A 3-D Computational Model To Investigate The Influence of Quorum Sensing And Antibiotic Treatment On Growth Dynamics Of Poly-Microbial Biofilms

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A Dissertation Submitted to

Indian Institute of Technology Hyderabad

In Partial Fulfillment of the Requirements for

The Degree of Master of Technology

भारतीय प्रौद्योगिकी संस्थान हैदराबाद

Indian Institute of Technology Hyderabad

Department of Chemical Engineering

June 2015

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Acknowledgements

Foremost, I would like to express my sincere gratitude to my advisor Dr. Parag D. Pawar, for the continuous support in my study and research, for his patience, motivation, enthusiasm and immense knowledge. His guidance helped me in all the time of research and writing of this thesis.

Beside my advisor, I would like to thank the rest of my thesis committee: Dr. Lopamudra Giri, Dr. Phanindra Jampana and Dr. Jyotsnendu Giri, for their encouragement, insightful comments and hard questions.

I am grateful to the office staff of Chemical Engineering Computational Lab, Indian Institute of Technology, Hyderabad for providing excellent computation facilities to complete this project. I would like to thank my senior Mr. Anil Rajapantul and Ms. Lakshmi Machineni for their assistance and support.

I am thankful to my friends Ms. Shital Yadav and Ms. Megha Gedam for their endless support, encouragement and true comments.

30.06.2015

Dedicated to

My parents

ABSTRACT

Biofilms are the surface associated cell assemblages encased with extra-cellular polymeric substances. They form 3 Dimensional heterogeneous structures with more than one bacterial species. In poly-microbial infections, high antibiotic resistance has been observed. The potential reasons for this resistance can be, genetic mutations, antibiotic diffusion limitation and quorum sensing.

We developed an individual based cellular automata model to study the effect of quorum sensing and antibiotic treatment on biofilms, formed by slow growing and less resistant species *Pseudomonas Aeruginosa* (*P. aeruginosa*) with fast growing and more resistant species *Staphylococcus Aureus (S. aureus)*. We investigated the growth dynamics of two species during different treatment hours with varying concentrations.

A synergistic interaction between two species was observed at different time steps in order to protect the whole biofilm. We observed that, biofilms can be completely eradicated at effective antibiotic concentration which is a function of time of exposure, cell number and antibacterial resistance of high resistant species. Development of virulence and protection by EPS has been observed in quorum sensing biofilms.

NOMENCLATURE

Y_{SE}	Yield coefficient of EPS
C_{Ab}	Antibiotic concentration (gm^{-3})
r_{Ab}	Rate of consumption of antibiotic by cell.
r_{BIC}	Rate of consumption of antibiotic at biofilm inhibitory concentration
$r_{\max}$	Maximum antibiotic consumption rate.
$K_{A_{\max}}$	Maximum specific growth rate of bacteria during antibiotic treatment
K_A	Half saturation coefficient
μ_{max}	Maximum growth coefficient in $hour^{-1}$
A_c	Auto-inducer concentration ($molm^{-3}$)
B_c	Biomass concentration in gm^{-3}
D_A	Diffusion coefficient of auto inducer
D_N	Diffusivity of nutrient
E_c	EPS concentration in gm^{-3}
K_q	Half saturation coefficient (gm^{-3})
Ks	Saturation coefficient (gm^{-3})
m	Maintenance coefficient
P(down)	Probability of down regulation of cell
P(up)	Probability of up regulation of cell
Y_{SB}	Yield coefficient of biomass
$Z_{A,d}$	Production rate of auto inducer by down-regulated cell ($molm^{-3}$)
$Z_{A,u}$	Production rate of auto inducer by up-regulated cell ($molm^{-3}$)
α	Spontaneous up regulation rate (hr^{-1})
β	Spontaneous down regulation rate(hr^{-1})
γ	Constant (m^3mol^{-1})

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Chapter 1

Literature survey

The development of biofilm is a series of complex but distinct and well-regulated molecular mechanism differ from organism to organism. The attachment of a small number of living cells anywhere along the system is all that is needed to initiate biofilm formation. Within few minutes, the adherent cells undergo exponential binary division. The new daughter cell spread upward from the attachment points, and embeds in EPS to form micro colonies. The biofilm structural and metabolic heterogeneity is influenced by intercellular signaling known as quorum sensing, in reaction to availability of nutrients in the immediate environment and growth conditions. Quorum sensing involves the production of signaling molecules to regulate bacterial population density or to initiate biofilm formation. The signal molecules are produced and released into the surrounding. Signal molecules can induce same bacteria to produce even more signal molecules, known as auto-induction. These bacteria also have a receptor that specifically detects the signaling molecules. When the auto- inducer binds with receptor, it activates transcription of certain genes. There is less possibility of a bacterium to detect 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. Because of these genetic changes, bacteria in the biofilm undergo behavioral changes and starts functioning as a single unit [1]. Certain behavioral changes in biofilm are: symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation and biofilm formation [2]. Quorum sensing can be observed in bacteria of single species as well as multiple species.

Under natural conditions biofilm grows as mixed culture of different species. In human body, the poly-microbial infection can range from simple skin infection to chronic pneumonia and life threatening cystic fibrosis, systemic shock and

sometimes organ failure [3, 4]. During hospital stay, 5-15% patients develop bacterial infection which prolongs the treatment, stay and ultimately cost [4, 5].

In individuals with poly-microbial infections, microbes often display synergistic interactions that can enhance their colonization, virulence, or persistence. One of the most prevalent types of poly-microbial infection occurs in chronic wounds, where Pseudomonas aeruginosa and Staphylococcus aureus are the two most common causes. Although they are the most commonly associated microbial species in wound infections, very little is known about their interspecies relationship. From literature it has been known that the gram positive bacteria *S. aureus* and the gram negative bacteria P. aeruginosa secretes same chemical signal molecule Acylated Homoserine Lactone (AHL)[6,7,8]. When threshold value of the AHL is reached then bacteria starts producing extra cellular polymeric substances, EPS. EPS constitutes 2/3rd of the total biofilm biomass [9]. It is glue like substance composed of mainly water (95-99%) along with bacterial polysaccharides, extra-cellular protein and DNA as well as excreted host cellular products such as muco polysaccharides, fibrin and collagen [10].

Poly-microbial cultures are highly relevant in the successive secondary infection in patients suffering from the genetic disease cystic fibrosis (CF). The poly-microbial infection leads to the most frequent (80%) cause of death of patients due to lung failure by immune reactions. CF patients are infected with more than one bacterial species mainly Staphylococcus aureus, Burkholderia cepacia, Psuedomonas aeruginosa or Haemophilus influenza. This mixed infection causes special difficulties for therapy, because tested efficacies for antibiotics against one strain cannot be transferred equivalently to the mixed culture. Also, experimentation with the patient is not possible. So it is better to study using computational model.

Chapter 2

Methodology

2.1 Model theory and description

To model growth dynamics of poly-microbial biofilm, an individual based Cellular Automata (CA) model [11-13] has been presented in which two species grow on surface substratum with aqueous medium of glucose as a source of nutrient. Each species consumes glucose from the surroundings at different rates, as a result nutrient depletion starts and bacterial biomass increases. When each bacterium gets double of its original biomass, it undergoes division resulting in growth of biofilm. However, biofilm diminishes because of death and detachment. A comprehensive description of different parts and processes of individual based CA model has been explained in next section.

2.2 Simulation domain

The simulation domain is a thin section discretized as a 3-D lattice. Each element of lattice is of 3 μm in length occupying total volume of 27 μm^3. This volume is sufficient to acquire biomass of a single bacterium [14]. Each element of the domain is occupied either by bacterium or by bulk liquid. From here onwards we will use the term entity to denote the key element of the biofilm. Entities are of two types, soluble and insoluble. Different rules are used to model these entities.

Simulation domain is bounded on one side by solid surface on which biofilm develops called as substratum. Initially, same numbers of two species are allowed to grow in 3 D domain with 10% variation in cell parameters. Because of this variation our model is stochastic and robust. A constant nutrient source is available above the mass transfer boundary which has been fixed from the top of biofilm at 6 μm. Mass

transfer boundary layer is defined as the layer through which the diffusion of nutrients or soluble entities takes place.

Three different boundary conditions have been imposed on the domain. Zero flux boundary condition at the bottom, assuming no entrainment of any entity through the substratum or no concentration gradient at substratum .Constant flux boundary condition above the mass transfer boundary, assuming constant value of soluble entity at the top. In order to avoid edge effects and to maintain continuity of biomass, the periodic boundary condition has been imposed on left and right edges, such that, if the biofilm goes past the boundary on side of the domain, it is wrapped to the corresponding opposite side of the domain.

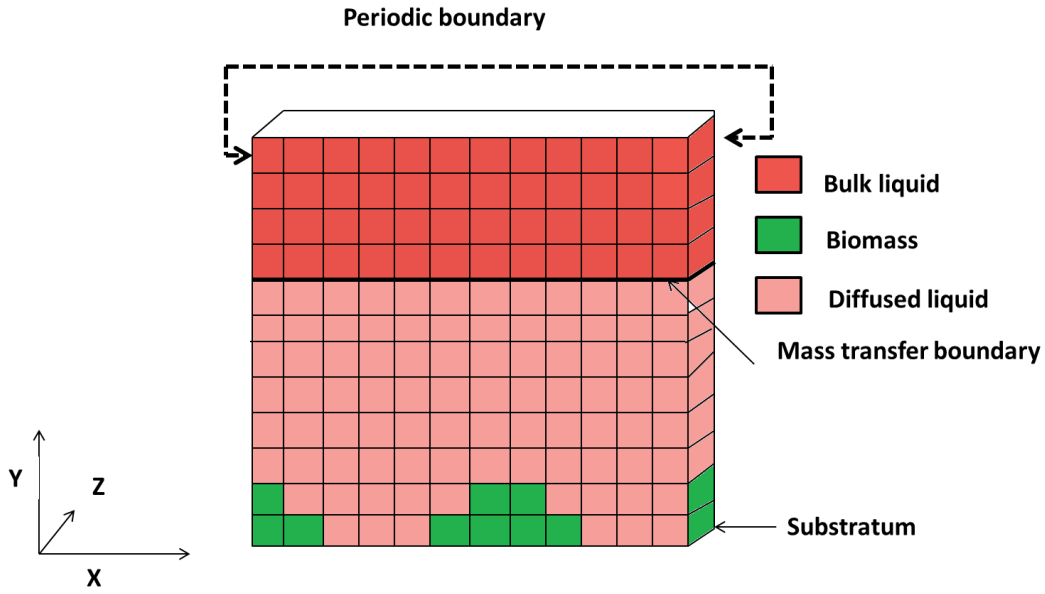

Figure 1.1: A Schematic of the modeling domain. Each element/cube can harbor one bacterial cell or an equivalent volume of liquid. The domain is bounded at the bottom by a sold surface (substratum) on which the biofilm can grow. In the mass transfer boundary layer (below the solid line) there is a substrate concentration gradient due to bacterial consumption of nutrients and diffusion of nutrients from the bulk liquid (above the solid line). In the bulk liquid the nutrient concentration is assumed to be constant. Since the concept of periodic boundaries is used, the

domain is represented as a cylinder (indicated by the dotted arrow at the top) where biofilm biomass at the right edge wraps around to the left edge.

