EFFECT OF EXPOSURE TIME, BACTERIA CONCENTRATION AND CULTURE AGE OF *ESCHERICHIA COLI* AND *BACILLUS SUBTILIS* ON THE GLASS SURFACE

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NASUHA BINTI IBRAHIM

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SUPERVISOR'S DECLARATION

We hereby declare that we have checked this thesis and in our opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Biotechnology).

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STUDENT'S DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged. The thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

Signature :
Name : NASUHA BINTI IBRAHIM
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Date : JANUARY 2013

Dedication

Dedicated To

My

Parents;

Mr.Ibrahim Bin Mamat

and

Mrs. Noor Asni Binti Awang

Sister;

Natasha Binti Ibrahim

Brothers;

Ismail Bin Ibrahim

Ikhwan Bin Ibrahim

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ABSTRACT

This thesis presents the effect of the bacterial adhesion on the glass surface (hydrophilic surfaces) at different time exposure and bacterial concentration. The ability of *Escherichia* coli and *Bacillus subtilis* to attach to the surfaces depends mainly on the interaction of hydrophobic domains. However, *E. coli* and *B. subtilis* have evolved in different ways in order to manipulate the hydrophobic effect for their adherence on the solid surface. On the other hand, the surface properties e.g surface charges are inherently important and often regulate the mechanism of the bacteria adhesion. Besides that, adhesions of bacteria were also affected by culture media, exposure time of bacteria on glass surface, age and bacterial concentration. Both bacteria have different surface characteristic which also affect adhesion on the glass surface. Both bacteria were suspended in the phosphate buffer solution (pH 7.1) at different cell concentration (abs). The solution was suspended into glass container containing glass slide. The glass-bacterial solution was shake at 100 rpm and 30^0C in the incubator shaker and sampling were done at 4 h, 8 h, 12 h and 24 h. From the researches that have been done *B. subtilis* easily adhere on the glass surface compared to *E. coli*, with 46.9% reduction in optical density reading observed at 600nm. *Bacillus subtilis* was exposed for 24 hour at cell concentration 0.8 abs. Meanwhile, *E. coli* result in less adhesion to the glass surface with only 29.8 % reduction in optical density. Yet, the time of exposure for *E. coli* was only 12 hour with cell concentration 1.0 abs.

ABSTRAK

Tesis ini membentangkan kesan lekatan bakteria pada permukaan kaca (permukaan hidrofilik) pada pendedahan masa yang berbeza dan kepekatan bakteria yang berbeza. Keupayaan *Escherichia coli* dan *Bacillus subtilis* untuk melekat pada permukaan bergantung terutamanya kepada interaksi domain hidrofobik. Walau bagaimanapun , *E. coli* dan *B. subtilis* berinteraksi dengan cara yang berbeza untuk memanipulasi kesan hidrofobik untuk pelekatan mereka di permukaan pepejal. Sebaliknya, sifat-sifat permukaan seperti caj permukaan sememangnya penting dan sering mengawal mekanisme lekatan bakteria . Di samping itu, pelekatan bakteria turut terjejas olehfaktor sekeliling, masa dedahan bakteria pada permukaan kaca , umur dan kepekatan bakteria. Kedua-dua bakteria tersebut mempunyai ciri permukaan yang berbeza yang juga mempengaruhi lekatan pada permukaan kaca. Kedua-dua bakteria dimasukkan dalam penyelesaian penimbal fosfat (pH 7.1) pada kepekatan sel yang berbeza (abs). Bakteria yang dicampur dengan Phosphate buffer solution (PBS) telah dimasukkan ke dalam bekas kaca yang mengandungi kepingan kaca. Kepingan kaca-bakteria digoncang pada 100 rpm dan 30 ° C dalam penggoncang inkubator dan pemerhatian pelekatan bakteria pada kepingan kaca dilakukan pada jam ke-4 , ke-8, ke-12 dan ke-24 . Dari kajian yang telah dilakukan *B. subtilis* lebih mudah melekat pada permukaan kaca berbanding dengan *E. coli*, dengan pengurangan 46.9 % dalam membaca ketumpatan optik diperhatikan pada 600nm . *B. subtilis* telah didedahkan selama 24 jam di kepekatan sel 0.8 abs. Sementara itu , *E. coli* kurang lekatan ke permukaan kaca dengan pengurangan hanya 29.8 % dalam ketumpatan optik. Namun , masa pendedahan bagi *E. coli* adalah hanya 12 jam dengan kepekatan sel 1.0 abs.

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LIST OF ABBREVIATION

E. coli	*Escherichia coli*
B. subtilis	*Bacillus subtilis*
OD	Optical Density
Abs	Absorbance
PBS	Phosphate Buffer solution
T_0	Time at 0^{th} hour
T_4	Time at 4^{th} hour
T_8	Time at 8^{th} hour
T_{18}	Time at 18^{th} hour
T_{24}	Time at 24^{th} hour

1 INTRODUCTION

1.1 Motivation and problem statement

A fundamental question often asked ‘why do microorganisms stick to a surface?’ The prime directive of microorganism is to reproduce and to do so they must assimilate nutrient in sufficient amount to ensure that the process is successful. Almost all biological processes require an aqueous environment including the transport of nutrient into the microbial cell. Bacteria adhesion is the initial step of colonization and formation of biofilm. It causes an accumulated biomass of microorganism and extracellular material on certain area of the solid surfaces, where it depend on a number of microbiological, physical, chemical and material-related parameters. The ability to stick onto a surface would immediately provide several advantages to ensure reproduction in a nutrient limiting environment. Microbial adhesion is not limited to hard, intimate surfaces, but applicable even to soft tissues. For instance, human skin intestinal and pulmonary lining and urinary tract are all colonizable by microorganism which may result in pathologies

Over the past few decades, biofilm formation has been observed in many industrial and domestic domains. Unfortunately, in most cases the growth of biofilms has been detrimental, where many industries suffers the ill-effects of biofilm growth which result in heavy costs in cleaning and maintenance. Industries such as maritime, dairy (Yoo, 2002), food (Ganesh. 1998), water systems (Bott, 1998), oil (Nemati, 2001), paper (Klahre, 2000), opticians (Liesegang, 1997), dentistry (Marotta, 2002) and hospitals (Halabi, 2001) which often involved billions of dollars for cleaning and maintenance services . Perhaps the environment where people are exposed to biofilms most frequently is the domestic environment (Baker, 2000). Product spoilage, reduced production efficiency, corrosion, unpleasant odours (malodours), unsightliness, infection, pipe blockages and equipment failure are examples of the detrimental effects of biofilms. For these reasons and the emergence of restrictive legislation regarding the effects of cleaning agents on the environment and to user health and safety (Commission Regulation EC No. 1048/ 2005),

there is a lot of industrial interest in developing materials and methods which can remove and actively prevent the formation of biofilms.

In the UK, it is estimated that 9 million cases of intestinal disease every year, much of which originates at home, where human excreta are the primary source of infection (Curtis, 2003). Estimates show that for every case of infectious disease reported to the Communicable Disease Surveillance Centre (CDSC), 136 unreported cases occur in the community causing considerable morbidity. In the food industry biofilms cause serious engineering problems such as impeding the flow of heat across a surface, increases in fluid frictional resistance of surfaces and increases in the corrosion rate of surfaces leading to energy and production losses. Pathogenic microflora grown on food surfaces and in processing environments can cross-contaminate and cause post-processing contamination (Verran, 2000). If the microorganisms from food-contact surfaces are not completely removed, they can lead to mature biofilm formation and so increase the biotransfer potential. Examples of the food sectors that pay particular attention to the possibility of cross-contamination are the milk industry (Chye, 2004) and the slaughter industry.

Virulence and pathogenicity of microorganisms is often enhanced when growing as a biofilm, and new strategies are therefore required to control biofilm formation and development. Many pathogenic microorganisms reside within biofilms, which biofilms cause additional problems when designing new anti-microbial agents. Novel strategies are necessary because of the limitations to these current treatments such as inadequate control supply, potential for disease transfer and compliance issue. The capability and high resistance of sessile microorganisms to inhibitors, eradication of biofilm often requires high concentration of disinfectants or antibiotics, causing severe environmental damages, multi-resistance emergence and nosocomial infections. Public health concerns, as well the economic loss associated to biofilm formation raise an urgent need for developing biofilm resistant systems.

