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In Silico Study for Emergent Spatial Structure of *Paracoccus denitrificans* and *Wolinella succinogenes* Biofilm under Denitrifying Condition

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Abstract: Influx of the nitrogenous compounds from different sources are responsible for the high nitrate levels in the drinking water, which is a serious problem as it leads to serious health hazards, therefore biological denitrification is considered as a good alternative for nitrate removal from drinking and industrial waste water. Biofilms reactors are involved for treatment of nitrate, which are efficient as well as cheap as denitrification involves anoxic condition. In this study, *Wollinella succinogenes* and *Paracoccus denitrificans* are chosen as organism's having growth rate of 13.92 d^{-1} and 11.52 d^{-1} respectively. Simulation was carried out for nitrate limiting condition and formate limiting condition, it was observed that *Wolinella* dominated biofilm in nitrate limiting condition suffocating *Paracoccus* in course, whereas in case of formate limiting condition, *Paracoccus* following altruistic strategy thrives in biofilm and *Wolinella* as a cheater strain survives benefiting from *Paracoccus denitrificans.*
Keywords: Biofilm, Denitrification, Individual-based model, *Wolinella succinogenes*, *Paracoccus denitrificans.*

Introduction

Nitrogen is a vital component of all living beings, extreme concentration of certain nitrogen species in some sections of the environment can lead to substantial environmental problems. Nitrate is released into wastewaters of chemical, fertilizer, gun powder industries, which if untreated find its way into environment contaminating ground water, revers and sea. Consumption of excessive amount of nitrate can lead to various health hazards like methamoglobinemia, gastrointestinal cancer, goiter, birth malfunctions etc., thus WHO has setup approved limit of nitrate and nitrite in drinking water to be 50ppm and 3ppm respectively [10]. Nitrate has a high redox potential, making it a better e^- acceptor in anaerobic conditions for certain bacteria, which makes its removal from wastewater simpler by biological denitrification. Biological denitrification is a dissimilatory process in oxygen limiting environment, where nitrate is reduced to di-nitrogen in four steps involving intermediates like nitrite, nitric oxide and nitrous oxide [9].

Biofilms are clumps of microbes attached to a substratum and held together by extracellular polymeric substance which acts as an adhesive and defensive matrix [8]. Biofilm development proceeds via formation of (i) monolayer, (ii) micro-colonies, (iii) mature biofilm and (iv)dispersal where some sessile cell returns to planktonic life style. Mature biofilm form complex structural design involving highly organized and well differentiated cells, which is also figuratively called city of microbes [7]. The major component of in biofilm matrix is water up to 97%, the precise structure of any biofilm is perhaps a distinctive feature of environment in which it grows [6]. Spatial structure is quantification of biofilm structure for better understanding and

describing biofilm systems. It is important for better understanding and describing biofilm systems. It is important for better understanding, elucidation and extrapolation of influence a biofilm may have on system [2]. Spatial structure of biofilm is very important for the denitrification process, as it affects the overall transformation rates, stability of operation, fluid fractional resistance, biofilm detachment and biofilm ecology [3].

Traditionally, population level models were used to model biofilms. The use of individual-based modelling (IbM) started with development of BacSim framework, which was inspired mainly as a more realistic approach that quantitatively includes physiology of individual cells [11-13]. Biofilm environment hold some interesting challenges for individual-based models, like space limitation in the environment leads to shoving and another fascinating feature of individual-based model is cells held together by extracellular polymeric substance (EPS) produced and excreted by cells [14]. Individual/agent based modelling involves the individual or agents to be modelled explicitly. In Individual-based modelling, individuals are discrete and unique entities which vary in more than one way such as locus, cell composition and metabolism [5]. IbM have also been employed to simulate biofilm using agents to represent not specific cells but relatively cluster of similar type. IbMs have provided several mechanistic explanation to phenomenon as biofilm fingering [4]. IbM have also been implemented to define and improve several biofilm reactor uses and biofilm control tactics centered on the dissolution of extracellular polymeric substance matrix [1].

Materials and Methods

Model explanation

When a quantitative model for function and structure is built, it is beneficial to build the models from sub models, each of which defines one of the numerous ongoing developments in biofilm, comprising (i) biomass progression and deterioration, (ii) division and spreading of biomass, (iii) transportation of substrate and reactions, (iv) detachment of biomass, and (v) biomass attachment. Attachment of the cells to substratum is an important process as it governs the colonization pattern of cells on substratum and likely colonization of bacteria from liquid phase to numerous locations.

In present study, iDynoMiCS framework is used for modelling the emergent structure of the biofilm. This framework and its components are described in detail by Lardon et al. [5] and the output files generated are XML files which are analyzed in R statistical environment. Algorithm is explained by pseudo code below.

