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PERSISTENCE AND COMPETITION OF DENITRIFYING BACTERIA IN A DUAL-STRAIN BIOFILM SUBJECTED TO A NATURAL WASTEWATER FLORA

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Abstract

Bioaugmentation for enhanced wastewater treatment performance depends on survival, maintained activity and retention of the inoculated microorganisms in the reactor. Biofilms could be suitable mediators of bioaugmentation due to the retention of the immobilized cells and the protection offered by the biofilm matrix. The overall objective of this study was to investigate the potential of biofilm-mediated bioaugmentation for enhanced denitrification in the presence of a natural wastewater flora. In order to select a suitable inoculum, single and mixed strain biofilms were investigated in sterile wastewater with respect to biofilm formation, denitrification activity and bacterial interactions. The dual strain biofilm formed by the denitrifying organisms *Comamonas denitrificans* and *Brachymonas denitrificans* displayed good biofilm formation and high denitrification activity and was thus further investigated. When exposed to a natural wastewater flora, the relative number of *C. denitrificans* and *B. denitrificans* cells in the biofilm decreased over time until it was stabilized at just below 20 % of the total population after 4 weeks. The selected strains were thereby shown to persist in the competition with natural wastewater flora, highlighting the potential of biofilm mediated bioaugmentation.

Key words – Biofilm, denitrification, fluorescent *in situ* hybridisation, wastewater treatment

Introduction

Biofilms are hydrated, surface attached clusters of cells embedded in extracellular polymeric substances (Stewart & Franklin, 2008) which offer their resident cells protection from the surrounding environment (Branda, *et al.*, 2005). Biofilm processes are commonly used for wastewater treatment since they possess advantageous features such as high cell retention time, facilitated solid-liquid separation, resistance to shock loadings, high active biomass concentration, ability to adsorb metals and organic material as well as high microbial diversity (Verma, *et al.*, 2006).

Introduction of specific bacterial species to wastewater treatment reactors in order to enhance the removal efficiency of nutrients, complex organic carbons or toxic compounds is called bioaugmentation (van Limbergen, *et al.*, 1998). For a successful bioaugmentation the introduced bacteria must survive, maintain active and be retained in the new environment (van Limbergen, *et al.*, 1998). Biofilm-mediated bioaugmentation is a technique with vast potential for wastewater treatment (Singh, *et al.*, 2006). Biofilm existence offers the selected microorganisms protection against both external dangers and washouts.

Denitrification is the process in which nitrogen is removed from wastewater by reduction of nitrate to nitrogen gas. A bioaugmentation mediated enhancement of denitrification activity can be desired at start up of new or interrupted treatment systems, in systems with deficient denitrification activity or in plants with an unstable performance. Denitrification key-organisms,

Comamonas denitrificans 110 and *Brachymonas denitrificans* B79 were previously isolated from wastewater treatment plants (Gumaelius, *et al.*, 2001, Leta, *et al.*, 2003) and were also shown to form biofilm under various nutritional conditions (Andersson, *et al.*, 2008, Andersson, *et al.*, 2009). The aim of this study was to investigate if the bacteria in a biofilm formed by the denitrification key-organisms could proliferate and compete when subjected to a natural wastewater flora.

Methods

Bacterial strains and culture medium

Two biomarker organisms for denitrification, *Comamonas denitrificans* 110 ATCC900937 and *Brachymonas denitrificans* B79 CCUG45880, previously isolated in our laboratory from Bromma wastewater treatment plant, Sweden, and Ethio Tannery wastewater treatment plant, Ethiopia, were used in the study (Gumaelius, *et al.*, 2001, Leta, *et al.*, 2003). In addition, *Acinetobacter calcoaceticus* ATCC23055, an organism commonly isolated from wastewater treatment plants and found to promote intergeneric coaggregation was also included (Andersson, *et al.*, 2008, Simões, *et al.*, 2008). Municipal wastewater collected at Henriksdals wastewater treatment plant in Stockholm was used as culture medium. The wastewater was divided into two parts one of which was sterile filtered twice (Sarstedt 0.2µm) and frozen at –20°C in 100mL aliquots (sterile-WW) and the other was frozen in aliquots without treatment (WW).

Culture conditions

The first experiment was prepared by inoculating fourteen identical 100ml Erlenmeyer flasks holding 30 Kaldnes K1 carrier units and 20ml stWW each, in duplicate, with 2mL overnight culture diluted to optical density 0.5 of single, dual and triple strain solution of *C. denitrificans*, *B. denitrificans* and *A. calcoaceticus*. The flasks were incubated aerobically for 28 days. Every other day the culture medium was replaced. Denitrification activity and biofilm formation was measured every week.

