Monitoring of physiological heterogeneity of microbial populations in environmental biotechnological processes

Saeid Rezaeinejad

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

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A thesis submitted to Nanyang Technological University in fulfilment of
requirement for the degree of Doctor of Philosophy

2012

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Dedicated to my beloved Mum and Dad

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Abstract

Most of the approaches to the studies of microbial populations in environmental engineering systems have presumed that a microbial population comprises cells with the same physiological properties. However, it is known that individual cells within a microbial population have different physiological states. The purpose of this study was to investigate physiological heterogeneity in the eukaryotic population of *Saccharomyces cerevisiae* and prokaryotic population of *Escherichia coli*, and also within microbial community (e.g., microbial granules) found in environmental engineering systems. The first objective was to identify different subpopulations of *S. cerevisiae* and *E. coli* populations using flow cytometry analysis of membrane integrity, membrane potential and respiratory activity. The second objective was to examine sensitivity of different subpopulations of bacterial cells to chlorine disinfectant. The third objective was to analyse spatial distribution of viable and dead cells within bacterial granule of *Pseudomonas veronii*. Fluorescent probes such as propidium iodide (PI), DiBAC₄(3) and CTC in combination with flow cytometry and confocal laser scanning microscope were used for the assessment of physiological states of microbial cells at single-cell level. This study revealed that for the assessment of cell viability of eukaryote *S. cerevisiae*, the most practical method was the determination of membrane integrity using PI. Two subpopulations with high and low activities of alcohol dehydrogenase were detected in the growing population of *S. cerevisiae*. The share of respiratory active cells of *E. coli* population under long-term starvation dropped to 5%, whereas approximately 50% of cells maintained the membrane potential. The results of cell viability determination by PI in combination with flow cytometry indicated that CT values required for 2-log inactivation (CT₉₉) for *E. coli* cell populations with $\mu=0.9\text{ h}^{-1}$ and $\mu=0.2\text{ h}^{-1}$ were 0.06 and 0.09 mg min l⁻¹, respectively. A thin stratum of dead cells (<20 μm) was observed in the outside edge of

granule produced from cells of *P. veronii*, after simultaneous staining with PI and DiBAC₄(3). The cells with highest respiratory activity were detected in some distance from the granule edge, whereas the cells with lowest respiratory activity were found in the core of the granule stained with CTC. The principal conclusion was that microbial population and community are heterogeneous in terms of cell viability and cell energetic states. Physiological states of individual cells partially determine the susceptibility of individual cells to chlorine disinfectant. The study of microbial physiology in environmental biotechnology and engineering could help to improve monitoring and control of environmental biotechnological processes.

Publications

Peer-reviewed journal publications

1. **Rezaeinejad, S. & Ivanov, V.** (2010) “Heterogeneity of *Escherichia coli* population by respiratory activity and membrane potential of cells during growth and long-term starvation”, *Microbiological Research*, 166 (2), 129-135.
2. Ivanov, V., **Rezaei Nejad, S.**, Yi, S., Wang, X.H. (2008) “Physiological heterogeneity of suspended microbial aggregates”, *Water Science and Technology*, 58, 2435-2441.

International conference publications (Oral presentation)

3. **Rezaeinejad, S.** (2010) “Application of green fluorescence protein marker in combination with fluorescence probes for monitoring physiological states of bacterial cells” *YWT Symposium, Singapore International Water Week*, Suntec, Singapore.
4. **Rezaeinejad, S. & Ivanov, V.** (2009) “Assessment of physiological heterogeneity of a population in multi-species microbial community by fluorescence techniques”, *International Conference BIOMICROWORLD*, Lisbon, Portugal.
5. **Rezaeinejad, S. & Ivanov, V.** (2009) “Enumeration of active cells in a bacterial population” *YWT Symposium, Singapore International Water Week*, Suntec, Singapore.

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List of Symbols\Abbreviations

Acrolein	Acrylaldehyde,2-propenal
ADH	Alcohol dehydrogenase
ATP	Adenosine 5'-triphosphate
BD	Becton Dickinson
BOX	Bis-oxonol
CER	CO ₂ evolution rate
CLSM	Confocal laser scanning microscopy
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
CTF	3-cyano-1,5 ditolyl formazan
DiBAC ₄ (3)	Bis-(1,3-dibutylbarbituric acid)-trimethine oxonol
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSMZ	Deutsche Sammlung Mikroorganismen Zellkulturen
E.C	Enzyme classification
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substances
FCM	Flow cytometry
FISH	Fluorescence <i>in situ</i> hybridization
G ₀	Gap0 of mitotic cycle
G ₁	Gap1 of mitotic cycle
G ₂	Gap2 of mitotic cycle
GFP	Green fluorescent protein
LB	Luria-Bertani
Log phase	Logarithmic phase
M	Phase of mitosis in mitotic cycle
MAR	Microautoradiography
mM	Milimolar
μM	Micromolar
MP\ΔΨ	Membrane potential

NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
nM	Nanomolar
OD	Optical density
PI	Propidium iodide
R	Region of flow cytometry dot plot
Rh123	Rhodamine 123
RNA	Ribonucleic acid
S	Phase of mitotic cycle with active DNA synthesis
SOUR	Specific oxygen uptake rate
YMPG	Yeast extract, malt extract, peptone, glucose

CHAPTER 1

INTRODUCTION

1.1 Overview

Microorganisms play a vital role in many environmental engineering processes, for example in the removal of pollutants from wastewater. However, in some cases microorganisms are known to create problems, for example biofouling in membrane systems in the treatment of drinking water. Physiological states of microbial cells constantly change upon their interaction with dynamic changes in biotic and abiotic environments. For example, microorganisms frequently encounter nutrient-excess or nutrient starvation, and shift between these two conditions in the environment.

A microbial population comprises cells with diverse physiological states and metabolic activities. Physiological heterogeneity of microbial population is mainly originated from cell cycle phases, age distribution of individual cells, and micro-environmental conditions (Quiros et al., 2007; Muller et al., 2010). More knowledge in physiology of the microorganisms in population and community levels may help in better exploitation of microorganisms in the removal of pollutants and eradication of the problems posed by microorganisms.

In the recent decades, the assessment of physiological states of individual cells in the single-cell level has been undertaken with advent of techniques such as flow cytometry and fluorescence microscopy. Viability is the most common physiological function, which has attracted the attention of microbial physiologists in the medical, environmental and biotechnological fields. However, microbiologists have not yet reached an agreement on the definition of “viability”. Indicator parameters such as membrane integrity, membrane potential and respiratory activity have been generally used to determine physiological states and

particularly the viability of individual microbial cells in a population and community levels. Various fluorescent probes have been applied to examine these indicator parameters in combination with flow cytometry and confocal laser scanning microscope (Joux and Lebaron, 2000; Muller and Nebe-Von-Caron, 2010).

Microbial physiology is a broad discipline, which includes studies of numerous microbial strains. Two Gram-negative bacterial strains of *Escherichia coli* (*E. coli*) and *Pseudomonas veronii* (*P. veronii*), and yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) were used as model microorganisms in the present study. The reason for choosing these microorganisms was that the *S. cerevisiae* is a eukaryotic model and *E. coli* is a prokaryotic paradigm. *S. cerevisiae* and *E. coli* have been also used to demonstrate key concepts of microbial physiology (Duboc et al., 1996). Therefore, these microorganisms are excellent candidates for study of the physiological heterogeneity in microbial population and community.

The major research approaches used in this thesis draw upon the fields of microbial physiology, environmental microbiology and environmental engineering. In this study, different parameters were used for assessing physiological heterogeneity of microbial populations and communities using the model microorganisms. Several research questions were raised to be answered. First, a comparative study was carried out to investigate what parameters are the most reliable in the evaluation of physiological heterogeneity in growing and starved microbial populations. Furthermore, it was investigated whether the bacterial cell populations in the oligotrophic condition are more heterogeneous in response to disinfectant agent of chlorine and whether there are any adverse effects of chlorine on functional analysis of cells. Finally, the study explored how the viable and the dead cells are spatially distributed within the engineered microbial aggregates of granule.

1.2 Aims and objectives of study

Aims

1. To assess physiological heterogeneity in eukaryotic and prokaryotic microbial populations.
2. To evaluate physiological heterogeneity in microbial granule.

Objectives

1. To identify different subpopulations in the population of *S. cerevisiae* growing as batch culture with regards to physiological parameters of membrane integrity, membrane potential and respiratory activity.
2. To identify different subpopulations in the population of *E. coli* during growth and starvation with regards to membrane potential and respiratory activity.
3. To examine the effects of specific growth rate on inactivation of *E. coli* cells with chlorine.
4. To assess spatial distribution of viable and dead cells within bacterial granule of *P. veronii*.

1.3 The structure of the thesis

This thesis consists of seven chapters. A short introduction is presented in this chapter. Introduction sections are also included in the chapter 3-6, which cover a review of the relevant topics in the respective chapter.

Chapter two provides a detailed review of the relevant literature with a focus on physiological heterogeneity of microbial populations and communities, origin and analysis of the heterogeneity. Functional parameters, analytical methods and

instruments for physiological studies of microbial cell were discussed in this chapter as well.

Chapters three to six were written as stand-alone chapters and partially or entirely have been published or submitted to peer-reviewed journals, these chapters examine the physiological heterogeneities of eukaryotic cells of *S. cerevisiae* population (Chapter three), prokaryotic cells of *E. coli* population (Chapter four), *E. coli* population in responses to disinfectant (Chapter five) and bacterial granule of *P. veronii* (Chapter six).

Chapter three demonstrates the physiological heterogeneity of yeast *S. cerevisiae* population in terms of viability and energetic states. A comparative study between different functional criteria for assessment of cell viability was carried out using various fluorescent probes.

Chapter four describes physiological heterogeneity of bacterial population of *E. coli* in terms of membrane potential and respiratory activity. This chapter further examines the heterogeneity of bacterial population during growth and starvation.

Chapter five explores how differently individual bacterial cells of *E. coli* population respond to disinfectant agent of chlorine based on their physiological states. The chapter examines the effect of specific growth rate of bacterial population on *E. coli* resistance to chlorine and also the effect of chlorine to viability assessment of *E. coli* cells.

Chapter six demonstrates physiological heterogeneity within bacterial granule of *P. veronii*. Spatial distribution of viable and respiratory active cells was discovered in the *P. veronii* granule.

Finally, an overall conclusion and future research directions are presented in Chapter seven.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview

Microbial physiology illustrates how cells feed, grow, reproduce, interact with other biotic and abiotic elements of ecosystem, and how cells survive and die in the ecosystem. It is known that microbial populations and communities are heterogeneous. The heterogeneity within a microbial population can be divided into at least four general categories: genetic differences, biochemical differences, physiological differences, and behavioral differences (Brehm-Stecher and Johnson, 2004). Physiological heterogeneity originates from progression through the cell cycle and describes physiological differences between individual cells of a population (Davey and Kell, 1996; Sumner and Avery, 2002; Quiros et al., 2007; Muller et al., 2010). Physiological heterogeneity is also originated by micro-environmental factors affecting cells located in different strata within a colony or biofilm (Budde and Jakobsen, 2000; Stewart and Franklin, 2008; Muller et al., 2010).

Physiological heterogeneity of population is an important characteristic of biological system. The physiological heterogeneities have important practical consequences for a number of human interests, including industrial and environmental biotechnology. Understanding of the importance of physiological heterogeneity of microbial cell populations requires the development of new tools and techniques. Microbiological studies have conventionally been focused on the population level. Therefore, the knowledge of microbial cell interaction to their environment (abiotic) and with each other (biotic) has been obtained mainly by deduction from population level data. The measurements at population level, which study only the average values of a population, are not capable of determining

physiological states of individual cells. The monitoring of microbial physiology at the single-cell level could illustrate microorganism's activities, and their interactions with other biotic and abiotic elements. The physiological characteristics such as viability and metabolic activity are fundamental states of each individual cell. Methods that are capable of analyzing these features at the level of the individual cell enable a more complete understanding of phenomena that are inaccessible to researchers using population-scale approaches (Brehm-Stecher and Johnson, 2004).

Major studies in microbial aggregates that are exploited in biological treatment of water and wastewater, have been focused on the conventional homogenous populations. However, individual cells in populations and communities may differ extensively from each other by their physiological states. Therefore, studies of the physiological heterogeneity in microbial community of biofilms or granules can give new tools to monitor biological water and wastewater treatments.

2.2 Physiology of cell cycle

It is well known that the physiological heterogeneity of cell population is partially related to cell cycle properties (Munch et al., 1992; Davey and Kell, 1996; Kacmar et al., 2004; Quiros et al., 2007). The cell cycle of both prokaryotes and eukaryotes involve the progression through a series of events, which enable the cell to complete a round of cellular vegetative reproduction known as division. Therefore, vegetative replication incorporates all of the events associated with the preparation, production and release of daughter cells (Wheals, 1987). The mitotic cycle of eukaryotes is divided into five phases G_0 , G_1 , S, G_2 and M (Pringle, 1981; Wheals, 1987). DNA synthesis occurs during S phase, while segregation of chromosomes during mitosis takes place at M phase. Organelles production and normal cell processes such as growth and development take place on two “gap” periods of variable length, which is also so-called G_1 and G_2 (Wheals, 1987). In yeast *Saccharomyces cerevisiae*, bud emergence corresponds to S phase, where DNA is replicated. Higher value of CO_2

evolution rate (CER) and oxygen uptake rate (OUR) occurs during S-phase when buds emerged (Duboc et al., 1996).

Various steps are characterized during the cell cycle progression. These result in significant changes in intracellular and molecular compositions of cell. The biotic environmental and cellular interactions are changing during progression of cell cycle (Munch et al., 1992). The knowledge about the interactions between cell cycle events and external signals is important in regards to understand the growth behavior and physiological states of a cell population.

During active growth, cell division rate is limited by the growth of an individual cell. However, the effect of growth substances on cell cycle mechanism as major extracellular signals is not completely known. There is correlation between specific growth rate and the levels of enzyme activities occurring in intact microbial cells (Van Hoek et al., 1998). There is oscillation in specific growth rate of microbial population during different phases of cell cycle (Duboc et al., 1996). The duration of the G1 phase decreases in the cell with higher specific growth rate (Duboc and Von Stockar, 2000). The specific growth rate of cell population is low during S-phase and high during the G2, M and G1 phases for a constant dilution rate (specific growth rate) (Duboc et al., 1996).

The oscillatory in metabolism of yeasts during the progression of cell cycle is well known (Duboc et al., 1996; Patnaik, 2003; Richard, 2003). There are also known significant changes of energy metabolism and biosynthesis rates during the phases of cell cycle (Richard, 2003; Lloyd and Murray, 2006; Ivanov et al., 2008). The higher catabolic activity of cells during S-phase of cell cycle is likely due to the hydrolysis of carbohydrate storage materials (trehalose, glycogen) of the cells (Munch et al., 1992; Duboc et al., 1996). Hence, progression during cell cycle is caused by the physiological heterogeneity of microbial cell population and also the existence of various cell subpopulations with different oxidative and energetic metabolism in a microbial cell population.

Metabolic oscillations in the levels of enzymatic activities are known in microbial populations (Richard, 2003). The energetic metabolism oscillates during progression through cell cycle (Duboc et al., 1996; Richard, 2003). Oscillation of alcohol dehydrogenase (ADH) was observed during cell cycle phases of *Saccharomyces cerevisiae* (Wittmann et al., 2005). There is a correlation between activity of specific enzyme such as alcohol dehydrogenase (ADH) and the level of energy in the cell. ADH (E.C. 1.1.1.1) is an oxidoreductase enzyme, which plays an important role in regeneration of NAD^+ (Nicotinamide Adenine Dinucleotide) and energy generation in the yeast and bacteria (Guedon et al., 2000). Microbial cell population might be heterogeneous in terms of levels of intracellular energetic states.

The application of fluorescence-based methods to investigate the microbial cell cycle is well established. Flow cytometry technique has been used in the cell cycle analysis and also characterization of various cell subpopulations in different cell cycle phases (Munch et al., 1992; Jayat and Ratinaud, 1993).

2.3 Oscillations of energetic metabolism in cell cycle

Oscillatory phenomena are widespread in biological systems. The oscillation is mainly exhibited in the levels of intracellular parameters of energetic metabolism such as ATP, NADH and trehalose, and also enzymatic activities. Metabolism oscillation is partially correlated with cell cycle phases (Duboc et al., 1996).

Formation of cytosolic NADH in cell is a consequence of glycolytic pathway. The rate of cytosolic NADH formation is dependent on the growth rate and rate of glycolysis. High growth rates and/or glycolytic fluxes are accompanied by high rates of NADH formation (Pahlman et al., 2001). The oscillation, which is not related to cell cycle, is called short period metabolic oscillation. In the short period oscillations, the biological forms of energy are as follows; acetate, pyruvate, ATP, NADH and glycogen. The short period oscillation also appeared in an aerobic

continuous culture of yeast in the absence of glucose, when ethanol was the only carbon source (Richard, 2003).

The question whether the metabolic changes during the cell cycle could be partially regulated at level of enzymatic activity remains unclear. The higher activity of trehalase enzyme (E.C.3.2.1.18), which catalyses the breakdown of trehalose, was observed during the mobilization phase rather than during storage phase in the yeast *Saccharomyces cerevisiae* (Muller et al., 2003). The activity of alcohol dehydrogenase in *S. cerevisiae* was also shown to oscillate during the mobilization phase (Wittmann et al., 2005). Pyruvate kinase activity, which catalyses the breakdown of pyruvate, was relatively low during G1 and S phases of cell cycle and very high during G2 phase of *S. cerevisiae* (Van Doorn et al., 1988).

The oscillatory behavior causes the physiological variations among cells in a population. The physiological heterogeneity of cell population might be originated from physiological variations among the cells due to oscillation.

2.4 Physiological response of cell to starvation

In the natural environment, microorganisms frequently encounter conditions with nutrients surplus, nutrients deficit and rapid shift between these two conditions. Microbial growth in batch culture is characterized by short period of fast growth due to availability of excess nutrients followed by long period of nutritional deprivation. Microorganisms are capable to survive in the absence of nutrients for long period (Conter et al., 2001). In order to survive in the new environmental conditions, microorganisms change their physiology and enter into the so-called “stationary phase” (Kolter et al., 1993). The physiological state of cell in stationary phase changes dramatically under nutrient depletion in terms of viability, metabolic activity, energetic states, size, volume and reproductive ability in comparison to exponential phase. The microbial aggregates under nutritional starvation show different physiological states as well as different types of adhesion and aggregation. The response of microorganisms in stationary phase to environmental stress also

differs from those in the exponential growth phase. Bacterial cells remain viable during the prolonged period of starvation in stationary phase and return to the exponential cell cycle when starvation is relived. Hence, the physiology of starved bacterial cells can be divided into three stages: entry into stationary phase, maintenance of viability, and exit from stationary phase. Bacteria upon facing starvation enter into a developmental program that results in metabolically less active cells (Kolter et al., 1993).

