2011 International Conference on Environment and BioScience
IPCBEE vol.21 (2011) © (2011) IACSIT Press, Singapore

Pedomicrobium Enumeration in Biofilm from an Expanded Bioreactor for Manganese Removal

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Abstract. The 'dirty water' complaints due to manganese (Mn) in the distribution system is a big issue not only due to aesthetics, but also because it causes laundry staining and restricted water flow. Discovered as suitable to be immobilized in biofilm reactors, *Pedomicrobium* was used for oxidation and removal of manganese in water to assist water authorities to improve and implement corrective measures. In this study, the diversity of *Pedomicrobium* from an expanded bed reactor was quantified by qRT-PCR. It was found that gravel and sand from the fluidized bioreactor bed contained 2.58 x 10^9 cells and 1.69 x 10^8 cells/gram respectively, whilst settled biomass from backwash water contained 1.8 x 10^9 of cells/mL. This showed that *Pedomicrobium* is able to naturally colonize the sand particles and produce active Mn-oxidizing biofilms.

Keywords: Dirty water, Manganese removal, Manganese oxidation, *Pedomicrobium*

1. Introduction

Accumulation of Mn oxides by microorganisms may lead to significant Mn deposition in natural environments. In pipelines and drinking-water distribution systems, deposition occurs where the build up increases frictional forces at the surface causing head loss at the turbines, reduced flow in distribution systems (Griffin, 1960) and Mn-related dirty water (Sly *et al*., 1988 and 1990a).

Nealson *et al.* (1980) discovered that Mn oxidation was enhanced when Mn-oxidizing bacteria were attached to surfaces, resulting in an increase in nutrient consumption and the release of Mn ions into the water. According to Sly *et al*. (1988), the biofilm formed by *Pedomicrobium* was affected by the initial attachment and its ability to grow beyond the deposited Mn oxide and remain dominant under high water velocity conditions. These cells make use of the nutrients and manganous ions that are constantly replenished by the water flow (Sly *et al*., 1988a and Marshall, 1980) which in turn enhanced the oxidation rate (Sly *et al*. 1988).

Sly *et al*. (1988) confirmed that *Pedomicrobium* is the dominant Mn-oxidizing bacterium in Mn depositing biofilms. It bound at the non-hyphal pole of the cell and grew by adhering to surfaces at the solid-liquid interface. This observation was related to the different nature, mechanisms and physiological activities of the microorganisms present in the high and low velocity biofilms. To sum up, higher velocity in water distribution systems meant greater deposition rate, as *Pedomicrobium* dominates the biofilm.

Various water authorities had exploited the ability of Mn-oxidizing bacteria to form biofilms in sand filters for the removal of Mn from drinking water (Mouchet, 1992). Sly and co-workers designed a fluidized bioreactor with *Pedomicrobium* immobilized on magnetite and this reactor successfully oxidized and removed Mn at laboratory scale (Sly *et al*., 1993) and pilot scale (Sly *et al*., 1997). In the plant scale experiments at the Hinze Dam, the research showed that the bioreactor could oxidize and remove Mn when operated with sand as the carrier (Brouwers et al., 2000) but removal may be better when operated as an

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expanded bed rather than as a fluidized bed. It was also discovered that *Pedomicrobium* colonized the sand particles naturally and it was not necessary to inoculate the bioreactor frequently.

This research aimed to use molecular methods to quantitate the *Pedomicrobium* in the biofilm on the sand particles and to determine diversity of bacteria and the diversity of *Pedomicrobium* in the biofilm.

2. Methodology

2.1. Bioreactor design and operation

A scaled-up demonstration of pilot plant based on the laboratory scale bioreactor of Sly *et al.* (1993) was constructed at Hinze Dam and was operated intermittently since 2004 in both batch and single pass continuous configuration (Fig 1). A combination of surface and bottom (hypolimnium) water was used to operate the column initially for a period of two weeks at a fluidising flow rate of less than 25-30 m/h. This served as optimal conditions for biofilm establishment, and a source for natural microorganisms including *Pedomicrobium* in the raw water.