In the second experiment biofilms of *C. denitrificans* and *B. denitrificans* were allowed to form on 200 Kaldnes K1 carriers units in sterile-WW for two weeks (100 rpm, 30°C). Culture medium and inoculum was added every other day. Then, one hundred carrier units were transferred to each of two 500 ml Erlenmeyer flasks. One flask was fed with sterile-WW and the other with WW. The flasks were incubated aerobically (100 rpm, 30°C) for 42 days. Culture medium was replaced every other day and each time viable counts were performed on the fresh WW and the discarded culture medium from both flasks. Denitrification activity, biofilm formation and bacterial diversity (FISH) in the biofilm were assessed once a week.

Denitrification activity test

Eight randomly selected carriers from each flask were rinsed with sterile PBS and transferred under sterile conditions to capped sterile glass tubes with 10 mL sterile-WW supplemented with 10mg L^{-1} $NaNO_2$-N. The nitrite concentration was measured every 30–60 minutes (Merck spectroquant reagent no. 1.14776.0001, Aquamate spectrophotometer) for 5 hours and the denitrification activity (mg NO_2-N L_{K1}^{-1} d^{-1}) was determined.

Biofilm formation

Four randomly selected carrier units from each flask were rinsed five times with sterile Milli-Q water to remove loosely associated cells, dried in room temperature for one hour and then submerged in 0.1 % crystal violet solution for 45 minutes. The excess crystal violet dye was thoroughly rinsed off with Milli-Q water and each carrier was submerged in 1mL 96 % ethanol for 5 minutes. Next, 200µL of the ethanol-dye solution was transferred to a 96 well microtiter plate in duplicates. The absorbance was measured at 590 nm using a Floustar-Optima spectrophotometer.

Fluorescent *in situ* hybridization (FISH)

Two randomly selected carrier units were rinsed with PBS, fixed with 4 % paraformaldehyde, detached using ultrasonication and hybridized as described previously (Andersson, *et al.*, 2008) using the oligonucleotide probes EUB338 (Amann, *et al.*, 1990) targeting all bacteria, DEN (unpublished) targeting *Comamonas denitrificans* and OTU6-178 (Juretschko, *et al.*, 2002) targeting *Brachymonas denitrificans*.

Results

In the first experimental set up the influence of interspecies interactions and biofilm formation on denitrification activity was investigated. As expected, the two denitrifying strains showed high denitrification activity while *A. calcoaceticus* did not denitrify to any significant extent (Figure 1). The dual strain biofilm with *C. denitrificans* and *B. denitrificans* had a higher denitrification activity than the corresponding single strains during the first two weeks. A subsequent stagnation of activity in the dual strain biofilm was then observed at the same

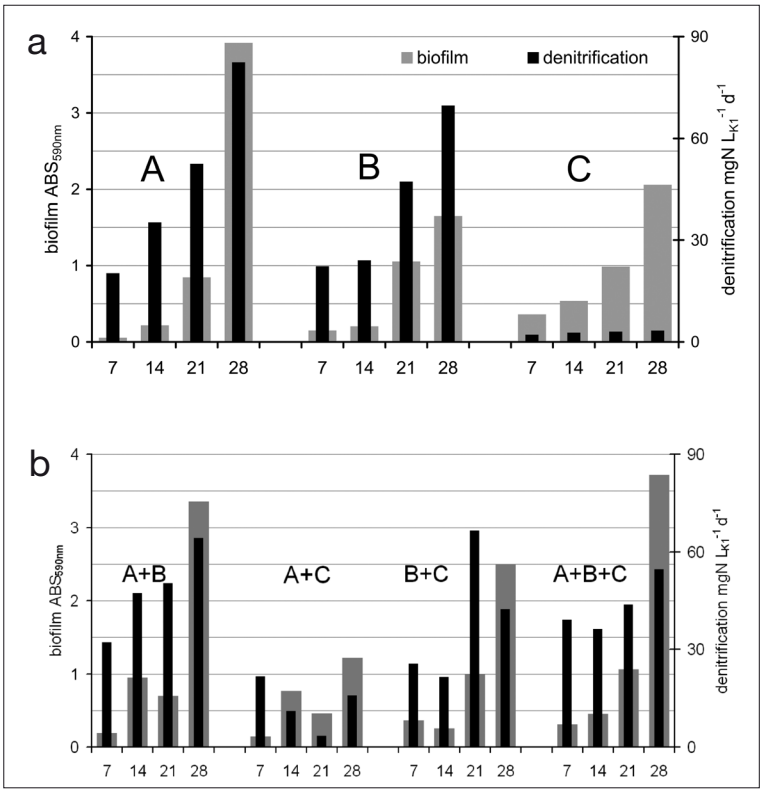

Figure 1. *The biofilm formation and denitrification activity of the single (a), dual and triple (b) strain cultures in experiment 1 using sterile WW.* **A** *is C. denitrificans*, **B** *is B. denitrificans and* **C** *is A. calcoaceticus. The x-axis shows the time (days).*