The metabolic activity of starved bacterial cells is greatly reduced. However, starved Gram-negative bacteria remain metabolically active and viable even after many weeks of starvation. In contrast, some of starved Gram-positive bacteria produce the dormant state of spores with sharp dropping of metabolic activity (Kolter et al., 1993). The physiological responses of microorganisms to energy source depletion and a rapid drop in ATP are varied in different phases of growth. Intracellular carbohydrate storage such as trehalose and glycogen also affects starvation tolerance. The cells with higher intracellular trehalose showed a better starvation tolerance (Plourde-Owobi et al., 2000). The availability of intracellular energy source, e.g. ATP, assists microorganisms under anaerobic condition to survive upon facing rapid carbon starvation. In contrast, high level of carbohydrate storage is not required to preserve fermentation capacity during carbon starvation under aerobic condition (Thomsson et al., 2003).

The response of microbial cells growing under anaerobic conditions to starvation is different from the response of aerobically grown cells. The aerobic microorganisms encountered to single nutrient starvation have showed higher tolerance to the carbon starvation than nitrogen starvation. Aerobically, nitrogen starvation causes a significant reduction in catabolic capacity than carbon starvation in yeast *S. cerevisiae* (Thomsson et al., 2003). The lack of tolerance for nitrogen starvation may be resulted from degradation of glucose uptake system in the cells, which are starved to nitrogen in the presence of carbon sources (Jorgensen et al., 2002; Thomsson et al., 2003).

Microbial cells upon facing starvation tend to alter their physiological state and become more resistance to physical and chemical stresses (Kolter et al., 1993). Changes in the cell envelope that are resulted from starvation reflect the need for protection from stressful environments (Baillie and Douglas, 1998). Growth rate is one of physiological changes in microorganisms upon starvation. The declining of growth rate and metabolic activities of microorganisms is known as survival strategies (Konopka, 2000). The level of some cytosolic proteins (Dps, and Fis) fluctuates in growing cells and stationary-phase cells. Fis protein is only abundant in growing cells, whereas Dps is only abundant in non-growing cells (Rockabrand et al., 1999).

Physiological states of microbial cells within the aggregates such as biofilms and microbial flocs of activated sludge also change under nutritional starvation (Steinberger et al., 2002). Cells within biofilms encounter to starvation showed a higher survivability. One of the main differences between planktonic microorganism growing in enriched laboratory media and biofilm in natural environment is the growth rate (Baillie and Douglas, 1998). Starvation of microbial cells is caused the cell surface changes, which enhance aggregation of microbial cells. The additional hydrophobic molecules on the membrane of starved cells increase the adhesion and aggregation in microbial flocs and biofilms (Watanabe et al., 2000).

Assessment of physiological changes of microbial cells during starvation by confocal laser scanning microscopy and flow cytometry techniques in combination with fluorescent probes has been done by several researchers (Lopez-Amoros et al., 1997; Kaprelyants and Kell, 1993; Hewitt et al., 1999). The effects of starvation on physiological parameters of bacterial cells such as; respiratory activity, membrane integrity and membrane potential have been assessed in various environmental conditions (Lopez-Amoros et al., 1997; Lisle et al., 1998; Rezaeinejad and Ivanov, 2010). Physiological heterogeneity was more distinctive in a microbial cell population encountered to starvation than exponentially grown population. Flow cytometric analysis of bacterial cells of *Micrococcus luteus* population starved for

long term of seven months showed a physiological heterogeneous population consisting viable, dormant, and dead cells subpopulations (Kaprelyants et al., 1996).

2.5 Physiological heterogeneity of microbial communities in environmental engineering systems

2.5.1 Physiological heterogeneity in microbial biofilms

Biofilms are the most common form of microbial growth in nature. Microbial biofilms are a cluster of microorganisms that are concentrated at an interface (usually solid-liquid) and typically surrounded by extracellular polymeric substance (EPS) matrix (Hall-Stoodley et al., 2004). In other words, biofilms are formed by the aggregation of microorganisms into multi-cellular structures that adhere to surfaces (Reynolds and Fink, 2001). There are five different stages in formation of biofilms; (i) initial, reversible adhesion; (ii) initial irreversible attachment with production of exopolysaccharides as an indication of the matrix elaboration; (iii) early maturation and acquisition of biofilm structure; (iv) mature biofilm and (v) dispersion of biofilms back to planktonic lifestyle (Ghigo, 2003).

The bacterial cells of microbial aggregates such as biofilms and flocs are in wide range of physiological states. It is known that bacteria growing in the biofilms as surface-associated communities are physiologically distinct from bacteria growing in a free swimming planktonic state (Stocks, 2004; Stewart and Franklin, 2008). Cells with diverse phenotypes within microbial aggregate express distinct metabolic pathways, stress responses and other specific biological activities. The biological activity within a biofilm is heterogeneous due to bioavailability of nutrients and oxygen gradients in different strata of cells. Mature biofilms contain concentration gradients of metabolic substrates and products. The presence of oxygen-concentration gradients from the surface to the core of biofilms cause physiological differences in different cells strata. Spatial physiological heterogeneity in biofilm is determined by using the expression of alkaline phosphatase (APase) as a physiological indicator in correlation with oxygen availability. APase production

was stopped immediately in the absence of oxygen, whereas APase production was resumed when the biofilm was exposed to oxygen (Xu et al., 1998; Drenkard, 2003). The concentration of any nutrient that is consumed in the biofilm will also decrease with increasing depth into the biofilm and distance from the nutrient source. The physiological heterogeneity is also due to the metabolic products concentration within the biofilm. In contrast to oxygen or nutrients, the metabolic product concentrations are higher in the biofilm interior and lower in the biofilm exterior (Stewart and Franklin, 2008).

In recent years, fluorescent physiological indicator stains, coupled with confocal scanning laser microscopy have been used to measure micro-scale physiological heterogeneity in biofilms. Application of LIVE/DEAD BacLight for cell membrane integrity and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) for measurement of respiratory activity of cells within biofilms were reported by several researchers (Wood et al., 1998; Hope and Wilson, 2003). Fluorescence *in situ* hybridization (FISH) has been used to characterize bacterial distribution in the biofilms (Jang et al., 2005; Stewart and Franklin, 2008). Microautoradiography (MAR) combined with FISH is a suitable technique to detect the physiological activities of individual cells within microbial aggregates. In the MAR, radiolabelled substrates are taken up by active cells and incorporated into cellular material. FISH-MAR coupled with isotope arrays could be used to determine the function of individual populations in microbial communities such as biofilm and also to reveal key physiological interactions between different members of these communities (Wagner et al., 2006).

From the environmental engineering perspective, biofilms are useful technology for biodegradation, controlling the rate of carbon and inorganic nutrient cycles and treatment of the wastes. However, biofouling, which is produced by biofilms, is major problem of water industry and main challenging for water membrane technologies (Baker and Dudley, 1999; Le-Clech et al., 2006). Therefore, elucidating bacterial physiology in the microbial aggregates is necessary for our understanding of biotechnological processes and bioreactor design, as well as other processes that are mediated by microorganisms (Stewart and Franklin, 2008).

However, reliable information on the physiological heterogeneity and population dynamic in environmental engineering systems such as biofilms and microbial flocs particularly related to cell cycle physiology is still missing. Study of physiological activities of different microorganism subpopulations within the biofilms, could help to enhance the biodegradation process efficiency. Technological advances for identifying and characterizing the physiological activities of bacteria at cellular level will provide insight into the behaviour of bacterial cells within the complex biofilm environment.

2.5.2 Physiological heterogeneity in microbial granules and microbial flocs

Microbial granules can be regarded as a compact microbial aggregate with a spherical outer shape. In fact, microbial granule is an aggregation of cells to form a fairly stable, contiguous, and multi-cellular association with various physiological states (Liu and Tay, 2004). The aerobic granulation has been applied in biological wastewater treatment (Tay et al., 2001). Microbial flocs are suspended aggregates of microorganisms within extracellular polymeric substances (EPS) matrix that grew in liquid suspension. A higher degree of microbial activity in granules than activated sludge flocs was reported (Jiang et al., 2002).

Microbial flocs have many similarities in terms of characteristics to biofilms, yet are not attached to solid surface. Bacterial cells within the microbial flocs structure are physiologically distinct and express distinct phenotypic traits in comparison with bacterial cells growing in a free swimming planktonic form (Hall-Stoodley et al., 2004). The rate of mass transfer between the medium and the granular matrix for the nutrients and metabolites may not be sufficient to provide normal cell metabolism at some distance from the edge of granule due to the dense aggregation of cells. This dense aggregation is an obstacle to oxygen penetration into the depth of granules (Ivanov et al., 2005). The oxygen gradients through the depth of microbial granules can form a physiologically heterogeneous microbial granule (Stewart and Franklin, 2008).

The microbial flocs and granules are heterogeneous population due to the presence of wide range of organic and inorganic compounds found in wastewater and no single species is capable to utilize all kind of substrates. However, despite such diversity, all microorganisms have common purpose of using catabolism to conserve free energy by distributing it among compounds, which can store and carry the energy, as it required in cell. In spite of same catabolism processes, microbial cells are heterogeneous in terms of level of free energy (Low and Chase, 1999).

It is known that activity of some enzyme such as phosphatase in microbial flocs is correlated with the fraction of intact, viable, and metabolically active cells. The correlation between physiologically active cells in activated sludge flocs and cells with high level of phosphatase was assessed by membrane integrity probes and microautoradiography (Van Ommen Kloeke and Geesey, 1999). The fluorescence *in situ* hybridization (FISH) coupled with MAR were applied to study the *in situ* physiology of microbial flocs in activated sludge (Nielsen et al., 2000).

2.6 Viability of microbial cell

There has not been yet any precise definition for “viability” as the most fundamental physiological state after decades of intensive studies. Viable cells can be generally defined as those cells that are capable of reproduction of similar viable progeny under suitable condition. Basically, the reproduction ability of cells is considered as the benchmark method for determination of viability, and could be determined by the plate count method (Roszak and Colwell, 1987). However, many microorganisms are not cultivated in laboratory media and also viable but non-culturable microorganisms are not counted in the plate count method (Pianetti et al., 2005). Therefore, there is an increasing interest in the development of rapid methods such as application of fluorescent probes for the determination of cell viability.

In the recent decades, criteria such as membrane integrity, maintenance of membrane potential and the presence of metabolic and respiratory activities have been progressively considered for assessment of cellular viability (Joux and Lebaron, 2000; Laflamme et al., 2004; Berney et al., 2007).

Cell with intact membrane is assumed intact cell and damage on membrane results to death of cell. When metabolic activity of cell is not detectable but integrity of essential envelope structures is perceived, it is called inactive cell. Dead cells can be detected until cell lysis, which are called ‘ghost’ cells. The cellular envelope is maintained in the ghost cell, whereas the nucleotide is lost (Joux and Lebaron, 2000). Viability as a physiological state parameter of bacterial cells in water environment has been assessed by molecular methods (Kahlisch et al., 2010).

In order to estimate the system productivity, biomass and substrate uptakes is essential to assess the viability of bacteria involved in engineered biological treatment processes (e.g. wastewater treatment process) (Biggerstaff et al., 2006). Monitoring of viability of yeast cells is an important quality control parameter that used during biotechnological processes to assess product quality (Deere et al., 1998).

2.7 Green fluorescence protein (GFP)

Green fluorescence protein (GFP) is a protein originated from jellyfish *Aequorea victoria*. GFP as marker and reporter genes has been used as tool in environmental monitoring of specific microorganisms (Jansson, 2003). GFP also is useful tool for monitoring of specific bacteria in microbial communities such as biofilms (Rice et al., 2003). GFP in combination with viability stains and flow cytometry has been used for monitoring of physiological state of cell (Lowder et al., 2000) and also quantification of metabolically active fraction of cell population (Jernberg and Jansson, 2002). The combination of GFP and PI as an alternative bacterial viability analysis, were shown better distinction of viable and dead than SYTO9/PI dual staining in bacterial cell population (Lehtinen et al., 2004).

2.8 Parameters of physiological activities of cell

2.8.1 Membrane integrity

The cell membrane is a fluid mosaic of lipids, proteins, and carbohydrates. The plasma membrane is vital for a cell. The membrane separates compartment inside the cell from the surrounding environment and regulates molecules exchange into and out of cell. Therefore, due to multiple functions linked to the plasma membrane (permeability barrier, transport, respiratory activity, etc.), loss of membrane integrity represents significant damage to a cell. The membrane integrity in the microbial cells is measured as an indicator of cell damage or cell death (Joux and Lebaron, 2000).

The assessment of membrane integrity of prokaryotic microbial cells such as bacteria is not easy due to their complex membrane structures. The envelope of Gram-negative and Gram-positive bacteria is different. The envelope of Gram-negative bacteria is comprised of three interacting layers; outer membrane, rigid peptidoglycan layer and the inner membrane (plasma membrane). Outer membrane layer does not exist in Gram-positive bacteria. Capsule, which is a highly hydrated polysaccharide layer outside bacterial cell, is present in some Gram-negative and Gram-positive bacteria (Joux and Lebaron, 2000).

Measurement of plasma membrane integrity is the most common assay for cell viability. This can be assessed either by the ability of a cell to prevent a fluorescent dye from entering it or the ability of a cell to retain a fluorescent dye within it. In the other words, membrane integrity analysis is based on the capacity of cells to exclude fluorescent dye compounds. The probes that are used at low concentrations do not normally cross intact membranes. The loss of cell membrane integrity as measured by uptake of membrane-impairment stain is generally considered irreversible. Plasma membrane becomes permeable to fluorescent dyes present outside of cell (Joux and Lebaron, 2000).

The stains used for the membrane integrity assessment generally label nucleic acids due to high concentrations of nucleic acids within a cell and large fluorescence enhancement exhibited by nucleic acid stains upon binding. DNA and RNA provide large numbers of intracellular binding sites that promote marked fluorescence enhancement of many different stains. Several nucleic acid stains are available with a variety of excitation and emission spectra, which allow cell viability to be assessed in combination with many different fluorescent probes (Roth et al., 1997). The most commonly used nucleic acid dyes are propidium iodide (PI), DAPI, Sytox green etc. Dyes that can penetrate and stain living cells have been described as vital stains (Joux and Lebaron, 2000).

LIVE/DEAD BacLight kit contains two nucleic acid stains of SYTO9 and PI, which has been widely used for detecting of dead and intact microbial cells simultaneously. The probes differ in their spectral characteristics and their ability to penetrate intact bacterial membranes (Boulos et al., 1999; Breeuwer and Abee, 2000; Berney et al., 2007). SYTO9 penetrates cells with both intact and damaged membranes, whereas PI only penetrates to cells with damaged membranes. When the dyes used in combination, cells with intact membrane show green fluorescence while cells with damaged membrane show red fluorescence. The emission properties of the stain mixture bound to DNA change due to displacement of one stain by the other and quenching by fluorescence resonance energy transfer (Stocks, 2004; Berney et al., 2007). In order to produce good results by using LIVE/DEAD kit, PI must be in sufficient excess to stain all nucleic acid present, whereas SYTO9 concentration can be decreased to attenuate the green emission and does not need to be in excess (Stocks, 2004). LIVE/DEAD BacLight kit has been applied not only for the differentiation of active and dead bacterial cells, but also for viability quantification of yeast *Saccharomyces cerevisiae* (Zhang and Fang, 2004). It is presumed by manufacturer, SYTO9 penetrates intact membrane of a large number of Gram negative and Gram positive bacteria (Joux and Lebaron, 2000).

The criterion of membrane integrity as a reliable indicator of viability is controversial matter. Since, it is reported that a significant percentage of heat-killed

E. coli cells was not stained by PI. One of the limitations of PI is low extinction coefficient and consequently relatively low fluorescence of this probe (Joux and Lebaron, 2000). LIVE/DEAD kit may not be ideally suited for testing of some environmental samples, because they adhere to substrate material as well as target molecule, resulting in increased non-specific binding and background fluorescence. The alternative combination of nucleic acid dyes, i.e. SYTO 13 and SYTOX Orange has showed less background fluorescence and non-specific binding than the commercial LIVE/DEAD BacLight kit upon testing environmental samples such as activated sludge (Biggerstaff et al., 2006). The other alternative dye to PI is SYTOX Green, which is highly fluorescent by binding to DNA. SYTOX Green is a high-affinity nucleic acid stain that does not penetrate through the membranes of intact viable cells yet easily cross cells with the impairment plasma membranes (Lebaron et al., 1998; Saint-Ruf et al., 2010). It is reported that SYTOX Green underestimate the fraction of dead cells in starved bacterial populations and overestimate the fraction of dead cells in antibiotic treated bacteria (Breeuwer and Abee, 2000).

Membrane integrity dyes are one of the most common approaches for assessing physiological states of biofilms. DNA fluorescent probes commonly have been used for determining of spatial distribution of viable and dead cells in biofilms and biogranules (Stewart and Franklin, 2008).

2.8.2 Membrane potential ($\Delta\Psi$)

Membrane potential ($\Delta\Psi$) is generated and maintained by concentration gradients of ions such as sodium, potassium, chloride, and hydrogen. The electrochemical potential occurring through the plasma membrane of metabolizing cell is generated by respiration or by ATP hydrolysis and extrusion of H^+ ions by H^+ ATPase or the electron transfer chain. In the other words, concentration gradients of H^+ , Na^+ , K^+ , Cl^- ions, which exist across the cell membrane, is caused electrical potential differences in most of prokaryotic and eukaryotic membranes cell. Membrane potential results the selective permeability of biological membranes to a variety of cations and anions due to differences of electric potential across the membrane.

Inside of cell in comparison to the outside of the cell is negatively charged (Padan and Schuldiner, 1987).

Membrane potential (MP) plays a critical role in microbial physiology. Membrane potential plays a central role in different cell life processes (ATP synthesis, active transport, mobility, regulation of intracellular pH, and etc.) (Joux and Lebaron, 2000). Circulation of proton across the plasma membrane plays a primary role in energy transduction of the prokaryotic cell. MP is involved in the generation of ATP as currency of cell energy (Padan and Schuldiner, 1987). MP is good indicator of physiological heterogeneity of cell population studies. The fluorescence probes for measurement of MP are appropriate to study physiological heterogeneity of cell populations.

In bacteria cells, which are lack of mitochondria, MP reflects both the state of energy metabolism and physical integrity of cytoplasmic membrane (Shapiro, 2000). In eukaryotic cells, mitochondrial $\Delta\Psi$ is reduced when energy metabolism is disrupted notably. Cytoplasmic $\Delta\Psi$ is also reduced to zero when the membrane is ruptured by chemical or physical agents (Joux and Lebaron, 2000; Shapiro, 2000). Cell with damaged membrane unable to maintain or generate electrochemical gradient and consequently cannot produce membrane potential, thus can be categorized as dead (Nebe-Von-Caron et al., 2000).