Established preliminary condition for biomass

Established preliminary condition for solutes in bulk compartment and biofilm microenvironment

Do

Biomass dynamics

1. Growth and deterioration
2. Division
3. Spreading
4. Disengagement

Solute dynamics

5. Mass balance in bulk compartment
6. Mass balance in biofilm

Time period, $t + \Delta t \rightarrow t$

While $t < t_{end}$

Model parameters

Kinetics involved in this model are Monod kinetics, simple inhibition and first order kinetics. The stoichiometric and kinetic parameters used in this model are depicted in table 1 and table 2 respectively, whereas the other parameters used in simulation protocol files are depicted in table 3.

Table 1: Values for stoichiometric coefficients

Symbol	Definition	Value	Unit	Reference
$Y_{P,ac}$	Yield of *Paracoccus* on acetate	0.3	$g.g^{-1}$	15
$Y_{W,fo}$	Yield of *Wolinella* on formate	0.076	$g.g^{-1}$	15
$Y_{P,fo}$	Yield of *Paracoccus* on formate	0.065	$g.g^{-1}$	15
$Y_{W,NO3}$	Yield of *Wolinella* on nitrate	0.313	$g.g^{-1}$	15
$Y_{P,NO3}$	Yield of *Paracoccus* on nitrate	0.182	$g.g^{-1}$	15
$Y_{P,NO2}$	Yield of *Paracoccus* on nitrite	0.228	$g.g^{-1}$	15

Table 2: Values for kinetic parameters

Symbol	Definition	Value	Unit	Reference
K_{NO3}	Nitrate half-rate saturation coefficient	1	$g.m^{-3}$	1, 23
K_{Ac}	Acetate half-rate saturation coefficient	4	$g.m^{-3}$	19, 23
K_{Fo}	Half-rate saturation coefficient for formate	4	$g.m^{-3}$	assumed
K_{NO2}	Half-rate saturation coefficient for nitrite	1	$g.m^{-3}$	19
$\mu_{m,W}$	Maximum specific growth rate of *Wolinella*	13.92	d^{-1}	17
$\mu_{m,P}$	Maximum specific growth rate of *Paracoccus*	11.52	d^{-1}	18
η_{no3}	Reduction factor under anoxic condition	0.2	-	16
η_{no2}	Reduction factor under anoxic condition	0.2	-	16
R_{decay}	Decay rate	0.08	d^{-1}	20

Protocol file. Protocol for the simulation experiments were written in XML. It involves defining simulator, input, worlds, reaction, solver, agent grid and species sections. Simulator mark-up defines few global simulation parameters for the run. Input section involves defining the initial conditions for the start of simulation by specification of bulk and agent conditions file to read in, parameters of input section is depicted in table 3. World section defines the environment simulation will model and it has two mark-ups computation and bulk. Reaction section defines all the reactions involved in simulation and defines which type of biomass carries out the reaction. Reactions in the protocol files are defined using stoichiometric and kinetic parameters, which are framed in a matrix depicted in table 4. Solver mark-up is involved in calculation of different features of the simulation. Agent grid manages all the alive agents in the given domain. Species subdivision is the final portion of protocol file, is used to define the species involved in the simulation, each species defined to be one of numerous classes and given different name and has some defining parameters.

Table 3: Parameters used in simulation

Symbol	Definition	Value	Unit	Reference
D_{Ac}	Diffusion coefficient of acetate	$1.045x10^{-4}$	$m^2\ d^{-1}$	22
D_{Fo}	Diffusion coefficient of formate	$1.218x10^{-4}$	$m^2\ d^{-1}$	-
D_{NO3}	Diffusion coefficient of nitrate	$1.468x10^{-4}$	$m^2\ d^{-1}$	-
D_{NO2}	Diffusion coefficient of nitrite	$1.468x10^{-4}$	$m^2\ d^{-1}$	-
D_{N2}	Diffusion coefficient of nitrogen	$1.624x10^{-4}$	$m^2\ d^{-1}$	-
ρ_B	Biomass density	200	$g.L^{-1}$	21
ρ_I	Inert density	200	$g.L^{-1}$	21
ρ_C	Capsule density	33	$g.L^{-1}$	21

Result & Discussion

In order to investigate the effect of various nutrient on the spatial structure of biofilm of *Paracoccus denitrificans* and *Wolinella succinogenes* in a denitrifying condition, a computer based simulation model was chosen and a series of simulations were carried out to see the effect of various limiting substrate on the growth and spreading of biofilm. In this study, acetate and formate were chosen as carbon source and electron donor during denitrification process, whereas nitrate and nitrite as electron acceptor. Nitrite formed in the process of denitrification by *Wolinella succinogenes*, which is not an efficient denitrifier when compared to *Paracoccus denitrificans*,which reduces the nitrate to di-nitrogen without accumulating substantial amount of intermediates.

