A 3D computational model for investigating the Spatial heterogeneity of Polymicrobial biofilm using K-means Clustering.

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Approval Sheet

This thesis entitled "***A 3D computational model for investigating the Spatial heterogeneity of Polymicrobial biofilm using K-means Clustering***" by Varun kumar Chiluveru is approved for the degree of Master of Technology from IIT Hyderabad.

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Abstract

Biofilm is considered for the cause of many microbial infections which range from Cystic fibrosis to middle ear infections. The biofilm mode of growth is more significant than planktonic as the biofilm mode of growth is protected from acidic PH, host immune responses, metal toxicity and antibiotics. Antibiotic resistance is becoming a bigger threat to mankind than cancer. The inter and intra species communications play a vital role in the growth dynamics and survival of the biofilm. The emergence of Small colony variants by phenotypic switching from *S.aureus* in presence of 4-Hydroxy-2-heptylquinoline N-oxide (HQNO) which is produced by *P.aeruginosa* play a superior role because of its inherent resilience and host adaptability.

K-means Cluster analysis is an important method in picturizing the heterogeneity of the biofilm, where the presence of low growing cell sub-populations are tolerant and resistant to antibiotics. The growth dynamics during the treatment gives a clear picture of sub-populations which are heterogeneous in terms of growth, substrate concentration, and antibiotic concentrations.

These studies will give an understanding of biophysical mechanisms for the antibiotic treatment in the polymicrobial biofilms. Prove the presence of subpopulations in the biofilm where nutrient and antibiotic infiltration was ineffective.

Nomenclature

μ_{max}	Maximum specific growth rate
m	Maintenance coefficient
Y_{BN}	Yield of biomass from nutrient
Y_{EN}	Yield of EPS from nutrient
Y_{HQNO}	Yield of HQNO from nutrient
$C_{N,bulk}$	Bulk nutrient concentration
$C_{Ab,bulk}$	Bulk antibiotic concentration
Di	Diffusivity of component 'i' in biofilm
Di,aq	Diffusion rate of 'i' in aqueous phase
Di,e/Di,aq	Relative effective diffusivity
Ki	Half-saturation coefficient
v	Local fluid velocity
α	Conversion rate for up-regulation (QS)
β	Spontaneous down-regulation rate (QS)
γ	Transition constant (QS)
α'	Conversion rate for SCV-switching (PS)
β'	Spontaneous WT-switching rate (PS)
γ'	Transition constant (PS)
$r_{A,u}$	Autoinducer production rate by up-regulated cells
$r_{A,d}$	Autoinducer production rate by down-regulated cells
$r_{HQNO,u}$	HQNO production rate by up-regulated cells
$r_{HQNO,d}$	HQNO production rate by down-regulated cells
$K_{B,max}$	Maximum specific reaction rate of antibiotic with respect to biomass
$K_{E,max}$	Maximum specific reaction rate of antibiotic with respect to EPS
$m_{OneCell}$	Mass of one cell
$C_{OneCell}$	Concentration of one cell
C_{BIC}	Bacterial inhibitory concentration
C_{max}	Concentration corresponding to maximum consumption rate
Δt	Time step used for CA

Contents

Chapter 1
Introduction

1.1 Overview

A biofilm is an aggregate of microorganisms in which cells adhere to each other and/or to a surface. They are formed on solid surfaces in contact with moisture or at air-fluid interfaces and can be widespread in natural, industrial and hospital settings [1][2]. Biofilms include bacteria, fungi, and protists. One common example of a biofilm is the dental plaque, a slimy buildup of bacteria that forms on the surfaces of teeth. Pond scum is another example. Biofilms have been found growing on minerals and metals. They have been found underwater, underground and above the ground. They can grow on plant tissues and animal tissues, and on implanted medical devices such as catheters and pacemakers.

1.2 History

1 Microbes and biofilms (which he called "scurf") were first reported by Antoine van Leuwenhoek in 1684.

2 Robert Koch is acclaimed as the father of microbiology; his work in the late 1800s resulted in the identification and cure of many bacterial diseases.

3 For many years after Koch's work, the study of microbiology focused on the "pure culture" paradigm in which microbes, such as bacteria, were isolated, cultured, and studied as free-floating cells (in planktonic form).

4 In mid to late 20th century, some microbiologists began to recognize that microbes most often were found in natural environments inhabiting heterogeneous colonies whose residents

included numerous other microbial critters, attached to surfaces by a common slimy substance—not as free-floating individuals of the pure culture paradigm.

5 By the late 20th century, biofilms had received enough attention that they began to be studied in earnest.

6 Since that time research has established that microbes that are part of biofilm exhibit properties that are different than their free-floating, planktonic counterparts and are generally much more resistant to treatment in a biofilm than they are in free-floating, planktonic form.

1.3 Biofilm vs. Planktonic mode of Growth

Small organisms that float or drift in great numbers in bodies of salt or fresh water is a planktonic organism. Biofilm-associated cells can be differentiated from their planktonic counterparts by a generation of an extracellular polymeric substance (EPS) matrix, reduced growth rates, and the up- and down-regulation of specific genes. [3] It has been suggested by some that as much as 40% of the genes of a bacterium may undergo up- or down-regulation in the transition from planktonic to biofilm state [1,4,5]. So, even though hereditarily they are the same, expression of some of the genes has undergone a dramatic shift and it is almost as if the two states (planktonic and biofilm) were entirely different organisms.

Biofilms offer resistance from a wide variety of environmental challenges like acidic pH, metal toxicity, antibiotics etc. [1] and shields against host immune responses.

1.4 Significance of Biofilms

Biofilms have been found to be involved in a wide variety of microbial infections in the body, by one estimate 80% of all infections.[6] Infectious which biofilm causes include bacterial vaginosis, urinary tract infections, catheter infections, middle-ear infections, formation of dental plaque, gingivitis, coating contact lenses and less common but more lethal processes such as endocarditis, infections in cystic fibrosis, and infections of permanent indwelling devices such as joint prostheses, heart valves, and intervertebral disc.[6-8].

Microbial infections can develop on all medical devices and tissue engineering constructs. 60-70% of hospital-acquired infections are associated with the implantation of a biomedical device [9].

1.5 Antibiotic Tolerance in Biofilms

Antibiotic tolerance is the ability of cells to survive the effect of an antibiotic due to a reversible phenotypic state. The applicability of these definitions is well suited for planktonic cells, while for biofilms, the situation is different. In biofilms, antimicrobial tolerance is related to the mode of growth of the biofilm. This is in contrast to bacteria growing in planktonic culture, which, usually, will show susceptibility to antimicrobials. Antibiotic tolerance is a natural state of biofilms.

Antibiotic tolerance mechanisms in biofilms include failure of antibiotics to penetrate biofilms, slow growth rate, altered metabolism, persister cells, oxygen gradients, and extracellular biofilm matrix.

1.6 Computational modeling

A computational approach that has revolutionized biofilm modeling is cellular automata (CA) [20-21]. CA are discrete models, in which space, time, and properties of the system can only have a finite number of possible states [21]. To date, the relative contributions of the parameters mentioned in section 1.2.1 have not been elucidated. Furthermore, previous work investigating the effect of cell death on biofilm development had considered 2-D systems [21]. Another study investigated the protection from antibiotic killing in biofilms based on a mechanism of localized nutrient limitation and slow growth; however, the computational model used for it was one-dimensional [32]. Hence, the data obtained from these studies may not be physiologically relevant.

1.7 Motivation

Recent work on biofilms is related to single species. Models used to explore the inter-species interactions are limited. The species present and the interactions between them critically influence the development and shape of the community. Cystic. Understanding these mechanisms will aid in the development of techniques for combating bacterial biofilms in clinical areas.

The emergence of small colony variants (SCVs) explains the antibiotic tolerance and inter-species interactions. Although SCVs have been known to exist for over a century, little attention was given to them originally as they were believed to be non-virulent and hence clinically insignificant. However, as their role in persistent infections began to unravel, it became crucial to thoroughly explore the mechanisms of SCV persistence and tolerance.

The understanding of biophysical mechanisms for the antibiotic resistance is the need of the hour. It has been reported that antibiotic resistance is going to be a bigger threat than cancer by 2050. K-means cluster analysis is a novel method for studying the heterogeneity of biofilm. My interest lies in to unravel the reasons for the antibiotic resistance in biofilms. The existence of micro niches, microcolonies make biofilm resistant to antibiotics. A detailed understanding of K-means clustering in biofilm structures may lead to the development of novel therapeutics and other potential biotechnological applications via the formation of synthetic biofilms.

1.8 Objective

1. To investigate how the cell-cell interactions (like competition, cooperation), the presence of SCVs, environmental heterogeneity etc. play a role in the antibiotic tolerance of biofilms.
   i) Especially the role of SCVs, HQNO and nutrient availability in the survival of biofilm

2. To study the various biophysical mechanisms for the antibiotic resistance in the polymicrobial biofilm using K-means Cluster analysis method, which helps in taking note of heterogeneity of the biofilm.
   (i) Existence of subpopulations in the biofilm, nutrient availability to these cells and antibiotic reach-out to these clusters

Chapter 2
Literature Review

2.1 Formation of Biofilm:

The formation of a biofilm begins with the attachment of free-floating microorganisms to a surface. While still not fully understood, it is thought that the first colonist bacteria of a biofilm adhere to the surface initially through weak, reversible adhesion through van der Waals forces and hydrophobic effects. Almost all species of microorganisms, not merely bacteria, have mechanisms by which they can adhere to surfaces and to each other. Depending on the environmental conditions, they can be as thin as a few cell layers or many inches thick. There are five stages in biofilm formation: (i) Conditioning film; initial attachment, (ii) Adsorption and irreversible attachment, (iii) Growth and colonization (iv) EPS production; biofilm formation, (v) Dispersion. The biofilm is held together and protected by a matrix of secreted polymeric compounds called EPS. EPS is an abbreviation for extracellular polymeric substance. The pathogens inside biofilm can communicate with each other to coordinate gene expression according to the density of their local population. This bacterial communication system to coordinate behaviors at the population level is known as quorum sensing.