Indirect estimation of $\Delta\Psi$ can be obtained by determining distribution of lipophilic ionic molecules between cells and suspending medium according to Nernst equation (Shapiro, 1990; Brauer et al., 2008). Voltage-sensitive dyes have been developed to measure MP in cell. Membrane potential dye depending on their charge either is accumulated in polarised (cationic dyes) or depolarised (anionic dyes) cells. Fluorescent probes applied in microbiology are either positively charged such as; rhodamine 123(Rh123) and the carbocyanines such as 3, 3-dihexyloxacarbocyanine iodide (DiOC₆(3)), 3,3-dithyloxacarbocyanine iodide (DiOC₂(3)), and 3,3'-di-propylthiadicarbocyanine iodide (DiSC₃(5) or negatively charged such as; bis-(1,3-dibutylbarbituric acid)-trimethine oxonol (DiBAC₄(3)), DiOC₆(3) and DiBAC₄(3).

Viable and active cells accumulate the cationic rhodamine 123 and cyanines dyes due to negative charge formation inside cell (Breeuwer and Abee, 2000).

Rhodamine 123 (Rh123) is a lipophilic, cationic dye commonly used to detect membrane potential (MP). However, staining with Rh123 often requires a pre-treatment step of cells, generally performed by adding EDTA, to permeabilise the outer membrane of Gram-negative bacteria. The pre-treatment conditions may vary depending on the environment condition (e.g., salinity) and between species. An additional problem is that Rh123 staining requires several cell washing steps, which are time consuming and may result in cell lost (Diaper et al., 1992). The membrane potential-dependent fluorescent dye Rh123 for mitochondrial staining is associated to level of cell energy metabolism. Amount of dye taken up is also directly related to the level of energy metabolism in a cell (Williams et al., 1999; Shapiro, 2003).

The cationic carbocyanine dyes (e.g., 3,3'-dihexyloxacarbocyanine; DiOC₆(3)) have also been used to estimate membrane potential of cell. These dyes do not require permeabilization and washing steps. However, nonspecific binding of carbocyanine dyes to hydrophobic regions of cell and quenching of the fluorescence of intracellular dye have been reported. Therefore, careful calibration of the staining procedure is required to avoid false positive signals (Diaper et al., 1992). DiOC₂(3) is a cationic carbocyanine dye, which is distributed across cellular membranes according to their charge (Shapiro, 2000; Andersen et al., 2007). Oscillations in mitochondrial membrane potential in yeast *S. cerevisiae* cell was reported by using DiOC₂(3) (Andersen et al., 2007).

Membrane potential can also be determined by the anionic lipophilic oxonol probes. Oxonol dyes are negatively charged and bind to the cytoplasmic membrane but do not accumulate in intact cells. Accumulation of Oxonol dyes inside bacterial cells is facilitated by a reduction in the magnitude of the MP. Bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC₄(3)] (BOX) has been reported to be useful to detect depolarised cells of numerous Gram-positive and Gram-negative bacterial species. In some protocols, Oxonol dyes combined with a pre-treatment of EDTA to

increase membrane permeability (Joux and Lebaron, 2000). Without treatment of cell for permeabilisation, the uptake of Oxonols might be more related to membrane integrity rather than membrane depolarisation (Nebe-von Caron et al., 1998). The simultaneous detection of depolarized and total cell population by combining [DiBAC4(3)] (BOX) with the nucleic acid dye SYTO17 has been suggested (Comas and Vives-Rego, 1997).

Applications of MP dyes to environmental samples are scarce and inconclusive, probably because of the effects of the permeabilisation step, which vary depending on species and also the interaction between charged dyes and chemical compounds present in the sample (Joux and Lebaron, 2000). Membrane potential dyes also have received little applications at the community level, because various pre-treatment steps are required for them.

2.8.3 Respiration activity

The respiratory activity, which takes place across the intact membrane of viable cell, is a metabolic reaction to get biochemical energy from fuel molecules such as carbon, and releasing of waste products. Oxidation-reduction (redox) reactions of cell results the releasing energy, which is conserved in the production of energy rich storage compound, such as ATP. In physiological studies the determination of metabolically active microbial cells within microbial populations and communities is an effective means for estimation of the biotechnological system productivity and also fully exploitation of microbial cells activities within the biotechnological processes.

Fluorescent probes for detection of respiratory activity in microbial cells have been developed based on the use of different tetrazolium salts. Tetrazolium salts are often used as indicators of microbial respiratory activity, as well as viability, particularly in physiological and ecological studies (Smith and McFeters, 1997). Fluorescent tetrazolium dyes are substituted electron acceptor of O₂ in redox reaction of cells. The dyes are reduced within the respiratory chain from a colorless complex to a

brightly colored intracellular formazan in active respiring microbial cells. Since electron transport is directly related to cellular energy metabolism in respiring cells, the reduction of tetrazolium compounds can be considered as indicator of cell activity. In other words, fluorescent tetrazolium dyes can act as an electron acceptor, and consequently respiring cells reduce tetrazolium dyes to their respective formazan products. 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) reduces to water-insoluble, red fluorescent of 3-cyano-1,5 ditolyl formazan (CTF), which enormously enhances the sensitivity of detection (Breeuwer and Abee, 2000; Joux and Lebaron, 2000).

The metabolic activity of microbial cell could be quantified under both aerobic and anaerobic conditions by CTC. Formazan deposition and consequently fluorescent signal or number of respiring cells may be enhanced by addition of substrate such as succinate, glucose, and intermediate electron carriers such as phenazine methasulfate during incubation with CTC (Thom et al., 1993). Various factors may effect on CTC reduction in a cell during incubation. tetrazolium reduction may be reduced by extracellular inorganic phosphate, at the concentration above 10 mM (Smith and McFeters, 1996). Depending on the lipophilicity, some formazan products can diffuse out of cell form extracellular deposits. This phenomenon could be suppressed by addition of cobalt ions, which form a complex with the formazan (Thom et al., 1993).

Toxic effects of CTC and its reduced components on bacterial metabolic activity in some cases have been reported. CTC reduction in cells might be inhibited due to its toxicity. The inhibition may cause underestimation of the viable and active cells fraction within a microbial population (Ullrich et al., 1996). The reduction of CTC also is inhibited by cyanide and other electron transport inhibitors (Rodriguez et al., 1992).

Detection of CTC in comparison to other tetrazolium salt dyes (e.g., INT) is simple technique due to red fluorescence of the reduced form (Ullrich et al., 1996). In the eukaryotic cells the mechanisms of tetrazolium (CTC) reduction to formazan is

primarily due to CTC reduction by membrane-bound NAD (P) H-dehydrogenase (NADH-D), and possibly superoxide anion in cells. In the prokaryotic cells, CTC is reduced prior to ubiquinone in the aerobic condition in the respiratory chain by succinate and NAD(P)H dehydrogenases (Smith and McFeters, 1997). In prokaryotic cells, information obtained using CTC to assess respiratory activity were varied in different assay condition and various microorganisms. For instance, it appears some metabolically active bacteria are not able to reduce CTC under certain conditions. This phenomenon could be related to low tetrazolium salt substantively (associated with cell components permeability), unique respiratory pathways (e.g. those bacteria which can respire, but do not contain a complex Kreb's cycle), or low cellular $\Delta\mu_{\text{H}^+}$ in some bacteria (Smith and McFeters, 1997).

Study of different physiological states related to the growth phases of bacterial culture were shown that during the lag and exponential phases, there was strong correlation between number of CTC-positive cells and total number of bacteria, as well as with the amount of CO₂ released. On the contrary, during the stationary phase due to a sharp decrease of cells respiratory activity, the number of CTC-positive cells also decreased, but not the same extent as respiratory activity reduction. Since, bacteria in stationary phase, physiologically can maintain enough energy to reduce CTC even when respiratory activity is decreased to a low level. However, in response to long-term stationary phase conditions, energy levels will decrease progressively in cells, which will finally result losing of their ability to reduce CTC to fluorescent formazan (Creach et al., 2003).

Application of CTC for direct microscopic visualization of actively respiring bacteria in environmental samples has been reported (Rodriguez et al., 1992; Biggerstaff et al., 2006). CTC has been applied widely for assessment of physiological activities of biofilms due to insolubility of reduced form of CTC (Yu and McFeters, 1994; Stewart and Franklin, 2008).

2.9 Analytical instruments for physiological assessment of cell based on fluorescence methods

The main advantages of rapid and direct methods based on fluorescence techniques are lack of incubation, high sensitivity (i.e., the number of molecules needed for detection is low), a high time resolution (approx. 10^{-8} s), and potential for analyses of individual cells in combination with flow cytometry (FCM) or image analysis such as confocal laser scanning microscopy (CLSM) (Breeuwer and Abee, 2000; Joux and Lebaron, 2000). Fluorescence technique based methods enable to detect physiological functions and integrity of membrane of microbial cells using fluorescent probes.

2.9.1 Flow cytometry (FCM)

Flow cytometry is a technique, which measures the physical and chemical characteristics of cells. The flow cytometry analysis of individual cells provides more useful information than traditional biochemical assays of entire population. Flow cytometry is useful technique to quantify particle-associated fluorescence. Flow cytometry could be applied to measure specific fluorescent stains in a microbial population. The characterization of single cells in population, in terms of distributions in their cellular properties such as protein content, DNA content, viability, enzyme activities could be done by FCM (Davey and Winson, 2003; Muller and Nebe-Von-Caron, 2010).

The characterization of physiological heterogeneity of a population requires growth-independent means of differentiation at single-cell level. Thus, techniques such as FCM provide a tool for microbial heterogeneity analysis (Davey and Winson, 2003; Czechowska et al., 2008; Muller and Nebe-Von-Caron, 2010). FCM as a tool has been used for understanding of cell population heterogeneity in response to environmental stresses (Kaprelyants et al., 1996; Czechowska et al.,

2008). Cell cycle analysis of microbial cells by FCM was performed by several researchers (Alberghina, 1991; Munch et al., 1992).

Determination of viability as one of the most fundamental properties of a cell is the common application of FCM. DNA is the most commonly stained cellular constituent in FCM analysis. The popularity of nucleic acid stains of PI and SYTO9 in combination with FCM for determination of viability have been increasing among the researchers in various fields (Zhang and Fang, 2004; Berney et al., 2007; Johnson et al., 2009). FCM is suitable technique to determine cell respiratory activity using redox dye of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Sieracki et al., 1999). FCM is also appropriate method for determination of membrane potential using stains such as anionic Oxonol dye of DiBAC₄ and cationic carbocyanine dye of DiOC2(3) (Novo et al., 2000).

Multi-parameter flow cytometric analysis for assessment of cell physiology has been applied in several studies. The combination of membrane potential, respiratory activity and membrane integrity for determination of viability and physiological state of cells by FCM provides much accurate and precise information (Hewitt and Nebe-Von-Caron, 2001a).

2.9.2 Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) is a technique to obtain optical images with high resolution. The main feature of CLSM is its ability to produce in focus images of thick specimens. In CLSM, a coherent light beam shine onto a specimen and in-focus light detected via a pinhole. A clear point image can be generated by CLSM, since all out of focus data are eliminated by the pinhole. CLSM permits to investigate of optical sections of relatively thick (> 10 µm) specimens (Furrer et al., 1997). CLSM is useful technique for visualization of microbial specimens such as biofilm and biogranule (Sanford et al., 1996). CLSM also has been used to quantify the viability of yeast *Saccharomyces cerevisiae* using LIVE/DEAD Backlight kit (Zhang and Fang, 2004). CLSM in combination with membrane potential and

respiratory activity fluorescence dyes were used for assessment of bacterial viability (Lopez-Amoros et al., 1997; Andersen et al., 2007; Kim et al., 2008). The CLSM technique in combination with membrane potential dyes has been used in physiological study of bacterial cells of biofilms (Wittmann et al., 2005).

2.9.3 Fluorescence spectrometry

Fluorescence spectrometry is a technique, which enable to measure the fluorescent intensity emitted by a specimen suspension when exposed to monochromatic light. The key parameters of fluorescence measured are intensity and wavelength of emission spectrum after excitation by a certain spectrum of light. These parameters are used to identify presence and amount of specific molecules in a cell. Fluorescence spectrometry is useful technique to characterize the environmental samples such as bacterial biofilms and microbial flocs (Wuertz et al., 2000).

2.10 Summary of literature review

1. Individual cells within a microbial population differ widely from each other in terms of their physiology and metabolic activity.
2. Physiological heterogeneity of cell population is partially originated from cell cycle phases, age distributions and micro-environmental conditions.
3. The energetic metabolism oscillation is exhibited as variations of ATP, NADH and trehalose, and also enzyme activities. Metabolic oscillation is partially correlated to cell cycle phases.
4. The oscillatory behaviour causes physiological variations among cells in terms of various oxidative and energetic capacities. Physiological heterogeneity of cell population might be originated from physiological variations among cells due to oscillation.

5. The physiological state of microbial cells changes significantly upon facing starvation.
6. Microbial cells within aggregates under nutritional starvation exhibit different physiological states.
7. Monitoring of physiological states and heterogeneity of microbial cell population is important in order to improve the efficiency of environmental biotechnological processes using biofilms, microbial granules and flocs.
8. The major cellular parameters for determination of physiological states of microbial cells are membrane integrity, membrane potential and respiratory activity.
9. Fluorescence probes in combination with flow cytometry, confocal laser scanning microscope and fluorescence spectrometry are useful techniques to characterize the physiological states of individual cells in a cell population as well as bacterial aggregates such as biofilms and microbial flocs.

CHAPTER 3

HETEROGENEITY OF *SACCHAROMYCES CEREVISIAE* POPULATION: VIABILITY AND ENERGETIC METABOLISM

3.1 Introduction

Individual microbial cells in a population are physiologically diverse due to the occurrence of cell cycle events or surrounding by inhomogeneous environment (Duboc et al., 1996; Kacmar et al., 2004; Quiros et al., 2007; Ivanov, 2010; Rezaeinejad and Ivanov, 2010). In a cell population, based on physiological classification, two major subpopulations could exist: 1) viable and non-viable cells, and 2) metabolic active and non-active cells. The efficiency of a biotechnological system mainly depends on the share of viable and metabolically active cells. Therefore, finding a proper method for assessment of viability, energetic states and metabolic activity of microorganisms could be invaluable in order to improve the biotechnological processes.

The yeast *Saccharomyces cerevisiae* has been commonly used as a research model microorganism to demonstrate key concepts of physiological studies. Determination of cell viability and metabolic activity of *S. cerevisiae* is important to control and optimize production of alcohol and other biotechnological products. *S. cerevisiae* was used for removal of heavy metals from effluent of wastewater (Machado et al., 2008). *S. cerevisiae* genotoxicity and cytotoxicity assays were appeared to be a reliable and sensitive tool for monitoring the toxic effects of industrial wastewater effluents (Schmitt et al., 2005).

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In terms of energetic states microbial cell populations are heterogeneous (Duboc et al., 1996). It is well known that microbial cells with high alcohol dehydrogenase activity are selectively killed by treatment with an appropriate alcohol such as allyl alcohol. Allyl alcohol (2-propen-1-ol) is oxidized by Alcohol dehydrogenase (ADH) to acrolein (acrylaldehyde, 2-propenal), a toxic aldehyde that binds to nucleic acids and kills the cell, whereas allyl alcohol itself is harmless to the cell. Cells with high energetic activity and high NAD^+ to NADH ratio upon exposing to allyl alcohol die due to production of toxic aldehyde of acrolein. Therefore, resistance of yeast to allyl alcohol reflects the activity of ADH and level of energy metabolism of cell (Wills, 1976; Lee et al., 2006).

Flow cytometry has been used as a proper technique for analysis of physiological characteristics such as viability and energetic states in *S. cerevisiae* population at single-cell level (Enfors et al., 2001; Boyd et al., 2003; Garcia et al., 2006). The major parameters for measurement of cell viability are membrane integrity, membrane potential, and respiratory activity (Diaz et al., 2010).

Several fluorescent probes and techniques have been developed for measurement of cellular parameters. The membrane integrity of cell is one of the key indicators of cell viability. Membrane integrity could be detected by dye-exclusion or dye-uptake. Viable cell with intact membrane is impermeable to fluorescent nucleic acid binding dyes such as propidium iodide (PI) due to its charge and structure (Shapiro, 2003). PI penetrates into cell with the damaged membrane and intercalates into double-stranded nucleic acid. PI in combination with SYTO9 is generally used for distinction of live and dead cells in prokaryotic as well as eukaryotic cells (Zhang and Fang, 2004; Berney et al., 2007; Ivanov et al., 2008; Tibayrenc et al., 2010).

FUN-1 in combination with Calcofluor White M2R (CFW) dye is exclusively used for detection of yeast cells viability (Cot et al., 2007; Essary and Marshall, 2009). Formation of cylindrical intravascular structures (CIVS) only in metabolically active yeast cells, which stained by FUN-1 dye, is deemed to distinct the viable cells from the dead cells (Millard et al., 1997; Cao-Hoang et al., 2008). The

formation of CIVS requires not only the membrane integrity but also metabolic activity of viable yeast cell (Henry-Stanley et al., 2004; Citiulo et al., 2009).

Membrane potential ($\Delta\Psi$) is also an important parameter for evaluation of cell viability. Membrane potential ($\Delta\Psi$) indicates membrane integrity as cell viability indicator and reflects rate of energy metabolism as cell activity indicator (Shapiro, 2000; Boyd et al., 2003; Rezaeinejad and Ivanov, 2010). Negatively charged oxonol dye of DiBAC₄(3) tend to be accumulated in the intracellular spaces of depolarized cells with interior-positive membrane potentials as well as dead cells with $\Delta\Psi=0$ and binds to the intracellular lipid-containing elements of cytoplasmic membrane (Breeuwer and Abee, 2000). Basically, yeast cell without an intact membrane is not able to generate membrane potential. In the eukaryotic cells of *S. cerevisiae*, DiBAC₄(3) enters cells against the electrochemical gradient across the cytoplasmic membrane (Shapiro, 2003).

In the physiological studies of microbial cells, the fluorescent redox probe of tetrazolium salts are commonly used as indicators of respiratory activity as well as cell viability (Hatzinger et al., 2003; Rezaeinejad and Ivanov, 2010). Fluorescent tetrazolium dye of 5-cyano-2,3-ditoly tetrazolium chloride (CTC) reduces within the electron transport system of respiratory chain and forms red fluorescent formazan product of “CTF” (Smith and McFeters, 1997; Sieracki et al., 1999; Breeuwer and Abee, 2004). Therefore, CTC reduction in a cell is considered as an indicator of cell respiration activity as well as cell viability (Creach et al., 2003; Stewart and Franklin, 2008).

The abovementioned physiological probes have been used widely to assess viability of *S. cerevisiae* cells. In this study, various dyes were used to determine viability of cells *S. cerevisiae* by their respiratory activity, membrane potential or membrane integrity.