time as the two single strain denitrification activities continued to increase. The dual strain biofilm involving *C. denitrificans* and *A. calcoaceticus* formed little biofilm at all time points and the denitrification activity displayed low values except for the measurement on day seven. The dual strain culture with *B. denitrificans* and *A. calcoaceticus* formed stronger biofilm on day 28 than the respective single strains, however the denitrification activity at this time point was lower than for pure strain *B. denitrificans*. The triple strain biofilm performed stably with a rather constant denitrification activity and increasing biofilm formation. The amount of formed biofilm was rather similar for all cultures until the measurement on week four when the biofilms formed by *C. denitrificans* in single strain culture, in dual strain culture with *B. denitrificans* and in triple strain culture showed a considerable increase (Figure 1).

The second experiment was set up to investigate the survival and competitiveness of an existing biofilm exposed to a natural wastewater flora. Based on the outcome of the first experiment a dual strain system comprising *C. denitrificans* and *B. denitrificans* was chosen. Two weeks old biofilms were then exposed to WW. The thawed WW contained an average of 2×10^5 CFU mL^{-1} and according to FISH analysis no bacterial cells in the WW were targeted by the probes OTU6-178 or DEN, ensuring that subsequent FISH analyses only discerned the proliferation of the initially inoculated population. The relative number of *C. denitrificans* and *B. denitrificans* cells decreased exponentially during the first four weeks to a relative abundance of just below 20 % of the total cells (Figure 2a). *C. denitrificans* was slightly more competitive in the presence of WW bacteria than *B. denitrificans* while the reference culture which was fed with sterile-WW displayed a fluctuating equilibrium between the number of *B. denitrificans* and *C. denitrificans* cells (Figure 2b). The denitrification activity in both cultures increased drastically on day 21 onwards (Figure 2c). The dual-strain biofilm displayed a higher denitrification activity than the mixed strain biofilm. The mixed strain biofilm on the other hand formed more biofilm (Figure 2d).

By plotting the denitrification activity against the biofilm formation a logarithmic relationship was found (Figure 3, Table 1) for all biofilm samples except single strain *A. calcoaceticus* and dual strain *A. calcoaceticus* and