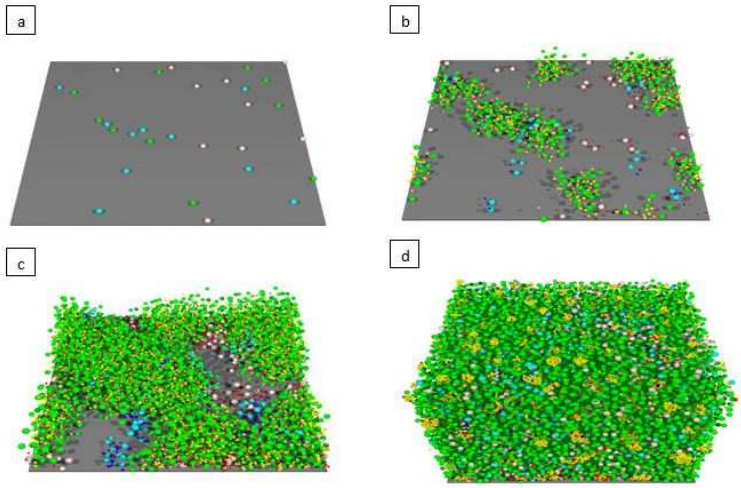

Figure 1: 3D simulation for emergent structure in a nitrate limiting condition. Simulated image has 3 species Green (*Wolinella*), Cyan (Formate utilizing *Paracoccus*) and White (Acetate utilizing *Paracoccus*). Image a, b, c, d are at time interval of 0, 5, 24 and 168h respectively.

Nitrate limiting condition

This case describes a nitrate limiting condition in which simulation was carried out. In this 10mM of nitrate was initially present in the system whereas 100mM of formate and acetate was fed. In case of nitrate limitation, it was observed that the *Wolinella succinogenes* have clearly dominated the biofilm, as seen in figure 2 (d – f), images are of various time interval 24 h , 72 h and 168h respectively. *Wolinella succinogenes* have a higher growth rate when compared to *Paracoccus denitrificans*, therefore it dominates the biofilm by producing EPS at faster rate than that of *Paracoccus* and pushing the cells to a higher nutrient zone and depleting the nutrients for *Paracoccus* leading to dominance of biofilm. Due to nitrate limitation, nitrogenous oxide production is less therefore *Wolinella* dominates and thrives in this condition.

Table 4: Matrix of stoichiometry and kinetic parameters

Process	S_{Ac}	S_{Fo}	S_{NO3}	S_{NO2}	S_{N2}	X_P	X_W	X_I	Rate
Paracoccus	$-1/Y_{P,Ac}$	$-1/Y_{P,Fo}$	$-1/Y_{P,NO3}$	$-1/Y_{P,NO2}$	$1/Y_{P,NO3}+ 1/Y_{P,NO2}$	1			R_P
Decay						-2		2	R_d
Wolinella		$-1/Y_{W,Fo}$	$-1/Y_{W,NO3}$	$1/Y_{W,NO3}$			1		R_W
Decay							-1	1	R_d

Note: negative sign in the matrix means consumption and positive means production of the metabolite or biomass.

$$R_W = \mu_{m,W}\eta_{no3}\frac{Sno3}{Sno3 + kno3}\frac{Kno2}{Kno2 + Sno2}\frac{Sfo}{Sfo + Kfo}X_W$$

$$R_P = \mu_{m,P}\eta_{no3}\,\eta_{no2}\frac{Sno3}{Sno3 + kno3}\frac{Sac}{Sac + Kac}\frac{Sno2}{Kno2 + Sno2}\frac{Sfo}{Sfo + Kfo}X_P$$

$R_d = R_{decay}X_W$ or $R_{decay}X_P$

Foramte limiting condition

This case describes a formate limiting condition for *Wolinella*and *Paracoccus*, formate is the carbon source which is utilized by *Wolinella succinogenes*, whereas *Paracoccus denitrificans* utilizes both formate as well as acetate present in the environment. In this condition, the nitrate is present in abundance, figure 2 (a – c) are the simulated images for this condition. We can see in the images figure 2 (a), which is a 24 h simulation result, clearly shows the dominance of *Wolinellasuccinogenes*, where as in figure 2 (b – c), which are 72 h and168 h simulated images for this condition, it is clearly seen that the dominance of *Paracoccus denitrificans* have increased over time, this is due to the denitrification, when *Wolinella succinogenes* reduces nitrate, it

accumulates nitrite and several other nitrogenous oxide intermediates which are inhibitory for it. *Wolinella succinogenes* is inhibited at 3mM nitrite concentration [15].

