Pergamon

Wat. Sci. Tech. Vol. 36, No. 10, pp. 1–8, 1997.

Printed in Great Britain.
0273–1223/97 $17·00 + 0·00

97-828

PII: S0273-1223(97)00636-7

RETENTION AND EXPRESSION OF RECOMBINANT PLASMIDS IN SUSPENDED AND BIOFILM-BOUND BACTERIA DEGRADING TRICHLOROETHENE (TCE)

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ABSTRACT

Exposure of plasmid recombinant microorganisms to an open environment, either inadvertently or intentionally, requires research into those fundamental processes that govern plasmid retention, transfer and expression. In the open environment, a majority of the microbial activity occurs associated with an interface, within thin biological layers consisting of cells and their insoluble extracellular polymer, layers known as *biofilms*. Current toxic wastewater or wastegas treatment reactors exploit bacterial biofilm systems for certain system operating advantages.

Using recombinant bacteria within a biofilm reactor to degrade xenobiotic wastes requires finding a suitable host to harbor and express the desired plasmid phenotype. Suitable host characteristics include: the ability to produce copious amounts of biofilm, resistance to waste-related injury and toxicity, and the ability to retain and express the desired plasmid during long term operation. This paper reports on a laboratory evaluation of factors governing plasmid retention and the expression of trichloroethene (TCE) degradative capacity in both suspended and biofilm cultures. © 1997 IAWQ. Published by Elsevier Science Ltd

KEYWORDS

Plasmids; bacteria; DNA; recombinant microorganisms; biofilm.

INTRODUCTION

Plasmids are non-chromosomal DNA sequences which possess their own origins of replication (replicons) and are capable of self-duplication. Two mechanisms will lead to the loss of the original plasmid DNA and subsequent gene expression: *(1)* structural and *(2)* segregational instability. Structural instability arises from physical changes in the plasmid DNA molecule and usually involves a deletion and/or insertion of a segment of plasmid DNA, or the rearrangement of DNA sequences within the plasmid. Segregational instability is the result of random, unequal partitioning of plasmid DNA molecules between daughter cells upon division, which can lead to a population of cells containing no plasmids. Typically, plasmid-free cells can exhibit higher turnover rates than their plasmid-bearing counterparts.

In an open environmental system, the majority of microbial activity is associated with interfaces, within thin biological layers known as biofilms. Biofilms are three-dimensional gelatinuous structures consisting of bacterial cells entrapped within a matrix of insoluble extracellular polysaccharides secreted by the bacteria themselves (Bryers, 1987). Use of plasmid microorganisms in open systems, either inadvertently or intentionally, mandates research into those fundamental organism/plasmid processes that influence plasmid retention, stability, and transfer in biofilm communities.

Plamid stability in biofilms

When compared to suspended cultures, biofilm and immobilized cells may experience increased plasmid stability for two reasons: (1) the proximity of immobilized cells to one another may improve intracellular transfer mechanisms and (2) the mass transfer limitations in biofilm and immobilized cell systems. Because biofilm cells exist in close proximity to one another, plasmid transfer by conjugation may be increased. The net rate of plasmid loss in the biofilm culture may be decreased when the number of plasmid transfers from plasmid-bearing cells to plasmid-free cells is included. Due to mass transfer limitations, there is also the tendency for growth rate gradients to develop within biofilm culture systems. Since plasmid segregational loss has been shown to be a function of growth rate, cells experiencing a slower growth rate due to nutrient and carbon source limitations may also experience decreased plasmid loss.

In gel immobilized whole cell cultures, researchers have found a net decrease in the rate of plasmid segregational loss (Leuven *et al.*, 1979; Stewart and Carlson, 1986; Saye *et al.*, 1987). In contrast to these reports, Huang *et al.* (1993) found the plasmid pMJR1750 in *E. coli* DH5 was segregationally less stable when the host was grown in a biofilm versus in suspension. Their results also reported changes in plasmid copy number between suspended and biofilm cultures which was attributed to the energy needed for production of the biofilm extracellular matrix polymers that might compete for plasmid maintenance/replication energy.