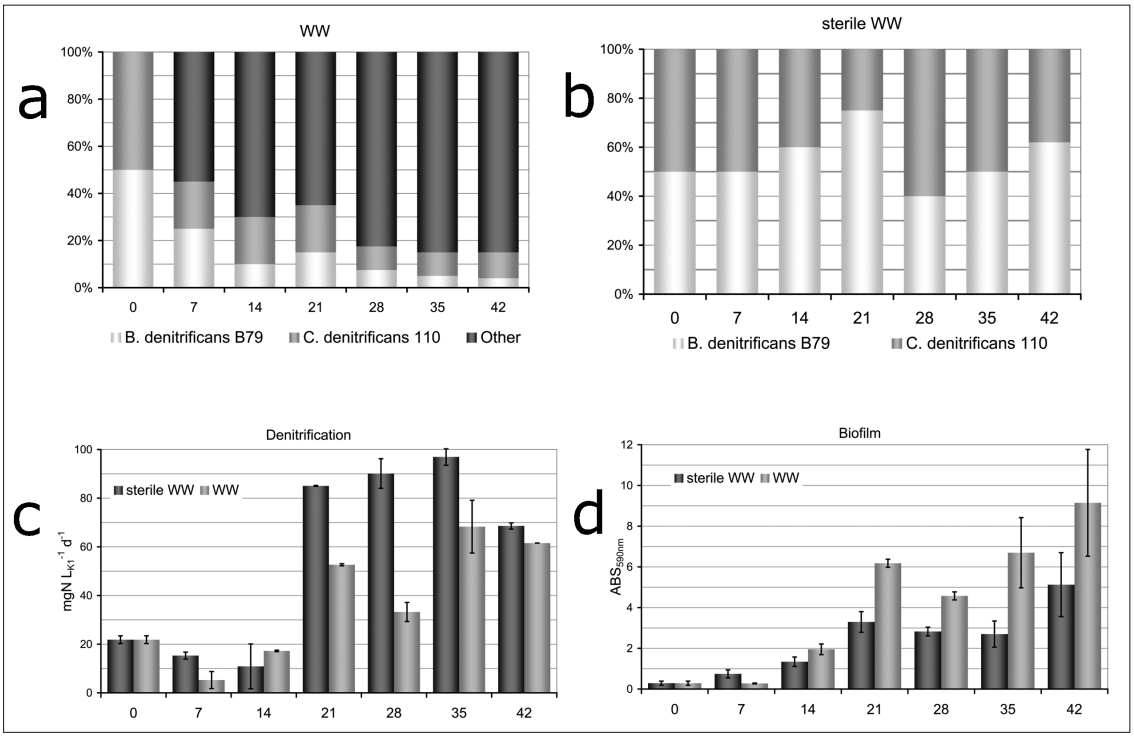

Figure 2. *The results from experiment 2. The composition of the microbial flora in the biofilms at different time points, as determined by FISH. (a) shows the system fed with WW and (b) the system fed with sterile-WW. Figure (c) shows the denitrification activity and (d) the biofilm formation at different time points in the two systems respectively. The x-axis shows the time (days).*

C. denitrificans. Dual strain biofilms of *C. denitrificans* and *B. denitrificans* fed with sterile-WW were assessed in both the first and second experiment giving rise to slightly diverging results.

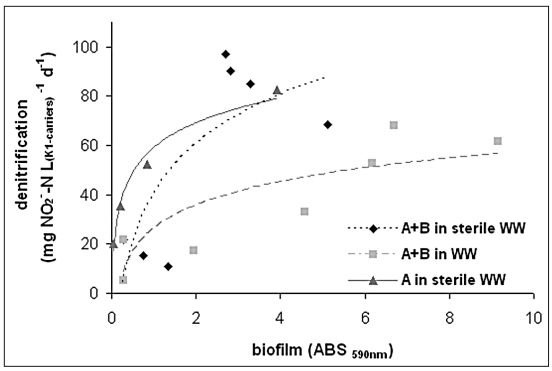

Figure 3. *The relationship between denitrification rate and biofilm amount for three of the assessed cultures where A is C. denitrificans and B is B. denitrificans. Adaptation of logarithmic curves (y = k×Ln(x) + m) gave high correlation coefficients for most cultures as can be seen in Table 1.*

Discussion

The present study investigated the proliferation and competition of two denitrifying strains in biofilm on suspended carriers subjected to a natural bacterial WW flora (experiment 2). First (experiment 1), a simplified system with dual and triple strain biofilms was used to determine which starting inoculum to use in the second experiment. The choice of Kaldnes K1 carriers as biofilm support was made based on (i) the carriers track record of successful full scale and pilot scale implementations (Dalentoft & Thulin, 1997, Welander & Mattiasson, 2003) (ii) its applicability for single stain biofilm growth (Andersson, *et al.*, 2008) and (iii) the inability of the high density polyethylene plastic to bind crystal violet dye.

The first experiment assessed the biofilm formation and denitrification activity as well as the influence of interspecies interactions in single, dual and triple stain cultures over a time period of four weeks. The dual strain biofilm comprising the two denitrifying strains was selected for the second experiment based on the consistently high denitrification activity. Both of the denitrify-

Table 1. *The denitrification rate (mg NO-N L(K1-carriers)$^{-1}$, d^{-1}, y, as a logarithmic function of the amount of biofilm (ABS_{590nm}), x, expressed as: y = k×Ln(x) + m (Figure 3). A lower k-value means that the denitrification activity is less dependent on the amount of biofilm while a high k-value conveys a stronger dependency between denitrification activity and biofilm formation. The m-value is the denitrification activity at Ln(x) = 0, i.e. when the absorbance (590nm) is one. R^2 is the correlation coefficient.*

Single, dual or triple strain biofilms formed by	k	m	R^2
Experiment 1			
C. denitrificans	14	59	0.98
B. denitrificans	18	54	0.93
A. calcoaceticus	–	–	–
C. denitrificans, B. denitrificans	11	51	0.96
C. denitrificans, A. calcoaceticus	–	–	–
B. denitrificans, A. calcoaceticus	6	44	0.40
C. denitrificans, B. denitrificans, A. calcoaceticus	7	44	0.91
Experiment 2			
C. denitrificans, B. denitrificans in sterile-WW	29	41	0.58
C. denitrificans, B. denitrificans in WW	14	26	0.72

ing strains displayed high activity also in single culture, however, the enhanced chance of survival and proliferation of at least one strain in a dual strain biofilm consortium was considered to be a potential advantage. Recent research suggest that *A. calcoaceticus* can act as a bridging organism in biofilm formation and development by means of protein-saccharide mediated interactions (Andersson, *et al.*, 2008, Simões, *et al.*, 2008). In this study limited synergistic effects on biofilm formation was seen in the presence of *A. calcoaceticus*, possibly explained by the differences in time frame between the studies. The referred studies were based on biofilms grown for 24–72 h while the present study dealt with considerably older biofilms, grown for 7–28 days. The bridging mechanism of *A. calcoaceticus* is probably more pronounced during the initial attachment phase of biofilm formation or in initial co-attachment of biofilm deficient strains.