The adhesion of bacteria on the solid surfaces have causes a lot of problems. Indeed the adhesive characteristics of natural human flora are now considered as a tool for preventing the adhesion of pathogenic bacteria to avert infection. To eliminate this

problem, studies on developing the anti-adhesive surfaces, incorporation of anti bacteria agent into medical device polymer, mechanical design alternative and produce antibiotic had bloomed significantly (Geesey, 2001; von Eiff *et al.*, 2002; Vincent, 2003; Lejeune, 2003). The attachment of microorganisms to surfaces and the subsequent biofilm development are very complex processes, affected by several variables such as surface roughness, chemical stability, hydrophobicity and surface charge (Donlan, 2002). In general, attachment will occur most readily on surfaces that are rougher, more hydrophobic, and coated by surface conditioning films (Martial, & Degraeve, 2008, Simo˜es, 2008). Properties of the cell surface, particularly the presence of extracellular appendages, the interactions involved in cell–cell communication and EPS production are important for biofilm formation and development (Parsek & Greenberg, 2005). An increase in flow velocity or nutrient concentration may also equate to increased attachment, if these factors do not exceed critical levels (Simo˜es, Sillankorva, et al., 2007).

1.2 Objective

In order to manipulate the occurrence of bacteria adhesion and biofilm formation, it is of important to study the factors that contribute to the bacteria adhesion on the solid surfaces. To study the factors that facilitates the adhesion of bacteria (*Escherichia coli* and *Bacillus subtilis*) on the glass surface (hydrophilic surfaces).

1.3 Scope

The scope have been drawn where bacteria characterization is characterized based on the types, morphology, size and shape. Besides that, the physical effects on bacteria adhesion; exposure time (4, 8, 12 and 24), bacterial concentration (0.8, 1.0 and 1.2) abs and culture age (16 and 66 hour).

2 LITERATURE REVIEW

2.1 Microorganism

2.1.1 Escherichia coli

Escherichia coli is a gram negative procaryote, non-spore forming rod. It may or may not be mobile. (Some rods are flagellated and some are not.) The organism is a facultative anaerobe and the optimal temperature for growth is at 37^{o}C. The optimum pH for growth is 6.0 to 8.0. However, growth can occur as low as pH 4.3 and as high as pH 9 to 10. *E. coli* is prokaryotic and capable of aerobic and anaerobic metabolism. *E. coli* is a heterotrophic organism, meaning that it obtains its food from a different source. This source is most often its host organism. They obtain carbon via biosynthesis of organic molecules that were ingested by their host. Carbon is very important to *E. coli* because the bacterial cell composed almost entirely of carbon molecules bound to other important elements. In response to changes in the temperature or the osmolarity of the environment, *E. coli* utilizes its ability to physically change the diameter of the porins found on the cell membrane. If there are larger nutrient molecules present, *E. coli* will enlarge in porin diameter of to allow the molecule to enter the organism. This also works in reverse in that if there are inhibitory molecules present, *E. coli* will decrease the diameter of the porins **(**Hu Amanda, 2002).

2.1.2 Bacillus subtilis

Bacillus subtilis cells are rod-shaped, gram-positive bacteria that are naturally found in soil and vegetation. *B. subtilis* grows best in the mesophilic temperature range where the optimal temperature is 25 to 35^{o}C (Stephen, 1998). Stress and starvation are common in this environment; therefore, *B. subtilis* has evolved a set of strategies that allow survival under these harsh conditions. For example, is the formation of stress-resistant endospores. Besides that, the other strategy is the uptake of external DNA, which allows the bacteria to adapt by recombination. However, these strategies are time-consuming. *B. subtilis* can also gain protection more quickly against many stress situations such as acidic, alkaline,

osmotic, or oxidative conditions, and heat or ethanol (Bandow, 2002). *B. subtilis* use their flagella for a swarming motility. This motility occurs on surfaces, for example on agar plates, rather than in liquids. *B. subtilis* are arranged in singles or chains. Cells arranged next to each other can only swarm together, not individually. These arrangements of cells are called 'rafts'. In order for *B. subtilis* to swarm, they need to secrete a slime layer which includes surfactin, a surface tension-reducing lipopeptide, as one of its components (Schaechter 2006).

2.2 Growth curve

Binary fission and other cell division processes bring about an increase in the number of cells in a population. Population growth is studied by analyzing the growth curve of a microbial culture. When microorganisms are cultivated in liquid medium, they usually are grown in a batch culture that is, they are incubated in a closed culture vessel with a single batch of medium. Because no fresh medium is provided during incubation, nutrient concentrations decline and concentrations of wastes increase. The growth of microorganisms reproducing by binary fission can be plotted as the logarithm of the number of viable cells versus the incubation time (Ingraham,2001).

2.2.1 Lag Phase

When microorganisms are introduced into fresh culture medium, usually no immediate increase in cell number occurs. This period is called the lag phase**.** However, cells in the culture are synthesizing new components. A lag phase can be necessary for a variety of reasons. The cells may be old and depleted of ATP, essential cofactors, and ribosome; these must be synthesized before growth can begin. The medium may be different from the one the microorganism was growing in previously. Here new enzymes would be needed to use different nutrients. Possibly the microorganisms have been injured and require time to recover. Whatever the causes, eventually the cells begin to replicate their DNA, increase in mass, and finally divide (Neidhardt, 2005).

2.2.2 Exponential phase

During the exponential (log) phase**,** microorganisms are growing and dividing at the maximal rate possible given their genetic potential, the nature of the medium, and the environmental conditions. Their rate of growth is constant during the exponential phase; that is, they are completing the cell cycle and doubling in number at regular intervals. The population is most uniform in terms of chemical and physiological properties during this phase; therefore exponential phase cultures are usually used in biochemical and physiological studies (Neidhart, 2005).

Exponential (logarithmic) growth is balanced growth**.** That is, all cellular constituents are manufactured at constant rates relative to each other. If nutrient levels or other environmental conditions change, unbalanced growth results. During unbalanced growth, the rates of synthesis of cell components vary relative to one another until a new balanced state is reached. Unbalanced growth is readily observed in two types of experiments: shift-up, where a culture is transferred from a nutritionally poor medium to a richer one; and shift-down, where a culture is transferred from a rich medium to a poor one. In a shift-up experiment, there is a lag while the cells first construct new ribosome to enhance their capacity for protein synthesis. In a shift-down experiment, there is a lag in growth because cells need time to make the enzymes required for the biosynthesis of unavailable nutrients. Once the cells are able to grow again, balanced growth is resumed and the culture enters the exponential phase. These shift-up and shift-down experiments demonstrate that microbial growth is under precise, coordinated control and responds quickly to changes in environmental conditions(Maloe,2005).

When microbial growth is limited by the low concentration of a required nutrient, the final net growth or yield of cells increases with the initial amount of the limiting nutrient present. The rate of growth also increases with nutrient concentration but in a hyperbolic manner much like that seen with many enzymes. The shape of the curve seems to reflect the rate of nutrient uptake by microbial transport proteins. At sufficiently high nutrient levels, the transport systems are saturated, and the growth rate does not rise further with increasing nutrient concentration (Maloe, 2005).

2.2.3 Stationary phase

In a closed system such as a batch culture, population growth eventually ceases and the growth curve becomes horizontal. This stationary phase usually is attained by bacteria at a population level of around 10^9 cells per ml. Other microorganisms normally do not reach such high population densities. For instance, protist cultures often have maximum concentrations of about 10^6 cells per ml. Final population size depends on nutrient availability and other factors, as well as the type of microorganism being cultured. In the stationary phase, the total number of viable microorganisms remains constant. This may result from a balance between cell division and cell death, or the population may simply cease to divide but remain metabolically active (Ingraham,2005).

Microbial populations enter the stationary phase for several reasons. One obvious factor is nutrient limitation; if an essential nutrient is severely depleted, population growth will slow. Aerobic organisms often are limited by O_2 availability. Oxygen is not very soluble and may be depleted so quickly that only the surface of a culture will have an O_2 concentration adequate for growth. The cells beneath the surface will not be able to grow unless the culture is shaken or aerated in another way. Population growth also may cease due to the accumulation of toxic waste products. This factor seems to limit the growth of many anaerobic cultures (cultures growing in the absence of O_2). For example, streptococci can produce so much lactic acid and other organic acids from sugar fermentation that their medium becomes acidic and growth is inhibited. Finally, some evidence exists that growth may cease when a critical population level is reached. Thus entrance into the stationary phase may result from several factors operating in concert (Neidhart,2005).

As we have seen, bacteria in a batch culture may enter stationary phase in response to starvation. This probably occurs often in nature because many environments have low nutrient levels. Procaryotes have evolved a number of strategies to survive starvation. Some bacteria respond with obvious morphological changes such as endospore formation, but many only decrease somewhat in overall size. This is often accompanied by protoplast shrinkage and nucleoid condensation. The more important changes during starvation are in gene expression and physiology. Starving bacteria frequently produce a variety of

starvation proteins**,** which make the cell much more resistant to damage. Some increase peptidoglycan crosslinking and cell wall strength. The Dps (D NA-binding *p* rotein from *s* tarved cells) protein protects DNA.