The environmental samples taken from the bioreactor (backwash water or washwater, gravel and sand) were collected aseptically in sterile 100 ml plastic containers and transported to the laboratory within 3 hours for analysis. Washwater samples were allowed to settle and the settled biomass was used for analysis.

Detection of *Pedomicrobium* in the samples was done by DNA extraction and conventional Polymeras Chain Reaction (PCR) and Quantitative Real Time PCR (qRT-PCR) to confirm the presence of *Pedomicrobium* in the samples.

2.2. Quantitation of *Pedomicrobium* by qReal-Time PCR

In this study, the qRT-PCR method developed by Wong (2004) was used and the results were presented as amplification curves that were used in quantitating the initial number of target templates in the samples. Due to the reciprocal relationship between the C_T value and the starting copy number of the template, the former was used for quantitation of the initial template number in the samples as it represented the PCR cycle at which an increase in fluorescence above a threshold could first be detected.

2.2.1 Dilutions of the cell suspension for standard curve

Five-fold dilutions of *Pedomicrobium* sp. ACM 3067 from 1.00 to 0.00064 was performed to determine the sensitivity of the probes and primers used and the relationship between cell number and C_T value. Samples of the dilution series were then used in direct microscopic enumeration counts and qRT-PCR to determine a standard curve and for estimation of cell numbers in the environmental samples.

3. Results and discussion

3.1. Direct Microscopic Enumeration Counts

Direct microscopic enumeration counts were conducted only on the undiluted and the first three dilutions in the series (0.2, 0.04 and 0.008). *Pedomicrobium* cells in clumps were counted as individuals on the assumption that each cell contained DNA even when in a clump. The number of cells in the undiluted sample was estimated by the method developed by Wong (2004).

1.00

			1085
		1102	
	1096		
1121			

$=4404 \times (1x5^0) \times 312{,}500$

$=1{,}376{,}250{,}000$ cells/mL

0.2

			364
		363	
	360		
353			

$=1440 \times (1x5^1) \times 312{,}500$

$=2{,}250{,}000{,}000$ cells/mL

0.04

			66
		63	
	75		
76			

$=280 \times (1x5^2) \times 312{,}500$

$=2{,}187{,}500{,}000$ cells/mL

0.008

			13
		13	
	14		
15			

$= 55 \times (1x5^3) \times 312{,}500$

$=2{,}148{,}437{,}500$ cells/mL

Average cells per milliliter of undiluted sample (from microscopic enumeration):-

= (Cells per mL in dilution 0.2, 0.04 and 0.008) / 3

= (2,250,000,000 + 2,187,500,000 + 2,148,437,500) / 3
= 2,195,312,500 cells/mL

Note: Cells per mL in dilution 1.00 was omitted as the value varied significantly from the figures obtained in the cell count of other dilutions, possibly due to clumping.

As 5 µl of template was used in each five-fold dilution for qRT-PCR, the number of cells per reaction was calculated using the following formula:-

$$\text{Cells per reaction} = \frac{\text{Average cells per mL x 5 µl}}{1000\mu\text{l}}$$

Based on the results of number of cells present in the standard 1.00, the number of cells in all dilutions was estimated by dividing by the dilution factor. The expected numbers of cells present in each dilution over the different dilutions are summarized in Table 1.

Table 1: Expected Number of Cells per 25 µl Reaction Estimated from Direct Microscopic Enumeration Counts

Dilution	Cells per 25 µl reaction (estimated)	Cells per 25 µl reaction (actual)
1.00 ($1/5^{0}$)	10,976,563	10,976,563
0.2 ($1/5^{-1}$)	2,195,313	2,250,000
0.04 ($1/5^{-2}$)	439,063	437,500
0.008 ($1/5^{-3}$)	87,813	85,938
0.0016 ($1/5^{-4}$)	17,563	-
0.00032 ($1/5^{-5}$)	3513	-
0.00064 ($1/5^{-6}$)	703	-
0.0000128 ($1/5^{-7}$)	141	-
0.00000256 ($1/5^{-8}$)	28	-
0.000000512 ($1/5^{-9}$)	6	-
0.000000102 ($1/5^{-10}$)	1	-

3.2. *Pedomicrobium* enumeration environmental samples

It was observed that the amplification curves of environmental samples occurred within those of the dilution series (as shown in Fig 1), thus dilution of the environmental samples was not required. The lower C_T value of the standard 1.00 indicated the higher amount of template present, which meant that the sooner the threshold level was reached for the assay to generate a particular amount of PCR products or fluorescence.