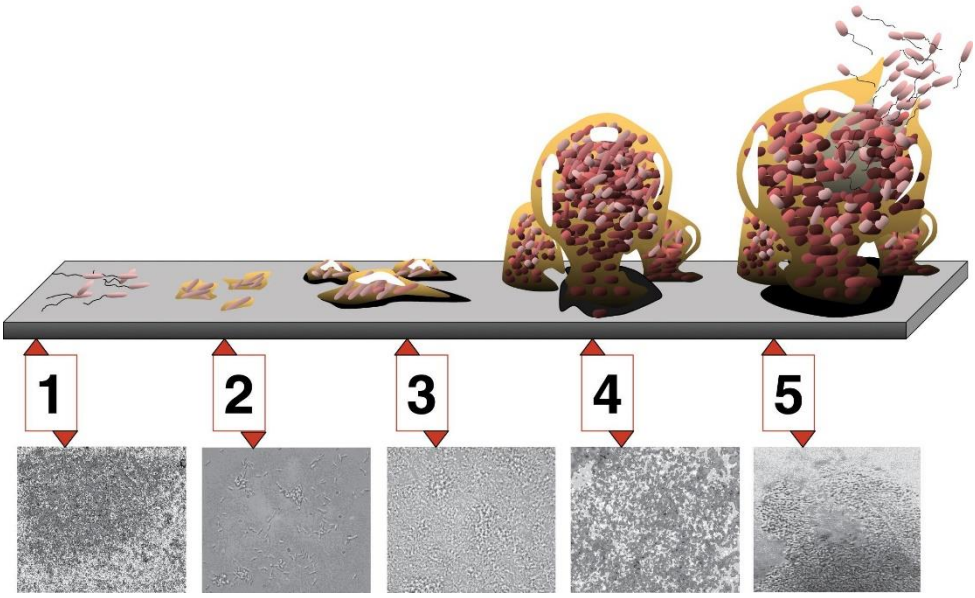

Fig 2.1: Five stages of Biofilm formation:(i) Conditioning film; initial attachment, (ii) Adsorption and irreversible attachment, (iii) Growth and colonization (iv) EPS production; biofilm formation, (v) Dispersion.

2.2 Quorum Sensing (QS)

Cell-to-cell signaling, known as quorum sensing, has been shown to play a role in biofilm formation. Bacteria that use quorum sensing constitutively produce and secrete certain signaling molecules called autoinducers. The autoinducer molecules bind to the appropriate transcription regulator(s) when the bacterial population reaches the quorum level that is, the signal concentration reaches a threshold concentration sufficient to facilitate binding to the receptor. These bacteria also have a receptor that can specifically detect the signaling molecule (inducer). When the inducer binds the receptor, it activates transcription of certain genes, including those for inducer synthesis. There is a low likelihood of a bacterium detecting its own secreted inducer. Thus, in order for gene transcription to be activated, the cell must encounter signaling molecules secreted by other cells in its environment. When only a few other bacteria of the same kind are in the vicinity, diffusion reduces the concentration of the inducer in the surrounding medium to almost zero, so the bacteria produce little inducer. However, as the population grows, the concentration of the inducer passes a threshold, causing more inducer to be synthesized. Activation of the receptor induces the up-

regulation of other specific genes, causing all of the cells to begin transcription at approximately the same time.

As a result, regulation by QS would allow the cells to save their resources under low-density conditions by expressing appropriate behavior only when it is effectual. This coordinated behavior of bacterial cells at the population level is useful in a variety of situations like biofilm formation [10,11], virulence [10,11], symbiosis [10], competence [10], antibiotic production [10], motility [10], etc. Studies have shown that this cell-cell communication via autoinducers can be both intra and inter-species as well [10,11].

2.3 Polymicrobial Biofilm

Biofilms, usually polymicrobial communities, which are commonly associated with chronic infection. Metagenomics has demonstrated that bacteria pursuing a biofilm strategy possess many mechanisms for encouraging diversity. By including multiple bacterial and/or fungal species in a single community, biofilms obtain numerous advantages, such as passive resistance, metabolic cooperation, byproduct influence, quorum sensing systems, an enlarged gene pool with more efficient DNA sharing, and many other synergies, which give them a competitive advantage. Routine clinical cultures are ill-suited for evaluating polymicrobial infections.

Biofilms can be formed by a single bacterial strain and most biofilm studies examine such cultures. However, a majority of natural biofilms are actually produced by multiple bacterial species.

It is clear that microbial life on earth is heavily biased toward multi-species communities, such as mixed biofilms. Microbial ecologists have been addressing this for quite some time, as exemplified by the active research into microbial mats. In contrast, medical microbiologists have, for many years, focused on studying mono-cultures of free-living microorganisms. Only recently has the prevalence of mixed-species biofilms and their involvement in various infections been appreciated. This

appreciation has highlighted the need for a better understanding of the interactions and dynamics within these mixed communities, which is necessary to successfully prevent or treat infections

involving mixed biofilms. Today, mixed infections are often treated using broad-spectrum antibiotics. In light of current knowledge described here, namely, that interactions between species influence the antibiotic resistance and pathogenicity of a mixed community, the composition of a mixed biofilm should be a key consideration when determining the course of future treatments.

The interactions between different species within a biofilm could be cooperative or competitive. One possible cooperative interaction is where one member provides conditions that promote survival of other members [12]. For instance, when anaerobic bacteria are grown along with aerobic bacteria in a mixed biofilm, the aerobic bacteria at the top, consume oxygen thereby providing anaerobic conditions within the deeper layers of the biofilm in which anaerobic bacteria can multiply [13]. Thus, in this case, even though anaerobic bacteria are sensitive to oxygen, they can survive and persist under aerobic conditions [14,15]. One possible competitive interaction is where one species actively inhibits the growth of others, by producing inhibitory compounds or consuming essential nutrients [12].

2.4 Proposed Mechanisms for Antibiotic Tolerance in Biofilms

The three primary theories explaining the reduced susceptibility of biofilms are: (i) Physical or chemical diffusion barriers to antibiotic penetration into the biofilm. (ii) Altered microenvironment and slow growth; most antimicrobials are effective in killing actively growing cells and nutrient concentration gradients within biofilms lead to gradients in microbial growth rate and activity. (iii) The emergence of biofilm-specific phenotype; a small subpopulation of microorganisms in a biofilm adopt a highly protected phenotypic state. These are dormant cells and are highly tolerant to killing by antibiotics. Example: persisters, small colony variants (SCVs). The dormancy of persisters can be observed by a typical biphasic antibiotic killing pattern with rapid killing of a bulk population in the initial stages, followed by a plateau where only the persister subpopulation remains alive or are slowly killed. This biphasic pattern is observed both with increasing antibiotic concentration and with increasing treatment time [16].

2.5 Small Colony Variants (SCVs)

Small colony variants (SCVs) are a slow-growing phenotype that is part of the normal life cycle of bacteria and often arise in response to environmental stresses, such as antibiotic treatment, starvation, host cationic peptides [17]. They are characteristically slow growing and therefore constitute a small fraction of the population from which they arise but continue to persist because of their inherent resilience and host adaptability [18].

Apart from slow growth rate (in the case of S. aureus, SCVs divide at about one-ninth the rate of wild-type cells [19]), typical characteristics of SCVs include atypical colony morphology, lack of pigmentation, reduced hemolytic activity, reduced coagulase activity, reduced carbohydrate utilization, low virulence potential reduced colony size (nearly one-tenth the size of the colonies associated with wild-type bacteria) [19]. They are capable of reversion to a wild-type or wild-type-like variant; thereby constituting a highly dynamic subpopulation serving as a reservoir for recurrent infections as illustrated in Fig. 2.1 [18]. Thus, phenotype switching enables bacteria to hide inside the host cell without inducing a strong host immune response.

SCVs are a challenging aspect in chronic infections such as cystic fibrosis in the lung, chronic obstructive pulmonary disease, osteomyelitis, diabetic foot ulcers, chronic wound infections, etc., where antimicrobials are administered during the acute phase of infection but fail to eradicate SCVs, which remain dormant within the host and later cause recurring infection. The occurrence of SCVs has been observed to be the highest in cystic fibrosis and osteomyelitis [19]. SCVs of S. aureus were identified in 24% of patients suffering from cystic fibrosis.

Chapter 3
Methodology

The domain geometry used in this model is a 3D model. A cellular automaton is a collection of cells on a grid of a specified shape. It involves set of rules (generally, some mathematical functions) for discrete time steps that determine the new state of each cell (in the next time step) based on the current state of the cell and the states of its neighboring cells. The rules are applied iteratively for as many time steps as desired.

3.1 Simulation Domain

Biofilm growth is simulated within the confined boxes. The bottom surface (square with side 120 µm) represents the stationary substratum upon which the biofilm develops. Periodic boundary conditions are applied in the horizontal directions, thereby eliminating edge effects, and ensuring continuity of biomass. A continuously replenished nutrient reservoir is placed at the top at a constant distance from the substratum. The interface between the nutrient reservoir and the biofilm domain is termed the diffusion boundary layer (DBL). It is assumed that the DBL has a constant thickness of 18 µm, and remains parallel to the substratum in the low-flow regime considered in this work. For the flow regime considered in this work, the nutrient concentration at a vertical distance of 18 µm from the highest cell in the biofilm was greater than 95 % of the bulk nutrient concentration, even at time points corresponding to the highest cell numbers.

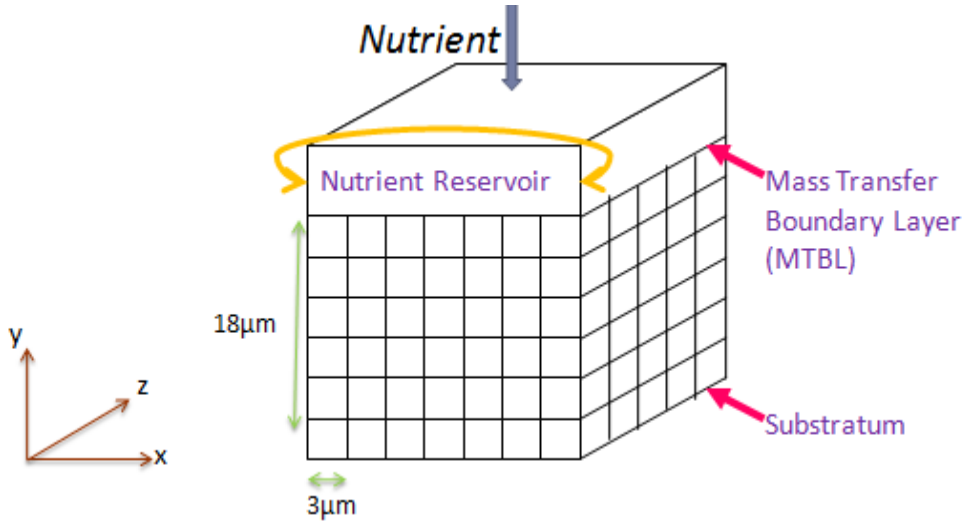

Fig 3.1: Schematic diagram of the model

3.2 Nutrient Transport and Uptake

Nutrient consumption rate was assumed to be a function of both C_B and C_N, as stated below:

$$\boldsymbol{r_N(C_N, C_B) = \left(\frac{\mu_{max}}{Y_{BN}} + m\right) C_B \left(\frac{C_N}{C_N + K_N}\right)} \qquad (3.1)$$

Here, μ_{max} is the maximum specific growth rate, Y_{BN} is the yield of biomass from nutrient (yield coefficient), m is the maintenance coefficient and K_N is the nutrient constant (half- saturation coefficient). μ_{max} and K_N are intrinsic parameters of the cell-nutrient system.