The aim of this chapter is to determine the different cell subpopulations in terms of energetic states in a population of *S. cerevisiae* and also the most efficient criterion for assessment of eukaryotic cell viability.

3.2 Materials and Methods

3.2.1 Microorganism and its cultivation

Strain of *Saccharomyces cerevisiae* (DSM 70449) was obtained from German Collection of Microorganisms and Cell Cultures (DSMZ). Cells were grown aerobically in the 100 ml flasks (Schott Duran, Germany) in the shaker incubator at 30°C and 200 rpm in a medium that contains 3 g of yeast extract, 3 g of malt extract, 5 g of peptone from soybeans, 10 g of glucose per 1 liter of distilled water (YMPG broth) (Difco™, Becton Dickinson, Sparks, MD, USA).

3.2.2 Sampling

Cells were washed twice with sterile phosphate buffered saline (PBS) (pH 7.2) by centrifugation for 10 min at 3398×g. The pellets were re-suspended in PBS. Concentration of cell suspension was adjusted to approximately 1×10^8 cells ml⁻¹ based on optical density of suspension at 600 nm.

The yeast cells were killed using 20% (v/v) allyl alcohol CH₂CHCH₂OH (Sigma-Aldrich) for 30 min incubation and used as control sample. The yeast cells were treated by addition of 1% (v/v) allyl alcohol, then incubated for 10 min at 30°C and used as test sample. After the incubation period, the cell suspensions were washed twice with PBS by centrifugation for 10 min at 4000 rpm (3398×g). Cells suspensions were re-suspended in 1 ml of PBS.

3.2.3 Staining protocols

LIVE/DEAD BacLight™ viability kit

LIVE/DEAD BacLight™ viability kit (Table 1) contains SYTO9, 3.34 mM, and propidium iodide (PI), 20 mM was obtained from Molecular Probes (Invitrogen, Eugene, Oreg.). The cell suspension were transferred to microcentrifuge tubes and stained with 1 µl of stock solution of dye mixture (one times of the SYTO9 (3.34 mM) and six times of the PI (20 mM)). The stained cell suspension after vortexing was incubated at 30°C in dark for 15 min. The suspension was washed twice in PBS and the pellet was re-suspended in PBS (Zhang and Fang, 2004; Rezaeinejad and Ivanov, 2010).

LIVE/DEAD Yeast viability (Fungolight) kit

LIVE/DEAD Yeast viability kit (Table 1) contains FUN-1 (Molecular Probes, Eugene, Oreg.), 10 mM solution dimethylsulfoxide (DMSO) and Calcofluor White M2R, 5 mM solution in water. For staining, the final concentration of FUN-1 and Calcofluor White M2R stains were 20 µM and 25 µM, respectively. The suspension was mixed thoroughly with stains and incubated at 30°C in dark for 30 min under shaking. The cell suspensions were washed twice with PBS and the pellet was re-suspended in PBS (Millard et al., 1997).

Staining of cells with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC)

The chemical properties of CTC are given in Table 1. An aliquot of 100 µl of the stock solution (50 mM) of CTC (Polyscience, Inc., Warrington, PA, USA) was added to a test tube containing 900 µl of sample and incubated for 1 h at 30°C in dark with agitation (200 rpm). Final concentration of CTC was 5 mM. The cell suspensions were washed twice with PBS and the pellet was re-suspended in PBS (Ullrich et al., 1996; Rezaeinejad and Ivanov, 2010).

Staining of cells with bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC₄)(3)

The chemical properties of DiBAC₄(3) are given in Table 1. Cells were stained with DiBAC₄(3) (Molecular Probes, Invitrogen, Eugene, Oregon, USA) at final concentration 1 μ M. The stock solution of DiBAC₄(3) in dimethyl sulfoxide (DMSO) at concentration of 250 μ M was stored at -20°C. An aliquot of 4 μ l of the stock solution was added to the test tube containing 996 μ l of cell suspension and incubated at 30°C in dark for 20 min subsequently. The pellet washed twice by centrifugation at 3398 $\times$ g for 5 min and re-suspended in 1 ml of PBS and analyzed by flow cytometry immediately (Boyd et al., 2003; Rezaeinejad and Ivanov, 2010).

3.2.4 Flow cytometry (FCM) data acquisition and analysis

Flow cytometry analysis was carried out using Becton Dickinson (BD) FACSCaliburTM flow cytometer with CELLQuestTM software for data acquisition. A total of 50,000 cells were counted, based on 488 nm excitation by 15 mW air cooled blue argon-ion laser. Fluorescence was measured using a band pass filter 530 $\pm$ 15 nm for green fluorescence of DiBAC₄(3) and SYTO9, and a band pass filter 620 $\pm$ 10 nm for red fluorescence of CTC and PI. Sheath flow rate was 10 ml min⁻¹ and sample analysis rate never exceeded 1000 events s⁻¹. Analysis of data was carried out using FlowJo V.7.2.5 software (www.flowjo.com).

Table 3.1 Fluorescent probes used for the measurement of yeast cell viability

Fluorochrome Measureable		Cellular parameter	Mode of action	Excitation Wave length (nm)	Emission Wave length (nm)
LIVE/DEAD BacLight™ viability kit	PI	Membrane integrity indicator and nucleic acid stain	Exclusion of PI and staining with SYTO9 in viable cell	488	623
	SYTO9	Nucleic acid stain		488	530
LIVE/DEAD Yeast viability kit	FUN-1	Metabolic activity	CIVS formation in metabolically active cells	488	620
	Calcofluor White			325	460
bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC ₄)(3)		Cytoplasmic membrane potential	Uptake by dead cells	488	525
5-cyano-2,3-ditolyl tetrazolium chloride(CTC)		Respiratory activity	Reduced to red fluorescent formazan product of “CTF” in respiratory active cells	488	630

3.2.5 Confocal laser scanning microscopy (CLSM) and image analysis

Stained cells were viewed using a Nikon A1R confocal laser scanning microscope (CLSM) (Nikon, Tokyo, Japan) with a 100× objective lens. Green and red fluorescences were excited by a 40 mW argon laser at 488 nm. Fluorescent light was separated with 570 nm splitting filter and detected in the channel with band pass filter 525/575 nm and in the channel with a band pass filter 595/645 nm.

3.2.6 Respirometry

Cells of *Saccharomyces cerevisiae* were grown in 100 ml volume DURAN[®] cylindrical culture bottles (Schott, NY, USA) in a shaker incubator at 30°C and 200 rpm, overnight. The oxygen uptake rate of samples was measured by dual gas (O₂/CO₂) respirometer (Columbus Oxymax ER, OH, USA). In order to measure the effects of different concentrations of allyl alcohol on respiratory activity of cells over the time, 0.05%, 0.1%, 0.2%, 0.3%, 0.5%, and 1% (v/v) of allyl alcohol were added to the sample. The bottles connected to the respirometer were kept on the shaker-water-bath incubator at 30°C. Samples were continuously aerated with air flow of 500 ml/min.

3.3 Results

3.3.1 Assessment of cell viability based on membrane integrity

Fig. 3.1 shows flow cytometry dot plots from the analysis of *S. cerevisiae* cells growing in batch culture and stained with PI and SYTO9 dyes. R1 is the subpopulation, which was stained with PI as a fraction of dead cells of *S. cerevisiae* population and R2 is the subpopulation stained only with SYTO9 as a fraction of live cells of population. The fraction of dead cells increased from 2% ± 1% in exponential phase to 40% ± 5 in the stationary phase after 3 days of batch culture (Table 3.2).

The sample of *S. cerevisiae*, which was stained with SYTO9 and PI, were tested using CLSM in order to determine the validity of flow cytometry data. Since, viable and non-viable cells emitted different fluorescents due to staining by LIVE/DEAD BacLight kit, therefore, the cells were differentiated visually to viable (green color) and dead (yellow color) in the depicted images by CLSM. The panel C of Fig 3.2 clearly shows the viable cells (in green) and dead cells (in yellow color).

Fig. 3.1 Viability analysis of *S. cerevisiae* cells in batch culture using LIVE/DEAD BacLight™ viability kit. Subpopulations of dead cells (region R1) and live cells (region R2): time of sampling (hours); a, 85; b, 36; c, 15; d control (dead cells). Red and green fluorescence are due to staining by PI and SYTO9, respectively. SYTO9 was measured in fluorescence channel FL1 and PI in FL3.

Fig. 3.2 Cells of *S. cerevisiae* strain stained with Live/Dead *BacLight* observed by CLSM; A, dead cells treated with 20% (v/v) of allyl alcohol for 30 min; B, sample of intact cells; C, sample treated with 1% (v/v) allyl alcohol for 10 min. The dead cells are differentiated by yellow color due to staining by PI and SYTO9 in panel C, whereas the live cells are green color due to staining by SYTO9 fluorescent dye.

3.3.2 Assessment of cell viability based on membrane potential

S. cerevisiae cells were stained with DiBAC₄(3) and analyzed by flow cytometry.

Fig. 3.3 Viability analysis of *S. cerevisiae* cells in batch culture by membrane potential Oxonal dye of DiBAC₄(3). Subpopulations of $\Delta\Psi^-$ cells (Q 1) and $\Delta\Psi^+$ cells (Q2) in batch culture of *S. cerevisiae*: time of sampling (hours); a, 85; b, 36; c, 15; d control (dead cells). Green fluorescence is due accumulation of negatively charged DiBAC₄(3) in the depolarized cells. DiBAC₄(3) was measured in fluorescence channel FL1.

Fig. 3.3 shows flow cytometry dot plots from the analysis of *S. cerevisiae* cells growing in batch culture stained with Oxonol dye of DiBAC₄(3). Q2 is the subpopulation of cells stained by green fluorescence dye of DiBAC₄(3) as a fraction of membrane-depolarized cells of *S. cerevisiae*. The fraction of dead and/or depolarized cells increased from 4% $\pm$ 1 in the exponential phase to 36% $\pm$ 3 in stationary phase after 3 days of batch culture (Table 3.2).

3.3.3 Correlation between different cell viability parameters

The correlation coefficient between the fraction of dead cells stained with PI and the fraction of depolarized cells stained with DiBAC₄(3) was 0.91 for the samples taken in different times of batch culture (Fig. 3.4).

Fig. 3.4 A correlation between the percentage of dead cells stained with PI and the percentage of membrane depolarized cells stained with DiBAC₄(3), which were sampled during batch culture of *S. cerevisiae* and analyzed by flow cytometry.

3.3.4 Assessment of cell viability based on respiratory activity

Fig. 3.5 Viability analysis of *S. cerevisiae* cells in batch culture by tetrazolium chloride (CTC) dye. Subpopulations of CTC⁺ cells (region R2) and CTC⁻ cells (region R1) in batch culture *S. cerevisiae*: time of sampling (hours); a, 64; b, 36; c, 15; d control (dead cells). Red fluorescence is due to CTC reduction. CTC was measured in fluorescence channel FL3.

Fig. 3.5 shows flow cytometry dot plots from the analysis of *S. cerevisiae* cells stained with CTC. There were two subpopulations detected in a population of *S. cerevisiae* namely, the CTC-reducing cells (CTC⁺) with red fluorescence and CTC-non-reducing cells (CTC⁻). The fraction of CTC⁺ cells dropped from 58% $\pm$ 5 in the exponential phase to 0% in the stationary phase, after 3 days of batch culture (Table 3.2).

Table 3.2 Assessment of viability of *S. cerevisiae* cells by fluorescent probes. A fraction of cells (mean $\pm$ standard deviation) stained with PI, DiBAC₄(3) and CTC in different samples from batch culture of *S. cerevisiae* cells. The data derived from flow cytometry dot plot events (e.g. Fig. 3.1, 3.2 & 3.4) of three independent experiments. Stains were used independently in aliquots of the same populations.

Time of cultivation (hours)	% PI stained cells	% DiBAC ₄ (3) stained cells	% CTC ⁺ cells
10	2 $\pm$ 1	4 $\pm$ 1	58 $\pm$ 5
15	3 $\pm$ 1	6 $\pm$ 2	52 $\pm$ 4
35	24 $\pm$ 4	10 $\pm$ 2	28 $\pm$ 3
65	35 $\pm$ 3	32 $\pm$ 4	10 $\pm$ 2
85	40 $\pm$ 5	36 $\pm$ 3	0

3.3.5 Assessment of cell viability based on yeast viability probe of FUN-1

S. cerevisiae cell population was stained by LIVE/DEAD Yeast viability kit for the analysis with CLSM. Fig. 3.6 shows the formation of CIVS in yeast cells of *S. cerevisiae* stained with FUN-1 and Calcofluor in the exponential phase. *S. cerevisiae* cells, which killed by allyl alcohol cannot form CIVS after staining with FUN-1. The orange-red CIVS forms within vacuolar space of cells with membrane integrity. Dead yeast cells only diffuse yellow-green fluorescence without forming of CIVS due to the lack of transport of FUN-1 from cytosol to the vacuole. The CIVS formation was observed in approximately 90% of cell population of *S. cerevisiae* in exponential phase of batch culture.

Fig. 3.6 Viability analysis of *S. cerevisiae* cells in batch culture using LIVE/DEAD. Yeast viability (Fungolight) kit. Cells were grown to mid-exponential phase. A, dead cells treated with 20% (v/v) of allyl alcohol for 30 min; B, sample of intact cells.

3.3.6 Subpopulations detected by activity of alcohol dehydrogenase (ADH)

Fig. 3.7 shows that about 10% of *S. cerevisiae* cells from exponential phase with higher activity of ADH, were killed by exposure to the non-toxic concentration of allyl alcohol.

Fig. 3.7 Flow cytometry analysis of *S. cerevisiae*, subpopulations using the Live/Dead BacLight™ viability kit. A, dead cells treated with 20% (v/v) of allyl alcohol solution for 30 min; B, sample of intact cells; C, sample treated with 1% (v/v) of allyl alcohol solution for 10 min.

3.3.7 Respiration activity of microorganisms during exposure to allyl alcohol

The oxygen uptake rate (OUR) of samples was measured by respirometer after exposure to different concentrations of allyl alcohol: 0.05%, 0.1%, 0.2%, 0.3%, 0.5%, and 1% (v/v).

Fig. 3.8 Effect of different concentrations of allyl alcohol on respiration rate of *S. cerevisiae* (% of initial oxygen uptake rate). Concentrations of allyl alcohol (v/v): 0% (♦), 0.05% (+), 0.1% (■), 0.3% (●), 0.5% (▲), 1% (×). Duration of treatment with allyl alcohol solution (10 min) showed by solid line.

Fig 3.8 shows that there was no reductions of O₂ uptake rate (OUR) in *S. cerevisiae* cells suspensions exposed to the different concentrations of allyl alcohol during the first 10 min in comparison to the OUR of control suspension sample. However, the percentage of the OUR reduction after 110 min exposure to 0.05%, 0.1%, 0.3%, 0.5%, and 1% (v/v) allyl alcohol, compared to the control sample (0% allyl alcohol) were 3%, 11%, 19%, 30%, and 34%, respectively.

3.3.8 Assessment of cell viability based on multi-parameter flow cytometry analysis

The staining of yeast *S. cerevisiae* population simultaneously with PI and DiBAC₄(3) probes showed three different cells subpopulations (Fig. 3.7). In the dot plot flow cytometric analysis three main subpopulations were present: 1) Cells subpopulation neither stained with PI nor DiBAC₄(3) in the lower left quadrant(Q4), which represents viable cells 2) Cells subpopulation in the upper right quadrant (Q2) stained simultaneously with PI and DiBAC₄(3), which are dead cells 3) Cells subpopulation in the lower left quadrant (Q3) only stained with DiBAC₄(3), which are depolarized cells. The fraction of depolarized cells was increased from 2% $\pm$ 1 to 12% $\pm$ 1 after 3 days culture, whereas the fraction of dead cells was increased from 5% $\pm$ 1 to 48% $\pm$ 3 after 3 days culture.

Fig. 3.9 Multiparameter analysis of *S. cerevisiae* cells in batch culture by simultaneous staining of PI and DiBAC₄(3). Green and red fluorescences are due to staining with DiBAC₄(3) and PI, respectively. Samples were taken at; a, 15 h; b, 36 h; c, 85 h; d control (dead cells).

3.4 Discussion

A comparative study among different fluorescent probes such as PI, DiBAC₄(3), CTC and FUN-1 that targeted different cellular functions and structures in combination with flow cytometry and confocal laser scanning microscope was carried out for assessment of cell viability of *S. cerevisiae*. The results revealed that all the fluorescent stains except CTC can exploit for determination of viability in *S. cerevisiae* cell population. However, PI and DiBAC₄(3) were the most statistically reliable dyes for the flow cytometric discrimination of viable cells from dead cells of *S. cerevisiae*.

The first prerequisite of cell viability is integrity of the cytoplasmic membrane. The membrane integrity can be examined by permeant and impermeant nucleic acid-specific fluorescent stains. PI is commonly used for discrimination of live and dead *S. cerevisiae* cells (Zhang and Fang, 2004). Assessment of membrane potential has been commonly used as an indicator of cell viability. The Presence of membrane potential is an indicator of cell viability, though the depolarization of membrane does not necessarily imply to the death of a cell. Boyd et al. (2003) used membrane potential Oxonol dye for measurement of *S. cerevisiae* cell viability. In this study, the flow cytometry analysis of *S. cerevisiae* cell population revealed that the percentage of dead cells detected by PI was approximately equal to the percentage of cells detected by DiBAC₄(3) in both exponential and stationary phases of batch culture (Table 3.2). The data of this study was in agreement with data observed by Attfield et al. (2000) in the *S. cerevisiae* population. There was a good correlation between the fractions of cell population stained with PI and the fraction of cell population stained with DiBAC₄(3) in *S. cerevisiae* population (Fig. 3.4). This relatively strong linear correlation between the fraction of cells stained with DiBAC₄(3) and PI in different phases of growth (Table 3.2), confirmed that the uptake of Oxonol dye of DiBAC₄(3) in the yeast *S. cerevisiae* cell is more related to membrane integrity than to membrane potential. This is in agreement with the report by Lopez-Amoros et al. (1997) in the bacterial cells. Therefore, DiBAC₄(3) dye could be used for assessment of yeast viability as well as PI.