2.3 Modeling of soluble entities

Inside the domain nutrient, antibiotic and auto inducer which is produced by bacterial cells are the soluble entities. The concentration of each soluble entity is modeled using discretized differential equation.

2.3.1 Nutrient diffusion-reaction equation

Let N_c (x, y, z, t) be the concentration of nutrient at any position in the domain at time t. The continuous nutrient source has been kept above the mass transfer boundary diffuses through the boundary layer and reaches to the bacterial cells which gets consumed by them and hence nutrient gradient is developed in the domain which can be given by the following equation [14] comprising of diffusion ,consumption and convection term.

$$\frac{\partial N_c}{\partial t} = D_s\left(\frac{\partial^2 N_c}{\partial x^2} + \frac{\partial^2 N_c}{\partial y^2} + \frac{\partial^2 N_c}{\partial z^2}\right) - r_s\left(N_c, B_c\right) - \nabla.\left(vN_c\right)$$

Where

N_c is the concentration of nutrient in g/m^3

B_c is the biomass concentration in g/m^3

D_s is the diffusivity of nutrient.

$r_s\left(N_c, B_c\right)$ is the consumption of nutrient by the bacterial cells which is used for internal metabolism and growth, given by Monod's growth model [14]:

Where,

$$r_s\left(N_c, B_c\right) = \left(\frac{\mu_{\max}}{Y_{SB}} + m\right) B_c \frac{N_c}{K_s + N_c}$$

μ_{max} is the maximum growth coefficient in hour^{-1}

Ks is the saturation coefficient in g/m^3 and

Y_{SB} is the yield coefficient defined as the amount of nutrient converted into the biomass to the amount of nutrient consumed.

2.3.2 Auto inducer production-diffusion equation

The *S. aureus* and *P. aeruginosa* species synthesizes auto inducer of same kind called Acyl Homoserine Lactones AHL. Each species produces auto inducer and at every time step the total auto inducer concentration is checked by the model. If auto inducer concentration is more than the threshold concentration defined, then cells are said to be up regulated. If the concentration is less than the threshold value, then the cells are down regulated. Up regulated cells produces auto inducer in double rate than the down regulated cells and also produces EPS. In our system *S. aureus* cells produces EPS.

The rate of transport of auto inducer is given by following equation [16]:

$$\frac{\partial A_c}{\partial t} = D_A\left(\frac{\partial^2 A_c}{\partial x^2} + \frac{\partial^2 A_c}{\partial y^2} + \frac{\partial^2 A_c}{\partial z^2}\right) + \frac{F}{l^3} - \nabla.\left(vA_c\right)$$

Where

F is the production of auto inducer by the cells.

$$F = \begin{cases} Z_{A,u} \dfrac{A_c}{K_q + A_c} \\ Z_{A,\mathrm{d}} \end{cases}$$

Where

A_c = Auto-inducer concentration (molm^{-3})

D_A = Diffusion coefficient of auto inducer

$Z_{A,u}$ = Production rate of auto inducer by up-regulated cell($molm^{-3}$)

$Z_{A,d}$ = Production rate of auto inducer by down-regulated cell($molm^{-3)}$

K_q = Half saturation coefficient ($gm^{-3)}$

v = Velocity vector

Inside the domain cells are getting converted from down regulated to up regulated type depending upon the threshold auto inducer concentration. The transition is given by following equations [16]:

$$Q^{+} = \alpha \frac{A_c}{1+\gamma\left(A_c + \overline{A_c}\right)} \qquad Q^{-} = \beta \frac{1+\gamma \overline{A}_c}{1+\gamma\left(A_c + \overline{A_c}\right)}$$

Where

Q^+ = Transition rate from down-regulated type to become up-regulated

Q^- = Transition rate from up regulate type to down regulated

Ac = Auto inducer concentration (gm^{-3})

$\overline{A_c}$ = Inhibitor concentration (gm^{-3})

β = Spontaneous down regulation rate (hr^{-1})

α = Spontaneous up regulation rate (hr^{-1})

γ = constant (m^3/mol)

The probability (P) of cells to be up regulated and down regulated is given as:

$$P(up) = Q^{+}\Delta t$$

$$P(down) = Q^{-}\Delta t$$

Where

P(up) = probability of up regulation of cell

P(down) = probability of down regulation of cell..

2.3.2 Antibiotic diffusion-consumption equation

Inside the domain antibiotic is released from top (above the mass transfer boundary) at particular time lasting up to certain hours. After diffusing through mass transfer boundary layer, it is consumed by bacteria. The rate of change of antibiotic concentration (C_{Ab}) at any position at any time is given by[15]:

$$\frac{\partial C_{Ab}}{\partial t} = D_{Ab}\left(\frac{\partial^2 C_{Ab}}{\partial x^2} + \frac{\partial^2 C_{Ab}}{\partial y^2} + \frac{\partial^2 C_{Ab}}{\partial z^2}\right) - r_{Ab}\left(C_{Ab}, B_c\right) - \nabla.\left(\vec{v}C_{Ab}\right)$$

Where r_{Ab} denotes the rate of consumption of antibiotic by the bacteria which can be obtained from Monod's growth model [15].

$$r_{Ab}\left(C_{Ab}, B_c\right) = K_{A_{\max}} B_c \frac{C_{Ab}}{K_A + C_{Ab}}$$

Where $K_{A_{\max}}$ denotes the maximum specific growth rate of bacteria during antibiotic treatment in per hour while K_A is the half saturation coefficient.

2.4 Modeling of insoluble entities

In our 3 D domain cell and EPS are insoluble entities. Insoluble entities have different behaviors like growth, division, death and detachment. These behaviors are interpreted inside the model with certain local rules.

2.4.1 Rules for growth of bacterial biomass

The nutrient consumed by bacterium which is not utilized for internal metabolism, gets converted into bacterial biomass B_c at a pre-determined efficiency factor called yield coefficient Y_{SB}

$$\frac{\partial B_c}{\partial t} = Y_{SB}\left(r_s\left(N_c, B_c\right) - mB_c\right)$$

Where

m is the maintenance coefficient.

Similarly, when *S. aureus* cells become up regulated, then the nutrient consumed which is not utilized for internal metabolism gets converted into EPS at a pre-determined efficiency factor called yield coefficient Y_{SE}.

$$\frac{\partial E_c}{\partial t} = Y_{SE}\left(r_s\left(N_c, B_c\right) - mB_c\right)$$

Where

E_c is EPS concentration in gm $^{-3}$.

2.4.2 Rules for cell and EPS division

When the bacterial biomass B_c gets double of its value than the original value, cell division occurs resulting in formation of two daughter cells are formed. One daughter cell is placed at the original position of mother cell and the other cell gets placed in the neighborhood of mother cell which offers least resistance. Least resistance is offered when the distance between two grids of the 3 D lattice is 1. As the cell division occurs biofilm starts growing and thus at each time step the height of the biofilm, concentration of the soluble entities is updated using discrete cellular automaton approach.

2.4.3 Rule for cell detachment

Detachment or dispersal of biofilm occurs when the cells present inside the domain loose contact with the substratum.

2.4.4 Rules for cell death:

Cell death can happen inside the biofilm due to three reasons:

1. Consumption to metabolism ratio falls below 0.15: During the biofilm growth bacteria voraciously consumes nutrient which starts depleting as the time progresses. If the rate of metabolism is more than the rate of substrate consumption then

bacterial growth will hamper. When the ratio of rate of consumption to rate of metabolism R falls below 0.15 we say that cell death is occurred [14]. Bacterium death occurs due to starvation.

$$R = \frac{r_s(S_c, B_c)}{mB_c}$$

2. Bacterium enters into stationary phase: Stationary phase is defined as the time phase when the entire nutrient consumed by bacterium is used for internal metabolism and no cell growth occurs. If the bacterium enters and remains in stationary phase for more than 24 hours then we say that bacterium death has occurred. We can say , cell death occurred due to old age.

3. Probability of killing by antibiotic: Bacteria die if for each time step, in the presence of antibiotic, each cell generates a random number less than equal to the probability of killing by antibiotic. The probability of killing is defined as:

$$P = \frac{r_{Ab} - r_{BIC}}{r_{\max} - r_{BIC}}$$

Where

r_{Ab} is rate of consumption of antibiotic by cell,

r_{BIC} is the rate of consumption of antibiotic at biofilm inhibitory concentration,

$r_{\max}$ is the maximum antibiotic consumption rate.

2.5 Cell parameters

We are using individual based CA model in which each bacterium exist as an independent entity with its own state and behavior. Each bacterium has its own set of parameter values, which is an independent copy of the list of default parameter values mentioned in Table 1. All the values in Table 1 are taken from the existing work done before. To increase the variability between the bacteria new parameter

values were generated for each new bacterium by random draws from a Gaussian distribution with a variation of 10% [14].

Table 2.1: Definition of variable and parameter values from the literature

Terms	Values	Units	References
Maximum specific growth rate	0.3125	hr	[14]
Time step for CA	1	hr	[14]
Element size	$3*10^{-6}$	m	[14]
Thickness of mass transfer boundary layer	$18*10^{-6}$	m	[14]
Biomass concentration		gm^{-3}	[14]
Diffusion coefficient of nutrient		m^2hr^{-1}	[14]
Diffusion coefficient in aqueous phase	$2.52*10^{-6}$	m^2hr^{-1}	[14]
Relative effective diffusivity	1/3		[14]
Biofilm strength	320		[14]
Shear stress	1-250		[14]
Half saturation coefficient	2.55	gm^{-3}	[14]
Maintenance coefficient	0.036	gn/gb/hr	[14]
Stationary phase	24-108	hr	[14]
Substrate concentration in	1,2,3,5	gnm^{-3}	[14]

bulk			
Maximum specific growth rate of antimicrobial	2.5	ga/gn/hr	[15]
Bulk antimicrobial concentration	10	gm^{-3}	[15]
Diffusivity of antimicrobials in the aqueous phase	$11.44*10^{-6}$	m^2hr^{-1}	[15]
Relative effective diffusivity of the antimicrobial in the biofilm	0.25	-	[15]
antibiotic Monod half saturation coefficient	1	gm^{-3}	[15]
Autoinducer diffusion coefficient	$1.998*10^{-6}$	m^2hr^{-1}	[16]
diffusivity	0.5		[16]
Production rate by up regulated cells	73800	$molhr^{-1}$	[16]
Production rate by down regulated cells	498	$molhr^{-1}$	[16]
Conversion rate	$7.8*10^{-17}$	$m^3hr^{-1}mol^{-1}$	[16]
Spontaneous rate	0.975	hr^{-1}	[16]
Transition constant	$7.9589*10^{-17}$	m^3mol^{-1}	[16]
Auto-inducer threshold concentration	$4.75909*10^{18}$	$molm^{-3}$	[16]

2.6 Model simulation

The state of the simulation domain is updated at discrete time steps. The dynamic of this update is described by the cellular automata rules, which represent the interaction of each element with its neighboring element in the domain. When time t=0 hr simulation progresses through the following steps:

1. The modeling domain is created and all cell parameters are established.
2. A fixed number of bacteria are allowed to colonize on the substratum at random locations.
3. The nutrient concentration field is generated at a fixed distance from the top of the biofilm and allowed to diffuse through the mass transfer boundary.
4. Bacteria consume nutrient, resulting in increase in biomass and production of signaling molecules.
5. Auto-inducer concentration field is generated.
6. Up-regulation status for individual cells is determined
7. If a cell is up-regulated, EPS is produced by *S. aureus* species.
8. Cell division and EPS division are performed.
9. Determination of bacterial death criteria using three rules.
10. Death operation is performed and dead bacteria are removed from the domain.
11. Identify bacteria that fulfill detachment criterion. Check for detachment and remove detached cell.
12. Check if the maximum number of simulation time steps has been reached and if not, move forward in time and perform steps 3 to 13 again.
13. Termination of simulation.