The nutrient concentration in the biofilm compartment depends on the balance between the rate of nutrient transport due to diffusion and convection, and nutrient uptake rate by the bacterial cells i.e given by:

$$\boldsymbol{\frac{dC_N}{dt} = -r_N(C_N, C_B) + D_N \sum \frac{\partial^2 C_{N,i}}{\partial x_i^2} - \nabla.(\bar{v}\, C_N)} \qquad (3.2)$$

Here, r_N is the volumetric rate of nutrient consumption. C_N and C_B represent nutrient and biomass concentration at each discretized element (x,y,z) and at each time point, t, respectively. DN is the diffusivity of nutrient in the biofilm, which is determined by multiplying the diffusion rate in the aqueous phase ($D_{N,aq}$) with the relative effective diffusivity $D_{N,e}/D_{N,aq}$, v is the local fluid velocity.

3.3 Spatial and Temporal development of Cellular Biomass

3.3.1 Growth

The nutrient that has been consumed is utilized for two purposes: cell growth and endogenous metabolism. The endogenous metabolism was assumed to be proportional to the concentration of biomass, i.e. it is given by m_{CB}. The nutrient that had not been consumed for endogenous metabolism was used for cell growth at an efficiency, Y_{BN}.

$$\frac{\partial C_B}{\partial t} = Y_{BN}(r_N(C_N, C_B) - mC_B) \tag{3.3}$$

3.3.2 Division

Bacterial cells consume nutrient; they keep on growing (biomass concentration increases) within their grid location. When the biomass of a bacterium reaches twice its native value, it divides into two daughter cells each having a new set of parameters. One of the two daughter cells remains in the original location of the mother cell, whereas the other one is placed in a neighboring element in the direction that offers least mechanical resistance. The Closest bacterium-free element is shifted by one grid element away from the mother cell, creating a bacterium-free element in the Moore neighborhood.

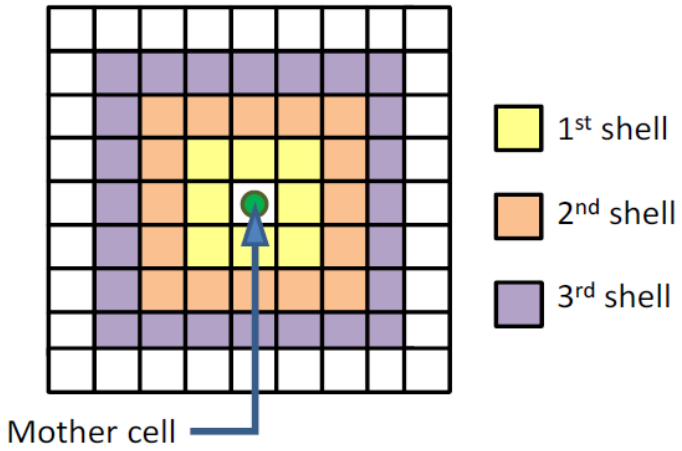

Fig 3.2: Least resistance path: Illustration of Chebyshev distance

With increasing distances from the mother cell, each direction is checked for free spaces during division and the first direction in which such a free space is found is considered to be the direction of least mechanical resistance. For example, consider a mother cell that is undergoing division. At first, the immediate neighboring elements i.e. the 1st shell (26 elements) as shown in Fig, will be checked for free spaces. If all of them are occupied, then the 2nd shell (125-27 = 98 elements) is checked and so on. Once an empty grid location is found, the entire line of cells between the mother cell and the closest free space is pushed away from the former by one element in the same line of direction so as to make space near the dividing cell where the second daughter cell is finally placed.

If two or more directions with equal mechanical resistance (i.e. directions having empty positions at equal distances from the mother cell location) are found, one of those is chosen at random.

3.3.3 Death

In the 3-D computational model that has been developed, there are three possible mechanisms by which a cell can die: (i) the cell has been in stationary phase for a predetermined number of hours (t_{SP},limit), or (ii) the ratio of nutrient consumption to endogenous metabolism (R) falls below a certain threshold, or (iii) due to antibiotic treatment. If the ratio, R (= r_N/m_{CB}), is greater than 1 then the cell exhibits net growth and if it is less than 1 then the bacterium has

entered stationary phase. This is recorded with an individual based counter, t_{SP}. If R is below 1 during one hour, t_{SP} increases by one. However, a bacterium also has the possibility to recover if R increases above 1 before it dies. Consequently, if R is above 1 during one hour, t_{SP} decreases by one. As soon as t_{SP} is greater than or equal to $t_{SP,limit}$, the cell dies.

Bacteria also die if R falls below a certain threshold value. This was done so as to account for bacterial death under circumstances of low or no nutrient concentration.
If the biofilm is under antibiotic treatment, then cells die based on certain probability which is a function of rate of consumption of antibiotic and bacterial inhibitory concentration.

A cell that is dead no longer consumes nutrient or divides; it neither plays any role in quorum sensing nor does it undergo phenotypic switching. Hence, it is removed from the simulation domain leaving a free space at its location.

3.3.4 Detachment

Detachment is an important stage in biofilm life. It is significant in terms of spreading and colonizing new surfaces thereby contributing to the propagation of contamination and infection in both clinical and public health settings. However, in the current computational model, this process was not considered in detail.

A cell detaches as soon as it loses contact with the substratum. The contact can be direct i.e. in contact with the substratum directly via EPS or indirect i.e. bound to the other bacteria. At the end of each time step, just like in the case of bacterial death, detached cells are also removed from the domain & are no longer tracked.

3.4 Quorum Sensing: Autoinducer Production and Transport

Every cell in the domain is modeled as being in either up or down-regulated state. Initially, during colonization, all the cells are in the down-regulated state. Cells are allowed to switch between the two states, at rates, dependent on the local autoinducer concentration. The transition rate from down- to up-regulated state was taken as [64]:

$$\boldsymbol{QS^{+}} = \boldsymbol{\alpha}\left(\frac{\boldsymbol{C_A}}{\boldsymbol{1+\gamma C_A}}\right) \tag{3.4}$$

Whereas, the transition rate from up- to down-regulated state was taken as [64]:

$$\boldsymbol{QS^{-}} = \boldsymbol{\beta}\left(\frac{\boldsymbol{1}}{\boldsymbol{1+\gamma C_A}}\right) \tag{3.5}$$

The probabilities of switching from one state to another within a time interval, Δt, was then taken as [64]:

$$\begin{aligned} \boldsymbol{P_u} &= \boldsymbol{QS^{+}\Delta t} \\ \boldsymbol{P_d} &= \boldsymbol{QS^{-}\Delta t} \end{aligned} \tag{3.6}$$

Here, α is the conversion rate for up-regulation, β is the spontaneous down- regulation rate, γ is the transition constant, CA is the local autoinducer concentration, P_u is the probability of a cell switching from down- to up-regulated state, P_d is probability of a cell switching from up- to down-regulated state. During the simulation, a random number is generated and if the probability of switching is greater than the said number, then the cell switches its state.

Up- and down-regulated cells are assumed to produce and release autoinducer molecules at constant rates of $r_{A,u}$ and $r_{A,d}$, respectively

$$\boldsymbol{r_A} = \begin{cases} \boldsymbol{r_{A,u}} \\ \boldsymbol{r_{A,d}} \end{cases} \tag{3.7}$$

Time evolution of autoinducer concentration within the biofilm is given by:

$$\frac{\partial C_A}{\partial t} - D_A \sum \frac{\partial^2 C_A}{\partial x_i^2} + \frac{r_A}{\Delta V} - \nabla.(\bar{v} C_A) \quad (3.8)$$

Here, r_A is the production rate (molecules/s) of autoinducer molecules, ΔV is the elemental volume, C_A is the local autoinducer concentration, D_A is the diffusivity of autoinducer in the biofilm, which is determined by multiplying the diffusion rate in the aqueous phase ($D_{A,aq}$) with the relative effective diffusivity $D_{A,e}/D_{A,aq,}$ v is the local fluid velocity.

3.5 Phenotypic Switching: HQNO Production and Transport

Our computational model consists of two model bacteria, *S. aureus* cell and *P. aeruginosa.* Every S. aureus cell in the domain is modeled as either wild-type or as an SCV. In fact, SCVs are treated as if they were an independent cell species since they are phenotypically different from the wild-type. Initially, during colonization, all the S. aureus cells are wild-type only. P. aeruginosa, on the other hand, has only one phenotype i.e. the wild-type.

P.aeruginosa is considered to release HQNO at two different rates down-regulated and up-regulated, Downregulation release is considered to be constant $r_{HQNO,d}$, while for up-regulation it is:

$$r_{HQNO,u} = r_{HQNO,d} + Y_{HQNO,N} * (r_N(C_N, C_B) - mC_B) \quad (3.10)$$

Here, $Y_{HQNO,N}$ is the efficiency at which a part of the nutrient, that had not been consumed for endogenous metabolism, was used for the production of HQNO molecules. Note that in the case presented here, $r_{HQNO,u}$, $r_{HQNO,d}$ and $Y_{HQNO,N}$ are valid only for P. aeruginosa.

Time evolution of HQNO concentration within the biofilm is given by:

$$\frac{\partial C_{HQNO}}{\partial t} = D_{HQNO} \sum_{i=1}^{3} \frac{\partial^2 C_{HQNO,i}}{\partial x_i^2} + r_{HQNO} - \nabla.(\bar{v} C_{HQNO}) \quad (3.11)$$

Here, r_{HQNO} (= $r_{HQNO,u}$ or $r_{HQNO,d}$) is the production rate of HQNO molecules, C_{HQNO} is the local HQNO concentration, D_{HQNO} is the diffusivity of HQNO in the biofilm, which is determined by multiplying the diffusion rate in the aqueous phase ($D_{HQNO,aq}$) with the relative effective diffusivity $D_{HQNO,e}/D_{HQNO,aq}$, v is the local fluid velocity.