Respiratory activity was reported as an indicator of cell viability (McDaniels et al., 2005). Although, presence of respiratory activity is a sign of cell viability, lack of respiratory activity may not suggest death of cell. Cationic lipophilic dye rhodamine 123 (Rh 123) were used to determine the respiratory activity of *S. cerevisiae* cells. A good correlation between distribution of Rh 123 fluorescence and the degree of respiratory activity was reported in yeast population by Porro et al. (1994). Tetrazolium dye of CTC as an indicator of oxidative metabolism has been widely used for detection of respiratory activity and in some cases for viability assessment in bacterial cells (Laflamme et al., 2004; Rezaeinejad and Ivanov, 2010). CTC was used efficiently for the assessment of respiratory activity in prokaryotic cells of *E. coli*. However, the share of CTC-reducing cells in the fraction of viable cells dropped noticeably after long term starvation of *E. coli* population. Therefore, it was concluded that CTC could not be used for determination of viability in bacterial cells (Rezaeinejad and Ivanov, 2010). Hence, the respiratory activity of cell generally may not be an appropriate criterion for its viability, particularly in case of extreme starvation. It was stated by Breeuwer and Abee (2004) that CTC reduction is not reproducible in yeast cells due to rejection of cells to uptake the dye. To our knowledge, this is the first time that CTC has been used effectively for determination of respiratory activity in the yeast *S. cerevisiae* cell (Fig. 3.5 and Table 3.2). However, it was observed that the fraction of CTC- reducing cells of *S. cerevisiae* was much lesser than the fraction of CTC-reducing bacterial population of *E. coli* reported in the previous studies for both exponential and stationary phases of batch culture (Pyle et al., 1995; Rezaeinejad and Ivanov, 2010). The underestimation of respiratory active yeast cells by CTC reduction and the smaller fraction of CTC-reducing cells in comparison to proportion of viable cells detected by PI, DiBAC₄(3) and FUN-1 in the same cell population indicated that CTC is not a suitable dye for the measurement of yeast cells viability (Table 3.2).

Red fluorescence cylindrical structures within the vacuoles of the metabolic active cells of *S. cerevisiae* after staining with FUN-1 were visualized by CLSM (Fig. 3.6). In both exponential and stationary phases of the batch culture, the percentage of viable cells stained with FUN-1 correlated with the percentage of viable cells

stained with PI and DiBAC₄(3) measured by FCM (Table 3.2). The determination of viability of yeast cells stained with FUN-1 using flow cytometry technique was reported by Prudencio et al. (1998). However, we suggest that FCM is not suitable technique for determination of yeast viability in combination with FUN-1 fluorescent probe. FCM analysis of *S. cerevisiae* population showed noticeably underestimation of red fluorescence generated by formation of CIVS in comparison to green fluorescence (data were not shown). The underestimation of viable cells by FUN-1 staining may be due to overlapping between green and red spectra in the flow cytometry analysis. Panel A in Fig. 3.6 shows that the red fluorescence of CIVS were surrounded by green fluorescence and presumably were masked by green color and only detected as green fluorescence in FCM and resulted in the underestimating of cells with CIVS in flow cytometry analysis. Therefore, it is not recommended to use FCM for the analysis of yeast cells stained with FUN-1.

It was hypothesized that microbial cell populations consist of two subpopulations: 1) subpopulation with high activity of ADH 2) subpopulation with low activity of ADH. The allyl alcohol is metabolically oxidized by ADH to toxic aldehyde named “acrolein (acrylaldehyde,2-propenal)”. The preliminary experiments on respirometry showed that there was not any significant decline in the OUR of cell suspensions during 10 min treatment by different concentrations of allyl alcohol less than 1% (v/v). Cells with the high level of ADH were intoxicated with the product of allyl alcohol oxidation. Therefore, the subpopulation of cells, which was shifted from the R2 region in the panel C to R1 (about 9% of events R2 was shifted to R1), corresponds to cells with high level of energetic metabolism (Fig. 3.7). Hence, two subpopulations with high and low activities of ADH were detected in cell population of *S. cerevisiae*.

The multi-parametric flow cytometric analysis for assessment of cell viability could be more practical. The simultaneous staining of yeast cells with probes targeting different cellular functions such as membrane potential and membrane integrity gives more accurate results for assessment of cell viability. Analysis of yeast cellular functions by multi-color fluorescence flow cytometry was carried out by

Achilles et al. (2006). The cell subpopulation stained with both PI and DiBAC₄(3) (Fig. 3.9, Q2) are dead cells without membrane integrity, whereas subpopulation stained only with DiBAC₄(3) (Fig. 3.9, Q3) are viable cells with depolarized membrane. Cells with depolarized membrane probably are in lower energy state and in the intermediate physiological states between life and death. Despite of presence of cells stained merely with DiBAC₄(3) (Fig. 3.9, Q3) there were not any cells, which stained only with PI (Fig. 3.9, Q1). This indicates that the accumulation of DiBAC₄(3) to cell is either due to the damaged membrane integrity or the depolarized membrane potential. This double staining of *S. cerevisiae* cell population revealed that the fraction of depolarized cells versus the dead cells increased noticeably from the exponential to stationary growth phases (Fig. 3.9). This functional analysis indicates the bigger fraction of the depolarized cells versus the dead cells in the late exponential phase was probably due to the drop of cell energy sources, which maintaining cell viability. The simultaneous staining of two probes with different functions could be a better method for distinction of cells with different physiological states. In this method, viable cell are clearly discriminated by one function versus the other function. Therefore, physiological states of individual cell in the multi-parametric analysis could be thoroughly discovered.

Based on the results obtained for *S. cerevisiae* cells stained by different fluorescence dyes, the physiological indices for assessment of viability could be evaluated in the order of membrane integrity, membrane potential, and respiratory activity. The simultaneous staining of yeast cells with probes targeting several cellular parameters such as membrane potential and membrane integrity may permit comprehensive determination of cells viability.

3.5 Conclusions

- PI and DiBAC₄(3) probes were the most relevant dyes for viability assessment of *S. cerevisiae* cells by flow cytometry.
- FUN-1 as probe for discrimination of viable and dead yeast cells was more reproducible using the microscopic technique than flow cytometry.

- Analysis of different functional and structural parameters for assessment of cell viability in *S. cerevisiae* population showed that level of viability could be evaluated as the sequence of following physiological parameters: membrane integrity, membrane potential and respiratory activity.
- Two subpopulations with high and low activities of ADH were detected in growing population of *S. cerevisiae*. The population of *S. cerevisiae* was heterogeneous in terms of energetic states.

CHAPTER 4

HETEROGENEITY OF *ESCHERICHIA COLI* POPULATION BY RESPIRATORY ACTIVITY AND MEMBRANE POTENTIAL OF CELLS DURING GROWTH AND LONG-TERM STARVATION

4.1 Introduction

Assessment of physiological states of individual bacterial cells can be useful in the monitoring of indicator bacteria in environment as well as bacterial populations in biotechnological process. The changes of bacterial cell physiological states during cell cycle and life cycle as well as interaction of cells with inhomogeneous microenvironment result in the formation of a physiologically heterogeneous cell population (Quiros et al., 2007; Stewart and Franklin, 2008). Physiological parameters such as cell respiratory activity and cell membrane potential are important to assess the physiological activity and viability of individual bacterial cell in a population.

Application of fluorescent probes for assessment of physiological state of individual cells analyzed by flow cytometry is the most useful analytical procedure to study the physiological heterogeneity of bacterial population (Joux and Lebaron, 2000; Brehm-Stecher and Johnson, 2004). Evaluation of viability as a physiological state of cell has attracted attention of microbiologists in all fields of medical, environmental and biotechnological microbiology. However, a unified definition for the term “viability” among the microbiologists does not exist.

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Conventionally, the terms of bacterial viability and culturability are used as equivalent in the environmental monitoring. However, existence of such physiological state as viable but non-culturable complicated the correlation (Oliver, 2009). Therefore, measurement of some cellular physiological parameters such as membrane integrity, respiratory activity and membrane potential by flow cytometry technique could be suitable for determination of cell viability. DiBAC₄, PI and CTC have been used for determination of viability and other physiological states of *E. coli* cells in the batch culture as well as in environmental samples (Braux et al., 1997; Canovas et al., 2007).

The tetrazolium dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) is often used to measure the respiratory activity of individual bacterial cells. CTC is reduced by electron transfer from the respiratory chain with the formation of a water-insoluble red fluorescent intracellular formazan (Smith and McFeters, 1996; Sieracki et al., 1999). Therefore, the reduction of CTC in cell is considered as an indicator of bacterial cell respiratory activity in environment (Rodriguez et al., 1992; Creach et al., 2003; Belkova et al., 2007).

The cell membrane potential ($\Delta\Psi$) plays a key role in different cellular processes such as energy transformation, active transport, and regulation of intracellular pH. Metabolically active bacterial cells with intact cytoplasmic membrane are electrically negatively charged in the interior with respect to their exterior (Joux and Lebaron, 2000). It is considered that membrane potential of bacterial cell reflects both the physical integrity of cytoplasmic membrane as viability indicator and activity of energetic metabolism as physiological indicator (Shapiro, 2000). Anionic lipophilic dye bis-(1,3-dibutylbarbituric acid) trimethine oxo (DiBAC₄(3)) has been used to determine membrane potential as well as the viability of bacterial cells (Lopez-Amoros et al., 1997; Haidinger et al., 2003). Negatively charged DiBAC₄(3) cannot penetrate into active cells due to their interior negative charge. Therefore, cells stained by DiBAC₄(3) are considered as depolarized cells (Breeuwer and Abee, 2000). However, it is not clear what indicator, CTC or DiBAC₄(3), can be

used for the evaluation of physiological heterogeneity of bacterial population during long-term starvation.

Green fluorescence protein (GFP) is known as a persistent fluorescent protein, which is originated from jellyfish *Aequorea aequorea* (Arana et al., 2003). Cells tagged with *gfp* gene are capable of producing a fluorescent protein, which fluoresces when it is excited by UV light or laser beam. The stable fluorescent protein of “GFP” enables the visualization and tracking of gfp-tagged bacterial cells in environmental samples. Green fluorescence protein (GFP) as genetic marker could be used for monitoring and tracking of specific bacterial strain in water environment. The gfp-tagged bacterial cells fluoresce after exciting by UV light regardless of being dead or viable. Fluorescence probes used for assessment of physiological states of bacterial cells in combination with the genetic marker help to evaluate physiological states of cells. The combination of PI or CTC with the gfp-tagged bacterial cells could be used for discrimination of dead from viable or respiring from non-respiring cells (Maraha et al., 2004). The knowledge obtained is vital in order to validate the efficacy of GFP as a marker for bacterial cells in environmental monitoring.

The aim of this chapter is to assess the physiological heterogeneity *Escherichia coli* cells during growth and starvation, which stained with CTC and DiBAC₄(3) fluorescent probes for the monitoring of respiratory activity and viability of cells.

4.2 Materials and Methods

4.2.1 Microorganism and its cultivation

Strain of *Escherichia coli*, ML 308, was obtained from German Collection of Microorganisms and Cell Cultures (DSMZ). In order to construct the gfp-tagged bacterial cells, the plasmid of pEGPlucTet was electroporated into *E. coli* cells using standard molecular biology methods. The green fluorescent protein (GFP)

gene contained in the gfp-tagged *E. coli* was under the control of a kanamycin-inducible promoter.

Cells were grown in 100 ml flasks (Schott Duran, Germany) Luria-Bertani broth medium (DifcoTM, Becton Dickinson, MD, USA) in a shaker incubator at 37°C and 200 rpm. The cell suspensions from exponential phase (3 hours of cultivation) and stationary phase (42 hours of cultivation) of batch culture were used for staining and flow cytometry analysis.

The bacterial cells, used for the starvation experiments, were washed twice with sterile phosphate buffered saline (PBS) (pH 7.2) by centrifugation for 10 min at 3398×g. The cells were re-suspended in PBS and starved in this buffer for 10 hours and 40 days. In the control sample, cells were killed by 30 min incubation in 20% (v v⁻¹) allyl alcohol (Sigma-Aldrich, MO, USA) solution. The allyl alcohol was removed by washing bacterial cells twice with PBS using centrifugation.

4.2.2 LIVE/DEAD BacLightTM viability kit

LIVE/DEAD BacLightTM viability kit containing SYTO9, 3.34 mM, and propidium iodide (PI), 20 mM, was obtained from Molecular Probes (Invitrogen, Eugene, Oregon, USA). The samples of cell suspension were stained with 1µl of stock solution of SYTO9, 3.34 mM, and PI, 20 mM, at 30°C in dark for 30 min. The suspension was then washed twice in PBS and the pellet was re-suspended in PBS for the further analysis (Berney et al., 2007).

4.2.3 Staining of cells with CTC

An aliquot of 100 µl of the stock solution (50 mM) of CTC (Polyscience, Inc., Warrington, PA, USA) was added to a test tube containing 900 µl of bacterial suspension and incubated with agitation of 200 rpm in dark at 30°C for 1 hour. The final concentration of CTC was 5 mM.

4.2.4 Staining of cells with DiBAC₄(3)

Cells were stained with DiBAC₄(3) (Molecular Probes, Invitrogen, Eugene, Oregon, USA) at final concentration 1 μ M. The stock solution of DiBAC₄(3) in dimethyl sulfoxide at concentration of 250 μ M was stored at -20°C. An aliquot of 4 μ l of the stock solution was added to the test tube containing 996 μ l of cell suspension, then incubated at 37°C in the dark for 20 min and washed twice by centrifugation at 3398 $\times$ g for 5 min, re-suspended in 1 ml of PBS and analyzed by flow cytometry immediately (Shapiro and Nebe-von-Caron, 2004).

4.2.5 Flow cytometry data acquisition and analysis

The flow cytometry measurements were carried out using Becton Dickinson (BD) FACSCaliburTM flow cytometer with CELLQuestTM software for data acquisition. A total of 50,000 cells were counted, based on 488 nm excitation by 15mW air cooled blue argon-ion laser. Fluorescence was measured using a band pass filter 530 $\pm$ 15 nm for green fluorescence of DiBAC₄(3), and a band pass filter 620 $\pm$ 10 nm for red fluorescence of CTC. Sheath flow rate was 10 ml min⁻¹ and sample analysis rate never exceeded 1,000 events s⁻¹. Analysis of data was carried out by using FlowJo V.7.2.5 software (www.flowjo.com).

4.2.6 Turbidity measurements

Microbial growth was determined by measurement of microbial suspension optical density at 600 nm (OD₆₀₀) using spectrophotometer DU 640B (Beckman Coulter, USA).

4.2.7 Respirometry

Cells of *E. coli* were grown in 100 ml DURAN[®] cylindrical culture bottles (Schott, NY, USA) in a shaker incubator at 37°C and 200 rpm. CO₂ production rate of the samples was measured by dual gas (O₂/CO₂) respirometer (Columbus Oxymax ER, OH, USA). The bottles connected to the respirometer were continuously aerated with air flow 500 ml min⁻¹ and were kept in the shaking water-bath incubator at 37°C.

4.2.8 Epi-fluorescence microscope

Bacterial cells stained with fluorescence probes were viewed using an Epi-fluorescence microscope (BX 60, Olympus Optical, Tokyo, Japan) with a Uplan FL 100X/ 1.3 oil objective. The microscope was equipped with a 100-W mercury lamp. The stained cells were viewed under blue light excitation (488 nm) using the 45G-530 and 45O-560 for green and red fluorescence, respectively. Images were obtained using a peltier cooled CCD camera mounted into the microscope.

4.3 Results

4.3.1 Physiological heterogeneity of *E. coli* population in batch culture

Growth curve was plotted by turbidity (optical density) measurement in the hourly intervals (Fig. 4.1) to determine exponential and stationary phases of batch culture. CTC and DiBAC₄(3) stains were used for determination of bacterial respiratory activity and viability in different phases of batch culture of *E. coli* (Fig. 4. 2 and Fig. 4.3).

Fig. 4.1 Optical density of cell suspension (▲), CO₂ production rate (●), and fraction of CTC⁺-cells (◆); fraction of ΔΨ⁺ cells (◇) in batch culture of *E. coli* cells.

The mean percentages $\pm$ standard deviations of CTC⁺-reducing cells (CTC⁺-cells) were $90\% \pm 1.2$ and $83\% \pm 0.8$ in the samples from the exponential and stationary phases, respectively (Fig. 4.1). The share of polarized cells (ΔΨ⁺- cells) was calculated by deduction of the share of depolarized cells (stained with DiBAC₄(3)) from 100%. The mean percentages $\pm$ standard deviations of ΔΨ⁺-cells were $92.4\% \pm 6.2$ and $73\% \pm 1.8$ for the samples from the exponential and stationary phases, respectively (Fig. 4.1).

The highest CO₂ production rate with a mean value of 0.12 mg min^{-1} was detected during the exponential growth phase between 4 to 9 hours of *E. coli* cultivation. The CO₂ production rate remained constant during the stationary phase of batch culture with mean value of 0.08 mg min^{-1} between 10 to 46 hours of the cultivation (Fig. 4.1).

Fig. 4.2 Subpopulations of CTC⁺-cells (R2) and CTC⁻-cells (R1) in batch culture of *E. coli* cells: a, 3 h; b, 10 h; c, 42 h; d control (dead cells). Red fluorescence is due to reduction of CTC.

Fig. 4.3 Subpopulations of $\Delta\Psi^+$ -cells (left dot plot) and $\Delta\Psi^-$ -cells (right dot plot) in batch culture of *E. coli* cells: a, 3 h; b, 10 h; c, 42 h; d control (dead cells). Green fluorescence is due to accumulation of DiBAC₄(3) inside cells.

Fig. 4.4 *E. coli* cells stained with DiBAC₄(3) observed by Epi-fluorescence microscope; dead cells treated with 20% (v/v) of allyl alcohol for 30 min. Dead cells were appeared as green color due to staining with DiBAC₄(3).

Fig. 4.4 shows the image of dead *E. coli* cells, which visualized by Epi-fluorescence as green due to accumulation of negatively charged DiBAC₄(3) probe in interior of cells. The image in Fig. 4.4 verified the flow cytometry data for determination of viability in *E. coli* cell population (Fig. 4.3, d).

The percentages of viable cells of *E. coli* population in the samples from the exponential and stationary phases of growth stained with LIVE\DEAD viability kit, were $98 \pm 1\%$ (mean $\pm$ standard deviations) and $85 \pm 3\%$ (mean $\pm$ standard deviations), respectively (Fig. 4.5).

Fig. 4.5 Subpopulations of dead cells (R1) and live cells (R2) in batch culture of *E. coli* cells: a, 5 h; b, 35 h; c, control (dead cells). The green fluorescence is due to staining of cells by SYTO9 and the red fluorescence is indicated the dead cells stained with PI.

A

B

Fig. 4.6 *E. coli* cells stained by Live/Dead BacLight observed by Epi-fluorescence microscope; A, intact cells; B, dead cells treated with 20% (v/v) of allyl alcohol for 30 min. Dead cells were appeared as yellow color due to staining with PI, which only penetrates to dead cells (panel B), whereas the live cells are green color of SYTO9 fluorescent dye (Panel A).

Viable *E. coli* cells, which were visualized by Epi-fluorescence microscope as green fluorescence in Fig. 4.6, A, validated the data from flow cytometry analysis (Fig. 4.5, R1). The dead cells of *E. coli* were observed as red fluorescence (Fig. 4.6, B) confirmed the data by flow cytometry analysis of *E. coli* population (Fig. 4.5, R2).