MATHEMATICAL ESTIMATION OF PLASMID SEGREGATION LOSS

Suspended cultures

A model for plasmid loss in continuously fed cultures was developed by Ollis (1982). A mass balance on a plasmid-bearing population in a chemostat accounts for cell growth rate, segregational plasmid loss which is directly proportional to cell growth rate, and loss of cells to their departure into the effluent. Ollis' model can be applied to a system where plasmid presence in a cell does not result in decreased cell growth rate (i.e., $\mu^+ = \mu^- = \mu$). In a steady-state chemostat system, Ollis' model predicts an exponential decay expression for plasmid-bearing cells (X^+) as a function of time

$$X^+(t) = X^+(t)*\exp(-\mathbf{p}*\mu*t)$$

where X^+ = concentration of suspended plasmid-bearing cells (number/L^3); X^-) = concentration of suspended plasmid-free cells, (number/L^3); μ^+ and μ^- = growth rate constants for plasmid-bearing and plasmid-free cells, respectively (t^{-1}); **p** = probability of segregational loss of plasmid per generation. This model was used to determine the probability of segregational plasmid loss in the suspended continuous cultures described below.

Biofilm cultures

Only one mathematical model has been developed to describe plasmid loss in biofilm cultures (Huang *et al.*, 1993). The model describes a culture of plasmid-bearing (B^+) and plasmid-free cells (B^-) growing in a biofilm and it accounts for *(1)* deposition of cells onto a surface; *(2)* attached cell growth, *(3)* plasmid loss due to segregation; and *(4)* detachment of B^+ and B^- biofilm cells. Huang *et al.*, solved their model for a system where the B^+ cells have a different growth rate than the B^- cells.

In the TCE system described below, there is no measurable growth rate differential between the plasmid-bearing and plasmid-free populations, so the above simplifications cannot be made. Consequently, the differential equations making up the Huang model must be solved using finite difference methods; this requires *(1)* an expression for the growth rate dependency on substrate concentration and *(2)* a biofilm detachment rate expression. An Andrews inhibitory substrate kinetic equation was determined from batch suspended culture studies (Sharp, 1995) discussed below. A kinetic expression for the detachment rate of biofilm cells, developed by Camper (1996), was used here.

Plasmid segregational loss factor, **p**, for the biofilm cultures was determined by comparing solutions of the differential material balance for B^+ and B^- cell mass balance to experimental data, with **p** being the only adjustable parameter.

MATERIALS AND METHODS

Plasmid mediated TCE degradation

Contamination of soil and groundwater by organic pollutants has been the focus of much research in recent years. Some of the most notable contaminants in America's aquifers are volatile organics, a large number of which are chlorinated aliphatic compounds. Trichloroethene (TCE - $ClC+CCl_2$) is a chlorinated ethene and a member of the chlorinated aliphatic family. TCE is a U.S. EPA priority pollutant and is one of America's most ubiquitous and recalcitrant groundwater contaminants (Love and Eilers, 1982).

Recently, a number of bacterial consortia and isolates capable of aerobic degradation of TCE have been discovered; the most notable are toluene oxidizing TCE degraders such as *P. putida* Fl and *B. cepacia* G4. The biochemistry of the TCE degradative pathway of *B. putida* Fl has been well characterized (Finette *et al.*, 1984; Gibson *et al.*, 1982; Subramanian *et al.*, 1985; Wackett and Gibson, 1988; Wackett and Householder, 1989). Results show that *B. cepacia* G4 degrades TCE via a plasmid-borne ($pTOM_{31c}$) cometabolic pathway that must be induced by one of the following: phenol, toluene, *m*-cresol, *o*-cresol, or catechol (Folsom *et al.*, 1990).

This paper reports on a laboratory evaluation of factors governing plasmid retention and the expression of TCE degradative capacity in both suspended and biofilm cultures.

All experimental methods and analytical techniques are provided in greater detail by Sharp (1995).

Bacterial strain

Transconjugant bacterium *Pseudomonas cepacia* 17616-pTOM31c (*Pc*17616-pTOM31c) was used in all of the toxicity, plasmid loss, and injury studies. *Pc*17616-pTOM31c transconjugant was obtained by solid surface transconjugation between *B. cepacia* PRl-pTOM31c and *P. cepacia* 17616 (Murgel *et al.*, 1991). Selective phenol-kanamycin agar plates of all plasmid-bearing strains were maintained and re-streaked every week and phenol-kanamycin selective slants were re-streaked every month.