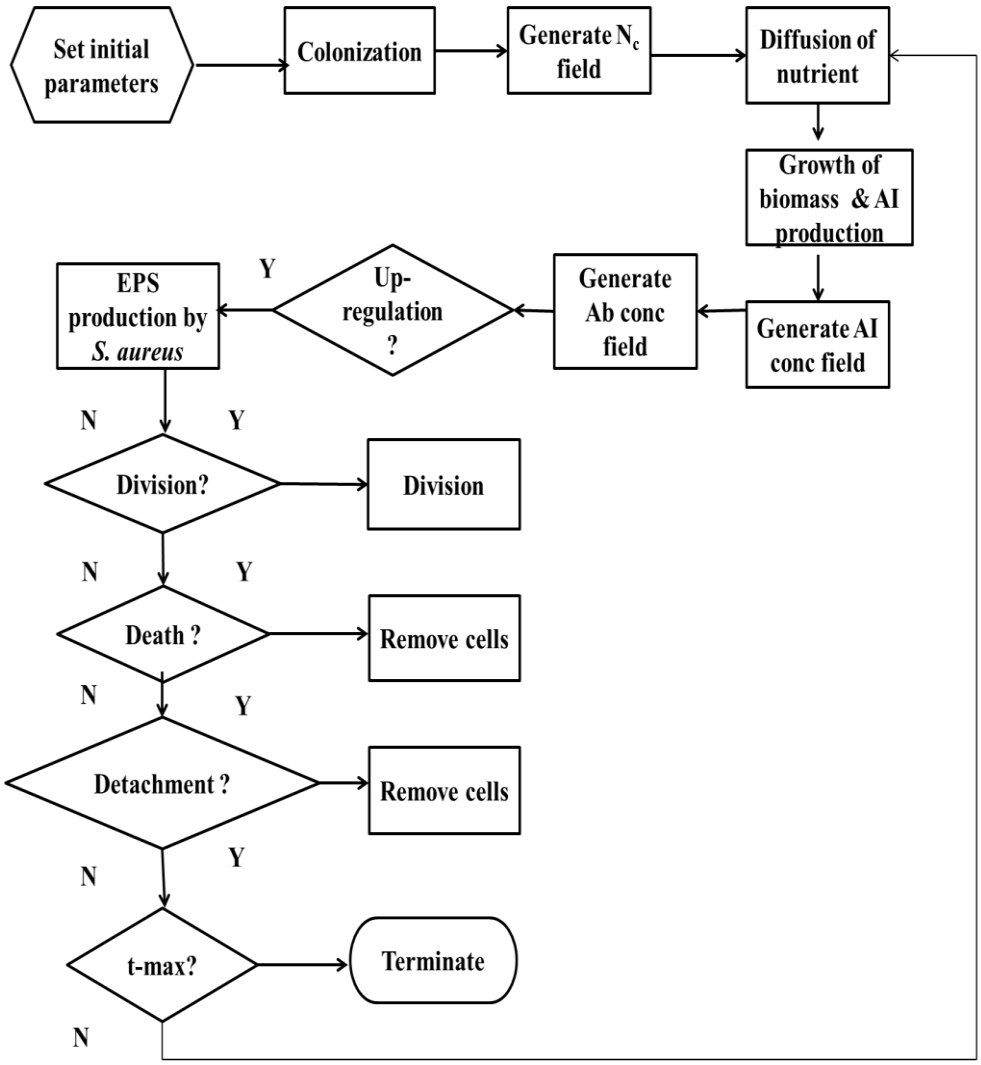

Figure 2.1: Flow-chart for the biofilm CA model, describing the sequence of processes during a typical simulation.

Chapter 3

Results and discussion

The main aim of this work was to investigate the role of quorum sensing during antibiotic treatment. Wide ranges of concentration were tested in order to obtain successful treatment. Periodic and continuous modes were compared in order to know better control. The quorum sensing biofilms were compared with non-quorum sensing. Following behaviors were observed.

1) Quorum sensing biofilms has greater biomass than non-quorum sensing biofilms.

First, we allowed, 2 cells of each species to grow on nutrient medium of 4 gm^{-3} concentration. It is already known that, total cell number is a direct function of nutrient concentration [14].We carried out all our simulations for 4gm^{-3} so as to fix one of our parameter. When we checked for total biomass present in domain for both the systems i.e. with and without quorum sensing, we found that the total biomass content of the quorum sensing biofilm is higher than the non-quorum sensing biofilm, see in fig.3.1.

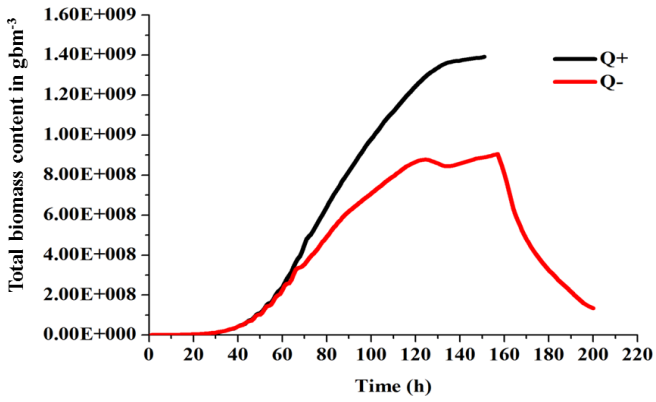

Figure 3.1: Total biomass over time for quorum sensing and non-quorum sensing biofilm grown at 4 gm^{-3} nutrient concentration.

Quorum sensing biofilm has EPS growing along with cells and that's the reason its biomass is higher.

When checked for growth dynamics of both the species , they grow in same manner as in non-quorum sensing biofilm. See fig.3.2 and 3.3. After 117 hr (approximately) *P. aeruginosa* species starts dying because of low consumption and metabolism ratio, which is already defined in section 2.4.4. Actually, *P. aeruginosa* species is slow growing and occupies the bottom positions in the domain, so first it will go in starvation, afterwards death of *S. aureus* species will start at 140^{th} hr (approximately).

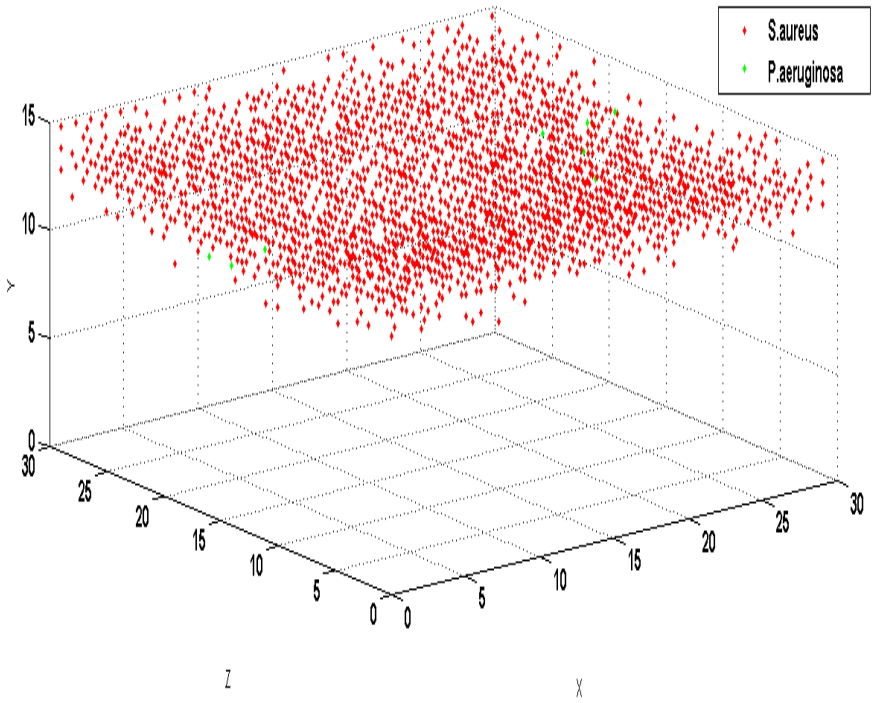

Figure 3.2: 3D graph showin position of cells in the domain at 200^{th} h for non-quorum ensing biofilm.

Because of death of bottom cells , non-quorum sensing biofilm will detach because of loosing contact with the subtratum. This detachment can be observed in non-quorum sensing biofilm prominently. Plese see fig. 3.4. , we can clearly observe the detachment at 160^{th} hr approximately.