Proteins called chaperone proteins prevent protein denaturation and renature damaged proteins. Because of these and many other mechanisms, starved cells become harder to kill and more resistant to starvation, damaging temperature changes, oxidative and osmotic damage, and toxic chemicals such as chlorine. These changes are so effective that some bacteria can survive starvation for years. There is even evidence that *Salmonella enterica* serovar Typhimurium *(S. typhimurium)* and some other bacterial pathogens become more virulent when starved. Clearly, these considerations are of great practical importance in medical and industrial microbiology (Neidhart,2005).

2.2.5 Death phase

For many years, the decline in viable cells following the stationary phase was described simply as the "death phase." It was assumed that detrimental environmental changes such as nutrient deprivation and the buildup of toxic wastes caused irreparable harm and loss of viability. That is, even when bacterial cells were transferred to fresh medium, no cellular growth was observed. Because loss of viability was often not accompanied by a loss in total cell number, it was assumed that cells died but did not lyse.

2.3 Mechanism of bacterial adhesion and development

Biofilm growth is governed by a number of physical, chemical and biological processes. There are a number of mechanisms by which numbers of microbial species are able to come into closer contact with a surface, attach firmly to it, promote cell–cell interactions and grow as a complex structure (Breyers & Ratner, 2004). Biofilm formation comprises a sequence of steps (Breyers & Ratner, 2004).

At present, processes governing biofilm formation that have been identified include (Fig. 1): 1. pre-conditioning of the adhesion surface either by macromolecules present in

the bulk liquid or intentionally coated on the surface; 2. Transport of planktonic cells from the bulk liquid to the surface; 3. Adsorption of cells at the surface; 4. Desorption of reversibly adsorbed cells; 5. Irreversible adsorption of bacterial cells at a surface; 6. Production of cell–cell signaling molecules; 7. Transport of substrates to and within the biofilm ; 8. Substrate metabolism by the biofilm-bound cells and transport of products out of the biofilm. These processes are accompanied by cell growth, replication, and EPS production; 9. Biofilm removal by detachment or sloughing (Breyers & Ratner, 2004). The attachment of microorganisms to surfaces and the subsequent biofilm development are very complex processes, affected by several variables (Table 1). In general, attachment will occur most readily on surfaces that are rougher, more hydrophobic, and coated by surface conditioning film (Martial, & Degraeve, 2008). Properties of the cell surface, particularly the presence of extracellular appendages, the interactions involved in cell–cell communication and extracellular polymeric substances (EPS) production are important for biofilm formation and development (Parsek & Greenberg, 2005). An increase in flow velocity or nutrient concentration may also equate to increased attachment, if these factors do not exceed critical levels (Simo˜es, Sillankorva, *et al*., 2007).

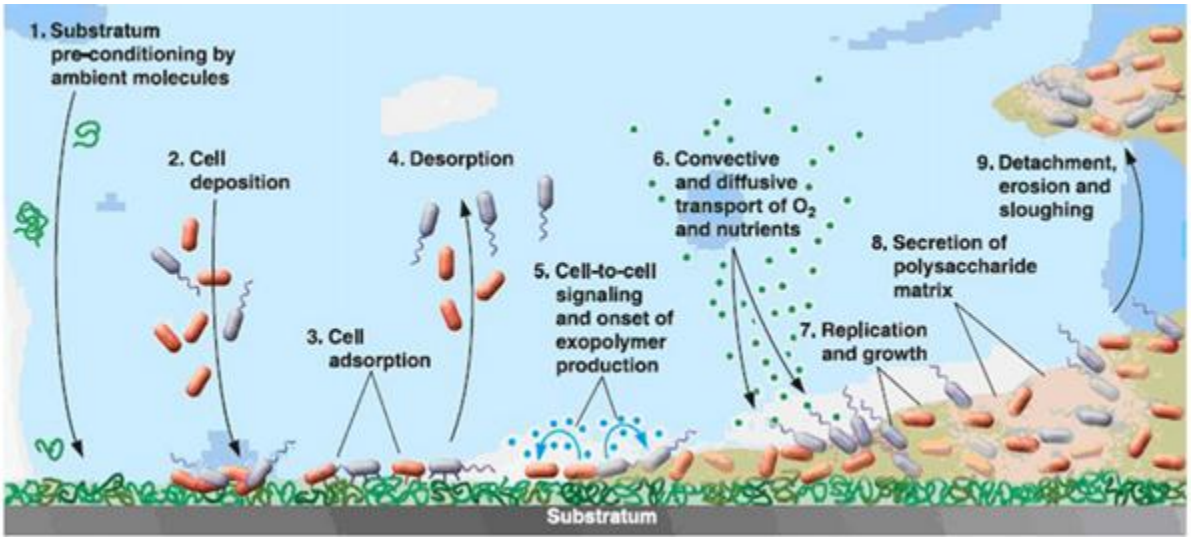


Figure 1: process of biofilm formation.

Table 2. 1: Variables important in cell attachment, biofilm formation and development (Donlan,2002)

Adhesion surface	Bulk fluid	Cell
Texture or roughness	Flow velocity	Cell surface hydrophobicity
Hydrophobicity	Ph	Extracellular appendages
Surface chemistry	Temperature	Extracellular polymeric Substances
Charge	Cations	Signalling molecules
Conditioning film	Presence of antimicrobial product	
	Nutrient availability	

2.3.1 The conditioning layer

The conditioning layer is the foundation on which a biofilm grows, and can be composed of many particles, organic or inorganic. Anything that may be present within the bulk fluid can through gravitational force or movement of flow settle onto a substrate and become part of a conditioning layer. This layer modifies substrata facilitating accessibility to bacteria. Surface charge, potential and tensions can be altered favorably by the interactions between the conditioning layer and substrate. The substrate provides anchorage and nutrients augmenting growth of the bacterial community.

2.3.2 Cell–cell communication

The driving force in bacterial community development is the self-organization and cooperation among cells, rather than the classical 'competitive' natural selection of individual microorganisms (Parsek & Greenberg, 2005). This concept becomes particularly apparent when examining bacterial biofilm communities (Parsek & Greenberg, 2005). Cell– cell signalling has been demonstrated to play a role in cell attachment and detachment from biofilms (Daniels *et al*., 2004). Bacteria are considered to be far from solitary microorganisms, and in fact are colonial by nature and exploit elaborate systems of

intercellular interactions and communications to facilitate their adaptation to changing environments (Fuqua & Greenberg, 2002). The successful adaptation of bacteria to changing natural conditions is dependent on their ability to sense and respond to the external environment and modulate gene expression accordingly (Daniels *et al*., 2004).

Quorum sensing is based on the process of auto induction (Eberhard *et al*., 1981). The process of quorum sensing provides a mechanism for self-organization and regulation of microbial cells (Parsek & Greenberg, 2005). It involves an environmental sensing system that allows bacteria to monitor and respond to their own population densities. The bacteria produce a diffusible organic signal, originally called an auto-inducer (AI) molecule, which accumulates in the surrounding environment during growth (Fuqua & Greenberg, 2002). Besides that, high cell densities result in high concentrations of signal, and induce expression of certain genes or physiological changes in neighboring cells (Parsek & Greenberg, 2005). A response to chemical signals in the process of cell communication is a concentration dependent process, where a critical threshold concentration of the signal molecule must be reached before a physiological response is elicited (Fuqua & Greenberg, 2002). Oligopeptides and N-acylhomoserine lactones (AHL) are major auto inducer (AI) molecules involved in intra-specific communication in Gram-positive and Gram-negative bacteria, respectively whereas boronated diester molecules (AI-2) are involved in inter-specific communication among both Gram-positive and Gram-negative bacteria (Parsek & Greenberg, 2005). Oligopeptides and N-acylhomoserine lactones (AHL) are the best characterized molecules (Ryan & Dow, 2008).

2.3.3 Population growth

As the stationary cells divide (binary division), daughter cells spread outward and upward from the attachment point to form clusters (Hall, 2002). Typically, such interactions and growth within the developing biofilm form into a mushroom-like structure. The mushroom structure is believed to allow the passage of nutrients to bacteria deep within a biofilm. After an initial lag phase, a rapid increase in population is observed, and cell growing exponential growth phase. This depends on the nature of the environment, both physically and chemically. The rapid growth occurs at the expense of the surrounding

nutrients from the bulk fluid and the substrate. At this stage the physical and chemical contribution to the initial attachment ends and the biological processes begin to dominate. Excretion of polysaccharide intercellular adhesion (PIA) polymers and the presence of divalent cations interact to form stronger bonding between cells (Dunme, 2002).