Plasmid

Plasmid pTOM31c is a 114Kb plasmid containing the TOM pathway. Plasmid pTOM31c constitutively encodes for toluene *ortho*-mon-oxygenase (tom A) and catechol 2,3 dioxygenase (C230) genes, as well as for all of the other genes needed for the aerobic, cometabolic mineralization of TCE (Shields and Reagin, 1992). In addition, pTOM31c contains a Tn5 transposon carrying the kanamycin resistance marker. A detailed map of plasmid pTOM31c can be found in Sharp (1995).

TFMP Assays

The *m*-trifluoromethylphenol (TFMP) assay is a colorimetric assay used to indicate the expression and activity of the TOM pathway (toluene ortho-mon-oxygenase and catechol 2,3, dioxygenase). The TFMP assay can be carried out in one of three ways: *(1)* as a suspended culture assay to give the specific activity of the TOM pathway for a suspended cell culture, *(2)* as a biofilm culture assay to determine the activity of the TOM pathway for a biofilm culture, or *(3)* as a colony assay to make a positive/negative determination of TOM expression and activity. pTOM specific activities can be expressed as the pTOM activity of the total culture or the true pTOM specific activity. The true pTOM specific activity is the total pTOM specific activity corrected for the fraction of plasmid-free, dead, and/or injured cells measured in a given culture. The correction is made by subtracting the fractions of biomass protein that are attributed to plasmid loss, cell injury, and toxicity from the total biomass, and then using that corrected protein value to determine the true pTOM specific activity.

Suspended culture plasmid stability and activity studies

Batch experiments using the transconjugant *P. cepacia* 17616-pTOM31c growing on non-selective acetate media were carried out to determine pTOM activities, acetate growth characteristics, and pTOM31c plasmid stability during batch growth. A series of growth studies at acetate concentrations ranging from 2 to 20 mM were performed. Plasmid pTOM31c activities were determined periodically during each batch experiment using the TFMP suspended culture assay and the TCE disappearance assay. Total. suspended plasmid-free (X^-), and suspended plasmid-bearing (X^+) cell concentrations were determined periodically throughout each batch study. Total cell numbers were determined by dilution plating on LCG agar plates.

Acetate-fed chemostat studies were carried out to determine the stability and activity of pTOM31c in *P. cepacia* 17616 during continuous culture. All continuous culture experiments were inoculated with a pure culture of *P. cepacia* 17616-pTOM31c cells harvested from a highly selective starter culture. Chemostats were run at a number of different dilution rates ranging from 0.06 to 0.19 h^{-1}. Steady state values for pTOM specific activity were determined every day for each dilution rate, using the suspended culture TFMP assay. Steady-state plasmid-bearing, plasmid-free and total cell counts were determinated daily for each dilution rate.

A set of pTOM selective, phenol-fed chemostat studies were performed to determine if phenol could be used to either stabilize the pTOM31c plasmid in *P. cepacia* 17616 or select for pTOM31c-bearing cells in continuous culture. Phenol concentrations were measured using a colorimetric phenol assay. Biomass measurements were made using a protein assay (BA protein assay; Pierce Co.). Plasmid loss was determined using the phenol selective direct colony transfer method.

Biofilm culture plasmid stability and activity studies

P. cepacia 17616-pTOM31c biofilm cultures were grown on non-selective acetate media to determine the stability and activity of pTOM31c in biofilm cultures. Biofilm cultures were grown in a rotating annular reactor (Sharp, 1995). Biofilm reactors were initially colonized under batch operation with high kanamycin selection (80 gamma or μg/ml) to insure the initial biofilm was essentially 100% plasmid-bearing cells. No suspended cells were added to the system after initial inoculation. Biofilm reactors were operated at two different influent acetate concentrations, 4 mM and 10mM acetate. Reactors were run at a dilution rate of at least 1.0h^{-1} (at least 5 times greater than the maximum growth rate of *P. cepacia* 17616) to minimize replication of detached biofilm cells.

Protein content and acetate concentrations of effluent and biofilm samples were determined periodically throughout an experiment. Plasmid-bearing, plasmid-free, and total cell counts in the effluent (X^+, X^-, and X_{total}) and biofilm (B^+ and B^-) samples from each annular reactor were determined every two days. Selective cell counts were used to determine the growth and plasmid loss characteristics of *P. cepacia* 17616-pTOM31c in biofilm culture. Plasmid pTOM31c specific activities of the biofilm cultures were determined using the TFMP assay on a 5ml aliquot of a homogenized biofilm sample.