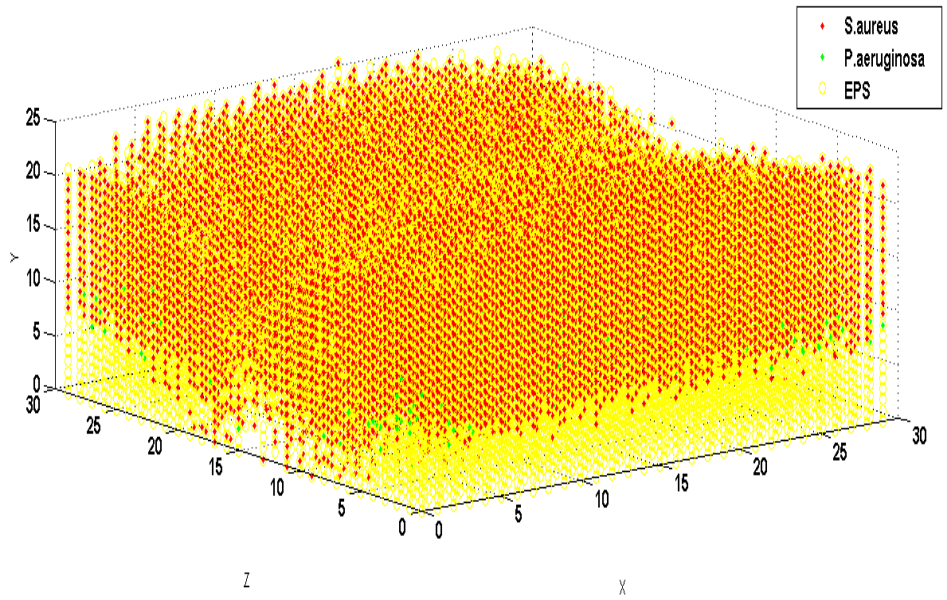

Figure 3.3: 3D graph showin position of cells and EPS in the domain at 151 th h for quorum ensing biofilm.

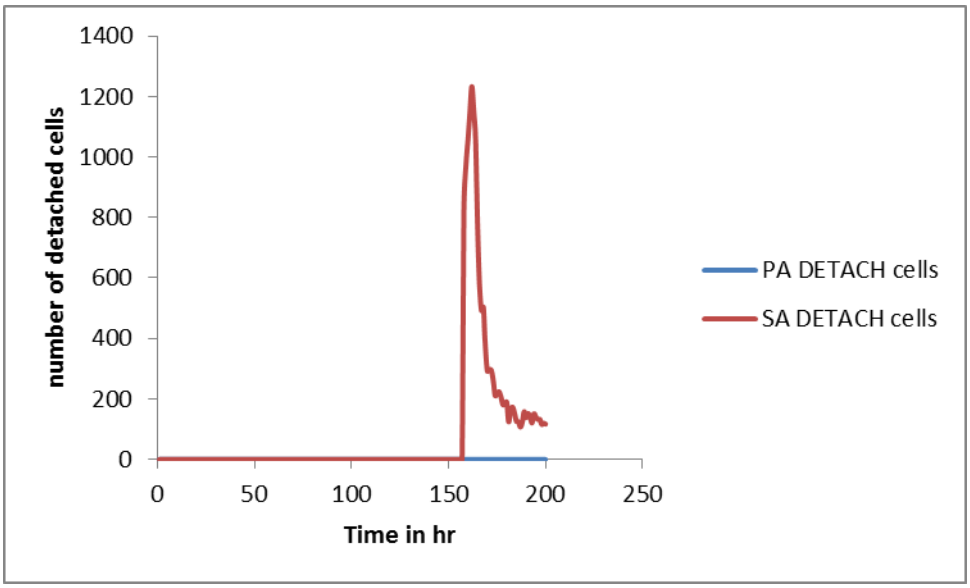

Figure 3.4: Number of detached cells over time for system without quorum sensing

However, for quorum sensing biofilm, EPS is present in the domain which occupies the place of died cells and helps to anchor the biofilm on subtratum. That's why,

non-quorum sensing biofilm may diminish because of detachment but quorum sensing biofilm wil never detach.

2) The concentration required to eradicate the biofilm completely, is a function of time duration of treatment.

In order to fix another parameter, we carried out continuous antibiotic treatment of 43 gm^{-3} for 24 hr and 48 hr duration when cell number was 10000. We found that, 48 hr continuous treatment could eradicate both the species but 24 hr continuous treatment couldn't, please refer fig. 3.5.

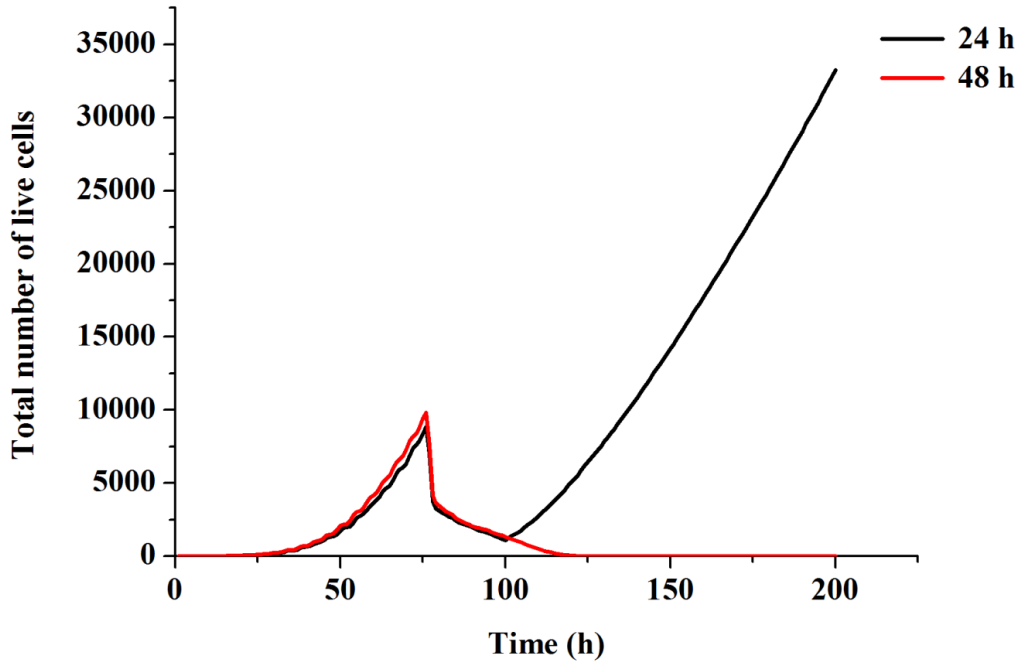

Figure 3.5: Total live cells over time for antibiotic concentration of 43gm^{-3} and continuous treatment done at 24hr and 48 hr.

From fig.3.5 we can also predict that, for fast treatment, more antibiotic concentration is required. So, for the purpose of safe treatment we preferred low antibiotic concentration with long duration of 48 hr. Safe treatment means, no damage to body tissues in neighbor with bacterial biofilm, because of high antibiotic concentration.

3) More antibiotic concentration is needed to eradicate quorum sensing biofilm. It is observed that virulence is build up in biofilms with quorum sensing.

We carried out wide range of simulations to get successful therapeutic treatment. We came across two remarkable concentrations differing by small amount but one could eradicate biofilm and one couldn't. We defined effective concentration as, the minimum antibiotic concentration at which both the species dies and the threshold concentration as, the maximum antibiotic concentration which biofilm can tolerate.

Following table depicts the effective and threshold values for both the system i.e. with and without quorum sensing.

It is clear from the effective concentration values from table 3.1, of both systems that, more antibiotic concentration is required to eradicate the quorum sensing biofilm. However, if you compare these values, you will find that effective concentration for non-quorum sensing biofilm is less than that for quorum sensing biofilm by certain factor. We will define here, virulence factor as,

Virulence factor = $[Q+Ab_{eff} - Q\text{-}Ab_{eff}] \div Q\text{-}Ab_{eff}$

Where

$Q+Ab_{eff}$ =effective concentration for quorum sensing biofilm.

$Q\text{-}Ab_{eff}$ = effective concentration for non-quorum sensing biofilm.

Table 3.1:.Response of quorum sensing and non-quorum sensing biofilm to continuous antibiotic treatment for 48 hrs.

Avg. cells at the start of treatment	**Time start (hr)**	**Time end (hr)**	**Q+**		**Q-**	
			Threshold Conc. (gm^{-3})	**Effective Conc. (gm^{-3})**	**Threshold Conc. (gm^{-3})**	**Effective Conc. (gm^{-3})**
2500	**48**	**96**	**39**	**40**	**29**	**30**
5000	**67**	**115**	**42**	**43**	**31**	**32**
10000	**77**	**125**	**42**	**43**	**33**	**34**

Figure 3.6 and 3.7 illustrates the difference between growth dynamics of biofilm at threshold and effective antibiotic concentration.

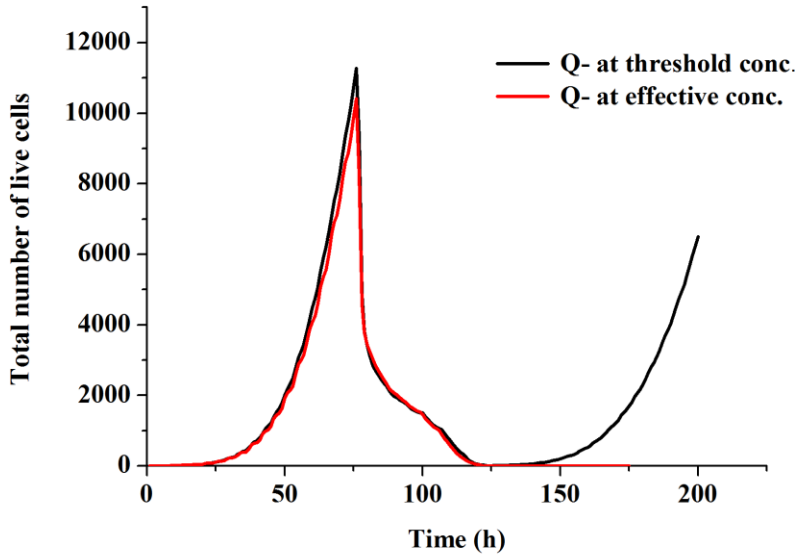

Figure 3.6 : total live cells over time in hr for quorum sensing biofilm treatment started at 10000 cell number.

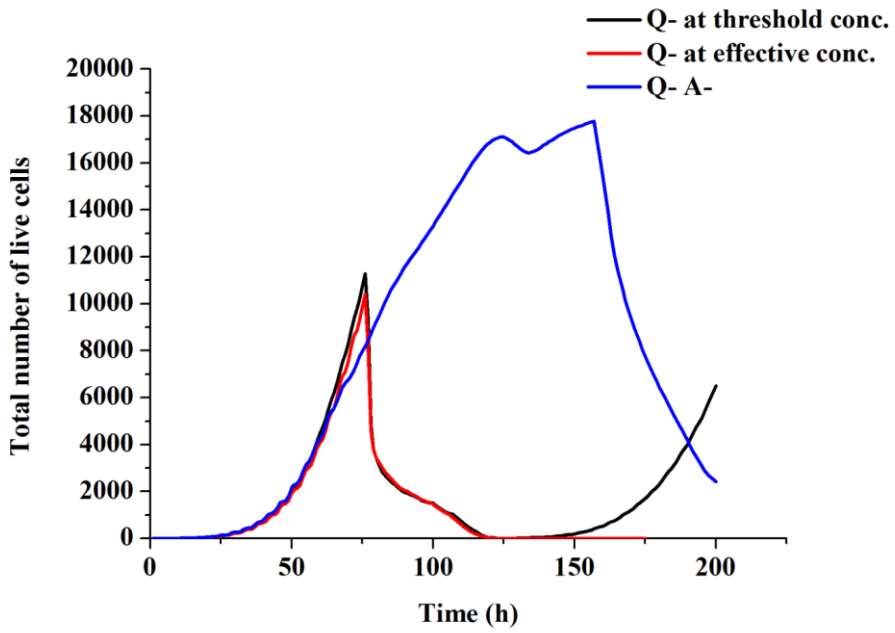

Figure 3.7 : total live cells over time in hr for non-quorum sensing biofilm treatment started at 10000 cell number.