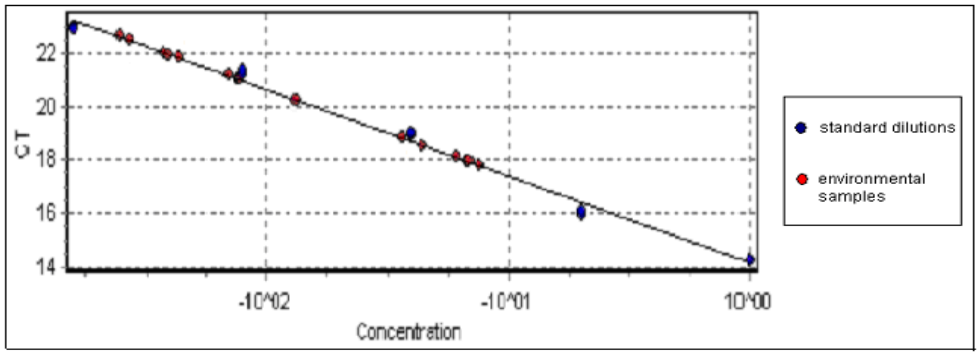

Fig. 1: Plot of environmental sample C_T values that fall within the C_T values of standard dilutions.

Fig.2 revealed that the plots of C_T values against log of the estimated bacterial number and against log of the actual bacterial number respectively. The graphs were quite similar except for the slope, which meant that either graph can be used for enumeration. The R^2 values for the two were 0.992138 and 0.993265.

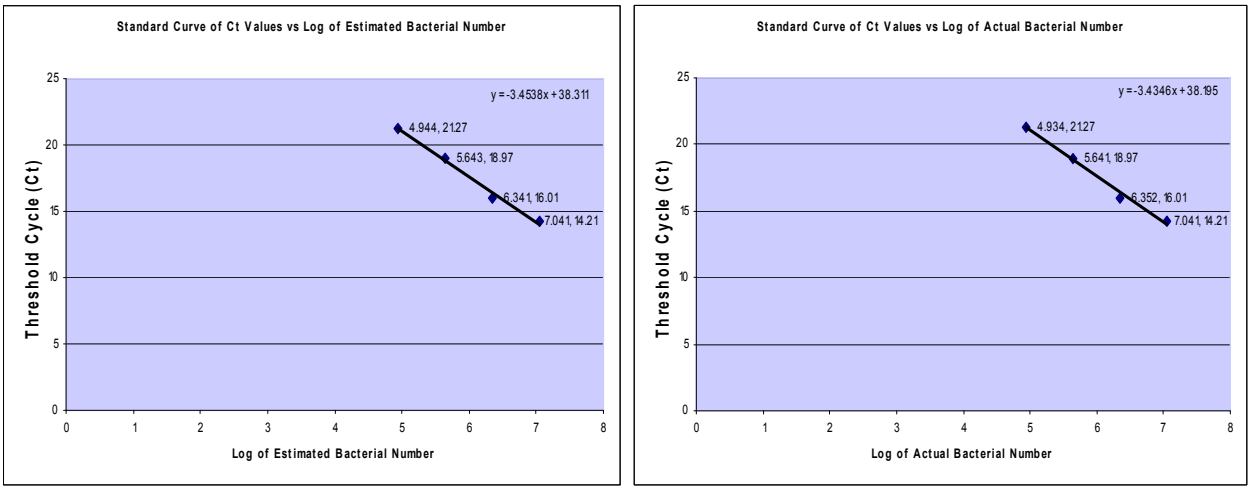

Fig. 2: Standard curve of threshold cycle versus log of estimated and actual bacterial number (standard 1.00 to dilution 0.0016)

There was a variation between replicates in both standard dilutions and environmental samples despite the same volumes of 5 µl being used, but generally replicates showed good reproducibility. An explanation to this lies in the clumping nature of *Pedomicrobium* besides operator error.