S. aureus cells are allowed to switch between the two phenotypic states, at rates, dependent on the local HQNO concentration.

The transition rate from wild-type to SCV state was taken to be:

$$\boldsymbol{PS^{+} = \alpha' \left(\frac{C_{HQNO}}{1+\gamma' C_{HQNO}}\right)} \tag{3.12}$$

whereas, the transition rate from SCV to wild-type state was taken to be:

$$\boldsymbol{PS^{-} = \beta' \left(\frac{1}{1+\gamma' C_{HQNO}}\right)} \tag{3.13}$$

The probabilities of switching from one phenotypic state to another within a time interval, Δt, was then taken to be:

$$\begin{aligned} \boldsymbol{P_{SCV}} &= \boldsymbol{PS^{+}\Delta t} \\ \boldsymbol{P_{WT}} &= \boldsymbol{PS^{-}\Delta t} \end{aligned} \tag{3.14}$$

Here, α' is the conversion rate for SCV-switching, β' is the spontaneous WT-switching rate, γ' is the transition constant, P_{SCV} is the probability of a cell switching from wild-type (WT) to SCV state, P_{WT} is the probability of a cell switching from SCV to wild-type (WT) state. During the simulation, a random number is generated and if the probability of switching is greater than the said number, then the cell switches its phenotypic state.

3.6 Death due to Antibiotics:

Antibiotic transport and uptake was modeled within each element of the simulation domain by using a discretized form of the three-dimensional convection-diffusion equation:

$$\frac{\partial C_{Ab}}{\partial t} = -\left(K_{B,max}C_B + K_{B,max}C_B\right)\left(\frac{C_{Ab}}{K_{Ab}+ C_{Ab}}\right) + D_{Ab}\sum_{i=1}^{3}\frac{\partial C_{Ab}}{\partial C_i^2} - \nabla.(\vartheta C_{Ab}) \quad (3.15)$$

Rate of antibiotic consumption by cells:

$$r_{Ab} = K_{B,max}C_B\left(\frac{C_{Ab}}{K_{Ab}+ C_{Ab}}\right) \quad (3.16)$$

Rate of antibiotic consumption by cells at a bacterial inhibitory concentration (C_{BIC}):

$$r_{BIC} = K_{B,max}\, C_{OneCell}\left(\frac{C_{BIC}}{K_{Ab}+ C_{BIC}}\right) \quad (3.17)$$

Maximum antibiotic consumption rate by cells:

$$r_{max} = K_{B,max}C_{OneCell}\left(\frac{C_{max}}{K_{Ab}+ C_{max}}\right) \quad (3.18)$$

The probability of cell death due to antibiotic consumption is given by:

$$P_{death} = \frac{r_{Ab}- r_{BIC}}{r_{max}- r_{BIC}} \quad (3.19)$$

During the simulation, a random number is generated and if the probability of death is greater than the said number, then the cell dies.

Table I. Model parameters

Parameter	Description	Value	Unit
Δx	Element length	3	μm
	Thickness of the DBL	18	μm
$N_j\ (j = x, z)$	Number of elements in the j^{th} direction	40	

N_0	Initial number of bacterial cells	6	
m	Maintenance coefficient	0.036	h^{-1}
μ_{max}	Maximum specific growth rate of *P. aeruginosa* bacterial population	0.213	h^{-1}
μ_{max}	Maximum specific growth rate of *S. aureus* bacterial population	0.3125	h^{-1}
Y_{NB}	Yield coefficient for biomass	0.45	
t_{SP}	Time in the stationary phase at which cell death occurs	24	h
R_{min}	Ratio of the rate of nutrient consumption to that of endogenous metabolism below which cell death occurs	0.15	
	Threshold biomass at which cell division occurs	2×10^{-12}	g
D_N	Diffusion coefficient of nutrient	0.84×10^{-6}	m^2h^{-1}
K_N	Monod saturation constant	2.55	gm^{-3}
$C_{N,bulk}$	Bulk nutrient concentration	4	gm^{-3}
	Threshold concentration at which EPS division occurs	33000	gm^{-3}
$r_{A,u}$	Autoinducer production rate by up-regulated cells	73800	$molecules\ h^{-1}$
$r_{A,d}$	Autoinducer production rate by down-regulated cells	498	$molecules\ h^{-1}$
α	Spontaneous up-regulation rate	7.89×10^{-17}	$m^3molecule^{-1}h^{-1}$
β	Spontaneous down-regulation rate	0.975	h^{-1}
γ	Transition constant	7.96×10^{-17}	$m^3molecule^{-1}$
D_{ab}	Diffusion coefficient of antibiotic	0.36×10^{-6}	m^2h^{-1}

K_{ab}	Antibiotic half-saturation coefficient	1	gm^{-3}
K_{abMax}	Maximum specific reaction rate of antibiotic with bacterial cell	2.5	h^{-1}
BIC	Biofilm inhibitory concentration	1- 64	gm^{-3}
K_{EMax}	Maximum specific reaction rate of antibiotic with EPS	0.25	h^{-1}

3.7 Heterogeneity of bacteria cells based on growth rates

The growth rate of a cell is evaluated by the change in biomass per hour. After 105 h growth rate of all cells (in absence of antibiotic treatment) are evaluated, Growth rates are in the range of -700 to 10,000 gmh^{-1}, where negative growth rate describes the decreasing of the biomass of the cell. The total cell population is classified into different growth rates. To understand the heterogeneity of cells, Cluster Analysis is used to segregate cell population into different growth rates.

3.8 Simulation for Cluster Analysis

K-Means cluster analysis is used for the clustering of cells. Number of clusters (K) to be formed and number of iterations (N) are given. All the cells at a particular time step are segregated into K clusters using the properties, three coordinates (x, y, z) and growth rate. K (ten) cell's coordinates and growth rate/antibiotic concentration/substrate concentration are randomly picked as centroids initially. Euclidean distance is calculated for each cell to all the random centroids. Euclidean distance using all four properties are used for the calculation of Euclidean distance.

Euclidean distance is given by:

$$Euclidean\ distance = \sqrt{(x_1 - x_2)^2 + (y_1 - y_2)^2 + (z_1 - z_2)^2 + (G_1 - G_2)^2}$$

Where x, y, z are the coordinates and G is the growth rate/antibiotic concentration/substrate concentration of two different cells in a particular time step. Based on the minimum Euclidean distance all the cells are segregated into ten clusters. After each iteration, the four properties of a cell are very much similar to other cells in the same cluster, whereas same four properties of one cluster are highly different from other clusters. All the cells go into one or the other cluster, no cell is left out of not being in any cluster. These clusters are termed as mathematical clusters.

3.8.1 Mathematical Clusters

Flowchart:

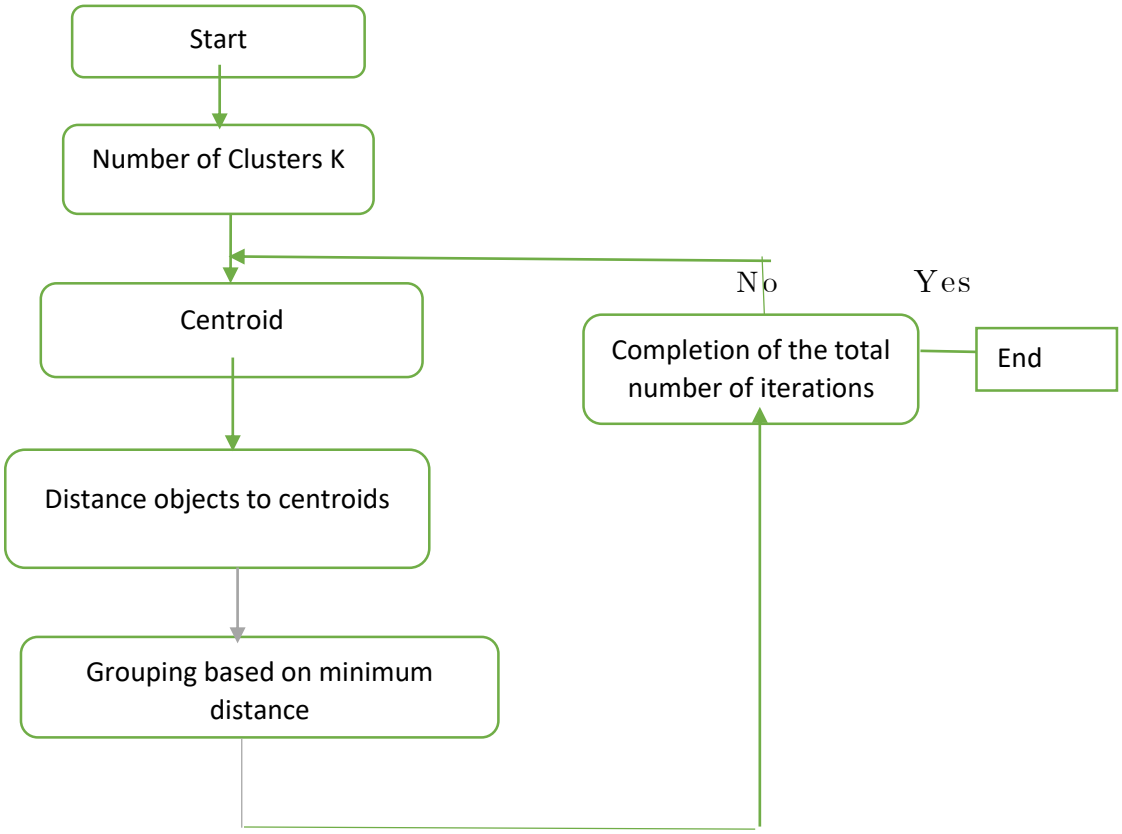

Fig 3.3: Flowchart illustrates the Algorithm for mathematical clusters

After completion of all iterations finally, ten clusters are formed. Ten mathematical clusters have ten centroids, the centroid for growth rate is the average the growth rate of all the cells in that cluster. Each mathematical cluster cells are again segregated into clusters which are physically in contact with each other, these clusters are termed as physical clusters. Standard deviation is also reported for all the cells in a single cluster to study the similarity of properties in a single cluster.

3.8.2 Physical Clusters

Flowchart: Physical clusters are calculated by calculating the Chebyshev distance between two cells when Chebyshev distance is equal to 1 then it is in contact with the cell in the immediate neighboring cubical space.