4.3.2 Physiological heterogeneity of *E. coli* population during starvation

E. coli cell populations under short and long term nutrient starvation were extremely heterogeneous in terms of respiratory activity and membrane potential.

Fig. 4.7 Changes in the size of subpopulation of CTC⁺ (□) and DiBAC₄(3)⁺ (■) of *E. coli* cells, during starvation for 10 hours and 40 days. Cells were taken from different phases of batch culture. Bars represent standard errors (n = 3).

The shares of CTC⁺-cells in *E. coli* population under short-term, 10 hours, nutrient starvation in the samples taken from the exponential and stationary growth phases of batch culture dropped from $92 \pm 1\%$ to $78 \pm 1\%$ and from $83 \pm 1\%$ to $33\% \pm 1\%$, respectively (Fig. 4.7). The share of $\Delta\Psi^+$ - cells also decreased to $72 \pm 1\%$ and $68 \pm 1\%$ in the samples taken from the exponential and stationary growth phases, respectively (Fig. 4.7). These results indicate that a bigger fraction of cell population in stationary phase lost their respiratory activity in comparison to exponentially growing cells. In contrast to significant drop in respiratory activity of cells, the changes in the percentage of $\Delta\Psi^+$ -cells after short-term starvation were not dependant on the phase of batch culture.

The share of CTC⁺ cells of *E. coli* under long-term, 40-days, nutrient starvation in the sample taken from the stationary growth phase dropped from $83 \pm 1\%$ to $5 \pm 1\%$, whereas the share of $\Delta\Psi^+$ -cells dropped from $72 \pm 1\%$ to $50 \pm 1\%$ in the same sample (Fig. 4.7).

4.3.3 Viability analysis of gfp-tagged bacterial cells

Viability and respiratory activity of gfp-tagged *E. coli* cell population were assessed by PI and CTC fluorescent probes in combination with flow cytometry.

Fig. 4.8 Viability analysis of gfp-tagged *E. coli* cells in batch culture stained with PI. Subpopulations of dead cells (region R1) and live cells (region R2): time of sampling (hours); a) 5; b) 20; c) 30; d) control (dead cells). Red fluorescence is due to staining with PI and green fluorescence is due to GFP, respectively. GFP was measured in fluorescence channel FL1 and PI in FL3.

Fig. 4.9 Respiratory activity analysis of gfp-tagged *E. coli* cells in batch culture by tetrazolium chloride (CTC). Subpopulations of CTC⁺ cells (region R2) and CTC⁻ cells (region R1): time of sampling (hours); a) 5; b) 20; c) 30; d) control (dead cells). Red fluorescence is due to CTC reduction. GFP was measured in fluorescence channel FL1 and CTC in FL3.

Fig. 4.10 gfp-tagged *E. coli* cells stained with CTC observed by Epi-fluorescence microscope. Cells were visualised as green due to GFP and red due to staining with CTC.

Figure 4.8 shows the flow cytometry dot plots from the analysis of gfp-tagged *E. coli* cells growing in batch culture stained with PI. In each panel, R1 is the subpopulation, which stained with PI as a fraction of dead cells, and R2 is the subpopulation of non-stained cells as fraction of viable cells of gfp-tagged *E. coli* population. The fraction of dead cells increased from $2\% \pm 1$ in the exponential phase to $15\% \pm 2$ in the stationary phase.

Figure 4.9 shows the flow cytometry dot plots from the analysis of gfp-tagged *E. coli* cells, which stained by CTC. Two subpopulations CTC-reducing cells (CTC^+) and CTC-non-reducing cells (CTC^-) were detected in a population of gfp-tagged *E. coli* (Fig. 4.10). The fraction of CTC^+ cells dropped from $92\% \pm 4$ in the exponential growth phase to $30\% \pm 2$ in stationary growth phase.

4.4 Discussion

This chapter is presented to argue cell activity and viability under growth and starvation conditions in *E. coli* cell population. The fluorescence dye of propidium iodide is commonly used for the assessment of bacterial cell viability. Cyanoditotyl tetrazolium chloride (CTC) as indicator of respiration activity and oxonol dye DiBAC4 (3) as membrane potential probe also were used to assess viability of bacterial cells.

It is known that the share of respiratory active (CTC⁺) cells in a bacterial population decreases during transition from exponential to stationary phases of batch culture (Creach et al., 2003). In this study, the fraction of CTC⁺ cells in the exponential phase was 90%, which is in agreement with the data by Pyle et al. (1995). The fraction of CTC⁺-cells in the stationary phase was 83% of cell population, which is higher than the value reported previously for *E. coli* population (Pyle et al., 1995). The minor changes in the percentage CTC⁺-cells of *E. coli* population during transition from exponential to stationary phase of batch culture can be correlated to the high rate of respiration and CO₂ production in stationary phase. The linear correlation between the share of respiratory active cells and the CO₂ production rate during the batch cultivation was observed in a bacterial population (Creach et al., 2003). However, in the present study, linear correlation between the CO₂ production rate and share of CTC⁺-cells was not found.

It was known that share of CTC⁺-cells decreased under starvation (Lisle et al., 1998; Cook and Bolster, 2007). It was shown in the present study that the decline in the share of respiratory active cells in the samples taken from the stationary phase was about three times greater than the sample taken from the exponential phase. This difference was probably due to lower storage of cell energy sources in the stationary phase than the cells in the exponential phase of batch culture.

DiBAC₄(3) and other membrane potential dyes have been used for discrimination between the viable and the depolarized (dead) bacterial cells (Amor et al., 2002).

There was a strong correlation between share of dead cells stained with PI and depolarized cells stained with DiBAC₄(3) in the different phases of batch culture of *E. coli* population (Fig. 4.1, Fig. 4.3 and Fig. 4.5).

The data in this thesis showed for the first time that almost 50% of *E. coli* cells are remained as $\Delta\Psi^+$ -cells (viable cell) after a long-term nutrient starvation of 40 days (Fig. 4.7). Meanwhile, only 5% of cells were detected as respiratory active (CTC⁺-cells), after 40-day starvation (Fig. 4.7). CTC is reduced by respiratory chain dehydrogenases in bacterial cells (Smith and McFeters, 1996). In long term starvation of bacterial cells, CTC reduction dropped in a larger extent most probably because of the drop in the specific activity of NADH dehydrogenases. In contrast, bacterial cells most likely maintain their proton motive force under long-term starvation. Therefore, under the long-term starvation, relatively small fraction of cell population lose their membrane potential in comparison to a significant drop in cell numbers with high respiratory activity. Although CTC fluorescent dye has been used to assess the bacterial cell viability (McDaniels et al., 2005), the data of present work showed that CTC is not a suitable dye for measurement of bacterial cell viability under long-term starvation period. However, CTC is an appropriate probe for the measurement of the physiological state of bacterial cells. This could be taken into account for the detection of viable and respiratory active cells during the flow cytometry monitoring of environmental and biotechnological processes. Flow cytometry enables the analysis of different parameters such as membrane integrity and membrane potential simultaneously. For instance, the double-staining of PI and DiBAC₄(3) could provide more information in discrimination of live and dead cells and also give better insight into diversity of physiological states of bacterial cells.

The flow cytometric analysis of gfp-tagged *E. coli* revealed that the percentage of non-respiring cells (CTC⁻) increased four times higher than the percentage of cells stained with PI considered as dead cell in the aliquots of the same populations in the stationary phase. It is speculated that losing the ability of respiratory activity in a cell is not necessarily identical to the loss of cell viability. In the other words,

although the presence of respiratory activity is a sign of cell viability, cell with lack of respiratory activity may not be considered as a dead cell.

The flow cytometry analysis showed that the green fluorescence protein (GFP) maintained persistently throughout the transition of cell from the exponential to the stationary phases of batch culture. However, as it expected the percentage of viable and respiratory active cells were changed in the different growth phases. These results demonstrated the applicability of the GFP marker for monitoring the physiological states of specific bacterial cells in environmental samples of water.

4.5 Conclusions

- DiBAC₄(3) as a probe for cell membrane potential is more suitable than CTC as a probe for cell respiratory activity for the detection of viable indicator bacteria in environment.
- Bacterial population was shown to be more physiologically heterogeneous upon facing to stress, e.g. starvation.
- Respiratory activity of cells dropped significantly in comparison to the membrane potential in a bacterial cell population under long-term starvation.

CHAPTER 5

HETEROGENEITY OF BACTERIAL CELL POPULATION AND SUSCEPTIBILITY TO DISINFECTANTS

5.1 Introduction

The presence of pathogenic microorganisms in drinking water is mainly due to contamination by human or animal wastes. The elimination of waterborne pathogens from drinking water is one of the main goals in water treatment. Chemical disinfection is an effective technique for deactivation of pathogenic microorganisms in drinking water.

Application of disinfectants such as chlorine is widespread in the treatment of water and control of microbial growth in water distribution system. Inactivation kinetics of bacterial cells by the disinfection of water with chlorine has been studied extensively for almost a century. Several disinfection kinetic models have been developed. All the disinfection kinetic models including Chick-Watson model are based on an assumption that treated microbial population as physiologically homogenous (Haas and Kaymak, 2003). However, it is well known that individual microbial cells within a population are not identical in terms of their physiological states (Quiros et al., 2007; Muller et al., 2010; Rezaeinejad and Ivanov, 2010). Therefore, it could be expected that the susceptibility to a disinfectant is different among the individual cells of a microbial population.

Physiological characteristics of microorganisms in water tend to be altered due to their exposure to dynamic changes in environment. Microorganisms in drinking water are deprived of access to essential nutrients. A microbial population under the nutrient-limited condition is extremely heterogeneous in terms of physiological states (Kaprelyants et al., 1996). Microbial cells enter a physiological state that

results in metabolically less active and more resistant to physical or chemical stresses, upon starvation (Kolter et al., 1993). Therefore, it could be also expected that there are different fractions of bacterial population with various susceptibility to disinfection in the drinking water.

Specific growth rate is one of the major physiological parameters depending on concentration of nutrients. Microorganisms adopt the slow growth rate strategy in order to survive upon encountering the limited availability of nutrients (Konopka, 2000). The physiological changes (e.g., changes in cell envelope compositions and formation of capsule), which are accompanied by slow growth rate of bacterial cells can help the bacteria to be less susceptible to disinfectant substances (Berney et al., 2006). Most of bacteria in the oligotrophic ecosystems enter a physiological state, where cells lose their culturability on standard laboratory media, but maintain their viability (Leclerc and Moreau, 2002; Oliver, 2009). This viable but non-culturable (VBNC) state of cell is considered as a survival strategy of bacterial cells in adverse condition (Del Mar Lleo et al., 2007; Du et al., 2007). Therefore, the effect of the initial physiological state of cells should be also taken into account in the studies of the cell inactivation by chemical disinfectants.

Microbial water quality guidelines are generally based on the viability of an indicator bacteria such as *Escherichia coli* (Field and Samadpour, 2007). The conventional technique of heterotrophic plate count (HPC) has been routinely used for evaluation of disinfection impact on microbiological quality of water (Pernitsky et al., 1995). The cultivation-based method may not be sensitive to the starved and stressed bacterial cells in oligotrophic systems of drinking water. Numerous water-born pathogens are not growing in laboratory culture. Culturable bacteria may enter into the temporarily state of “VBNC” in response to certain conditions. The VBNC physiological state of bacterial cells is not recoverable by conventional microbiological test of HPC (Leclerc and Moreau, 2002). Therefore, a technique such as flow cytometry could be used as an alternative method to the conventional cultivation-based technique for analysis of physiological states of bacteria at single-cell level. Flow cytometry can be also used for determination of bacterial cells

inactivation kinetics by chemical agents in water. Flow cytometry method is more effective than the culture-based method for evaluation of microbial quality of potable water due to higher degree of sensitivity and detection limit, less time required to obtain results, and detection of non-culturable bacteria (Hoefel et al., 2003; Czechowska et al., 2008; Hammes et al., 2008). Therefore, flow cytometry has been used in combination with fluorescent probes for determination of bacterial cells inactivation (losing viability) during chemical disinfection of water (Phe et al., 2005; Cunningham et al., 2008).

The viability of microbial cells at single-cell level could be assessed based on physiological indices such as; integrity of cell membrane, maintenance of cell membrane potential and presence of respiratory activities (Joux and Lebaron, 2000; Muller and Nebe-Von-Caron, 2010; Rezaeinejad and Ivanov, 2010). Cytoplasmic membrane integrity of cell is the most common assay for cell viability. The loss of cell membrane integrity can be measured by uptake of membrane-impairment dyes, e.g., Propidium iodide (PI) (Berney et al., 2007). A cytoplasmic membrane, which is damaged by exposure to oxidant, e.g., chlorine, becomes permeable to membrane-impairment fluorescent dye of PI and the dye intercalates with double-stranded nucleic acid (Phe et al., 2005). However, severe damage in nucleic acids of bacterial cells by highly chlorinated water may results in an underestimation of the inactivated cells determined by PI (Phe et al., 2004). Membrane potential ($\Delta\Psi$) is another key parameter, which indicates cell plasma membrane integrity as viability indicator and reflects the level of energy metabolism of cell (Shapiro, 2000; Rezaeinejad and Ivanov, 2010). Negatively charged oxonol dye of DiBAC₄(3) accumulates in the intracellular space of a cell with interior-positive membrane potential or a dead cell with $\Delta\Psi = 0$, and binds to the intracellular lipid-containing elements (Diaz et al., 2010). The fluorescent redox probe of tetrazolium salts are commonly used as an indicator of respiratory activity as well as viability (Hatzinger et al., 2003; Rezaeinejad and Ivanov, 2010). Fluorescent dye of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) is an alternative to oxygen terminated electron acceptor and forms a red fluorescent formazan product of “CTF” (Sieracki et al., 1999). CTC

was used to examine the antimicrobial effects of chlorine in bacterial cells (Kim et al., 2008).

In this study, in order to determine the inactivation of *E. coli* cells by chlorine at single-cell level, the fluorescent probes of PI, DiBAC₄(3) and CTC were used in combination with flow cytometry. The aims of this chapter are:

- 1) To assess the effects of specific growth rate on the inactivation of *E. coli* cells by chlorine.
- 2) To examine the adverse effects of chlorine on physiological indices of cell viability.

5.2 Materials and methods

5.2.1 Bacterial strain and cultivation conditions

Strain of *Escherichia coli*, ML 308, was obtained from German Collection of Microorganisms and Cell Cultures (DSMZ). Cells were grown in 100 ml of Luria-Bertani broth medium (DifcoTM, Becton Dickinson, MD, USA) in the flasks (Schott Duran, Germany) placed in a shaker incubator at 37°C and 200 rpm. Bacterial cells were sampled after 5 hours of cultivation (fast-growing cells) and 24 hours of cultivation (slow-growing cells) by centrifugation for 10 min at 3398×g. Cells were washed twice with sterile phosphate buffered saline (PBS) (pH 7.2) by centrifugation for 10 min at 3398×g and adjusted to concentration of 10⁸ cells ml⁻¹ in reverse osmosis water.

5.2.2 Turbidity measurements and growth rate calculation of bacterial population

The microbial growth was determined by measurement of optical density at 600 nm (OD₆₀₀) of cell suspension in every one hour interval using spectrophotometer DU

640B (Beckman Coulter, USA) versus a blank, which was the same medium without cells at 600 nm. The specific growth rates (μ) were calculated for linear parts of growth curves, using following equation; $\mu = \Delta \ln OD_{600} / \Delta t$, where t is time.

5.2.3 Chlorine measurement procedure

Chlorine solution was prepared with an aqueous sodium hypochlorite (NaClO) reagent (commercial solution of liquid chlorine bleach, GB Chemicals, Singapore) at pH 7 with demand-free filtered (0.2 μ m, Minisart, Sartorius) Mill-Q water. The concentration of free chlorine in a freshly prepared standard solution was measured by DPD (*N, N*-diethyl-*p*-phenylenediamine, Sigma-Aldrich, USA). The DPD reagent was prepared by dissolving 1.1 g l⁻¹ DPD sulphate in Mill-Q water. The standard solution of chlorine were prepared by constructing a standard curve in the range of 0.001-1 mg l⁻¹ for measurement of Cl₂ by DPD method with a spectrophotometer (DU 640B, Beckman Coulter, USA). A red intermediate spectrum forms by the reaction between DPD and free chlorine rapidly, which was measured at 515 nm with spectrophotometer DU 640B (Beckman Coulter, USA) straight away (Moberg and Karlberg, 2000).

5.2.4 Chlorine treatment of bacterial cells

Aliquots of cell suspensions in Mill-Q water (pH 7) were spiked with different concentrations of chlorine ranging from 0.001 to 0.01 mg l⁻¹ and incubated for different times (3, 6 and 10 min) at room temperature (25°C). In the control sample, cells were killed by a solution with 1 mg l⁻¹ free chlorine for 10 min incubation. The residual chlorine neutralized by the addition of sodium thiosulfate and the cells suspension washed twice with PBS by centrifugation for 10 min at 3398×g. A non-chlorinated control sample was prepared in the same conditions. CT values (“C” is chlorine concentration in mg l⁻¹ and “T” is contact time in min) were used to calculate the bacterial inactivation.

5.2.5 Staining of cells with LIVE/DEAD BacLight™ viability kit

LIVE/DEAD BacLight™ viability kit, containing SYTO9, 3.34 mM, and propidium iodide (PI), 20 mM, was obtained from Molecular Probes (Invitrogen, Eugene, Oregon, USA). The aliquots of 1 ml of chlorinated and non-chlorinated *E. coli* cells suspensions were stained with 1 µl of stock solution of SYTO9, 3.34 mM, and 6 µl of stock solution of PI, 20 mM, at 30°C in dark for 30 min. The cells suspensions were washed twice in PBS subsequently and the pellet was re-suspended in PBS for analysis by flow cytometry (Rezaeinejad and Ivanov, 2010).

5.2.6 Staining of cells with CTC

An aliquot of 100 µl of the stock solution (50 mM) of CTC (Polyscience, Inc., Warrington, PA, USA) was added to a test tube containing 900 µl of bacterial cells suspension and incubated in dark at 30°C for 1 hour. Final concentration of CTC was 5 mM (Rezaeinejad and Ivanov, 2010).

5.2.7 Staining of cells with DiBAC₄(3)

The stock solution of DiBAC₄(3) (Molecular Probes, Invitrogen, Eugene, Oregon, USA) in dimethyl sulfoxide at concentration of 250 µM was stored at -20°C. The chlorinated and non-chlorinated cells were stained with DiBAC₄(3) at final concentration 1 µM. An aliquot of 4 µl of the stock solution was added to a test tube containing 996 µl of cells suspension, then incubated at 37°C in dark for 30 min and washed twice by centrifugation at 3398 ×g, re-suspended in 1 ml of PBS and analyzed by flow cytometry immediately (Rezaeinejad and Ivanov, 2010).

5.2.8 Flow cytometry data acquisition and analysis

Flow cytometry measurements were done using Becton Dickinson (BD) FACSCalibur™ flow cytometer with CELLQuest™ software for data acquisition.

