledgements

The authors would like to acknowledge the Thai government for financial support. We also wish to thank Miss Srikarnjana Kloyroeng, Maejo University, Thailand, for providing *Pseudonomas* sp. used in this work.

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