The biofilm continues to grow after the end of antibiotic treatment done at threshold concentration. It is observed that, at low cell number, *P. aeruginosa* species dies completely by antibiotic treatment for threshold value, but *S. aureus* species still continues to grow after treatment and mono-species biofilm developes fully on surface.

4) Effective antibiotic concentration is independent of total number of cells present in domain rather it is function of antibiotic resistance of bacterium.

With reference to **table 3.1** up to cell number 10000, the effective antibiotic concentration values for quorum sensing biofilm is around 40 to 43 gm^{-3} and for non-quorum sensing it is around 30-34 gm^{-3}. Effective concentration is seems to be independent of number of cells present, but it is found that , it takes more time to eradicate *S. aureus* species than *P. aeruginosa*. The reason behind this can be, *S. aureus* is comparatively more resistant than *P. aeruginosa* species.

Figure 3.8 reports the response of *S. aureus* species and *P. aeruginosa* species to antibiotic treatment done at threshold concentration.

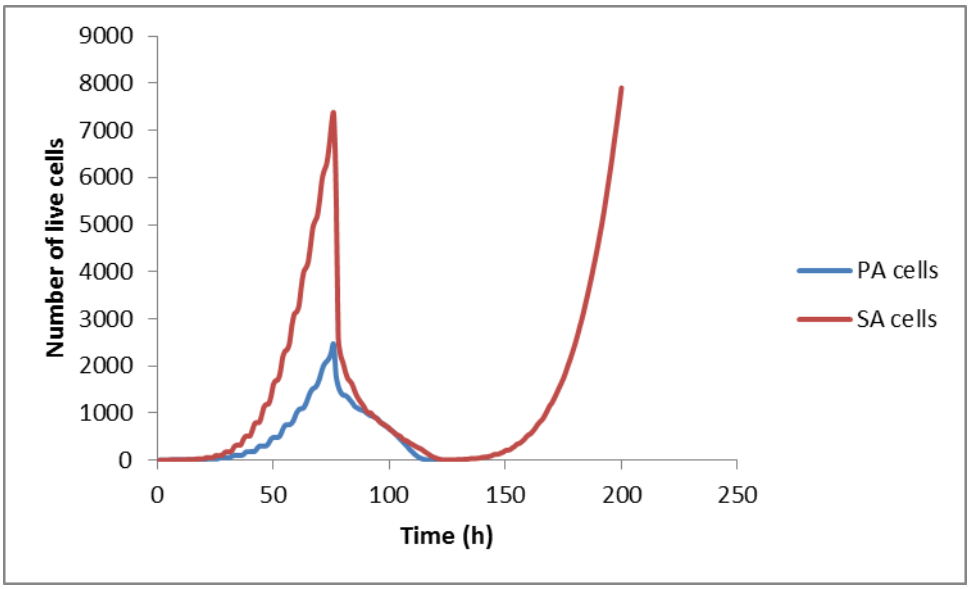

Figure 3.8: Number of live cells over time for quorum sensing biofilm at threshold concentration of 43gm-3 when treatment started at 10000 cell number.

5) If treatment started earlier, less resistant and slow growing species eradicates completely whereas it becomes very difficult to eradicate same species when treated late. A strong co-ordination is observed between two species in order to protect whole biofilm.

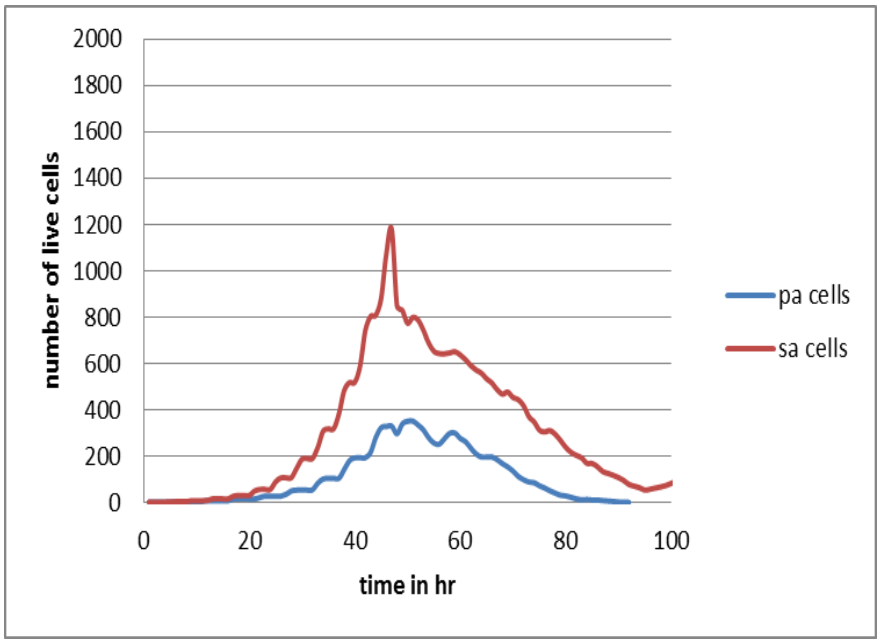

Figure 3.9: Number of live cells over time plotted for two species, for 30 gm^{-3} antibiotic treatment started at 48 hr.

At early treatment with low antibiotic concentration of 30 gm^{-3} *P. aeruginosa* cells dies because of being less resistant species and also from the 3-d graphs it is observed that *P. aeruginosa* cells are not getting advantage of quorum sensing .

Figure 3.10 illustrates the effect of quorum sensing on early treatment. In our system, *S. aureus* species is producing EPS after the cells gets up regulated. In the beginning, the EPS will be selectively placed above *S. aureus* Species and as the time progresses with increase in total cell number, EPS will occupy places above *P. aeruginosa* species as well as vacant grids inside the defined simulation domain.

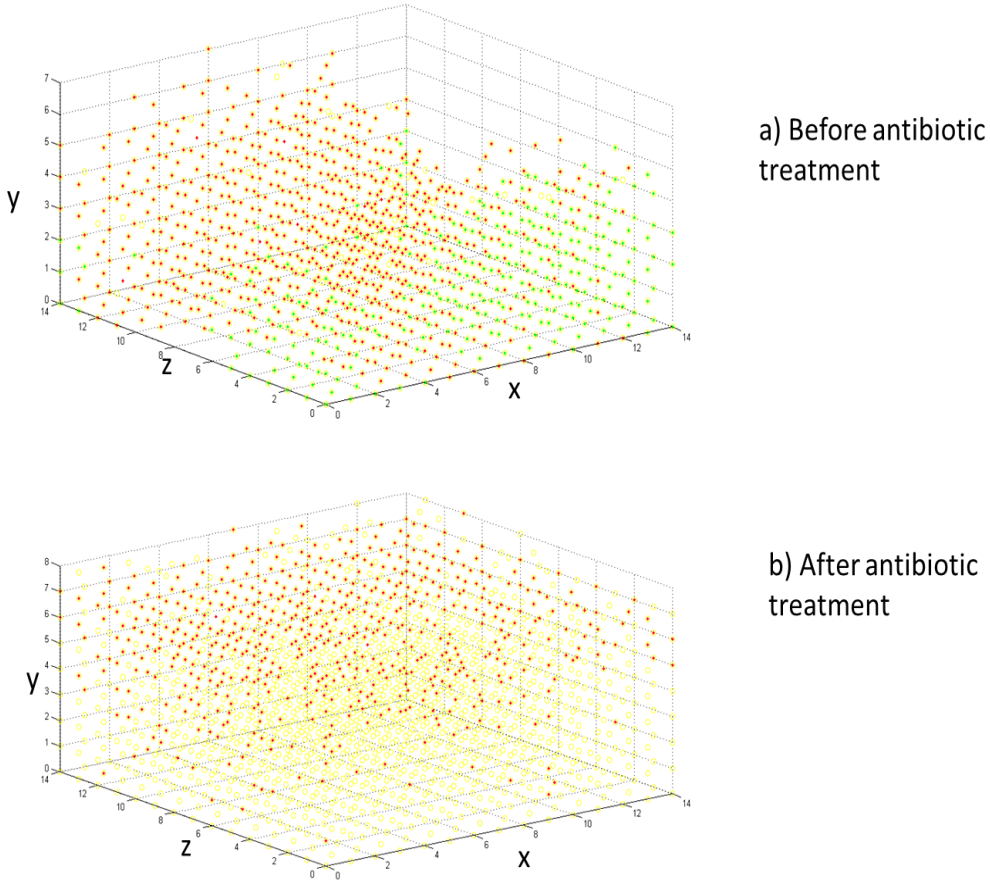

Figure 3.10: 3-D graph showing position of cells and EPS in the domain at the start of early treatment at 46 hr. Green dot represents *P. aeruginosa* species, red dot represents *S. aureus* species and yellow circle represents EPS.

When we checked the response of *P. aeruginosa* cells to the same antibiotic concentration carried out at higher cell number than that used for above case, we found that *P. aeruginosa* species could resist the antibiotic for the continuous treatment of 48 hrs.

Figure 3.11 denotes the difference in behavior of *P. aeruginosa* species for same antibiotic concentration started at different time steps. One is done at 48 hr and other is done at 67 hr.