A total of 50,000 cells were counted, based on 488 nm excitation by a 15mW air cooled blue argon-ion laser. Fluorescence was measured using a band pass filter 530 ± 15 nm for green fluorescence of DIBAC₄(3), and a band pass filter 620 ± 10 nm for red fluorescence of CTC and PI. Sheath flow rate was 10 ml min⁻¹ and sample analysis rate never exceeded 1,000 events s⁻¹. The analysis of data was carried out by using FlowJo V.7.2.5 software (www.flowjo.com).

5.2.9 Statistical analysis

Statistical analysis was performed by standard method of Pearson 2-tailed test using the mean of three measurements. All data evaluations were performed using PASW statistics 18 (SPSS Inc., USA).

5.3 Results

Three different probes in combination with flow cytometry were used for assessment of bacterial inactivation by treatment with chlorine. Two *E. coli* cell populations with specific growth rate of 0.2 h^{-1} (slow growing) and 0.9 h^{-1} (fast growing) were exposed to free chlorine with concentration ranges from 0.001 to 0.01 mg l^{-1} in different contact times of 3, 6 and 10 min.

5.3.1 Inactivation analysis by membrane integrity probe

Fig. 5.1 shows the flow cytometry dot plots from the analysis of *E. coli* cell population with $\mu = 0.2 \text{ h}^{-1}$, which were exposed to 0.01 mg l^{-1} of free chlorine in different contact times and stained with SYTO9 and PI simultaneously. There was a clear separation between cells subpopulation (R2) stained only with green fluorescent dye of SYTO9 (live cells) and the cells subpopulation (R1) stained with red fluorescent dye of PI (inactivated cells) as well.

In slow growing population, the fractions of cells inactivated by 0.01 mg l^{-1} of free chlorine solution after 3 and 10 min exposure were 71 % and 99%, respectively (Fig. 5.1).

Fig. 5.1 Flow cytometry dot plots of viability analysis of *E. coli* cells ($\mu=0.2 \text{ h}^{-1}$) in batch culture by LIVE/DEAD BacLight™ viability kit. Subpopulations of dead cells (region R1) and live cells (region R2); *E. coli* cells exposed to chlorine concentration of 0.01 mg l^{-1} in contact time (min) ; a, 3; b, 10; c, control sample (live cells); d control sample (dead cells). Red and green fluorescence are due to staining by PI and SYTO9, respectively. SYTO9 fluorescence was measured in fluorescence channel FL1 and PI fluorescence in FL3.

Fig. 5.2 Inactivation kinetics of *E. coli* cells grown in batch culture at specific growth rate of 0.2 h^{-1} (dash dot line ●) and 0.9 h^{-1} (solid line ◆) with chlorine. Inactivation analysis of *E. coli* cells (losing viability) were done by LIVE/DEAD BacLight™ viability kit in combination with flow cytometry. Each datum point is ratio of the percentage of surviving cells to the percentage of initial viable cells from the total population in logarithmic scale. Error bars represents standard error of the mean measurement ($n=3$).

In order to compare the effect of free chlorine on the *E. coli* cell population with lower specific growth rate (μ) of 0.2 h^{-1} and higher specific growth rate (μ) of 0.9 h^{-1} , the inactivation curves were plotted by log inactivation versus CT values (Fig. 5.2). The data for inactivation of *E. coli* cells in Fig. 5.2 were derived from the flow cytometry analysis of cells stained with membrane integrity probe of PI. The CT values required for 2 log inactivation (CT_{99}) for *E. coli* cell populations with $\mu=0.9 \text{ h}^{-1}$ and $\mu=0.2 \text{ h}^{-1}$ were 0.06 and $0.09 \text{ mg min l}^{-1}$, respectively (Fig. 5.2). The log inactivation for *E. coli* cell populations with $\mu=0.9 \text{ h}^{-1}$ and $\mu=0.2 \text{ h}^{-1}$ at CT value of $0.06 \text{ mg min l}^{-1}$ were 2.1 ± 0.07 (mean $\pm$ standard error) and 0.68 ± 0.04 (mean $\pm$ standard error), respectively (Fig. 5.2).

Fig. 5.3 Inactivation kinetics of *E. coli* cells grown at specific growth rate of 0.2 h^{-1} exposed to different concentration of free chlorine and different contact times (min); 3 (long dash dot line $\diamond$), 6 (dot line $\bullet$) and 10 (solid line $\blacktriangle$) measured with LIVE/DEAD BacLight™ viability kit in combination with flow cytometry. Each datum point is ratio of the percentage of surviving cells to the percentage of initial viable cells from the total population in logarithmic scale. Error bars represents standard error of the mean measurement ($n=3$).

Fig. 5.4 Inactivation kinetics of *E. coli* cells grown at specific growth rate of 0.9 h^{-1} exposed to different concentration and different contact times (min); 3 (long dash dot line $\diamond$), 6 (dot line $\bullet$) and 10 (solid line $\blacktriangle$) measured with LIVE/DEAD BacLight™ viability kit in combination with flow cytometry. Each datum point is ratio of the percentage of surviving cells to the percentage of initial viable cells from the total population in logarithmic scale. Error bars represents standard error of the mean measurement ($n=3$).

The effects of chlorine on *E. coli* cell populations with different specific growth rates were determined during exposure to a range of chlorine concentration from 0.001 to 0.01 mg l⁻¹ for the contact time of 3, 6 and 10 min (Fig. 5.3 and Fig. 5.4). There was no significant differences between log inactivation curves for both populations with $\mu=0.9$ h⁻¹ and $\mu=0.2$ h⁻¹ during their exposure to different concentration of chlorine and contact time of 3 min (Fig. 5.3 and Fig. 5.4). The log inactivation was increased for three-fold in the *E. coli* cell population with $\mu=0.9$ h⁻¹ than cell population with $\mu=0.2$ h⁻¹ during contact time of 6 min and chlorine concentration of 0.009 mg l⁻¹. Both *E. coli* cell populations with $\mu=0.9$ h⁻¹ and $\mu=0.2$ h⁻¹ were inactivated by 2 log (99% of inactivation) after 10 min exposure to chlorine concentration of 0.009 mg l⁻¹ (Fig. 5.3 and Fig. 5.4).

5.3.2 Inactivation analysis by membrane potential probe

Fig. 5.5 shows flow cytometry dot plots for *E. coli* cells with $\mu=0.2$ h⁻¹, which were exposed to 0.01 mg l⁻¹ of free chlorine for different contact times and stained with DiBAC₄(3). Region of Q2 in Fig. 5.5 indicates a cells subpopulation of slow-growing *E. coli* population, which includes dead and membrane depolarized cells stained by green fluorescence dye of DiBAC₄(3). The fractions of dead and/or depolarized cells (Q2) of an *E. coli* cell population exposed to 0.01 mg l⁻¹ of free chlorine during 3 and 10 min were 73% (Fig. 5.5a) and 90% (Fig. 5.5b), respectively. The *E. coli* cells killed by solution of 1 mg l⁻¹ free chlorine as a positive control, were deemed to be stained with DiBAC₄(3), but were not stained (Fig. 5.5d).

Fig. 5.5 Flow cytometry dot plots of viability analysis of *E. coli* cells ($\mu=0.2 \text{ h}^{-1}$) in batch culture by membrane potential dye of DiBAC₄(3). Subpopulations of $\Delta\Psi^+$ cells (Q 1) and $\Delta\Psi^-$ cells (Q2): *E. coli* cells exposed to residual free chlorine concentration of 0.01 mg l^{-1} in contact time (min); a, 3; b, 10; c, control sample (live cells); d control sample (dead cells). Green fluorescence is due to accumulating of DiBAC₄(3) on the dead/depolarized cells. DiBAC₄(3) fluorescence was measured in fluorescence channel FL1.

Fig. 5.6 shows inactivation curves for *E. coli* cell population with $\mu=0.9 \text{ h}^{-1}$ and $\mu=0.2 \text{ h}^{-1}$, which were plotted as log inactivation of cells versus CT values. The data for inactivation of *E. coli* cells in Fig. 5.6 were derived from flow cytometry analysis of cells stained with membrane potential probe of DiBAC₄(3). The inactivation curves plotted for slow and fast growing cell population show that the population with $\mu=0.9 \text{ h}^{-1}$ was more susceptible to chlorine than the population with $\mu=0.2 \text{ h}^{-1}$ at different CT values (Fig. 5.6). Therefore, log inactivation of *E. coli* cell population determined by DiBAC₄(3) dye for each data point (Fig. 5.6) was underestimated in comparison with the log inactivation determined by PI (Fig. 5.2). The log inactivation of *E. coli* cell population with $\mu=0.9 \text{ h}^{-1}$ determined by DiBAC₄(3) at CT value of $0.09 \text{ mg. min l}^{-1}$ was 1.5 ± 0.09 (mean $\pm$ standard error), whereas the log inactivation for the same population determined by PI was 2.2 ± 0.17 (mean $\pm$ standard error).

Fig. 5.6 Inactivation kinetics of *E. coli* cells grown in batch culture at specific growth rate of 0.2 h^{-1} (dash dot line ●) and 0.9 h^{-1} (solid line ♦) with chlorine. Inactivation analysis of *E. coli* cells (losing viability) were done by membrane potential dye of DiBAC₄(3) in combination with flow cytometry. Each datum point is ratio of the percentage of surviving cells to the percentage of initial viable cells from the total population in logarithmic scale. Error bars represents standard error of the mean measurement ($n=3$).

5.3.3 Inactivation analysis by respiratory activity probe

Fig. 5.7 shows the flow cytometry dot plots from the analysis of *E. coli* cells with $\mu=0.2 \text{ h}^{-1}$, which were exposed to 0.01 mg l^{-1} of free chlorine in different contact times and stained with CTC, subsequently. There were two subpopulations detected in a population of *E. coli* cells; the CTC-reducing cells (CTC^+) with red fluorescence (Fig. 5.7c, R1) and CTC-non-reducing cells (CTC^-) (Fig. 5.7c, R2). The fraction of respiratory active cells during 3 and 10 min exposure to 0.01 mg l^{-1} free chlorine were 55% and 2%, respectively.

Fig. 5.7 Flow cytometry dot plots of viability analysis of *E. coli* cells ($\mu=0.2 \text{ h}^{-1}$) in batch culture by respiratory activity indicator of CTC. Subpopulations of CTC⁺ cells (region R2) and CTC⁻ cells (region R1): *E. coli* cells exposed to residual free chlorine concentration of 0.01 mg l^{-1} in contact time (min); a, 3; b, 10; c, control sample (live cells); d control sample (dead cells). Red fluorescence is due to CTC reduction. CTC was measured in fluorescence channel FL3.

Fig. 5.8 Inactivation kinetics of *E. coli* cells grown in batch culture at specific growth rate of 0.2 h^{-1} (dash dot line ●) and 0.9 h^{-1} (solid line ♦) with chlorine. Inactivation analysis of *E. coli* cells (losing viability) were done by tetrazolium chloride (CTC) dye in combination with flow cytometry. Each datum point is ratio of the percentage of surviving cells to the percentage of initial viable cells from the total population in logarithmic scale. Error bars represents standard error of the mean measurement ($n=3$).

The inactivation curves for slow- and fast growing cell populations during their exposure to different concentrations of free chlorine for different contact times are shown in Fig. 5.8. These data were derived from flow cytometry analysis of cells stained with respiratory activity dye of CTC. The log inactivation determined by CTC method for *E. coli* cell population with $\mu=0.9 \text{ h}^{-1}$ and $\mu=0.2 \text{ h}^{-1}$ at CT values of $0.06 \text{ mg min l}^{-1}$ were 1.83 ± 0.09 (mean $\pm$ standard error) and 0.88 ± 0.04 (mean $\pm$ standard error), respectively (Fig. 5.8).

Fig. 5.9 Log inactivation of cells based on membrane integrity test versus log inactivation of cells based on respiratory activity assay.

There was a clear linear correlation with correlation coefficient of 0.98 (p value >0.0005) between log inactivated of *E. coli* cells measured by evaluation of membrane integrity with PI and log inactivated of *E. coli* cells measured by respiratory activity using CTC (Fig. 5.9).

5.4 Discussion

The finding of this research suggested that genetically identical bacterial cells can exhibit different levels of susceptibility to chlorine due to their physiological differences. The susceptibility of bacterial cells to chlorine was associated with specific growth rate of a population. Membrane integrity and respiratory activity of cells were two suitable cell parameters for assessment of inactivation of *E. coli* cells by chlorine using flow cytometry technique.

5.4.1 Specific growth rate and bacterial cells susceptibility to chlorine

The higher resistance of slow-growing rather than fast-growing cells of genetically identical bacterial cells to chlorine was reported by Taylor et al. (2000). The results of this study clearly demonstrated that susceptibility of *E. coli* cells to chlorine was strongly correlated to the specific growth rate of population (Fig. 5.2, Fig. 5.3 and Fig. 5.4). The data show higher susceptibility to chlorine in the faster-growing bacteria cells (Fig. 5.2), which are in consistent with the data of Taylor et al. (2000). Chlorine concentration and contact time are two main variables in the kinetics of bacterial cells inactivation. CT value required to achieve 2-log inactivation (CT_{99}) in slow-growing population of *E. coli* was $0.1 \text{ mg min l}^{-1}$ (Kim et al., 2008), $0.085 \text{ mg min l}^{-1}$ (Cho et al., 2010) and $0.09 \text{ mg min l}^{-1}$ (Taylor et al., 2000). In this study, CT_{99} for slow-growing population of *E. coli* (Fig. 5.2) was approximately same as the previous studies.

It was reported that a fraction of *E. coli* cell population can enter a “persister” state with lower growth rate and higher resistance to stress such as antibiotic treatment (Balaban et al., 2004). The differences of log inactivation between *E. coli* cell populations with $\mu=0.9 \text{ h}^{-1}$ and $\mu=0.2 \text{ h}^{-1}$ at CT value of $0.06 \text{ mg min l}^{-1}$ were suggested the presence of a subpopulation of *E. coli* cells with low susceptibility to chlorine in the later one (Fig. 5.2). There were approximately three subpopulations in respect to sensitivity to chlorine; 1) cells with lower resistance to chlorine, which after 3 min exposure to 0.009 mg l^{-1} chlorine were deactivated (existed in both slow and fast-growing populations); 2) cells with moderate resistance to chlorine, which after 6 min exposure to 0.009 mg l^{-1} chlorine were deactivated (existed merely in fast-growing population); 3) cells with higher resistance “persister” to chlorine, which after 6 min exposure to 0.009 mg l^{-1} chlorine were not deactivated (existed only in slow-growing population) (Fig. 5.3 and Fig. 5.4). The presence of a resistant cell subpopulation in the slow-growing *E. coli* population to chlorine at sub-lethal CT value of $0.06 \text{ mg min l}^{-1}$ suggested that the slow-growing population was more heterogeneous with respect to sensitivity to chlorine.

Chlorination plays an important role in the wastewater treatment as well as drinking water treatment. One of the parameters in calculation of chlorine demand of water is the function of chlorine as an antimicrobial agent (Warton et al., 2006). The growth rate of bacterial cells is associated with the nutritional conditions of environment (Brown et al., 1990). Bacterial cells in eutrophic system of wastewater with rich-nutrient condition have basically higher growth rate than their genetically identical counterparts in oligotrophic system of drinking water with a low-nutrient environment. Since, bacteria with higher growth rate are more susceptible to the chlorination. It could be speculated that for the inactivation of genetically identical waterborne pathogenic bacteria, the chlorine demand in wastewater treatment system is generally lower than the treatment systems of drinking water.

5.4.2 The effect of chlorine on the cell viability indicators

Chlorine as nonselective oxidant reacts with various extrinsic and intrinsic cellular components and disrupts major metabolic processes of cells. The predetermined parameters for the assessment of inactivation of *E. coli* cells by chlorine were membrane integrity, membrane potential and respiratory activity. However, it was revealed that the chlorine itself had substantial adverse effects on membrane potential parameter of cell (Fig. 5.5d). The adverse effects on the assessment of viability in chlorinated *E. coli* cells using membrane integrity and respiratory activity parameters appeared to be negligible.

It was stated that free chlorine continuously penetrates into cell and reacts with intracellular components without involving significant surface damage and changes in cell permeability (Cho et al., 2010). The interior of membrane of intact bacterial cells are negatively charged due to the existence of membrane potential (Novo et al., 2000). Therefore, the electrically neutral hypochlorous acid (HOCl) formed by reaction of chlorine with water, can penetrate into the intact cells rather than negatively charged form of hypochlorite ions (OCl^-). Hypochlorous acid (HOCl) as a multi-targeted reactor may damage cell membrane due to reaction with unsaturated bonds of membrane lipids. Therefore, disrupting lipid bilayers of

bacteria membrane by chlorine could increase the membrane permeability and result in the inactivation of cells.

The DiBAC₄(3) dye was reported as a robust viability indicator for bacterial cells (Rezaeinejad and Ivanov, 2010). The DiBAC₄(3) probe was used to assess viability in chlorine disinfected *E. coli* cells (Lisle et al., 1999). The present study revealed that the viability of chlorinated bacterial cells could not be assessed effectively by DiBAC₄(3) probe. *E. coli* cells killed by a lethal concentration of chlorine were hypothetically deemed to accumulate the DiBAC₄(3) and to be detected as the green fluorescence (Fig. 5.5d, Q2). However, neither highly chlorinated (Fig. 5.5d) nor non-chlorinated cells (Fig. 5c) were detected by DiBAC₄(3). The same sample stained with LIVE/DEAD kit showed almost 100% of cells were stained with PI (Fig. 5.1d, R1). The entire population of *E. coli* cells lost their membrane integrity upon their exposure to 1 mg l⁻¹ chlorine for 10 minutes, but nucleic acids were not damaged. In bacterial cells losing membrane integrity is not reversible and results losing viability or cell death (inactivation) (Nystrom, 2003). Bacterial cells were supposed to accumulate dye of DiBAC₄(3) in their intracellular space upon losing their viability. The lipid components of membrane in the highly chlorinated cells (Fig. 5.5d) might be damaged by multi-targeted oxidizing agent of chlorine, and might lose their ability to bind to the lipophilic dye of DiBAC₄(3). Therefore, underestimation of log inactivation by DiBAC₄(3) dye (Fig. 5.6) was probably due to damaging of lipid components of membrane by chlorine in a fraction of *E. coli* cell population, which disable the cell components to bind with the dye.

The strong linear correlation between log inactivated measured by PI and CTC (Fig. 5.9) indicates that chlorine has not any adverse effects on the assessment of viability by respiratory activity indicator. However, it was reported that the respiratory activity in bacterial cells dropped significantly in long-term starvation (Rezaeinejad and Ivanov, 2010). Therefore, we suggest that measurement of membrane integrity by PI in combination with flow cytometry might be the most practical alternative method for evaluation of bacterial cells inactivation by chlorine.