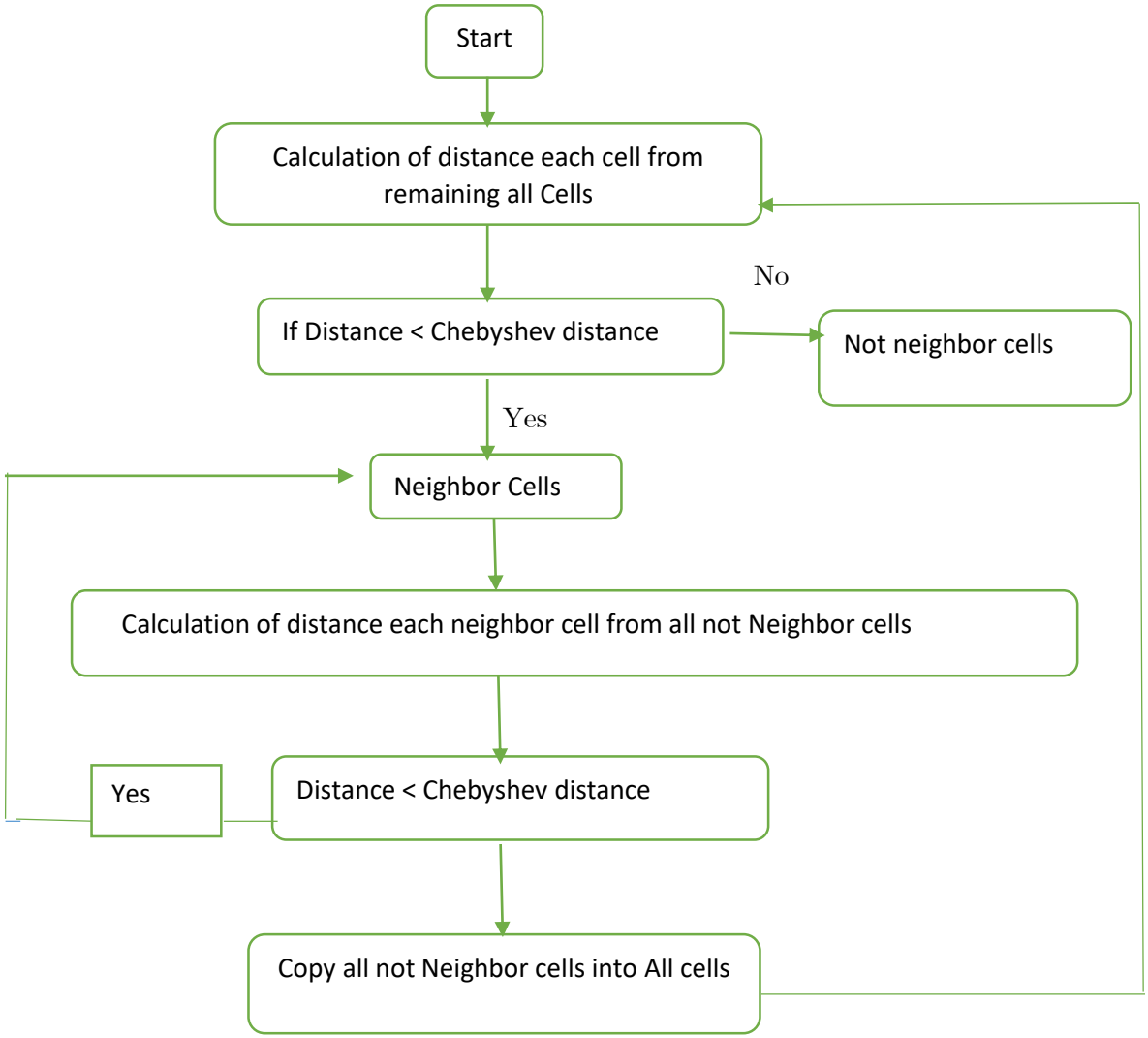

Fig 3.4: Flowchart illustrates the Algorithm for physical clusters from the cells in mathematical clusters

Chebyshev distance is given by:

$$\boldsymbol{D = \max(|x_1 - x_2|, |y_1 - y_2|, |z_1 - z_2|)}$$

Where x_1, y_1, z_1 are the coordinates of the first cell and x_2, y_2, z_2 are the coordinates of the second cell. A cell in the 3D model has 26 elements as the Chebyshev distant elements, cell present in any of these elements is considered as in-contact cell and is part of the physical cluster. Only physical cluster with more than ten cells is considered as a minimal cluster size.

Chapter 4

Results and Discussions

A 3D computational model to investigate the biophysical mechanisms of antibiotic resistance in polymicrobial biofilm, Cluster analysis plays a vital role in describing the heterogeneity of the model. All the runs were conducted at QS- (without quorum sensing), biofilms at a nutrient concentration of $4gm/m^3$ and at an antibiotic concentration of $9gm/m^3$. All the runs were in no EPS production. Role of SCVs was studied and reported. Initially, to study the heterogeneity in the biofilm, cluster analysis was conducted for growth rates. Since, it is not possible to present all the simulation results, here we present only a few. MATLAB was used as the software tool.

4.1 Small Colony Variants(SCVs) change the growth dynamics of the biofilm.

We simulated the growth dynamics of biofilm over a period of 300 hours in both absence and presence of SCVs. We observed that in the presence of SCVs the biofilm survived. The SCVs were resilient and prevent sloughing of biofilm. In presence of SCVs, cells survive even in the low nutrient concentration which help biofilm adhere to the substratum and avoid sloughing. In absence of SCVs cells die at the substratum which results in detachment of biofilm.

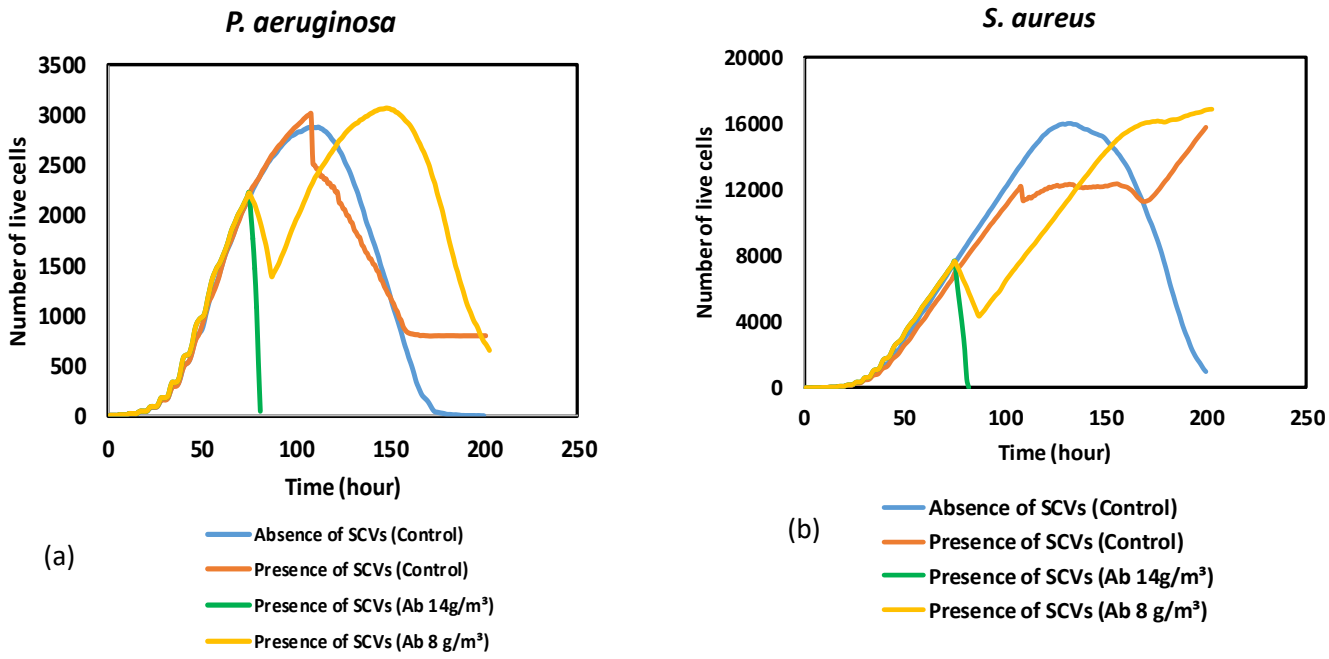

Fig 4.1: Growth dynamics of biofilm in presence and absence of Small Colony Variants(SCVs). The number of live cells as a function of time *P. aeruginosa* (a), *S. aureus* (b). Parameters for the above simulations are: Nutrient concentration = 4g/m^3, Antibiotic = 0 to 14 gm/m^3, μ_{max} = 0.213 for *P. aeruginosa*, μ_{max}= 0.3125 for *S. aureus*, μ_{max}= 0.3125/9 for *S. aureus* SCVs, m = 0.036, Y_{NB} = 0.4

We observed three important points in the plots Fig 4.1 (a) and (b). First, the life of *P. aeruginosa* is lesser than that of *S. aureus*. Second, in presence of SCVs, biofilm survive leads to monomicrobial biofilm because of resilient character of SCVs. Third, Biofilm treated with a lower antibiotic (sub-mbec) in presence of SCVs have a prolonged life (even higher life than control), where mbec mean minimum biofilm eradication concentration.

To validate these observations SCVs count as a function of time and HQNO concentration over the same period was studied.

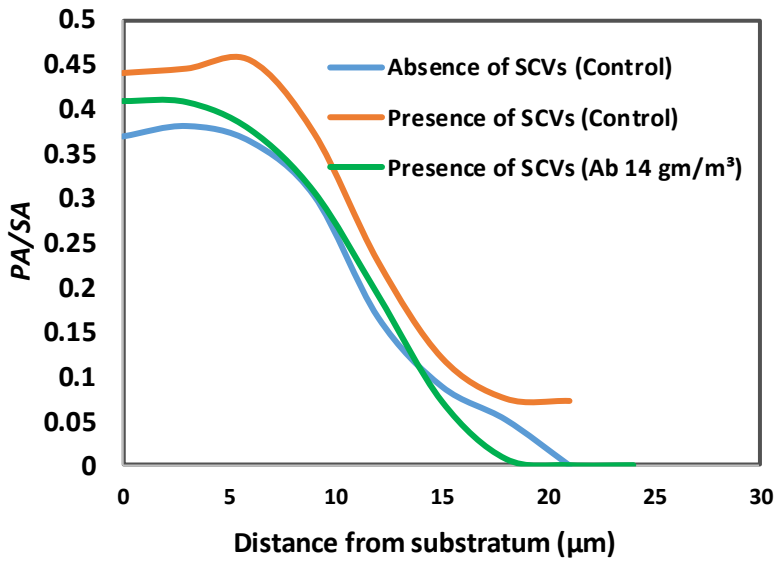

Fig 4.2: PA/SA mean Ratio of (*P. aeruginosa cells to S. aureus* cells). Spatial distribution of *P. aeruginosa and S. aureus* cells.