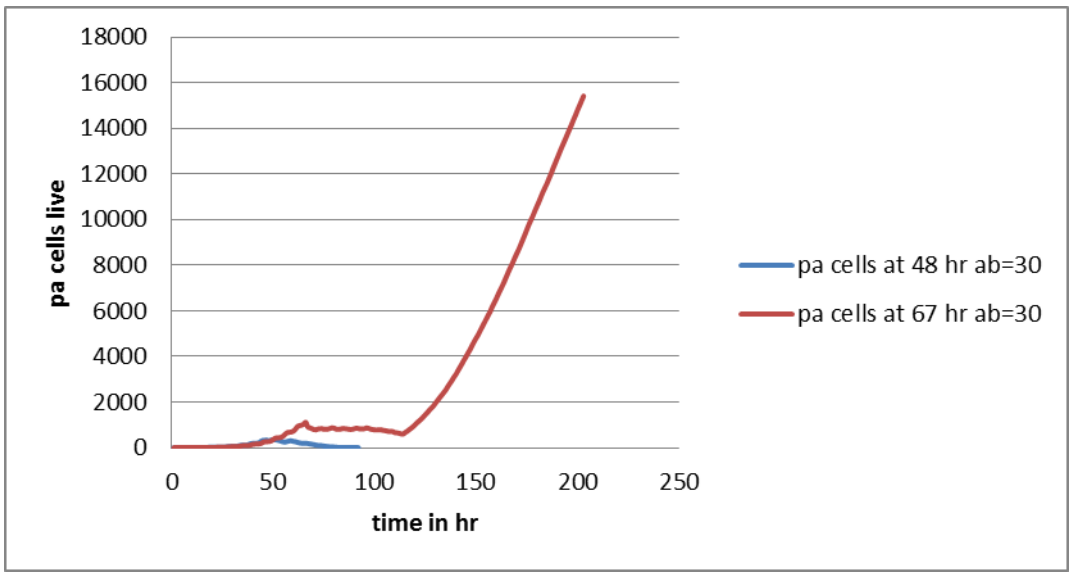

Figure 3.11: Number of live *P. aeruginosa* cells over time plotted for antibiotic concentration of 30 gm^{-3} with release time a) 48hr and b) 67hr.

In figure 3.10, *P. aeruginosa* species died at early treatment because of low resistivity. But it can resist the same antibiotic concentration when treated late. Here, quorum sensing comes into picture as a cell density dependent phenomenon.

When cell population is high, high amount of auto-inducer is synthesized in the biofilm, which triggers the cells to be up regulated.

The antibiotic treatment is highly influenced by the quorum sensing as, it is not only depends upon resistance of species but also co-ordination between these two species.

EPS, which is glue like in structure, holds the cells in the domain. It is observed that almost 70 % population of biomass is occupied by the EPS inside the domain. In our system, EPS can consume the antibiotic up to certain concentration, but it cannot die because of consumption. So, EPS will persist in the domain for whole time.
As our simulations are of stochastic nature, EPS will be placed randomly inside the domain in vacant grids as well as locations above the cells.
In figure 3.12 we tried to show the position of cells and EPS before the start of treatment and after the end of treatment.

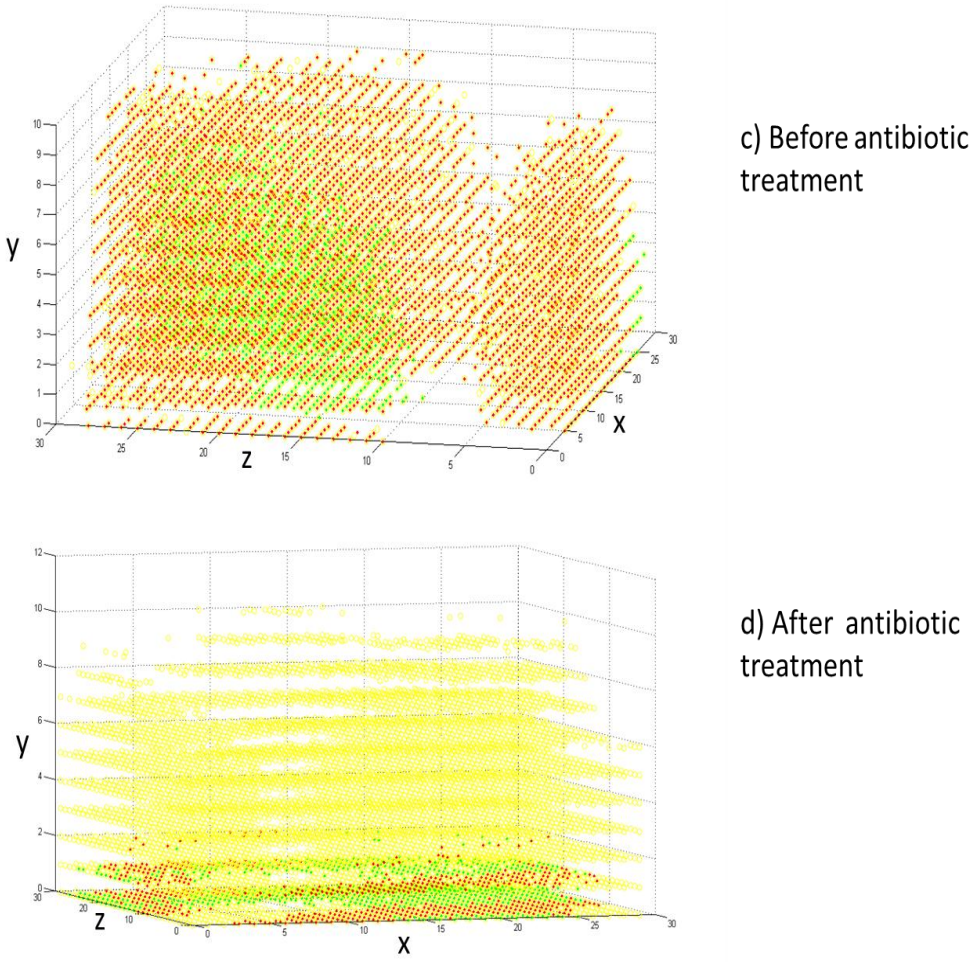

Figure 3.12: 3D graph showing position of cells and EPS in the domain before the antibiotic treatment. Green dot represents *P. aeruginosa* species, red dot represents *S. aureus* species and yellow circle represents EPS.

Figure 3.12 proves **quorum sensing as a population dependent phenomenon as well as providing protection to both species by synthesizing EPS**.

Continue with the behavior of slow growing and less resistant species, *P. aeruginosa*, at one concentration done at different ages of poly-microbial biofilm, we came across the case where more number of *P. aeruginosa* species is present in the system than *S. aureus* species. For this case, almost 20000 total cells were present in the domain where, *S. aureus* cells were nearly 15000 and *P. aeruginosa* cells were just nearly 5000 only.

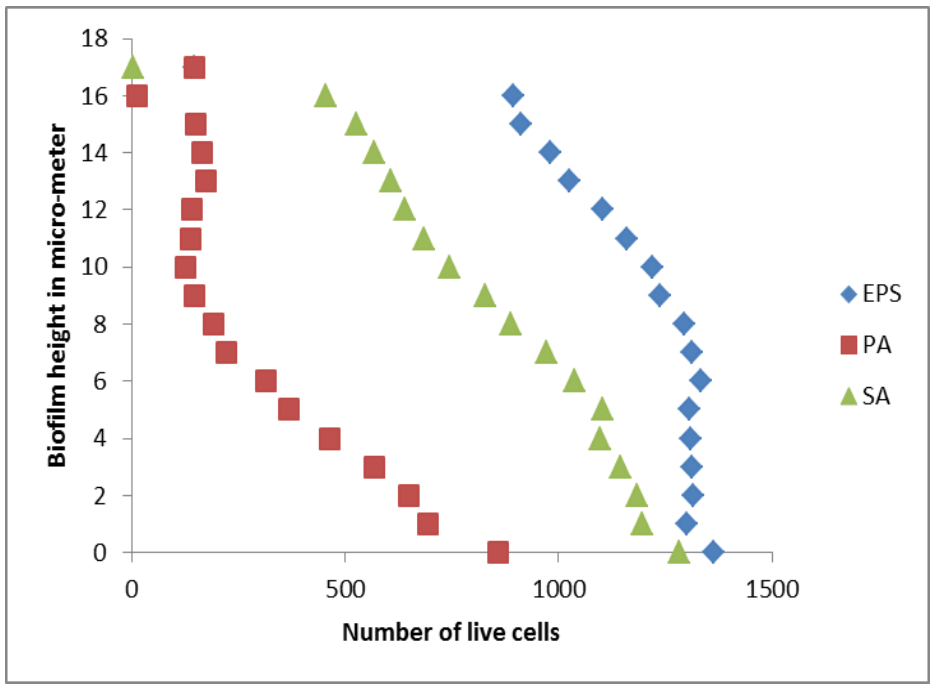

Figure 3.13: Fraction of biofilm height vs number of cells and EPS present in domain at 97hr for quorum sensing biofilm.

From figure 3.13, it is obvious that EPS occuopies bottom position as well as surface and its population is very high as compared to the bacterial species.*S.Aureus* cells are also seems more in number than *P. aeruginosa* cells and occurs almost all locations in the biofilm height. Whereas , *P. aeruginosa* cells are less in number and highly placed in bottom.

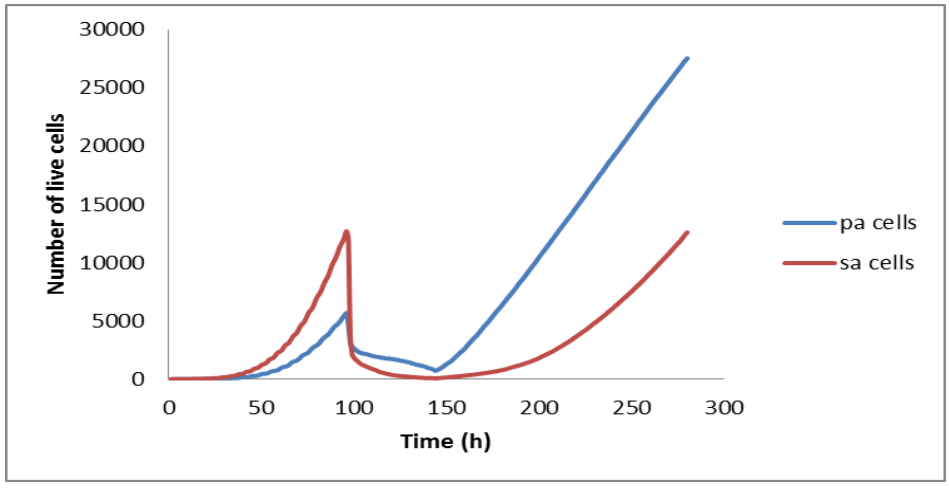

Figure 3.14: number of live cells over time for antibiotic treatment started at 97 hr.

Figure 3.14 illustrates the number of each species present in the biofilm after the antibiotic treatment of 30 gm^{-3} started at 97 hr.

Looking through above results we can conclude that, if antibiotic concentration is less than the effective concentration, then response of slow growing and less resistant, *P. aeruginosa* species is different at different ages of biofilm.

For early treatment, *S. aureus* species specifically controls the growth dynamics of whole biofilm. For very late treatment i.e. at 97 hr or at high cell number, *P. aeruginosa* species controls the growth dynamics of biofilm while if treatment started at average cell number 10000, then both species controls the growth dynamic.Here, we can strongly observe the synergistic interaction between two species.

6) If treatment started early to the commencement of quorum sensing, it is possible to eradicate the quorum sensing biofilm at low antibiotic concentration.