2.3.4 Final stages of biofilm development

The stationary phase of growth describes a phase where the rate of cell division equals the rate of cell death. At high cell concentration, a series of cell signaling mechanisms are employed by the biofilm, and this is collectively termed quorum sensing (Bassler, 1999). Quorum sensing describes as a process where a number of auto inducers (chemical and peptide signals in high concentrations, e.g. homoserine lactones) are used to stimulate genetic expression of both mechanical and enzymatic processors of alginates, which form a fundamental part of the extracellular matrix. The death phase sees the breakdown of the biofilm. Enzymes are produced by the community itself which breakdown polysaccharides holding the biofilm together, actively releasing surface bacteria for colonisation of fresh substrates.

2.4 Microbial Cell Surface Architecture

Since it is the microbial cell surface that largely determines the adhesion process it is necessary to describe a typical organization of the cell wall. Generally, a complete cell envelope possesses a number of functions (strength conferring, shape maintenance, molecular sieving, etc**.**) which can be provided by a single structural unit (Gram-positive bacteria) or by several layers with specialized functions (Gram-negative bacteria).

2.4.1 Gram-positive Bacteria

In Gram-positive bacteria**,** the stress-bearing component of the cell envelope that supports the internal turgor pressure of the cell is a thick, covalently cross-linked peptidoglycan-containing layer (Hancock, 1990). Other macromolecules such as polysaccharides, teichoic acids (secondary cell wall polymers), and proteins covalently linked to the peptidoglycan, penetrate its complex network. The relation between the amount of peptidoglycan (at least 40% by weight of the layer) and the total amount of anionic secondary polymers (remainder of the layer) with the outermost chains projecting into the surrounding fluid is generally maintained. So, the cell wall of Gram-positive bacteria is thought to be a covalently linked heteropolymeric structure overlaying and protecting the cytoplasmic membrane (Loeb, 1985). However, associated non-covalently with this structure are chemical components that represent extracellular products of the cell (glycocalyx). These are amphiphiles (lipoteichoic acids) that may retain an association with the cell membrane, wall-associated assemblies of glycoprotein forming regularly structured surface arrays (S-arrays) or capsules (‘slime layers ’) composed of an extracellular polysaccharide fibrous material.

2.4.2 Gram-negative Bacteria

While the cell wall of Gram-positive bacteria consist primarily of the relatively uniform single peptidoglycan-based layer, the cell wall of Gram-negative bacteria is multilayered and structurally and chemically more complex**.** Gram-negative bacteria possess a highly organized asymmetric outer membrane in which a bilayer of phospholipid (inner leaflet, 20-25%), lipopolysaccharide (oqter leaflet, 30%), and outer membrane protein (45-50%) constitute a permeability barrier with pores (ionic transmembrane channels) formed of aggregates of proteins (Hancock, 1991). So, the outer face of the outer membrane in the so-called smooth form (lipopolysaccharide consisting of a hydrophobic lipid component, a core polysaccharide, and 0-antigenicall y specific polysaccharide side chains) is hydrophilic. Interestingly, ‘rough’ mutants (lacking the core as well as the 0-polysaccharide portion of the lipopolysaccharide) are more hydrophobic and much more sensitive to hydrophobic molecules. Moreover, in Gram-negative bacteria, between the

outer cell membrane and the inner cytoplasmic membrane, there is a periplasm space filled with a macromolecular gel made up of a thin peptidoglycan layer in which periplasmic proteins and other molecules (lipoproteins) are distributed(Marshal,1985). Also, Gram-negative bacteria produce a wide variety of glycocalyces (glycoprotein S-arrays and polysaccharide capsules) closely associated with the cell surface.

2.5 Environmental factors influencing biofilm development

2.5.1 Effect of temperature

The optimum temperature for a microorganism is associated with an increase in nutrient intake resulting in a rapid formation of biofilm (Stepanovic, 2003). Nutrient metabolism is directly associated and dependent on the presence of enzymes. So it may be fair to say that the formation of a biofilm is dependent on the presence and reaction rates of enzymes, which control the development of many physiological and biochemical systems of bacteria. Temperature is correlated with the reaction rate of enzymes and the development of the cells. Optimum temperatures result in the healthy growth of the bacterial populations. Conversely, a temperature away from the optimum reduces bacterial growth. This is due to a reduction in enzyme to reaction rates. In addition, environmental temperature affects the physical properties of the compounds within and surrounding the cells. Fletcher (2001) reported the effect of temperature on attachment of stationary phase cells. Shown that a decrease in temperature reduced the adhesion of bacteria on the substrate. It is believed that the effect was due to a decrease in the bacterial surface polymer at lower temperatures as well as effects such as reduced surface area.

However, Herald and Zottola (1988) observed that the presence of bacterial surface appendages was dependent on temperature. At 35 ^{o}C cells were shown to have a single flagellum whilst at 21 ^{o}C they had two to three flagella and at 10 ^{o}C, cells exhibited on flagella. This may suggest that the initial interaction between the bacteria and substrate may increase with a lowering of temperature, increasing the likelihood of adhesion. Perhaps the more uniform properties of polysaccharides at lower temperatures increase the possibility of biofilm adhesion, because of many microbial polysaccharides undergo

transition from an ordered state at lower temperatures and in the presence of ions, to a disordered state at elevated temperature under low ionic environments.

2.6 Bacterial adhesion to surfaces

2.6.1 The influence of surface roughness.

Since the report in 1940 for Heukelekian (1940), has been known that the surface characteristics are an important factor for the bacterial adhesion and development. Until today this is central research area for the control of bacterial biofilm related disease. The adhesion of bacteria to a surface depends on a number of microbiological, physical, chemical, and material-related parameters, on surface topography has been widely produced as a parameter influencing bacterial adhesion (Flint, 1997). Contact with a solid surface induces the expression of a bacterial enzyme, which catalyzes the formation of exo-polysaccharides that promote colonization and protection. Thus, the modification of surfaces can be done to reduce attachment surfaces to limit the adhesion of microorganism e.g. electropolishing of stainless-steel. Several parameters or measures have been used to characterize the material surface based on two-dimensional characteristics such as the Ra (roughness average), Rt (is the maximum peak to valley height in the sample length), and Rz values (the average maximum profiler height) (Chiffre, 1990).

Amongst the most widely used is the surface roughness Ra value (which is the arithmetical mean deviation of the profile) and an Ra value of 0.8 µm or less has been recommended for dairies and, in general, for food contact surfaces. Although widely used, the Ra value will typically not characterize features of the surface such as soft or sharp topography or the presence of scratches or porosities During recent years, scanning electron microscopy (SEM) and atomic force microscopy (AFM) have been used to give a three-dimensional visualization of the surface topography including AFM determination of three-dimensional topographical parameters in the nanometer range (Stout, 1993).

2.6.2 Specialized attachment structures/surface properties of the cell

Cell surface hydrophobicity and the presence of extracellular filamentous appendages may influence the rate and the extent of microbial attachment. The hydrophobicity of the cell surface is important in adhesion because hydrophobic interactions tend to increase with an increasing non-polar nature of one or both surfaces involved, for example the microbial cell and the adhesion surface (Donlan, 2002). According to Drenkard and Ausubel (2002), the ability of bacteria to attach to each other and to surfaces depends in part on the interaction of hydrophobic domains. On the other hand bacteria and other microorganism have evolved many different ways to use the hydrophobic effect in order to adhere to surface (Doyle, 2002). Surface charges are inherently important for bacteria adhesion to the surface. In addition, bacteria may be affected by culture media, nutrients and age, the surface charge would also dependent on those parameters. Since it is the microbial cell surface that largely determines the adhesion process it is necessary to describe typical organization of the cell wall.

2.6.3 Electrostatic, Hydrophobic and Bridging Effects of Cell Surface Components

The reversible initial stage results from complex physicochemical interactions among the cell, the surface and the liquid phase (Kim and Frank, 1994). These interactions are caused by the surface charge (Hogt *et al*., 1985; Dickson and Koohamaraie, 1989), the hydrophobicity (Dahlback *et al*., 1981; Van Loosdrecht *et al*., 1987) and electron acceptor and electron donor (Van Oss, 1993) of interacting surfaces. The role of electron-donor/electron acceptor, i.e. Lewis acid-base proper- ties, in the interaction between two materials has been widely studied (Van Oss and Visser, 1992). Their importance in polar aqueous media has been underlined and reviewed by Van Oss (1993). Several studies (Boulangé-Petermann *et al*., 1993; Van Oss, 1993) have reported that the electron-donor/electron acceptor plays a crucial role in the microbial adhesion phenomenon. It should be noted that the energy of these interaction may be twice as much as that produced

by the Lifshitz-van der Waals interactions (LW) or electrostatic interactions (EL) usually described in the DLVO theory (Van Oss, 1996).