The *P. aeruginosa* cells were more dominant in the lower layers of biofilm and *S. aureus* cells above them. The ratio of PA/SA less than 1 mean dominance of *S. aureus* cells. Factually the presence of *P. aeruginosa* in the top layers was negligible. This was the trend in all the cases mentioned with different colors in the plot. To examine the nutrient availability in the biofilm we essentially evaluated the corresponding nutrient.

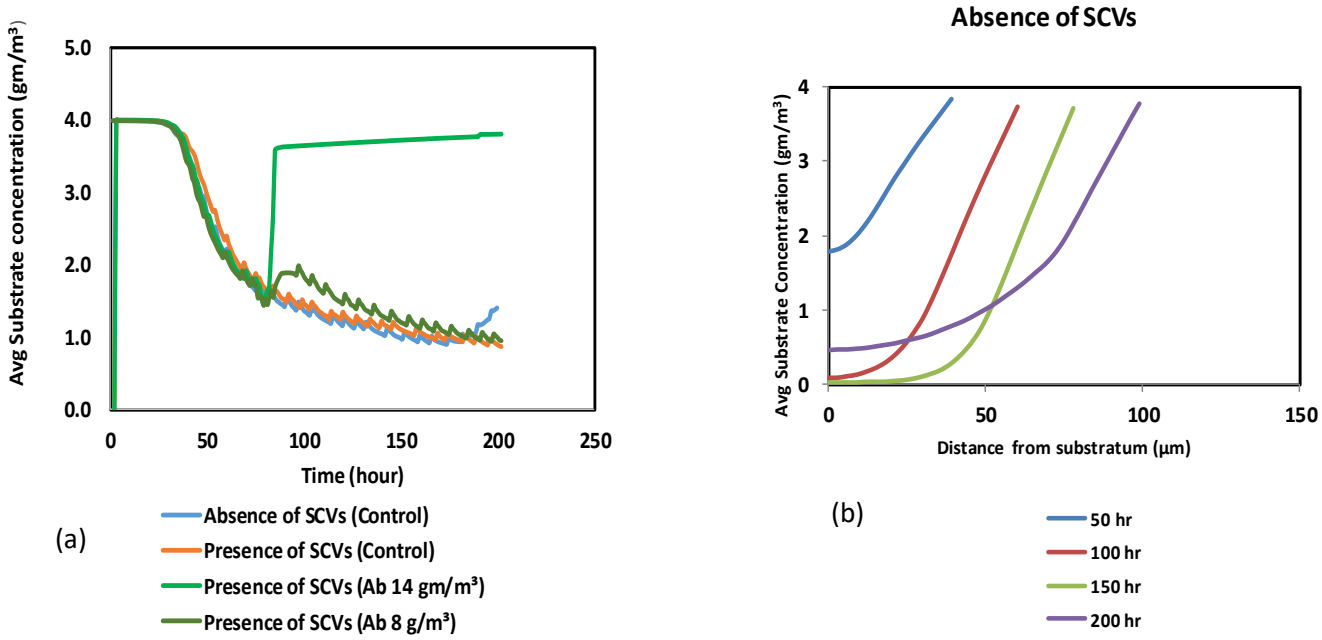

Fig (4.3): Spatial and temporal plots for the nutrient in all the cases. Temporal distribution of nutrient(a) and Spatial distribution of nutrient in the case of no SCVs.

The nutrient availability at various time-steps had different trends. The penetration of nutrient a during the 100 to 180 hours is not effective (Fig 4.3 (b). The spatial distribution of nutrient concentration was plotted against distance from substratum to inspect the nutrient availability near the substratum where most of the *P. aeruginosa* was located. The availability of nutrient to *P. aeruginosa* was meagre, resulting in starvation. *P. aeruginosa* cells are dead in the bottom layers which substantiates the observation 1. Secondly, the SCVs are resilient and play its role in biofilm survival, to validate this we evaluated the number of SCVs and HQNO concentration over the period of time.

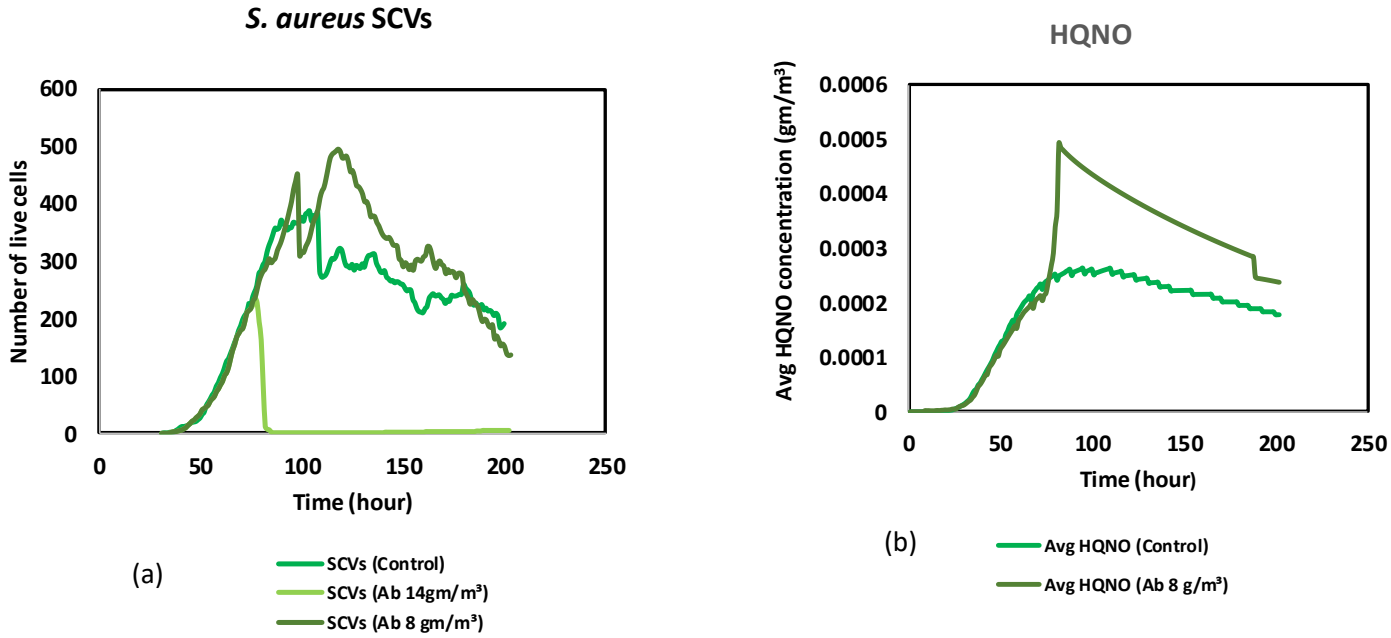

Fig 4.4: SCVs growth dynamics in all cases as mentioned in the legend (a), HQNO concentration over the period of time (b)

In (Fig. 4.4). The increase in the number of SCVs was the reason for the survival of biofilm. To inspect the reason, HQNO concentration is evaluated. The HQNO concentration exhibited a steep increase in the concentration until 80 hours, after 100th hour concentration gradually decreased. Since HQNO is released by *P. aeruginosa* it is understood that decrease in the live cells of *P. aeruginosa,* the reason for the decrease in the concentration of HQNO.

4.2 Clustering reveals, the presence of subpopulation cells resistant to antibiotics:

Cluster analysis is a technique whose primary purpose is to group objects (e.g., respondents, products, or other entities) based on the characteristics they possess. Cluster analysis was performed for biofilm based on growth rate. It was known that subpopulations of low growing bacteria cells are resistant to antibiotics. To understand this phenomena clustering using K-means method was performed. Interestingly, Clustering based on growth rate revealed the heterogeneity in the biofilm.

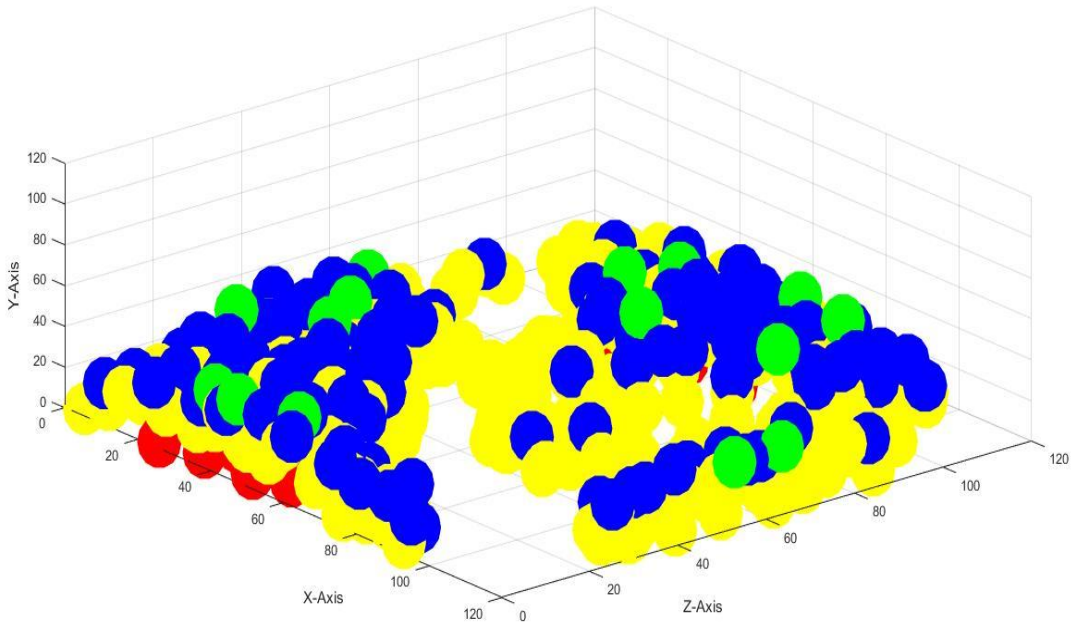

Fig 4.5: 3D image of physical clusters. Negative growth rate clusters (red), Low growth rate clusters (yellow), Intermediate growth rate clusters (blue) and high growth rate clusters (green).