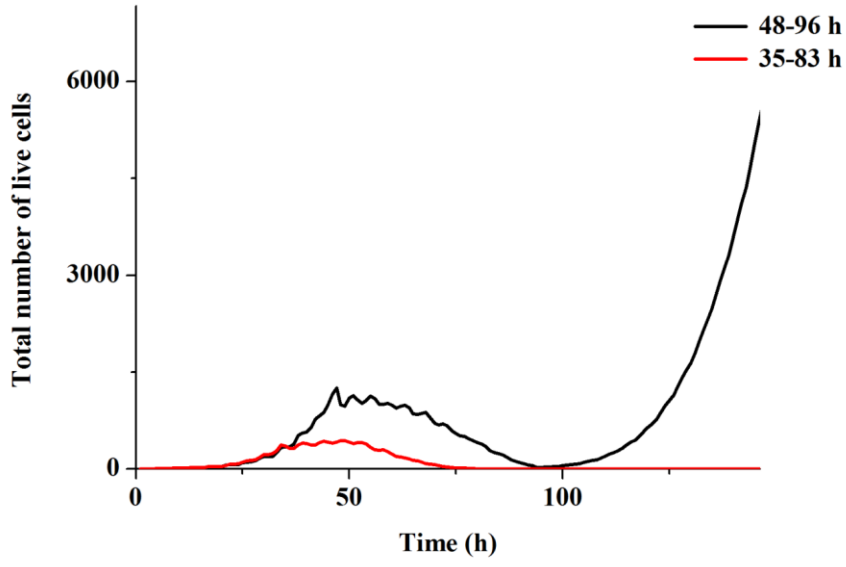

Figure 3.15: Total live cells vs time for antibiotic concentration of 35gm^{-3} and duration of 48 hr with time start of treatment a) at 48 hr and b) at 35 hr.

From above discussion, we came to know that EPS protects the biofilm from antibiotics. EPS is observed to be placed above the cells inside the domain. The effect of quorum sensing increases as the number of cells increases. So, we checked the time of treatment at which less auto inducer will be found and less antibiotic will require for eradication of whole biofilm.

7) Surface cells die faster than the bottom cells.

As the time advances the fast growing species occupies the top position in the domain while slow growing species grows at bottom. When cell numbers reaches up to 20000 cells and if we release antibiotic of same concentration used as earlier then we found that less resistant and slow species has more cells present in the biofilm than other species.

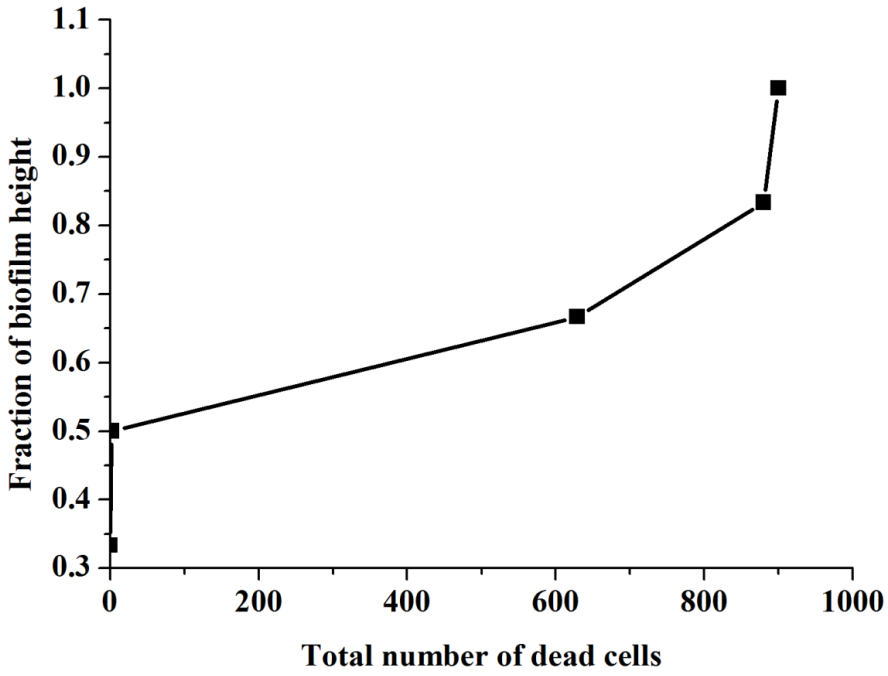

Figure 3.16: Fraction of biofilm height vs total number of dead cells for antibiotic treatment of 30 gm-3 carried at 20000 total cell numbers.

From above fig.3.16 it is noticed that after 145th hr more *P. aeruginosa* cells are present. The reason for this can be less penetration of antibiotic to the bottom and high possibility of death of surface cells by continuous antibiotic diffusion.

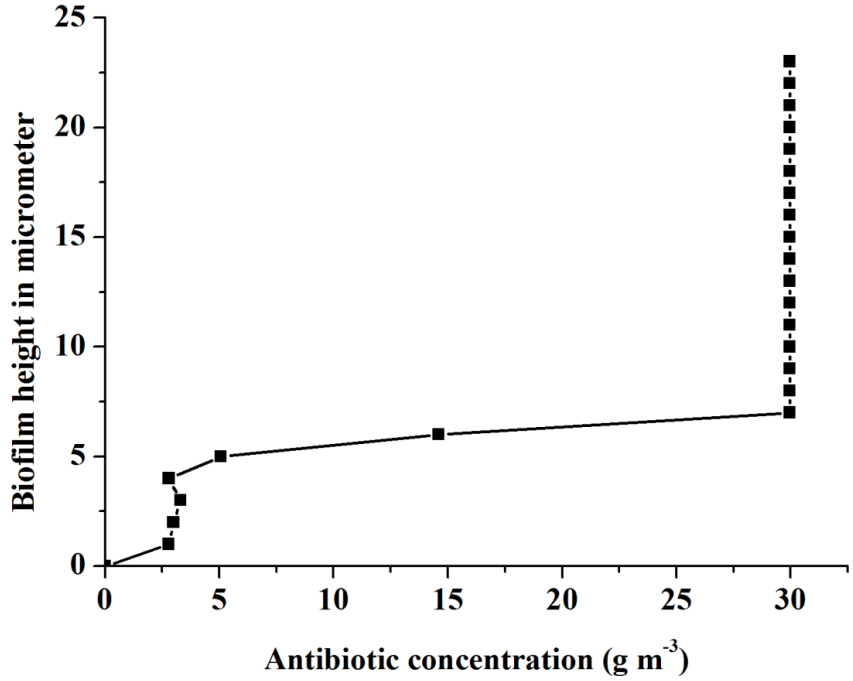

Figure 3.17: Average antibiotic concentration vs biofilm height for antibiotic treatment of 30 gm^{-3} started at 20000 cell number.

As we can see in fig.3.17, average antibiotic concentration present at height 7 to 23 µm is nearly 29.97 gm^{-3} and for such a high concentration compared with between 6 to 0 µm, cell death is observed to be more for biofilm height fraction above 0.6, as shown in figure 3.16.

We observed that effective antibiotic concentration is dependent of diffusion height.

8) In quorum sensing biofilm, slow growing species is protected by fast growing species but same behavior is not found in non-quorum sensing biofilm.

In consistent with result explained in section 5, we observed that there is no interaction between *P. aeruginosa* and *S. aureus* species in biofilm without quorum

sensing. Figure 3.18 and 3.19 gives the number of live cells plotted for both species at 30 gm^{-3} concentration at same time step but vast difference is observed in their response, recorded for quorum sensing and non-quorum sensing system. For non-quorum sensing biofilm, *P. aeruginosa* species dies as it is less resistant and *S. aureus* species survives because it could resist the antibiotic, please refer fig. 3.19.

For quorum sensing biofilm, as shown in fig.3.18, more number of *P. aeruginosa* cells observed to be present in the domain than *S. aureus* species. The only explanation for this behavior can be, *P. aeruginosa* species is being protected by the *S. aureus* species present at surface. EPS is the protective measure for the quorum sensing biofilm, adopted by the micro-bacteria in order to protect the biofilm from environmental changes, surfactants, hydrodynamic shear and antibiotic treatment.

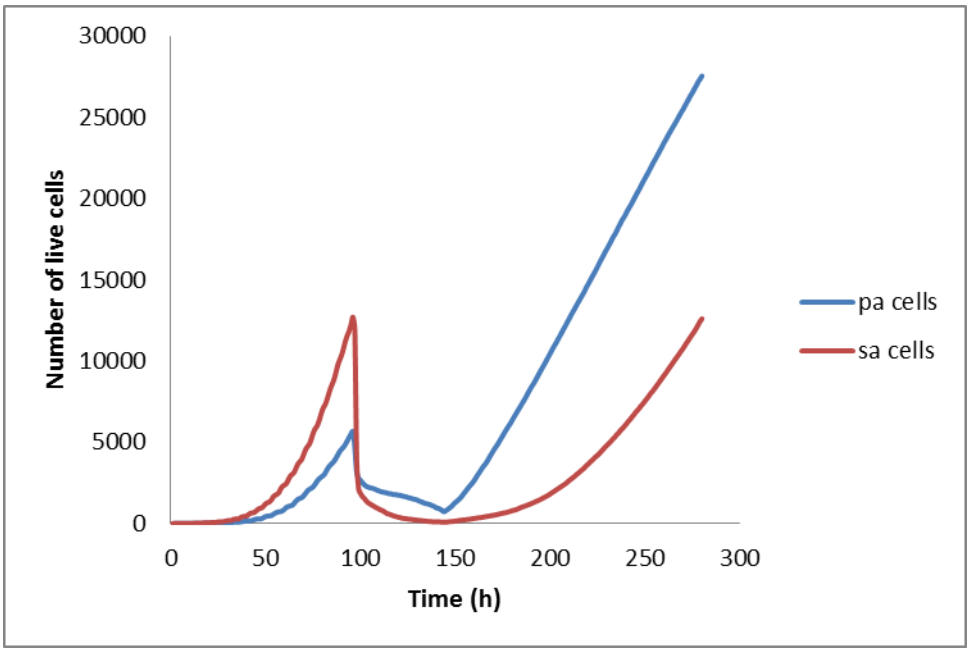

Figure 3.18: Number of live cells over time for quorum sensing biofilm, when 30gm^{-3} antibiotic released at 10000 cells.

So, from the above discussions, it is clear that for successful treatment of non-quorum sensing biofilm, the effective concentration value depends on the antibacterial resistance offered by high resistant species. However, in case of quorum sensing biofilm, effective resistance is not only depends on the resistance offered by high resistant species but also it depends on virulence factor increased in the domain because of the quorum sensing phenomenon.