In 1996, Bellon-Fontaine *et al.* developed a new method-namely M.A.T.S (Microbial adhesion to solvents), to determine the electron donor/electron acceptor microbial cell properties. It was based upon the comparison between microbial cell affinity to a monopolar solvent and a polar solvent with the same LW surface tension component. This technique appears to be more useful than contact angle method (Van Oss *et al*., 1988), which requires specific and elaborate equipment. Microbial cell surface hydrophobicity is recognized as one of the determinant factors in microbial adhesion to surface (Van Loosdrecht *et al*., 1987). These properties are often evaluated by hydrophobic interaction chromatography, contact angle method, aqueous phase partitioning poly-ethyleneglycol/ dextran (PEG/DEX) and microbial adhesion to hydrocarbon (M.A.T.H). The latter technique is generally performed using p-xylene, hexadecane, octane and toluene. So, it can be a useful method to measure the cell surface hydrophobicity.

The cell surface physicochemical properties can be modified depending on surface cell structures (Ljunjh and Wadstrom, 1984; El Ghmari *et al*., 2002) or environmental factors such as temperature, medium composition, ionic strength and pH. Many workers have described the effects of these environmental parameters on hydrophobicity and charge (Beck *et al*., 1988; Herben *et al*., 1990; Van Der Mei *et al*., 1993; Latrache *et al*., 1994; Braindet *et al*., 1999a; Latrache *et al*., 2000). Literature data (Rouxhet and Mozes, 1990) reported that the hydrophobicity and charge were insufficient to explain the adhesion phenomenon. So the involvement of electron donor/electron acceptor properties could also be important in explaining this phenomenon (Van Oss *et al*., 1988). Despite the fact that the electron donor/electron acceptor properties play an important role in adhesion phenomenon, limited data concerning the effects of environmental parameters on these properties have been published (Braindet *et al*., 1999a; 1999b)

2.7 Measurement of microbial growth

There are many ways to measure microbial growth to determine the growth rates and generation times. Either population number or mass may be followed because growth leads to increase in both. Here are most commonly employed techniques for determining the population sizes are examined briefly and the advantages and disadvantages of each noted.

2.7.1 Measurement of cell mass

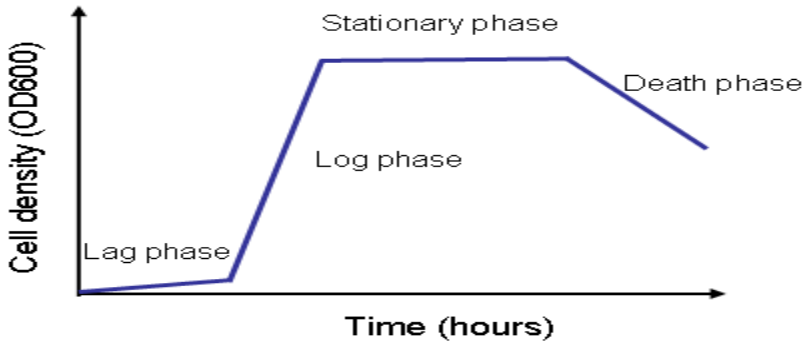


Figure 2.7.1: Growth curve

Numbers of bacteria can be estimated by two most widely used methods which are viable plate count and spectrophotometric analysis. In liquid culture, the medium appears more and cloudier as the bacteria increase in number by division. A tube of bacteria will tend to reflect light so that less light is transmitted through the tube. A spectrophotometer can measure the amount of light passing through the tube, or conversely the amount of light absorbed. These measurements of turbidity or optical density (OD) are not direct measurements of bacterial numbers, but an indirect measurement of cell biomass that includes both living and dead cells. As the bacterial cell population increases, the amount of transmitted light decreases, increasing the absorbance reading on the spectrophotometer. If one takes readings of the same culture over time, the absorbance readings will increase as the cell number increases.

There are some limitations with this method, though. A growth curve that includes the lag, log, and stationary phase will take several hours to complete and the relationship between cell number and absorbance will begin to deviate from linearity at high cell densities. In the Figure 2 it shows the idea of how the turbidity measurements correspond to actual numbers, more than a million cells/ml needed to be presented in order to get even a trace of a measurement on the spectrophotometer (Sauer and Camper, 2001)

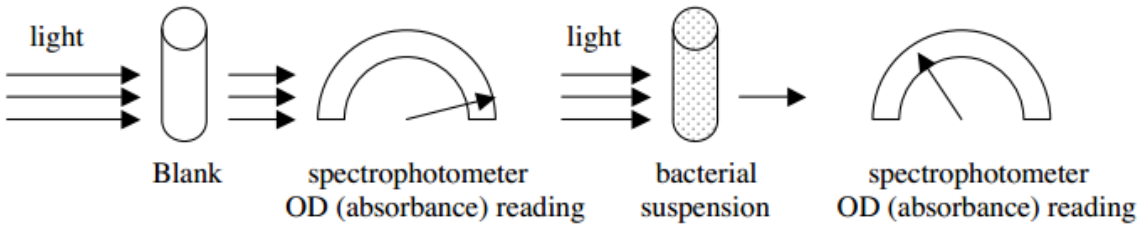


Figure 2.7.2.1: Spectrophotometric determination of cell densities

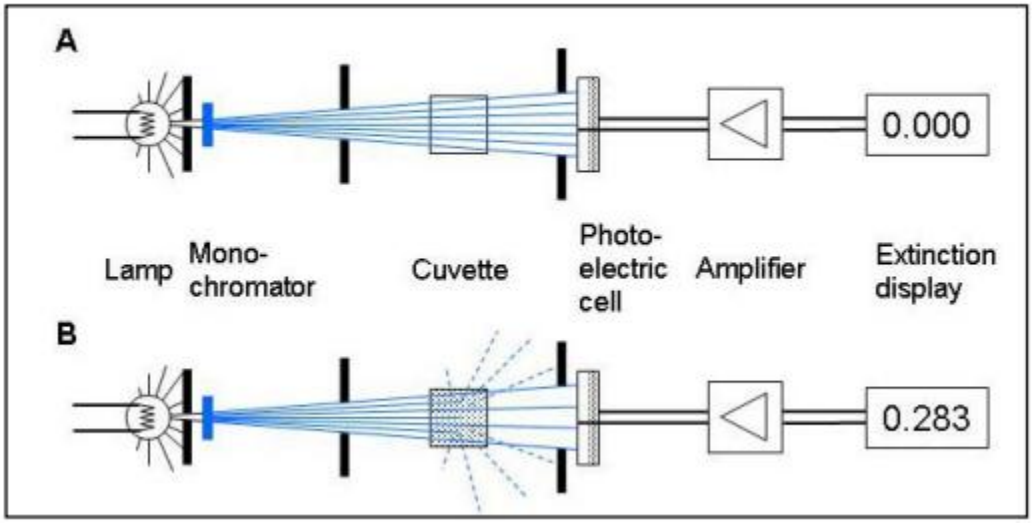


Figure 2.7.2.3 : Measurement of the OD of a culture

2.7.3 Measurement of cell numbers

Plating techniques are simple, sensitive and widely used for viable count bacteria and other microorganism in samples of food, water and soil. The purpose of plate counting is to estimate the number of cells present based on their ability to give rise to colonies under specific conditions of nutrient medium, temperature and time. Theoretically, one viable cell (viable defined as able to multiply via binary fission under the controlled conditions) can give rise to a colony through multiplication. However, solitary cells are the exception in nature, and most likely the progenitor of the colony was a mass of cells deposited together.