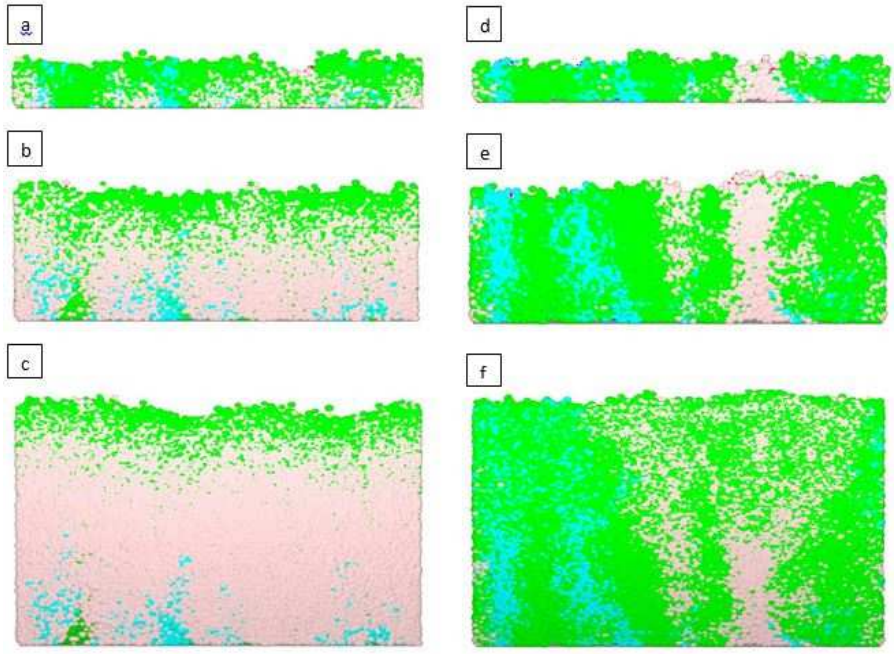

Figure 2: 2D simulation for the spatial structure of denitrification in varying condition (a – f). Simulated images have three species, *Wolinella*(green), *Paracoccus* acetate utilizing (white) and Formate utilizing *Paracoccus* (cyan). Images a – c, are of formate limited condition and d – f, are of nitrate limiting condition.

Our model have delivered an insight into the spatial structure emergence and evolutionary behavior of the organisms in a microbial biofilm community. The present work focused on the evolutionary behavior during nutrient limitation conditions in a biofilm microenvironment. Figure 1 (a – d) clearly show the biomass spreading of *Wolinella* over time dominating *Paracoccus* in the biofilm, when nitrate is limited in the microenvironment and this is because, *Wolinella succinogenes*is a cheater strain which grows faster utilizing nutrients at higher rate and depleting it for the growth of *Paracoccus denitrificans,* under nitrate limited condition *Wolinella* grows producing EPS and pushing its cells in higher nutrient zones and suffocating *Paracoccus,* this condition can't prevail for long and leads to biofilm collapse.

Paracoccus denitrificans follows altruistic behavior, it has low growth rate but high yield which is good for biofilm growth and survival. In high nitrate condition, *Wolinella succinogenes* dominates initially but due to inefficient nitrate reduction, it accumulates nitrite which is inhibitory for it can lead to its inhibition in a biofilm, but due to presence of *Paracoccus denitrificans* which is a very good denitrifier, reduces the nitrite accumulated by *Wolinella succinogenes* making environment better for thriving of *Wolinella.* For a better biofilm, altruistic behavior is important and it is evident from this model that a mixed biofilm is better than a single species biofilm as they cooperate for the survival but the cooperative behavior of all biofilm forming organisms may not be cooperative after all.

Nomenclature

- $\mathbf{R_P}$ Rate of reaction for *Paracoccus*
- $\mathbf{R_d}$ Rate of death of cells (*Paracoccus* and *Wolinella)*
- $\mathbf{R_W}$ Rate of reaction for *Wolinella*
- $\mathbf{X_W}$ *Wolinella* biomass concentration
- $\mathbf{X_P}$ *Paracoccus* biomass concentration
- $\mathbf{X_I}$ Inert biomass concentration (*Paracoccus* and *Wolinella)*
- $\mathbf{S_{ac}}$ Concentration of acetate
- $\mathbf{S_{fo}}$ Concentration of formate
- $\mathbf{S_{NO3}}$ Concentration of nitrate

S_{NO2} Concentration of nitrite
S_{N2} Concentration of nitrogen.

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