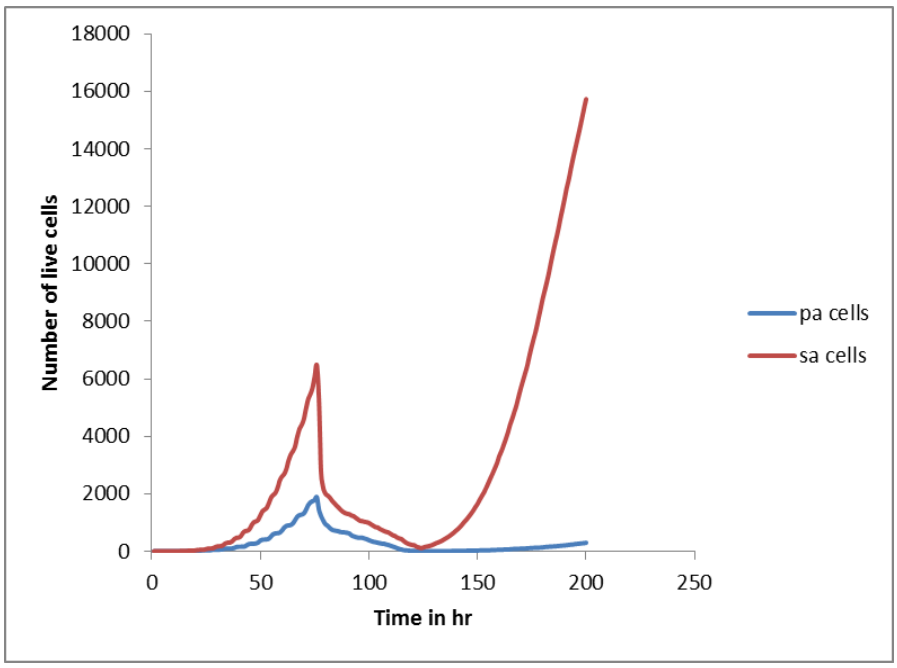

Figure 3.19: Number of live cells over time for non-quorum sensing biofilm, when 30 gm^{-3}antibiotic released at 10000 cells.

9) Continuous treatment has better control over biofilm death dynamics than periodic treatment.

As said earlier, the effective antibiotic concentration depends upon the duration of treatment. For continuous 48 hr of treatment quorum sensing biofilm eradicates completely, but if total 48 hr treatment is given periodically then it is not possible to eradicate the biofilm, refer fig.3.22. We carried out periodic treatment of continuous 12 hr at the interval of 4 , 6 and 12 hr respectively for non-quorum sensing as well as quorum sensing biofilm, but failed to eradicate the biofilm at effective concentrations obtained for both the cases. So, it is advised to go for continuous mode for long duration and low concentration rather than periodic mode.

One of the reasons for failure of periodic treatment is, bacterium regains its resistance in interval gap. Figure 3.22, reports the growth curves for the continuous and periodic treatment carried out for non-quorum sensing biofilm.

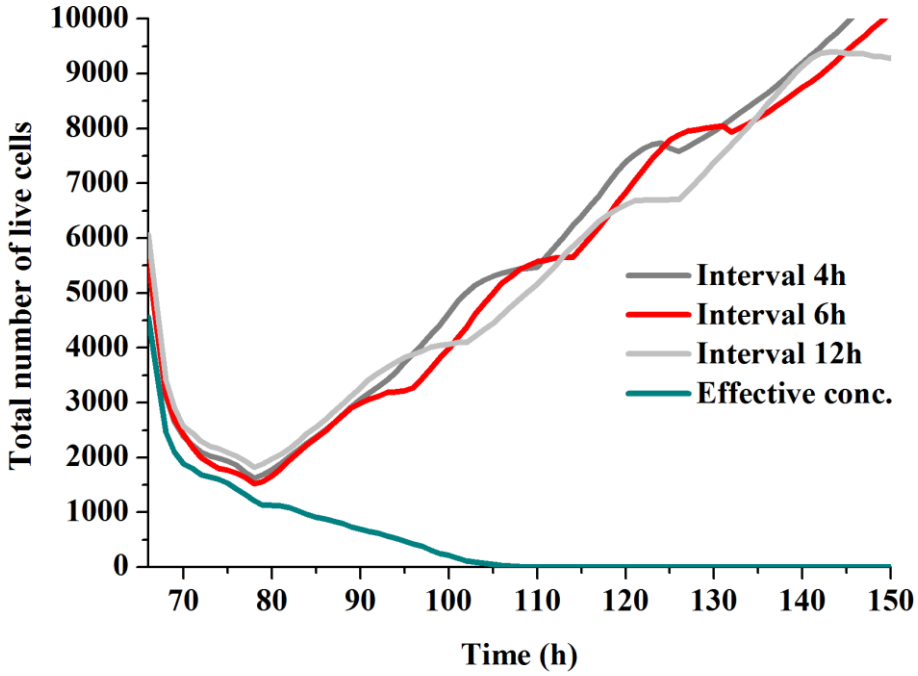

Figure 3.20: Total number of cells vs time for antibiotic treatment started at 67 hr with a) continuous treatment for 48 hrs and periodic treatment for 12 hrs with gap of b) 4 hrs b) 6 hrs and c) 12 hrs, for non-quorum sensing biofilm.

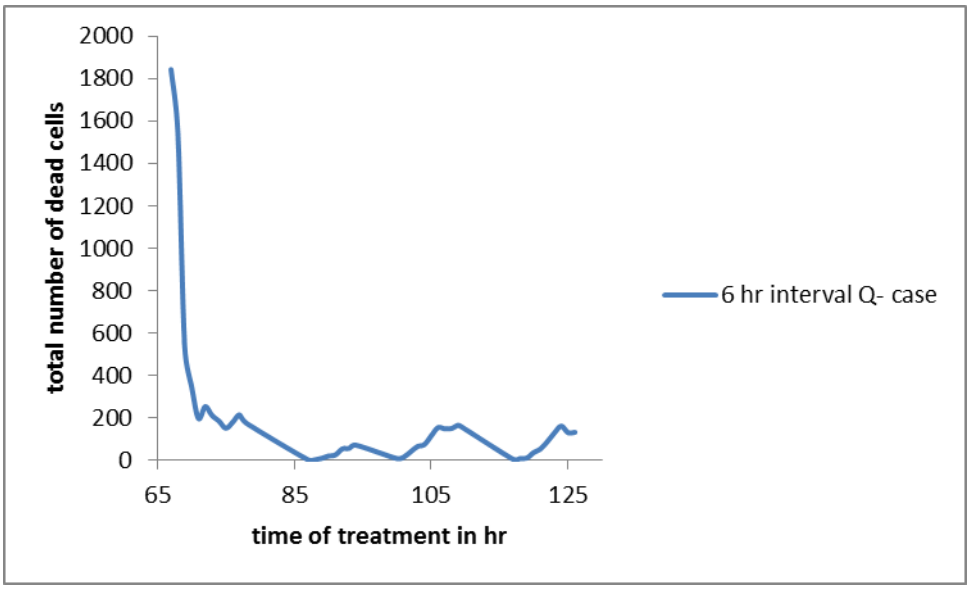

Figure 3.21 : Total number of dead cells over time for antibiotic treatment started at 67 hr with interal gap of 6 hrs.

From fig. 3.23 we can observe that maximum cell death ocured at initial 12 hours continuous tretment. However, after the gap of 6 hours , very less cells are observed to be died in the biofilm. Cells are observed to get persistant with the antibiotic environment after the treatment interval.

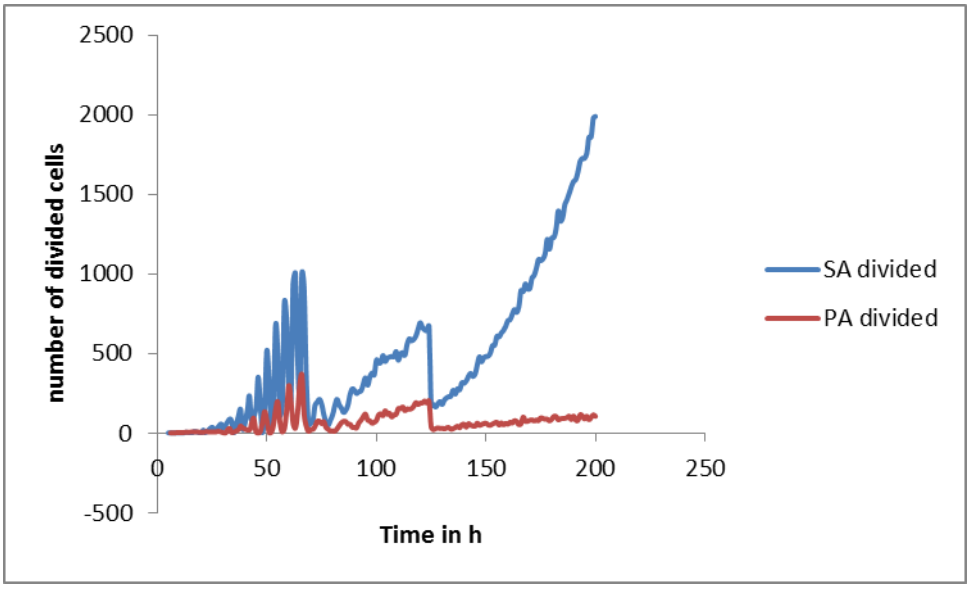

Figure 3.22: Number of divided cells over time.

In figure 3.24 cell divisions after the anttibiotic treatment are faster than before treatment.

We can say that administration of antibacterial , influences the cell division after the interval. So, it is ineffective to choose periodic mode for the eradication of biofilms. Further, high antibiotic concentration will need to remove poly-microbial infection.

Chapter 4

Conclusion

Quorum sensing biofilms are eradicated at more concentrations than non-quorum sensing biofilms. The reason for this can be development of virulence in quorum sensing biofilms. EPS protects the biofilms from erosion and sloughing. Successful therapeutic treatment is obtained at effective concentration which is a function of time duration of treatment, cell number and biofilm height. If time of exposure of antibiotic is less then high antibiotic concentration required for complete death of biofilm.

A synergistic interaction is observed between two species in order to protect the whole biofilm. Surface cells are found to protect the bottom cells. Slow growing and less resistant species dies in early treatment whereas, same species when treated late, has higher contribution in biofilm. Biofilms enters in protected mode and are virulent than non-quorum sensing biofilms.

Chapter 5

Future work

In future, we would focus on, auto-inducer inhibitor and its influence on death dynamics of quorum sensing biofilm. From literature, it has been found that furanone acts as inhibitor in gram negative bacterial species [16]. We will study the release of furanone at different ages of biofilm and antibiotic treatment at that time.

Further, *S. aureus* species is found to release surfactant which dismantles the EPS and cells gets dispersed from assemblage, in order to build new biofilm on new surface. We will study effect of antibacterial when surfactant is released, because cells will start detaching from the EPS and at this peak time it will be easy to destroy planktonic cells at low concentration.

We will also study, the effective antibacterial concentration required to eradicate two species when both the species produces EPS. We will optimize the duration and concentration of antibacterial for periodic and continuous mode.

FIGURES

TABLES

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