To quantify viable cells a plate count is done. A sample of bacteria is diluted in a sterile medium until the numbers are very low. This diluted sample of bacteria is then transferred onto an agar plate and spread out evenly so that each cell is separate from the others. Each viable cell will continue to divide into a discrete colony of millions of bacterial cells which can now be seen with the naked eye. These colonies can then be counted. Keeping in mind that each colony arose from a single cell that was plated onto the agar, the number of colonies can be used to determine the number of bacterial cells present in the original culture. . The numbers of colony forming units (CFU's) are divided by the product of the dilution factor and the volume of the plated diluted suspension to determine the number of bacteria per ml that were present in the original solution.

$$\frac{\text{Number of CFU}}{\text{Volume plated (mL) x total dilution used}} \longrightarrow \frac{\text{Number of CFU}}{\text{mL}}$$

2.8 Bacteria characterization

2.8.1 The Gram Staining

Microorganisms found in pharmaceutical and healthcare environments require identification in order to determine the species. This is important so that the origin of contamination can be assessed and the origin of contamination determined. This is commonly performed by using a standing technique called the Gram stain, which is based is a type of "phenotypic identification method" and it undertaken so that the microbiologist can understand the general profile for microorganisms.

The first step of most identification schemes is to describe the colony and cellular morphology of the microorganism. Colony morphology is normally described by directly observing growth on agar, where the colony will appear as a particular shape (such as raised, crenated, spherical and so on) and the colony will have a particular pigment. Some microbiologists will attempt to identify the microorganism based on such visual identification. This is not normally encouraged as considerable experience is required to do this and the variety of microflora cannot be characterized with any degree of accuracy. Furthermore, the characteristics of a microorganism are often dependent upon the type of culture medium used. Nevertheless, a description of the morphology can assist with further stages of identification (Micheal, 2010).

Cellular staining provides important information relating to the composition of the microbial cell wall, as well as the shape of the organism. Of these, the most frequently used method is the Gram stain.The Gram stain method employed includes the four-step technique: Crystal violet (primary stain); iodine (mordant); alcohol (decolorizer); and safranin (counter stain). Done correctly, Gram-positive organisms retain the crystal violet stain and appear blue; Gram negative organisms lose the crystal violet stain and contain only the counter-stain safranin and thus appear red. Common pitfalls in this method are that heat fixation may cause Gram-positive cells to stain Gram-negative and older cultures may give Gram-variable reaction; using too much decolorizer could result in a false Gram-

negative result and not using enough decolorizer may yield a false Gram-positive result (Sadle, 2011)

The Gram reaction is based on the differences in the cell wall composition for the two cellular 'groups'. The bacteria that retained the stain (the Gram-positive bacteria) have a higher peptidoglycan and lower lipid content than those that do not retain the stain (the Gram-negative bacteria). The effect of the solvent is to dissolve the lipid layer in the cell wall of the Gram-negative bacteria, thereby causing the crystal violet to leach out; whereas for Gram-positive bacteria the solvent dehydrates the thicker cell walls, blocking any diffusion of the violet-iodine complex, which closes the pores of the cell and retains the stain. There is now several automated Gram stain devices available on the market that can reduce the labour requirement required when performing several multiple Gram stains and, possibly, improve accuracy. In addition to the difference based on cell wall, microscopic examination of the stains allows the cellular shape to be determined. Bacteria commonly fall into categories of coccus (spherical), rod, vibrio (curved), spirilla (spiral), and plemomorphic (viable).

2.8.2 Fundamental Principles of Scanning Electron Microscopy (SEM)

Accelerated electrons in an SEM carry significant amounts of kinetic energy, and this energy is dissipated as a variety of signals produced by electron-sample interactions when the incident electrons are decelerated in the solid sample. These signals include secondary electrons (that produce SEM images), backscattered electrons (BSE), diffracted backscattered electrons (EBSD that are used to determine crystal structures and orientations of minerals), photons (characteristic X-rays that are used for elemental analysis and continuum X-rays), visible light (cathodoluminescence-CL), and heat. Secondary electrons and backscattered electrons are commonly used for imaging samples: secondary electrons are most valuable for showing morphology and topography on samples and backscattered electrons are most valuable for illustrating contrasts in composition in multiphase samples (i.e. for rapid phase discrimination).

2.9 Cells Surface Hydrophobicity/Microbial Adhesion to Solvents (CHS/MATs)

Microbial adhesion to solid surfaces depends on reversible and subsequently irreversible interaction. The reversible initial stage results from complex physiochemical interactions among the cell, the surface and liquid phase. These interactions are caused by the surface and electron acceptor and electron donor (Van Oss, 1993) of interacting surfaces.The role of electron-donor and electron-acceptor in the interaction between two materials has been studied using Lewis acid-base properties. Their importance in polar aqueous media has been underlined and reviewed by Van Oss (1996). Several studies have been reported that the electron donor or electron acceptor plays a crucial role in the microbial adhesion on the surfaces.

In 1996, Bellon-Fontaine has developed a new method which is microbial adhesion to solvents (MATHs) to determine the electron donor or electron acceptor microbial cell properties. It was based upon the comparison between microbial cell affinity to monopolar solvent and a polar solvent with the same surface tension component. Microbial cell surface hydrophobicity is recognized to be one of the determinant factors in microbial adhesion to surface. These properties are often evaluated by hydrophobic interaction chromatography, contact angle method, aqueous phase partitioning poly-ethyleneglycol/dextran (PEG/DEX) and microbial adhesion to hydrocarbon. To measure the cell surface hydrophobicity the latter technique is generally performed using p-xylene, hexadecane, octane and toluence. This method will identify the Gram-positive or Gram negative bacteria that attach to the surface.

3 METHODOLOGY

3.1 Preparation of Culture Medium

3.1.1 Preparation of nutrient broth

8 g of nutrient broth powder was weighed and added into 1 L of distilled in a 1L Schott bottle. The powder was dissolved completely in the water and sterile at 121°C for 20 minutes.

3.1.2 Preparation of nutrient agar

23 g of nutrient agar powder was weighed and added to 1 L of distilled in a 1 L Schott bottle. The powder was dissolved completely and sterile at 121°C for 20 minutes.

3.1.3 Preparation of agar plates

15-20 ml of a warm sterile nutrient agar was poured into petri plate. The nutrient agars are allowed to harden.

3.1.4 Preparation of agar slants

5 ml of a warm sterile nutrient agar was pipette into or universal bottle. Universal bottle was placed in a bend position and allow the nutrient agar to harden in this position.

3.1.5 Stock culture preparation

One loopful of bacteria was taken from bacteria stock obtained from central lab and streak on agar plate. The agar plate then incubated at 37^{o}C in inverted position.

3.1.6 Working culture (inoculums preparation)

For *E. coli* three loopful of bacteria was taken from stock culture and added into 60 ml nutrient broth in 150 ml conical flask. It was incubated at 37^{o}C and 180 rpm.

For *B. subtilis* three loopful of bacteria was taken from stock culture and added to 60 ml nutrient broth in 150 ml. The mixture was then incubated at 37^{o}C and 180 rpm.

3.2 Phosphate Buffer Saline (PBS) preparation

0.802 ml 1 M K_2HPO_4, 0.198 mL 1 M KH_2PO_4, 1.0 ml 0.1 M $MgSO_4$, 0.85 g NaCl and 1.0 ml 5 M KCl in 97 ml dissolved distilled water and autoclaved at 121^{o}C for 20 minutes.

3.3 Growth curve and Colony Forming Unit (CFU) preparation

5 ml of bacteria was taken from the inoculums and added into 45 ml nutrient broth in 150 ml conical flask. The optical density of the solution is then checked whether the absorbance is 1.0 or not. If the absorbance not reaching 1.0 more nutrient broth added until it reach required absorbance value. If the absorbance value is less than 1.0 more bacteria solution is added. When 1.0 absorbance obtained, 5 ml of the solution was pipette out into 45 ml nutrient broth in 150 ml conical flask and incubate according to the specific parameters.

3.3.1 Growth curve

1.5 ml of seed culture was pipette out into cuvette for every 2 hours until 24 hours. The wavelength for both bacteria *E .coli* and *B. subtilis* was set at 600nm.

3.3.2 Colony forming unit (CFU)

10 µl of sample solution was dilute in 0.99 ml distilled water. The dilution of 10^2 was obtained the procedure was repeated to create dilution of 10^4. For dilution 10^5, pipette out 0.1 ml from sample 10^4 dilution into 0.9 ml steriled distilled water. For *E. coli* the dilution undergo until 10^5. For *B. subtilis* the dilution undergo until 10^6. 5 µl of the dilution was pipette into nutrient agar and streaked by using hockey stuck in aseptically way. The nutrient agar is then incubated at 37^oC in inverted position. The colony is then measured.

3.4 Bacteria characterization

3.4.1 Determination of bacteria size under light microscope

One loopful of stock bacteria were taken from inoculum stock and smeared on the surface of clean glass slide. The glass slide was dried in the room temperature and slide through the flame. The smear was then covered with crystal violet for one minute, followed by washing with distilled water from a wash bottle. The smear was cover with Gram's iodine for one minute. The iodine was removed by tilting the slide and squirting water above over the smear followed by decolorization with 95% ethyl alcohol for 20 second. The smear was immediately washed with distilled water. Finally, safranin was added for 30 second and re-flushed the slide with distilled water. The slides were dry with a paper towel or adsorbent paper and the slide were examined under light microscope.