RESULTS

Batch suspended culture studies

Batch growth data for X^- and X^+ cells were correlated using Andrews substrate inhibition kinetics. Values found by experiment were as follows: maximum growth rate (μ_m = 0.49 h^{-1}), half-saturation constant (K_s = 3.5 mM acetate, inhibition constant (K_i = 9.1 mM acetate), and biomass to substrate yield ($Y_{x/s}$ = 0.25 gBiomassC/gAcetateC). There was no significant difference between the acetate growth rates of the X^- and X^+ *P. cepacia* 17616 cultures.

A series of batch studies under non-selective growth conditions were run at different acetate concentrations (2mM to 40mM) to determine the stability of pTOM31c in its new host *P. cepacia* 17616. There was no plasmid loss detected for any of the acetate concentrations tested over the 10 generations observed in batch growth.

Repeat batch studies using pTOM-selective phenol medium were performed to determine plasmid loss under selective growth conditions. As in the non-selective plasmid loss studies, plasmid loss was not measured in the selective batch growth studies. However, cell injury caused by growth on the selective phenol medium was noted and accounted for about 10-15% of the total population. These results were the first indication that phenol was injurious to pTOM bearing strains.

Continuous suspended culture studies

Selective and non-selective continuous culture studies were conducted to determine the retention and activity of pTOM31c in *P. cepacia* 17616 as functions of growth rate.

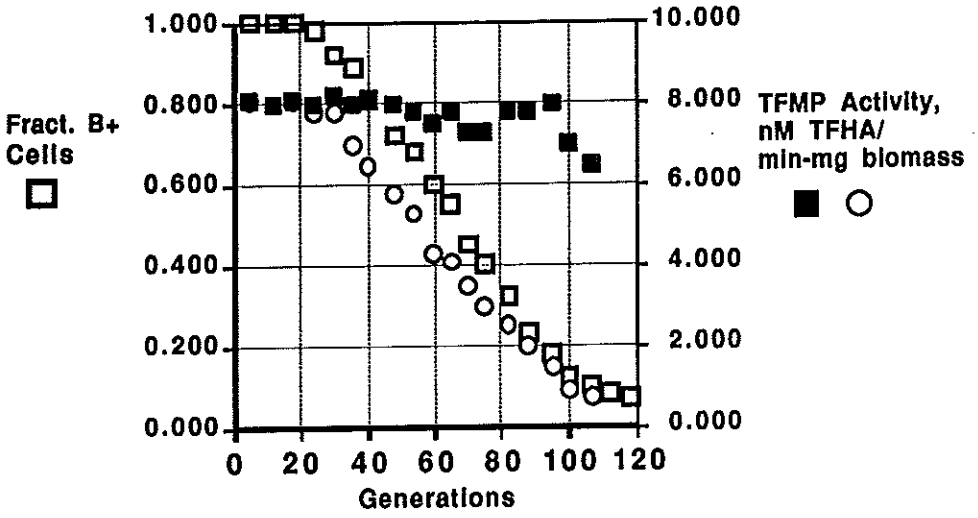

Figure 1. Plasmid bearing fraction, total TFMP and specific TFMP activity for *P. cepacia* 17616-$pTOM_{31c}$ in chemostat culture: dilution rate = 0.17h^{-1}.

Figure 1 exemplifies data for the *P. cepacia* 17616 X^+ cell fractions and pTOM specific activities in chemostat culture at a dilution rate of 0.17h^{-1}. Similar results were observed at dilution rates of 0.065 and 0.10h^{-1}. Results indicate that considerable plasmid loss occurred at each dilution rate and that the total pTOM specific activity decreased with elapsed time as did the fraction of plasmid-bearing *P. cepacia* 17616 cells. True pTOM specific activity is defined as the pTOM activity per plasmid-bearing cells only. True pTOM specific activity remained relatively constant throughout each of the continuous culture studies, indicating that the plasmid-bearing 17616 cells can maintain essentially the same pTOM activity during prolonged continuous growth. Figure 1 also indicates that plasmid loss did not become measurable until

approximately 25 cell generations. This lag in plasmid loss is an artifact of the direct colony transfer method and the highly selective continuous culture start-up conditions which selected for plasmid-bearing cells at the beginning of each chemostat study. Plasmid loss was determined using the selective direct colony transfer method and the plasmid loss factor, **p**, was found using a plasmid loss model (Eqn. 1). Values for the segregational loss factor, **p**, were approximately 0.03 for all three dilution rates examined.