We started with 10 mathematical clusters, then calculated the physically in contact cells and grouped them as physical clusters. On studying these physical clusters, it revealed the hindrance

of low growing clusters by high growing clusters. To report these clusters centroids of the clusters were reported and plotted as growth rate vs distance from the substratum.

4.3 Clustering at the zeroth hour of antibiotic treatment:

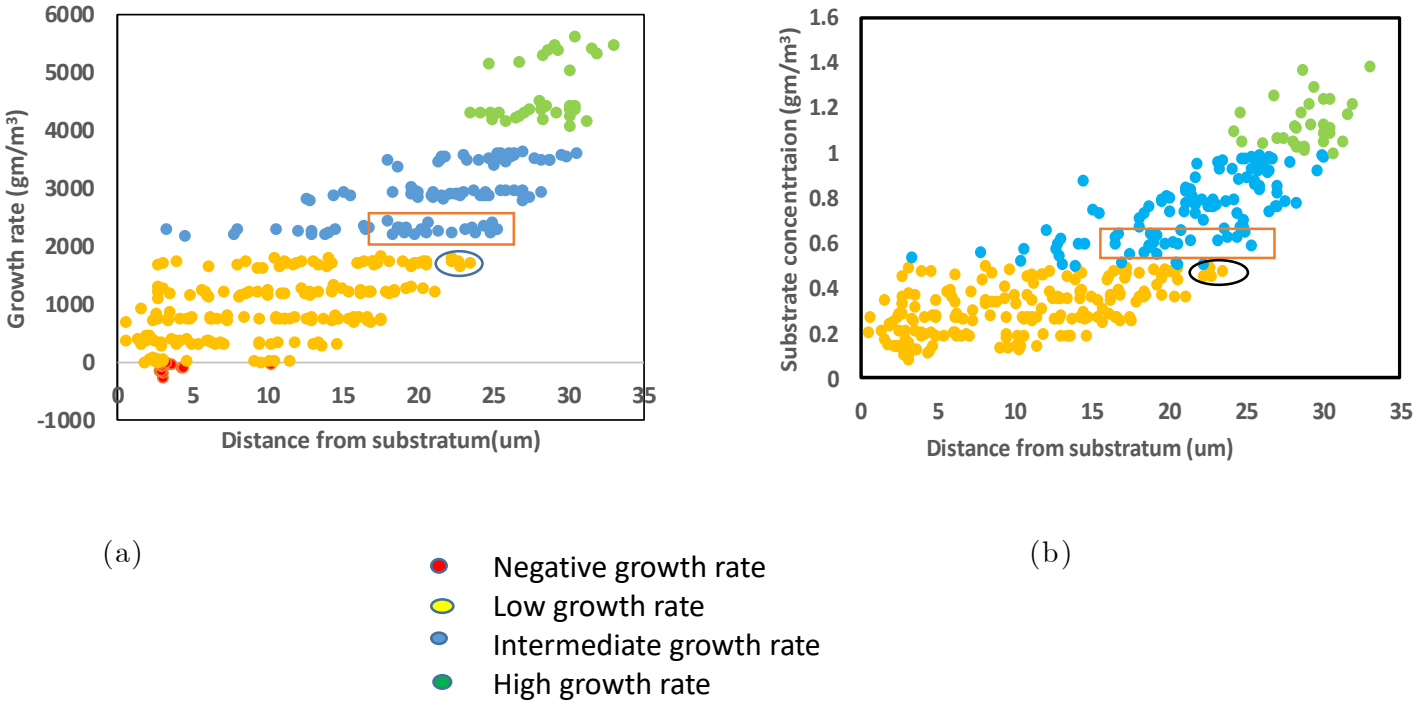

Fig 4.6. **Spatial plot of physical clusters for growth rates (a), physical clusters for substrate concentrations (b).**

Clusters, as shown in (Fig.4.6), were categorized into four groups: Negative value clusters (red), Low concentration clusters (orange 0-2000 growth rate), Intermediate concentration clusters (blue 2000 – 4000 growth rate), High concentration clusters (green above 4000 gm/m^3).

Antibiotic treatment was initiated when the total number of live cells was ten thousand i.e. time-step 80. Biofilm was subjected to 5 gm/m^3 antibiotic treatment continuously. Cluster analysis was performed just before antibiotic treatment, in the 6th hour of treatment and in the 12th hour of the treatment. The growth rate was higher among the top layers of the biofilm and the growth rate of biofilm decreases as the distance from substratum decreases. Factor being the nutrient availability, which was lesser at the lower layers (Fig 4.6 b).

The growth based-clusters revealed that low growing clusters (orange clusters encircled by blue) are surrounded by high growing clusters (marked by a red color rectangle). To inspect the nutrient availability for corresponding clusters at the same instance of time, nutrient concentration against distance was calculated. Validating the fact that low growth rate clusters are covered-up by high growth clusters, the nutrient concentration for the corresponding clusters was low and surrounded by high nutrient concentration pockets. Making it difficult for the substrate to reach out. As shown in the (Fig 4.6 b) (marked in red for high nutrient clusters and blue for the low nutrient cluster).

Fig 4.7: 2D sliced image of growth based clusters, blue cells are high growth rate (above 2000 gm/m^3) and low growth rate cells orange (below 2000).

4.4 Cluster analysis at midway (6th hour) of antibiotic treatment (5 gm/m3)

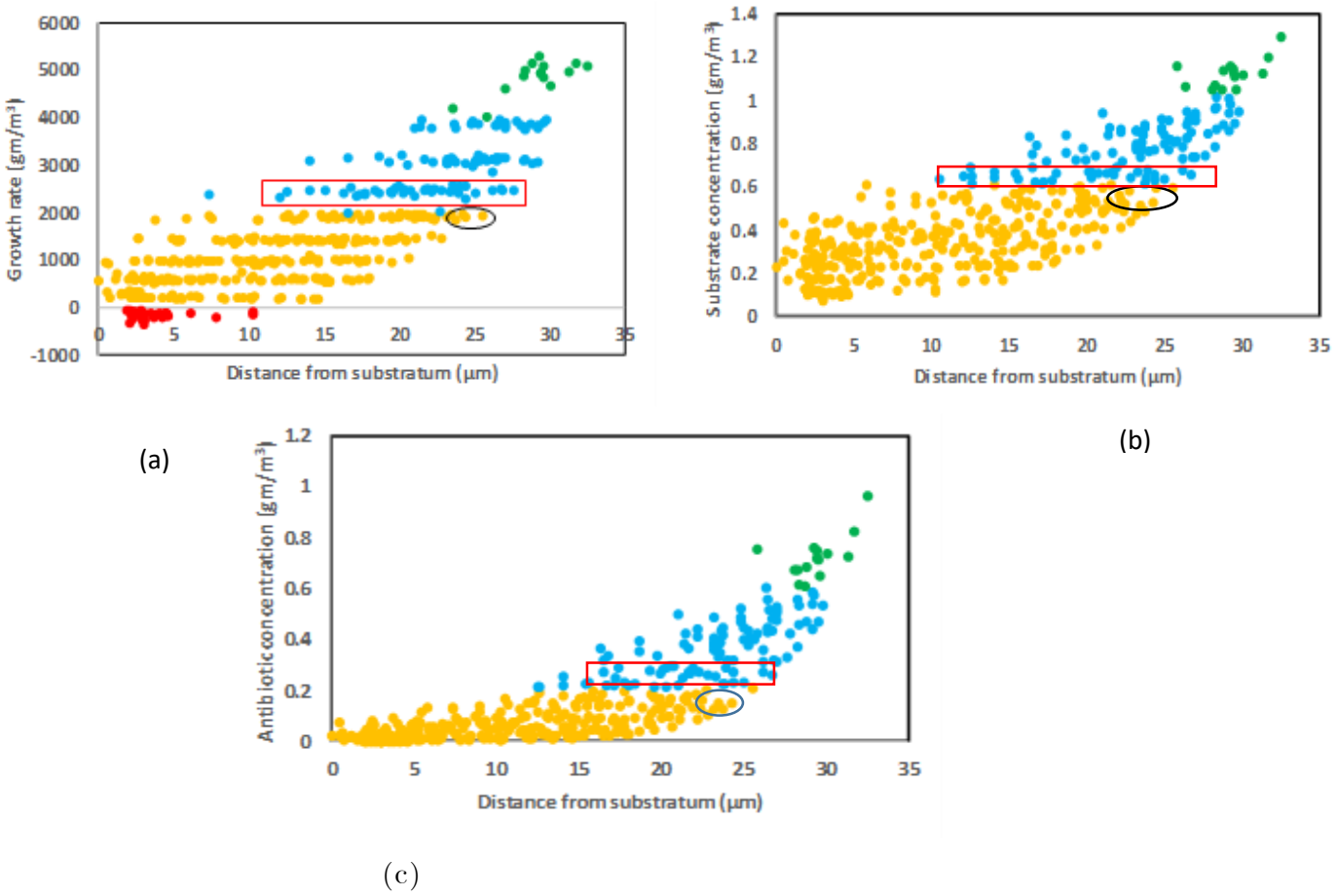

Fig 4.8 Physical clusters at the 6th hour of antibiotic treatment for growth rates (a), Substrate concentrations (b) and Antibiotic concentrations (c).

From (Fig.4.8), In the 6th hour of antibiotic treatment, we observed a higher number of low growing cells. The low growing cell subpopulations were surrounded by high growing cell population. High growing clusters were marked by a red color rectangle and low growing clusters encircled by black.

To investigate the dramatic heterogeneity of biofilm growth dynamics, we tracked the substrate concentration at the same location of biofilm for the same instant, nutrient availability for the same coordinates was calculated. The Substrate is not distributed uniformly across the volume (Fig 4.8 b), interestingly low substrate concentration volumes (orange) were enclosed by high concentration volumes (blue). Cells present in the volumes of these grids were less accessible to

nutrient and grow at a slower rate. To understand in more detail about the antibiotic susceptibility of these clusters, we studied the antibiotic concentration of the biofilm.

The (Fig 4.8 c) revealed that antibiotic penetration in these locations was less effective. The low antibiotic concentrated clusters (yellow) were surrounded by high antibiotic concentration clusters (blue) was observed. Reaction-diffusion barrier for antibiotic penetration can be the reason for lower antibiotic concentration pockets presence.