3.4.2 Determination of the cell surface using Scanning Electron Microscope (SEM)

Cells at the exponential and stationary state were harvested from a liquid sample by centrifuged for 10 minute at 10 000 rpm. The supernatant was then discarded and replaced with the same amount of Nacl solution and centrifuge again for 5 minutes. The Nacl supernatant was then discarded again and the cell pellet was re-centrifuged twice with distilled water. Cells were transferred onto glass slide and dried at room temperature. The

cells were coated with platinum where working chamber were flushed several times with argon gas.

3.5 Cell Surface Hydrophobicity/Microbial Adhesion to Solvent (CSH-MATs)

Two bacteria were studied which *B.subtilis*, and *E.coli* for each growth conditions, bacteria were cultured for 16 hour and 66 hour. After culture, the cells were harvested by centrifugation for 15 min at 10000 rpm. The harvested cell was dilute in 60 ml PBS solution at optical density 1.0 (abs). Chemical products (hexadecane, diethyl ether and hexane) having a highest purity grade were obtained commercially. The optical density of suspended will be measured. The optical density measured at 600 nm of the bacterial suspension before mixing. The mixture of cell suspension and solvent were 1:1 ratio 3 ml solvent was added to 3 ml of cell suspension. The mixture was vortexes for 20 second and allowed to separate at room temperature for 10 minutes. Each experiment was performed in triplicate by using three independently prepared cultures. The optical density of the cell suspension was measured using UV-Vis Spectrophotometer.

3.6 Bacteria adhesion on glass through the exposure time and cell concentration

Single colony obtained from 24-hours agar plate- culture was inoculated into shake flask containing 30 ml medium and incubated at 30^{o}C for 16-18 hours. After the incubation, the seed culture was centrifuged at 10000 rpm for 10 minute. The supernatant was removed and the cell pellet was re-centrifuged two times with NaCl for cell washing. Cell precipitate was resuspended in fresh PBS solution and make up to an OD of 1.0. It was then transferred into a container containing glass slide. The solution was shake at 100 rpm and 30^{0}C in the incubator shaker. The adhesion of the bacteria will be observed in the constant time (4, 8, 12, 24 hours) at optical density 1.0(abs) for both *E.coli* and *B.subtilis*. When the best time was obtained using optical density (abs) at 1.0, both bacteria were

repeated with optical density at 0.8 and 1.2 (abs) in order to study the effect of bacteria concentration on the adhesion mechanism.

3.7 Bacteria adhesion on glass through the effect of culture age.

Single colony obtained from 24-hours agar plate- culture was inoculated into shake flask containing 30 ml medium and incubated at 30^{o}C for 16-18 hours. After the incubation, the seed culture was centrifuged at 10000 rpm for 10 minute. The supernatant was removed and the cell pellet was re-centrifuged two times with NaCl for cell washing. Cell precipitate was resuspended in fresh PBS solution and make up to an OD of 1.0. It was then transferred into a container containing glass slide. The solution was shaking at 100 rpm and 30^{0}C in the incubator shaker. The adhesion of the bacteria will be observed after 16th and 66th hours.

4 RESULT AND DISCUSSION

4.1 Bacteria's Characteristics

4.1.1 Gram staining

In order to study the characteristic of *E. coli* and *B. subtilis* gram staining method was used to identify the characteristic for both bacteria by using electronic microscope. Major differences between gram-negative and gram-positive bacteria depend on cell wall structure. From the observation of *E. coli* under light microscope using gram staining method was pink and proved that that *E. coli* was gram negative bacteria. Gram negative bacteria are bacteria that do not retain crystal violet dye in the gram staining protocol (Baron, 1996). In gram staining test, a counterstain (safranin) was added after crystal violet and coloring all gram negative bacteria with pink color. The counterstain was used to visualize the otherwise colorless gram negative bacteria whose much thinner peptidoglycan layer does not retain crystal violet. While color for *B. subtilis* using the same method under light microscope was violet and proved for gram positive bacteria. Gram-positive organisms are able to retain the crystal violet stain because of their thick peptidoglycan layer, which is superficial to the cell membrane.

Table 4.1 indicates that the size of *E. coli* at exponential phase was 1.23±0.07 while the size of *B. subtilis* was 1.69±0.18. While the size of *E.coli* was 1.59±0.09 while the size for *B. subtilis* was 2.00±0.06. It clearly shown that length of *B. subtilis* is longer than *E. coli*. Besides that, the size of bacteria at death phase was longer compared to exponential phase with 0.36 differences for *E .coli* and 0.31 differences for *B. subtilis.* From figure 4.1, it clearly shown that the size for *B. subtilis* was a little bit longer than *E. coli* that observed under light microscope at 5.0 µm. Theoretically, bacteria at exponential phase cells are rod-shaped while decline phase cells are more spherical (Zambrano, 1993). Supposedly, size of bacteria at exponential phase should be longer than the death phase. This might happen due to the agglomeration of bacteria at the death phase. The image observed under light microscope at 100x seen to be longer.

Table 4. 1: Size of bacteria at exponential and death phase

Bacteria	Size at exponential phase (µm)	Size at death phase (µm)
Escherichia coli	1.23±0.07	1.59±0.09
B*acillus subtilis*	1.69±0.18	2.00±0.06

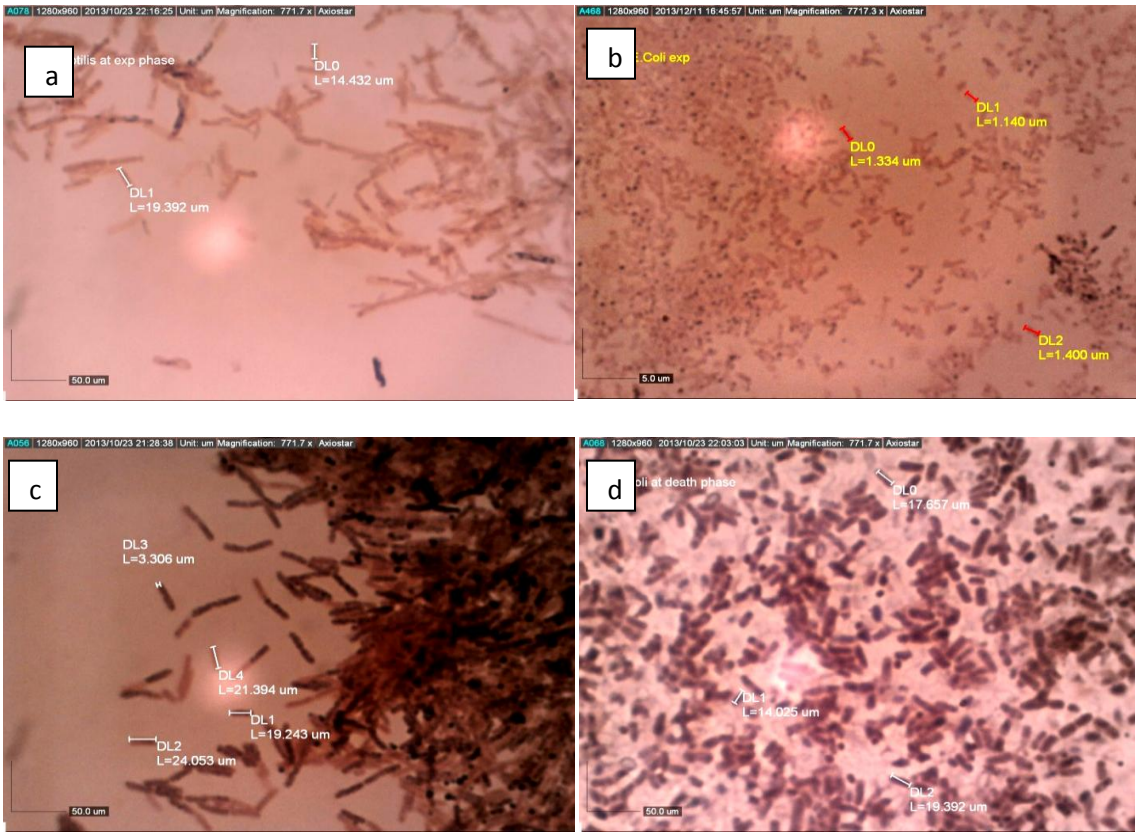


Figure 4. 1: (a) *B. subtilis* at exponential phase; (b) *E. coli* at exponential phase ; (c) *B. subtilis* at death phase; (d) *E. coli* at death phase using light microscope under 100x magnification.