Phenol-fed continuous culture studies were performed to determine if phenol could be used to select for pTOM bearing cells. Experiments demonstrated that there was no measurable plasmid loss during continuous culture fed 2 mM phenol. These studies demonstrate that phenol can be used to select for pTOM-bearing cells either by decreasing the plasmid loss factor of 17616pTOM31c or by deterring the growth of plasmid-free cells resulting in the wash-out of the plasmid-free cells.

Biofilm studies

A series of non-selective biofilm growth studies using a rotating annular reactor (Sharp, 1995) were performed to determine the activity and stability of plasmid pTOM31c in *P. cepacia* 17616 biofilm cultures. Biofilm growth studies were also used to evaluate the ability of *P. cepacia* 17616-pTOM31c to produce a biofilm when grown on a non-selective, non-competitive acetate medium.

Two rotating annular reactors were operated at different acetate inlet concentrations, 4.8 mM and 8.2 mM, respectively. Reactors were run at a dilution rate that was five time greater than the maximum 17616 acetate growth rate of approximately 0.2/h. The reactors were inoculated as a batch system with a high plasmid selection (~ 120 gamma kanamycin) and were allowed to proceed through a complete batch growth curve before medium flow was initiated. Analysis of the reactors was carried out every three days after inoculation and included: acetate analysis of reactor effluents using ion chromatography and determination of the plasmid-free and -bearing cell concentrations, and total cell concentrations of both the effluent and biofilm. Effluent samples were also analyzed for pTOM specific activity using the liquid culture TFMP assay.

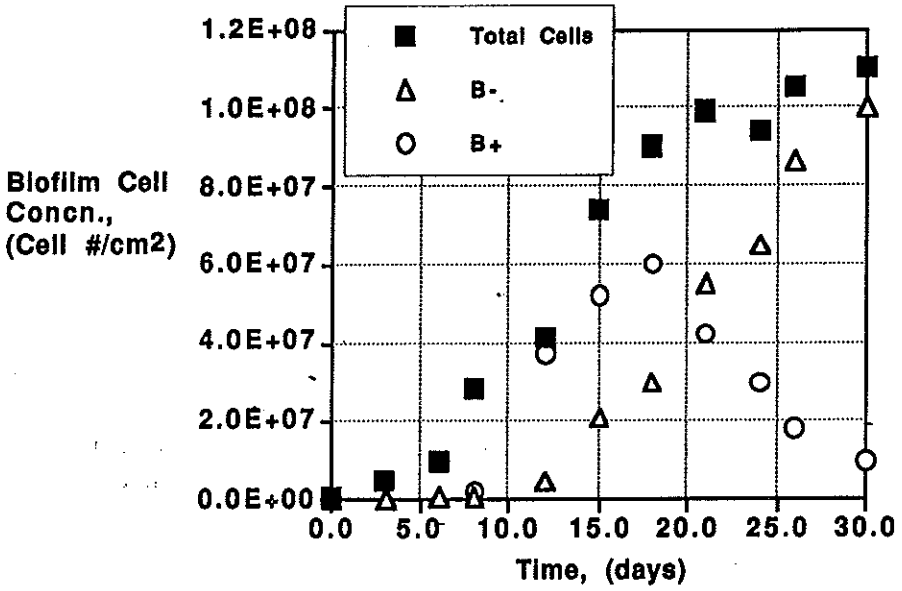

Figure 2. Biofilm cell concentrations of plasmid-bearing, plasmid-free, and total biofilm cells in a biofilm reactor fed 4.8 mM acetate.

Figure 2 exemplifies data for the biofilm population dynamics during one of the two of the annular reactor studies, at an inlet acetate concentration of 4.8 mM. It can be seen that initially as the total biofilm cell numbers accumulate, the B^+ cells increase accordingly. However, as time proceeds, the number of B^- cells

increases and eventually the B^+ cells reach a maximum and decline due to segregational loss while the B^- cells continue to increase and dominate the biofilm population.

The population composition of the detached biofilm material in the reactor effluent was similar to those found in the biofilm. X^- cell fractions in the effluent and biofilm B^- cell fractions were essentially the same in both annular reactor experiments. The effluent population dynamics mirrored those of the biofilm culture, which indicates that there was no apparent preferential detachment of B^- or B^+ cells from the biofilm.