By substituting the C_T values of environmental samples into the equation, the cell numbers per reaction could be calculated as per Table 2.

Table 2: Calculation of cell numbers in environmental samples

y	=	-3.257x + 14.132
Log bacteria no.	=	-3.257 x C_T + 14.132
Bacteria no.	=	$10^{-0.3257 \times CT + 14.132}$

Washwater (average)	Gravel	Sand
No. of bacteria per reaction^	$= 10^{-0.3257 \times CT + 14.132}$	
$= 10^{-0.3257 \times 20.19 + 14.132}$	$= 10^{-0.3257 \times 18.48 + 14.132}$	$= 10^{-0.3257 \times 22.12 + 14.132}$
= 35,984,626 cells per 5 µl	= 129,737,044 cells per 5 µl	= 8,462,837 cells per 5 µl
∴ 7,196,925 cells per 1 µl	∴ 25,947,408 cells per 1 µl	∴ 1,692,567 cells per 1 µl
Cells per 1 ml of DNA sample	**= Eluted amount x cells per 1 µl***	
= (50µl x 7,196,925 cells)	= (50µl x 25,947,408 cells)	= (50µl x 1,692,567 cells)
= 3.6 x 10^8 cells in 200 µl of settled biomass of washwater	= 1.29 x 10^9 cells in 500 mg of gravel sample	= 8.46 x 10^7 cells in 500 mg of sand sample

^ 5 µl of extracted DNA was used per reaction

*1 ml of extracted DNA was eluted out into 50µl of DES.

From the results, it can be seen that all the samples from the bioreactor had high numbers of *Pedomicrobium* cells indicating that *Pedomicrobium* had become established in the biofilm. The settled biomass in the washwater samples contained 1.8 x 10^9 per mL of settled biomass, obtained by backwashing the sand and gravel at a water velocity of 70 m/hour. The gravel and sand samples contained approximately 2.58 x 10^9 cells and 1.69 x 10^8 cells per gram. This correlated well with the visual observation that showed more biofilm on the gravel than on the sand particles, although the sand had a higher surface area. This result may be explained by the gravel being in the bottom of the reactor and potentially having access to more nutrients than the sand above. However, it is more likely due to collisions of the sand particles and the biofilm having less protection from abrasion.

The number of *Pedomicrobium* detected on sand particles was higher than the results obtained by Wong (2004) which was 3.5 x 10^6 cells per mL of sand. These results indicated that *Pedomicrobium* was able to naturally colonize sand and gravel in the bioreactor and form effective Mn-removing biofilm.

4. Conclusion

The presence of Mn in water has extensive consequences, ranging from low aesthetic quality and flavour of the water, restricted pressure of water and increased costs. Owing to the ability of *Pedomicrobium* to oxidize and deposit Mn that causes 'dirty water' problems, various methods were developed to combat it via its biotechnological application. Water velocity is found to have a significant influence to the nature and physiological activity of biofilms. Biofilms on the solid surface could use up the nutrient at the liquid-solid interface thereby increasing Mn oxidation rate.

Due to the difficult DNA recovery, a modified genomic DNA extraction might be required, because extraction from pure cultures is different from extraction from environmental samples. However, the standard curve plotted in this study was successfully used in the cell enumeration of *Pedomicrobium* sp. The results showed that the backwash water contained 1.8×10^9 per mL of settled biomass whereas the gravel and sand contained 2.58×10^9 cells and 1.69×10^8 cells per gram respectively. These results showed that *Pedomicrobium* can successfully colonize the fluidized bed sand particles in the bioreactor without the need for frequent inoculation and produce active Mn-oxidizing biofilms.

5. Acknowledgements

The authors would like to thank Dr. Mark Fegan of The University of Queensland, The Gold Coast City Council, Dr. David Dixon of CSIRO, Geoff Hamilton and Barry Chiswell, team of the Sly Lab (Eric Chang, Eileen Koh, David Liu) as well as Rick Webb of the EMS lab and Bob Simpson, the RT-PCR expert.

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