The second experiment assessed the proliferation of a *C. denitrificans* and *B. denitrificans* dual strain biofilm in competition with a natural WW flora. Proliferation of both *C. denitrificans* and *B. denitrificans* was seen in competition with natural WW microorganisms. A stable fraction of just below 20 % of the total population was eventually reached with *C. denitrificans* constituting the major part. The lower denitrification rate in the WW fed system compared to the system fed with sterile-WW most likely depends on the presence of non-denitrifying strains in the WW fed system, reducing the overall nitrite reduction ability of the biofilm. However, denitrification could also be inhibited by metabolic products secreted by some of the other microorganisms in the community. The biofilm formation on the other hand was enhanced by the presence of natural WW microorganisms. Previous studies have shown that multi species consortia often promote biofilm formation and stability (Burmolle, *et al.*, 2006, Andersson, *et al.*, 2008).

The reproducibility of long term biofilm studies is generally low, even if the experimental conditions are kept constant (Lewandowski, *et al.*, 2004). The multitude of factors affecting biofilm formation and development are difficult, not to say impossible, to control (Branda, *et al.*, 2005). Thus it is not surprising that *C. denitrificans* and *B. denitrificans* dual strain biofilm generated slightly different results in the two experimental set ups (experiment 1 and 2). However, the deviation between the experimental set ups (reactor volumes, number of carriers and start up culture time) may be sufficient for the differences in the obtained results.

In general, the denitrification rates in the microbial biofilms used in this study was low, maximum value 97 mg N L(K1 carriers)$^{-1}$ d^{-1} corresponding to 0.4g N m^{-2} d^{-1}. This is roughly a tenfold lower than results obtained by Welander and Mattiasson (Welander & Mattiasson, 2003) using the same carriers at 20°C fed with 5g L^{-1} acetate. Denitrification is an anoxic respiratory process, meaning it is directly proportional to microbial activity. The main factors influencing the denitrification activity within a biofilm are the biochemical conversion rate, the carbon (C) to nitrogen (N) ratio of the supplied medium and the transport of substrates into the biofilm (Henze, *et al.*, 2002, Persson, *et al.*, 2006). At a C:N ratio above 3.4, carbon should theoretically not be the limiting substrate. The measured COD value of the

thawed WW was 220 mg O_2 L^{-1}. If all organic carbon is assumed to be represented by acetate this would correspond to a C:N ratio of around 9 (1g acetate = 1.07 g COD, (Farabegoli*, et al.*, 2003)). However, the biologically available fraction of COD is probably considerably lower than 220 mg O_2 L^{-1} and composed of more complex molecules than acetate, which could convey carbon limitation and as a result low denitrification rates. Another factor which might have negatively influenced the denitrification rate is the concentration of molecular oxygen in the culture medium. No active oxygen removal was made (e.g. by nitrogen gas) resulting in a slowly decreasing oxygen concentration in the medium, retarding the denitrification rate.

The logarithmic dependence of denitrification activity on biofilm formation suggested occurrence of substrate diffusion limitations in thicker biofilms. The different *k*- and *m*-values in the formulas describing the assessed biofilms (Table 1) show that substrate limitation occurred in thinner biofilm for the tripe strain cultures while the single and dual strain cultures formed by the two denitrifying strains had a higher denitrification activity per biofilm unit as well as a continuous increase in denitrification activity also in thick biofilms.

The results presented here provide preliminary results on the retention, proliferation and competitiveness of two denitrifying key-organisms in biofilm fed by natural wastewater.

Conclusion

Co-culturing of the two denitrifying organisms *C. denitrificans* and *B. denitrificans* in sterile-WW resulted in strong biofilms with high denitrification activity. A pregrown biofilm of these two strains subjected to a natural wastewater flora showed the ability of the strains to proliferate and compete within the biofilm. The potential of biofilm mediated bioaugmentation with respect to both survival and retention was thus confirmed to be high. Studies in larger systems over a longer time period is required for further optimizations.

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