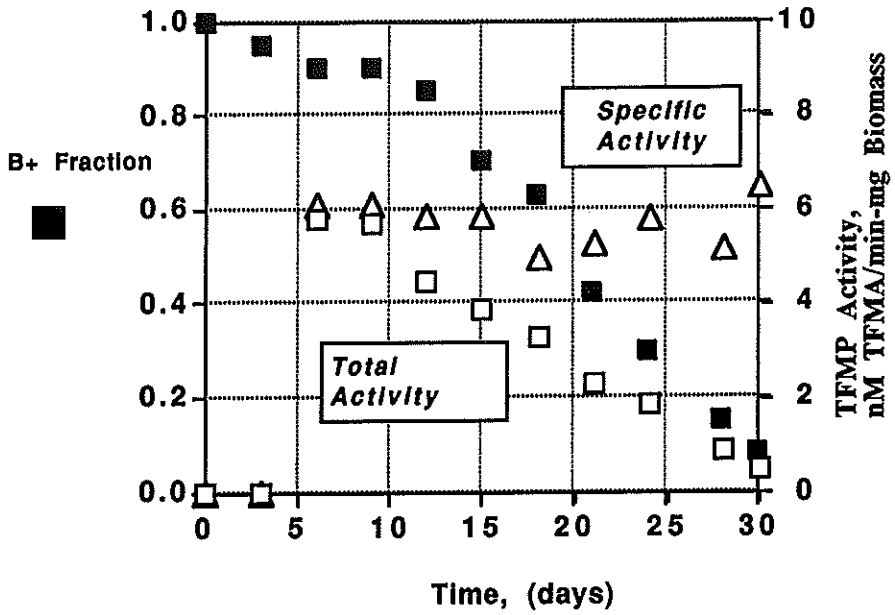

Figure 3. Plasmid-bearing cell fraction in biofilm and resultant total and specific TFMP activity. Biofilm reactor feed concentration 4.8mM acetate.

Figure 3 exemplifies the biofilm plasmid-bearing cell fractions, total pTOM specific activities, and true pTOM specific activities over time in the annular reactor experiment at 4.8 mM inlet acetate concentration. Results of the experiment at an inlet acetate concentration of 8.2 mM were similar. Independent of the initial acetate concentration, the total pTOM specific activities in both biofilm reactors decreased in accordance with the decline in the B^+ cell population.

However, the true specific activities of the B^+ cells remains relatively constant as indicated by the true pTOM specific activity.

Cell population dynamics obtained during these biofilm studies were utilized to determine the plasmid loss factor, **p**, for *P. cepacia* 17616-pTOM31c in biofilm cultures. Contrary to the results for the suspended culture experiments, calculations for biofilm cultures indicated that **p** was a slight function of biofilm cell growth rate with $\mathbf{p} = 0.03$ at a growth rate of $0.20 h^{-1}$ and $\mathbf{p} = 0.036$ at a growth rate of $0.25\ h^{-1}$.

CONCLUSIONS

Kinetic values for cell growth, substrate utilization, growth inhibition and cell yields were measured for *Pseudomonas cepacia* cultures both devoid of and containing the pTOM31c plasmid.

Batch studies using a non-selective substrate (acetate) at two different concentrations demonstrated that the plasmid was not lost although the selective substrate, phenol, was injurious to up to 15% of the pTOM31c-carrying cells in the population.

Continuous culture studies indicated that phenol could be used to select pTOM31c-bearing cells.

Biofilm studies were carried out using two rotating annular reactors operating with different concentrations of non-selective (acetate) media. The observed decrease in total pTOM specific activities in the reactors was independent of influent acetate concentrations and also decreased with the decay in numbers of plasmid-bearing cells. The true specific activities of plasmid-bearing cells however remained constant. The plasmid loss factor (p) for *P. cepacia* 17616-pTOM31c was 0.030 when specific growth was 0.20 h^{-1} and 0.036 when specific growth rate was 0.25 h^{-1}.

ACKNOWLEDGEMENTS

The authors would like to recognize the support provided for the research reported here awarded by The Center for Biofilm Engineering, a National Science Foundation Engineering Research Center, under their award EEC-89 07 039.

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