Correlation between the specific growth rate of bacterial cells and sensitivity of cells to disinfectants is just one aspect related to disinfection kinetic studies from the physiological point of view. There are many important unanswered questions regarding growing resistance of pathogenic bacteria in environment by the alteration of their physiology or phenotype. The role of physiological states of bacterial cells on their susceptibility to disinfection should be explored in more details by taking advantages of techniques such as flow cytometry. In future, water microbiology laboratory should undergo vital amendment with a need for rapid technique of flow cytometry to replace conventional HPC plate counts method. The new rapid methods, which are being improved in research laboratories can be exploited in general water microbiology laboratories for public health. This can lead to a profound improvement in the way, which environmental microbiology laboratory generally works.

5.5 Conclusions

- Physiological state of individual bacterial cell in a population of genetically identical cells determines the level of susceptibility to chlorine.
- There was a correlation between specific growth rate and sensitivity of *E. coli* cells to chlorine disinfection. The slow-growing *E. coli* cells ($\mu=0.2 \text{ h}^{-1}$) was more recalcitrant than fast growing cells ($\mu=0.9 \text{ h}^{-1}$) to chemical inactivation.
- The adverse effects of chlorine on viability indicator of membrane integrity and respiratory activity was insignificant, whereas highly chlorinated *E. coli* cells could not stained by DiBAC₄(3) dye as membrane potential viability indicator.
- Flow cytometry provides a tool for evaluation of kinetics and mechanism of bacterial disinfection by chemical agent, and as a result could be applied as an alternative technique for cultivation-based heterotrophic plate counts in general laboratory for assessment of microbial quality of water.

CHAPTER 6

PHYSIOLOGICAL HETEROGENEITY IN MICROBIAL GRANULES

6.1 Introduction

Microbial biofilms, granules and activated sludge flocs have an important role in water and wastewater treatment systems. Although, the structural heterogeneity (Ivanov, 2006; Ivanov et al., 2008), chemical heterogeneity (Werner et al., 2004) and genetic heterogeneity (Kirisits et al., 2005; Stewart and Franklin, 2008) in biofilms and granules have been well documented, limited studies have been done in the physiological heterogeneities within microbial communities. It is known that individual microorganisms within these microbial aggregates are not identical in terms of physiological states (Xu et al., 1998; Ivanov et al., 2008, 2010; Simoes et al., 2008; Stewart and Franklin, 2008; Muller et al., 2010).

Physiological heterogeneity in microbial aggregates is usually attributed to the genotypic diversity and gradients of nutrients and metabolites inside the cell aggregate. However, even within a cellular aggregate of pure culture the physiological heterogeneity of bacterial aggregates can also be detected. The packing of cells within the microbial aggregate causes the concentration gradient of nutrients from the periphery to the core. Therefore, the nutrients are depleted in the core of biofilms due to the limitation of access of the substrate by diffusion. Oxygen also is depleted from the upper layer toward the core of biofilms. Metabolic products in contrast with nutrients and oxygen are more concentrated inside than outside biofilms. In general, heterogeneity enforced by micro-environmental conditions and chemical heterogeneity caused the physiological heterogeneity of microbial aggregates (Stewart and Franklin, 2008; Muller et al., 2010).

Physiological heterogeneity of microbial aggregates is also caused by cell cycle phases, life cycle or age distribution of microbial cells (Muller et al., 2010).

More knowledge describing the physiological heterogeneity within biofilms and biogranules is vital in understanding their key functions and in formulating control strategies of processes such as wastewater treatment. Quantification of physiological heterogeneity of microbial aggregates may help control problems associated with biofilms such as biofouling. Aerobically growing aggregates of *Pseudomonas veronii* (*P. Veronii*) strain can be used effectively for wastewater treatment (Ivanov et al., 2006; Ivanov et al., 2008). Genetically modified microorganism such as GFP-tagged bacteria as indicator enables detection of specific bacteria strain in environmental sample such as water. Molecular probes such as PI and CTC in combination with fluorescence microscope are suitable approach for assessment of physiological heterogeneity of biofilms and biogranules (Tay et al., 2003; Stewart and Franklin, 2008).

The fluorescence based technique of confocal laser scanning microscope (CLSM) was used in analysis of microbial granules. Molecular probes of PI, DiBAC₄(3) and CTC were used to study the physiological heterogeneity of aerobic biogranule. The aim of this chapter is to assess the distribution pattern of respiratory active, viable and dead cells within bacterial granule of *Pseudomonas veronii*.

6.2 Materials and Methods

6.2.1 Microorganism, selection of seeds to facilitate formation of microbial aggregates

Strain of *Pseudomonas veronii*, wild type, DSM No 11331, was obtained from German Collection of Microorganisms and Cell Cultures (DSMZ). In order to construct gfp-tagged bacterial cells, electrocompetent *P. veronii* cells were prepared using standard molecular biology methods. The plasmid of pJBA28 was electroporated into the electrocompetent *P. veronii* cells. The green fluorescent protein (GFP) gene contained in the gfp-tagged *P. veronii* was under the control of a kanamycin-inducible promoter.

Bacterial cells of *P. veronii* were grown in 100 ml flasks (Schott Duran, Germany) in Nutrient broth medium (Oxoid, Hampshire, England) in a shaker incubator at 30°C and 150 rpm. An aliquot of cells suspension was sampled after overnight culture. In order to select the best aggregates, 1 ml of cells suspension was diluted in 9 ml of phosphate buffer solution (PBS) in a conical tube and left quiescent for 10 min to settle down the larger aggregates. An aliquot of cell suspension and aggregates from the bottom of conical tube was transferred in Nutrient broth medium and incubated for 48 hours in shaker incubator at 30°C and 150 rpm. An aliquot of the cell suspension was sampled and washed twice with sterile phosphate buffered saline (PBS) (pH 7.2) by centrifugation for 10 min at 3398×g. The above procedure for selecting the best aggregates was repeated at least 10 times to select rigid aggregates as seed.

6.2.2 Cultivation and formation of aerobic granule

The selected colony of *P. veronii* strain from the seed that prepared in the abovementioned procedure was transferred to 500 ml flasks (Schott Duran, Germany), which consisted of Nutrient broth medium (Oxoid, Hampshire,

England). The granules in batch culture were formed in shaker incubator at 30°C and 150 rpm by daily replacement of fresh medium in aseptic condition. The microbial granules of *P. veronii* were produced up to size of 1000 µm within 10 days of cultivation.

6.2.3 Staining of microbial granules with PI and DiBAC₄(3) simultaneously

Propidium iodide (PI), 20 mM and DiBAC₄(3) 250 µM were obtained from Molecular Probes (Invitrogen, Eugene, Oreg.). The microbial granules were transferred to microcentrifuge tubes and stained with 10 µl of solution of dye mixture (6 µl of the PI (20 mM) and 4 µl of DiBAC₄(3)). The microbial granules were incubated at 30°C in dark for 1 hour. The granules were washed twice in PBS and re-suspended in PBS.

6.2.4 Staining of microbial granules with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC)

An aliquot of 100 µl of the stock solution (50 mM) of CTC (Polyscience, Inc., Warrington, PA, USA) was added to a test tube containing microbial granules and incubated for 1 hour at 30°C in dark with agitation (150 rpm). The granules were washed twice with PBS and re-suspended in PBS.

6.2.5 Confocal laser scanning microscopy (CLSM) and image analysis

Stained cells were viewed using a Nikon A1R confocal laser scanning microscope (CLSM) (Nikon, Tokyo, Japan) with a 100× objective lens. Green and red fluorescences were excited by a 40 mW argon laser at 488 nm. Fluorescent light was separated with 570 nm splitting filter and detected in the channel with a band pass filter 525/575 nm and in the channel with a band pass filter 595/645 nm. The NIS-Elements-AR software was used for analysis of depicted image by CLSM.

6.3 Results

6.3.1 Assessment of distribution of respiratory active cells in aerobic granule

The spatial pattern of respiratory activities within the cellular aggregate (granule) of *P. veronii* stained with CTC was analyzed by CLSM.

Fig. 6.1 Aerobic granule of *P. veronii* stained with CTC and visualised by confocal laser scanning microscope, the scale bar indicates 200 μm.

Fig. 6.2 Spatial distribution of respiratory active cells stained with CTC, distance between granule-nutrient interface stratum on the periphery of the granules to the core of *P. veronii* granule.

Fig. 6.1 shows the respiratory activity patterns within the *Pseudomonas veronii* granules by red fluorescence of CTC reduction product. The spatial pattern of respiratory activities within the cellular aggregate (granule) of *P. veronii* stained with CTC was analyzed by CLSM (Fig. 6.2). It demonstrated the presence of three distinct layers: 1) the outer layer of cells with very low respiratory (about zero) and, probably, growth activities in the depth of 20 μm from the edge of the aggregate; 2) the intermediate layer of cells with the highest respiratory, and probably, growth activities between 20 to 200 μm from the edge of the cellular aggregate; 3) cells with the lowest respiratory and growth activities in the core of cellular aggregate.

6.3.2 Assessment of distribution of dead cells in aerobic granule by multi-parametric staining

The granule of *P. veronii* was stained with PI and DiBAC₄(3) simultaneously. The spatial distribution of dead cells within the granule was analyzed by CLSM.

Fig. 6.3 Aerobic granule of *P. veronii* stained with DiBAC₄(3) and PI simultaneously and visualised by confocal laser scanning microscope, the scale bar indicates 200μm.

Fig. 6.4 Spatial distribution of dead cells stained with DiBAC₄ from one edge to the opposite edge of aerobic granule of *P. veronii*.

Fig. 6.5 Spatial distribution of dead cells stained with PI from one edge to the opposite edge of aerobic granule of *P. veronii*.

Fig. 6.3 shows the *P. veronii* granule image, which was stained simultaneously with PI and DiBAC₄(3) for assessment of spatial distribution of dead cells and depicted by CLSM. Fig. 6.4 shows that there is a sharp peak of green fluorescent intensity in the periphery and relatively low fluorescent intensity on the interior of granule of *P. veronii* due to staining with DiBAC₄(3). The same pattern of fluorescent intensity profile was observed in Fig 6.5 due to staining of the *P. veronii* granule with PI. A thin stratum of dead cells (<20 µm) observed in the outside edge of the granule (Fig. 6.4 & Fig. 6.5), while the interior of granule was shown with relatively lower but heterogeneous distribution of dead cells in different strata of the *P. veronii* granule.

6.3.3 Assessment of distribution of dead cells in gfp-tagged *Pseudomonas veronii* aerobic granule

Fig. 6.6 shows the image of gfp-tagged *Pseudomonas veronii* granule, which was stained with PI and visualized by CLSM. The entire cells of gfp-tagged *P. veronii* exhibit bright green fluorescence in the granules due to producing of green fluorescence protein. Dead gfp-tagged *P. veronii* cells exhibit red fluorescence due to staining with PI. Fig 6.7 shows the spatial distribution of live and dead cells gfp-tagged *P. veronii* in the granule. The distribution of live bacterial cells was relatively higher in the periphery than the core of the granule.

Fig. 6.6 Aerobic granule of gfp-tagged *P. veronii* stained with PI and visualised by CLSM, green fluorescence is due green fluorescence protein and non-stained cells, and red fluorescence is due to staining of cells with PI. The scale bar indicates 100 μm .

Fig. 6.7 Spatial distribution of dead cells stained with PI (■) and live cells (▲) from one edge of to the opposite edge of gfp-tagged *P. veronii* aerobic granule. The solid trendline is for data of live cells (▲) and dash trendline is for data of dead cells (■) with 10 periods of moving average.

6.4 Discussion

Pure culture biofilms and granules are more appropriate than complex biofilms to study physiological heterogeneity. Pure culture biofilms of *Pseudomonas aeruginos* (Stewart et al., 1993) and *Vibrio paraheamolyticus* (Lawrence et al., 1991) were used for structural heterogeneity studies. However, to my knowledge it is for the first time that the pure culture of biogranules is formed to study physiological heterogeneity.

In the present study, the physiological heterogeneity of cellular aggregates of *Pseudomonas veronii* in terms of respiratory activity were visualized by CTC staining coupled with the CLSM technique. The results show that respiration and growth activities are not homogenously distributed inside the bacterial aggregate of *P. veronii*. The faster-growing cells generally lie on the periphery, visualized with lower respiratory activity comparing to the highest respiratory active cells located in some distance from the periphery of granules. The presence of non-respiratory active cells in the core of granules might be attributed to the dormant state of cell or cells with the lower state of energy, which are unable to reduce CTC to its fluorescence product within CLSM detection limit.

It is known that slow respiring and growing cells are most resistant to toxic substances and adverse conditions. Therefore, the low respiratory activity of the outer layer of cells indicates the lower sensitivity of the microbial aggregates to toxic substances in comparison with the sensitivity of the suspended cells. The cells in deeper layers of granules are less susceptible to unpleasant condition such as toxic chemicals or other environmental stresses due to their lower growth rate and also the reaction-diffusion limitation of toxic chemicals to deeper lying cells. Since, metabolically active cells contribute bigger shares in the system productivity and biomass turnover processes. Therefore, the presence of respiratory active cells in deeper strata of granule may contribute more in biotechnological processes.

A narrow stratum of dead cells similar to a membrane for biogranules was observed in the edge of granule by multi-parametric staining (Fig. 6.4 and Fig. 6.5). This was in agreement with Fig. 6.2, in which a narrow stratum of cells with almost zero respiratory activity was similarly observed in the edge of the granule. Individual bacterial cells are dynamic within the granule and most probably dead cells migrate from the interior to the exterior of the granule to detach from granule. This stratum of dead cells on the periphery of the granule before detachment temporarily provide a protective shield against detrimental effects of surrounding environment in the inner strata of the granule for active and viable members of community.

GFP requires oxygen for activation of fluorescence (Cubitt et al., 1995). However, it has reported that the application of GFP for aerobic bacteria was not restricted in very low concentration of oxygen (Hansen et al., 2001). To my knowledge, in this study for the first time the GFP tagged granules were produced. Dead cells and viable cells were distributed heterogeneously within gfp-tagged *P. veronii* aerobic granule from edge to core of the granule.

The changes in physio-chemical and biological environment of bioreactor determine the physiological heterogeneity of granule. Therefore, more knowledge about physiological heterogeneity within biofilms and granules give useful insight to direct attempts for optimization and control of the processes that mediated by biofilms and granules in the treatment of water and wastewater.

6.5 Conclusions

- Staining of bacterial strain of *P. veronii* granule by fluorescent dye of CTC showed that even bacterial granules of pure culture are physiologically heterogeneous in terms of respiratory activity.
- A narrow stratum of dead cells, similar to a skin for protection of viable and active cells in the interior strata was observed in the edge of *P. veronii* granule.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

This research has been carried out to contribute to our knowledge of microbial physiology in population and community levels with standpoint of environmental microbiology and biotechnology fields. A number of conclusions have been drawn and future study directions emerged from this research.

7.1 Conclusions

The structural and functional single-cell analysis for assessment of physiological heterogeneity of microbial populations and communities were carried out effectively using flow cytometry and confocal laser scanning microscope. Analysis of different functional parameters coupled with various fluorescent probes revealed that membrane integrity is the most practical indicator for the assessment of viability in *S. cerevisiae* cell population. PI as a membrane integrity stain and DiBAC₄(3) as the membrane potential probe were used efficiently for assessment of cell viability in batch culture of *S. cerevisiae*. Multi-parametric flow cytometry using PI and DiBAC₄(3) simultaneously was effective to discriminate between different physiological states. Several subpopulations of intact polarized, intact depolarized and dead cells were detected by the multi-parametric analysis of *S. cerevisiae* population. The yeast *S. cerevisiae* cell population was heterogeneous in terms of energetic states. Two subpopulations with high and low activities of ADH were determined in growing population of *S. cerevisiae*.

Bacterial population of *E. coli* was heterogeneous in terms of membrane potential and respiratory activity under growth and starvation conditions. *E. coli* cell population upon encountering to starvation was more physiologically heterogeneous than growing *E. coli* cell population. The share of respiratory active *E. coli* cells after long-term starvation dropped to 5%, while about 50% of cells detected viable

by membrane potential and membrane integrity indicators. Bacterial cells in environment are often facing long-term starvation. This leads the respiratory activity of cells to drop significantly. Hence, respiratory activity is not a proper criterion for assessment of viability of bacterial cells in environmental samples.

One of the potential engineering aspect addressed in this study was whether chlorine disinfection of drinking water has any detrimental effects on viability assessment of bacterial cells. The adverse effects of chlorine on viability indicator of membrane integrity and respiratory activity was insignificant, whereas the membrane potential of highly chlorinated *E. coli* cells could not be detected by DiBAC₄(3) probe. There were different subpopulations in terms of the level of susceptibility to chlorine in *E. coli* cell population. Physiological states of individual microbial cells in a population of genetically identical cells determine the level of susceptibility to antimicrobial agent such as chlorine. There was a correlation between specific growth rate and sensitivity of *E. coli* cells to chlorine disinfection. The slow-growing *E. coli* cells were more recalcitrant than fast growing cells to chemical agents. Flow cytometry provides a tool for evaluation of kinetics and mechanism of bacterial inactivation by disinfectants. Therefore, flow cytometry could be used as an alternative technique for cultivation-based heterotrophic plate counts in general laboratory for assessment of microbial quality of water.

The other relevant theme to environmental engineering was the assessment of physiological heterogeneity in self-aggregated *P. veronii* granule. Staining of *P. veronii* granule by fluorescent dye of CTC showed that even pure bacterial granules are physiologically heterogeneous in terms of respiratory activity. A narrow stratum of dead cells was observed in the edge of *P. veronii* granule by simultaneous staining of the granule with PI and DiBAC₄(3). The highest respiratory active cells were located in some distance with periphery of granule and cells with lower respiratory activity were observed in the core of granule. The narrow stratum of dead cells in the outer surface of granule can probably protect viable and high

respiratory active cells in the interior edge of the granule from detrimental and toxic effects of substrates.

7.2 Recommendations for future work

This research underscores the importance of physiological studies to the environmental engineering processes of bio-granulation and also inactivation of pathogenic bacteria in drinking water. It may not be possible to use the knowledge of physiological studies to directly improve the efficacy of engineered biotechnological process of microbial granules or disinfection process of drinking water. However, more knowledge about physiology of individual cells in microbial populations and communities will help in understanding the role of microbial physiology in the treatment of waste and also bacterial survival against antimicrobial agents.

Physiological heterogeneity of microbial populations and communities is partially enforced by micro-environment conditions. Future work should elucidate in detail the role of environmental parameter, e.g. starvation and salinity, in physiological adaptation of individual microbial cells of environmental matrix. Functional parameters for analysis of physiological states of individual microbial cells by techniques such as flow cytometry should be applied as an alternative approach to define thoroughly the physiological state of “viability”. Multi-parametric flow cytometry should be improved for better discrimination between different physiological states of microbial cells.

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