4.5 Cluster analysis at the 12th hour (last hour) of antibiotic treatment (5 gm/m^3)

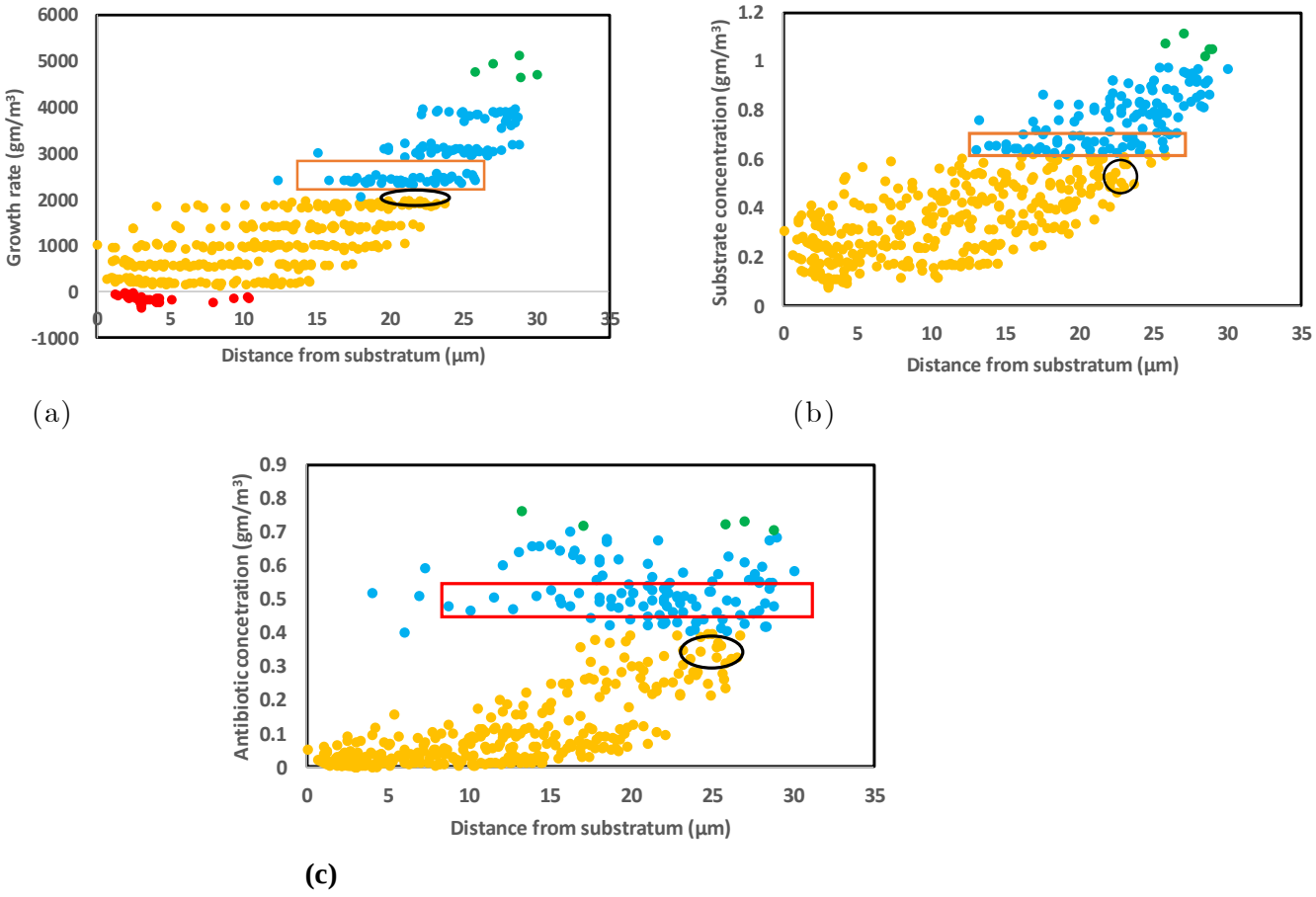

Fig 4.9: Spatial distribution of physical clusters based on growth rate (a), Substrate concentration (b), Antibiotic concentration (c).

Fig 4.9 (a) depicts physical clusters with 10 strips. Each strip formed from each mathematical cluster. Observing each strip it was evident that each mathematical cluster contains almost same

growth rate but over a range of biofilm height. The rectangle marked clusters are the intermediate growth rate clusters hindering low growth rate cluster or clusters in yellow color. This is one example of it. There are many instances and locations of this hindrance of low growing cells by high growing cells. It creates a diffusion barrier for substrate and antibiotic penetration. The reaching out of antibiotic was ineffective, making these cells as persister cells i.e. resistant to antibiotics. To validate this observation, the corresponding nutrient and antibiotic concentrations were assessed (Fig 4.8 b & c). The nutrient availability was tracked for all clusters the graph substantiated the fact that nutrient availability was low for low growing cells.

Similarly, the antibiotic penetration was ineffective.

4.6 A 3D individual-based model result in the natural heterogeneity of the biofilm:

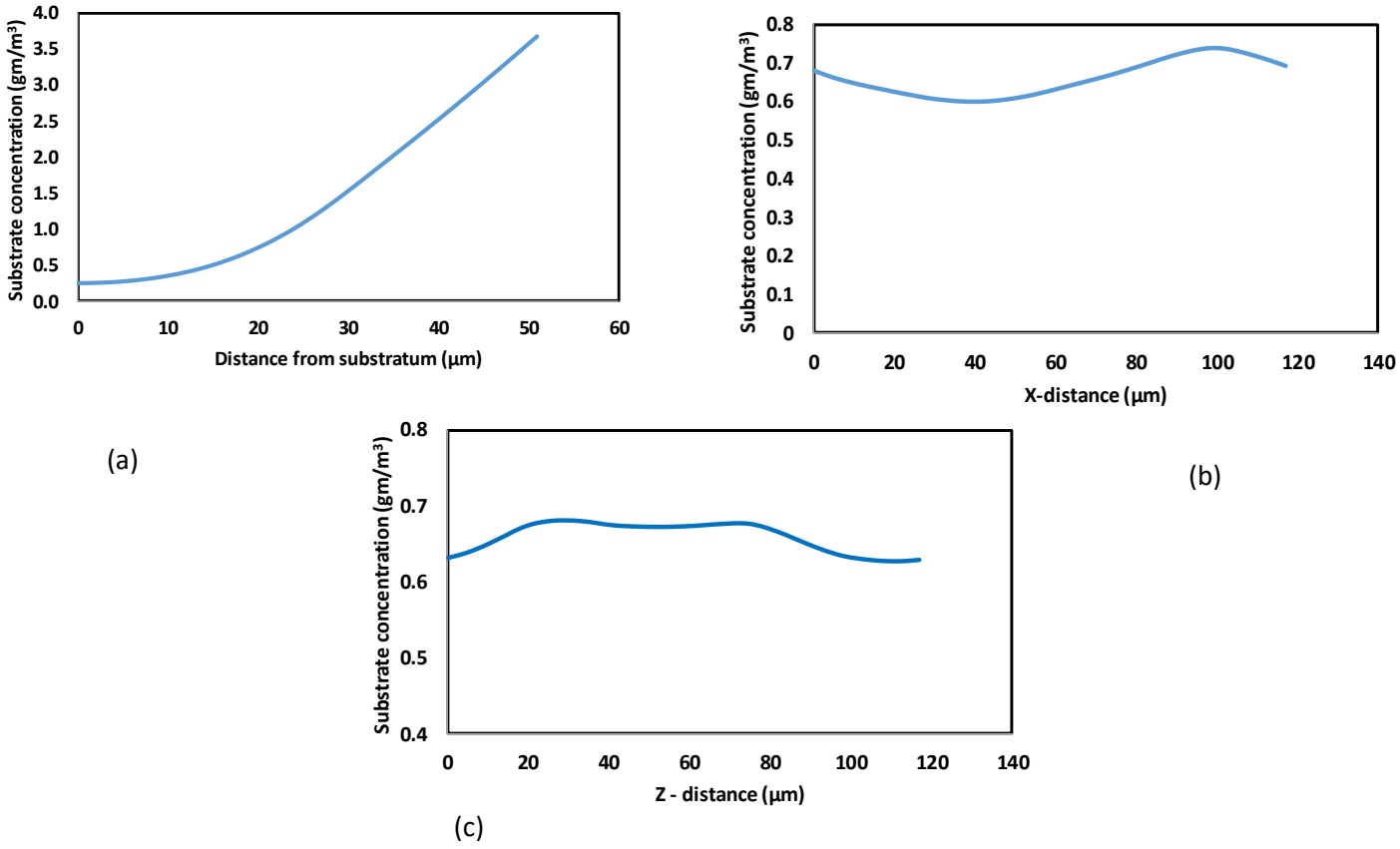

Fig 4.10 Gradients of substrate (nutrient) in all the directions, distance from biofilm (a), in x-direction (b), in z-direction (c).

Since this model is a 3D individual based model there exists gradients in all the directions. It was expected in y-direction because of nutrient and antibiotic diffuses from the top. The gradients in the Y and Z directions were important and showed up because the model was a 3D individual-based model. The gradients in all the directions had made the model to produce heterogeneity in the biofilm.

Chapter 5

Conclusions

A 3-D individual-based model was developed to investigate the physical mechanisms for antibiotic resistance in polymicrobial biofilms. The model enriched the ideas about the survival of biofilm in presence of Small colony variants (SCVs). This model could be used for any species which switches between two different growing phenotypes that prolong the life of biofilm.

Emergence of SCVs play a vital role in the survival of biofilm, they switch between two growth rates. SCVs have typical characteristics like inherent resilience and host adaptability, atypical colony morphology, a higher tendency for adhesion, capable of reversion to a wild-type, reduced carbohydrate utilization, low virulence potential and increased resistance to aminoglycosides(antibiotics). Possessing these characteristics SCVs help in the survival of biofilm.

Scientists have found only a few subpopulations are resistant to antibiotics. To investigate the fact, we started off with K-means clustering to segregate the cells into different growth rate clusters. K-means clustering helps in classifying cells into different growth rates, which are helpful in studying heterogeneity. Low growing cells are hindered by high growing cells. Antibiotic resistant microcolonies were discovered, reaching out of substrate and antibiotic was less effective because of reaction-diffusion barrier. Presence of altered microenvironment and micro-niches in the biofilm is found using K-means clustering.

Chapter 6

Future Work

The focus of research could be on the heterogeneity of biofilm. to understand the microcolonies which were resistant to antibiotics in more detail. To investigate and track the clusters throughout the life of biofilm. To study the nutrient and antibiotic penetration into the biofilm.

Our other focus could be on the performing K-means clustering species wise. Species- wise would give a clear understanding of growth rate in detail and then understand the microcolonies and the altered microenvironment in the biofilm.

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