4.1.2 Observation under Scanning Electron Microscope

E. coli and *B. subtilis* at exponential phase were also observed under scanning electron microscope (SEM) for clear view. Figure 4.2 shows that the shape and size of *B. subtilis* was vary due to the budding activities. During the exponential phase**,** microorganisms are growing and dividing at the maximal rate possible given their genetic potential, the nature of the medium, and the environmental conditions. Their rate of growth is constant during the exponential phase; that is, they are completing the cell cycle and doubling in number at regular intervals (Ingraham, 2005). For *E. coli*, the image was barely seen under SEM due to difficulties to observed bacteria using glass slide because of the conductive properties of the glass slide. Supposedly *E. coli* undergoes the same process as *B. subtilis* during exponential phase. Unfortunately due to the difficulties the process was hardly seen.

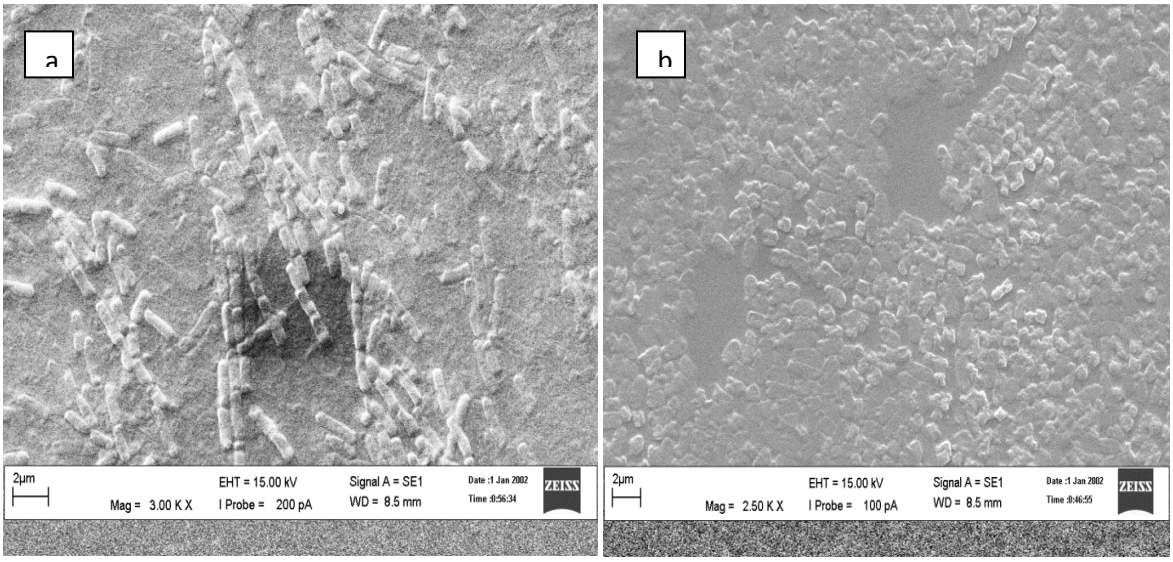


Figure 4. 2**:** (a) *B.subtilis* (b) *E.coli* at exponential phase observed under Scanning Electron Microscope (SEM)

4.1.3 Cells Surface Hydrophobicity/Microbial Adhesion to Solvents (CHS/MATs)

The wet ability of a surface is now more generally expressed in reverse sense and is referred to as hydrophobicity. However, it has been shown that solvent test measures a complicated interplay of hydrophobic and electrostatic interactions (Van der Mei *et al*., 1993). On the other hand, bacteria and other microorganism have evolved many different ways to use the hydrophobic effect in order to adhere to substrata (Doyle, 2000). Dodecane, is acidic solvent which exhibit negligible basic character when pure (Synder, 1974) while hexane and ethyl acetate, are strongly basic solvent (Synder,1974). Hydrophobicities were reported as the percentage of total cell partitioned into hydrocarbon (Pembrey,1999).

The higher affinity to dodecane was an indicative of the predominance of basic properties on the cell surface, while higher adhesion to the basic solvent ethyl acetate compared to hexane indicated that the cell surface presented more acidic properties. The hydrophobicities can be indicated by the percentage of *E. coli* and *B. subtilis* partitioned to acidic solvent (dodecane) and basic solvent (ethyl acetate). The percentage of *B. subtilis* when the cell partitioned into chloroform was higher compared to *Escherichia coli*. This is happen due to the high electron donating property of bacteria (Bellon,1996) and indicates the *B. subtilis* as hydrophobic. On the other hand, the percentage of *E. coli* partitioned into ethyl acetate was higher compared to *B. subtilis* due to high electron accepting property (Bellon, 1996) and indicates *E. coli* as hyrdrophilic. Bacteria to cell interactions are complex and varied, but include lectin- like, electrostatic and hydrophobic mechanisms. When a surface hydrophobicity of a bacterial cell is increased, the charge on the cell surface is also reduced. This has the effect of diminishing the repulsive forces which normally exist between two negatively charged bodies and increases the chances of adhesion (Johnson, 2000). The fimbrial adhesions found in pathogenic *E. coli* are predominantly composed of hydrophobic aminoacids (Anandkumar, 2012), these increase the surface hydrophobicity and reduce the cell surface charge (Johnson,2000). Bacterial cell adhesions can be ranked on the basis of their hydrophobicity with recognized

pathogenic *E. coli* showing a greater surface hydrophobicity (Johnson,2000) than non pathogens.

Table 4.2 also shows that the percentage of cell partitioned into hydrocarbon different at stationary phase. Mainly percentage of bacteria partitioned to solvent decrease at stationary phase. Exception when *E. coli* with hexane and *B. subtilis* with ethyl acetate.

Table 4. 2: Optical density of bacteria suspension into hydrocarbon

Bacteria	**Solvent/Cell stage**	**Exponential state Adhesion %**	**Stationary state Adhesion %**
Escherichia coli	Hexane	5.994±0.00	7.57±0.01
	Dodecane	16.58±0.00	5.02±0.00
	Ethyl Acetate	25.27±0.03	11.93±0.03
Bacillus subtilis	Hexane	11.10±0.00	7.01±0.00
	Dodecane	10.46±0.00	8.22±0.00
	Ethyl Acetate	11.93±0.03	13.32±0.03

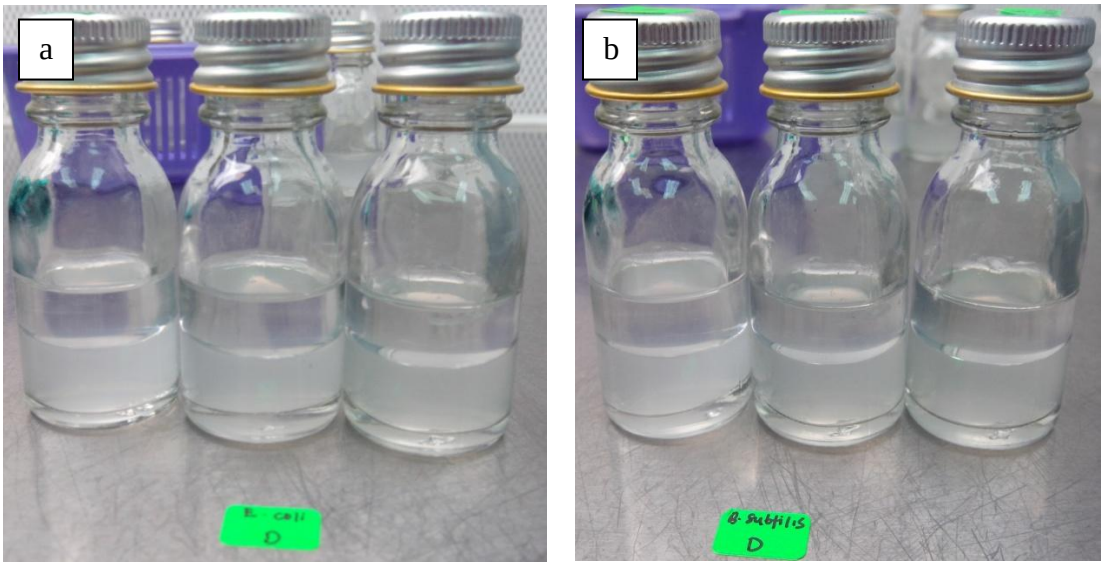


Figure 4. 3: (a) *E.coli* suspension into dodecane (b) *B.subtilis* suspension into dodecane at 16 hour fermentation

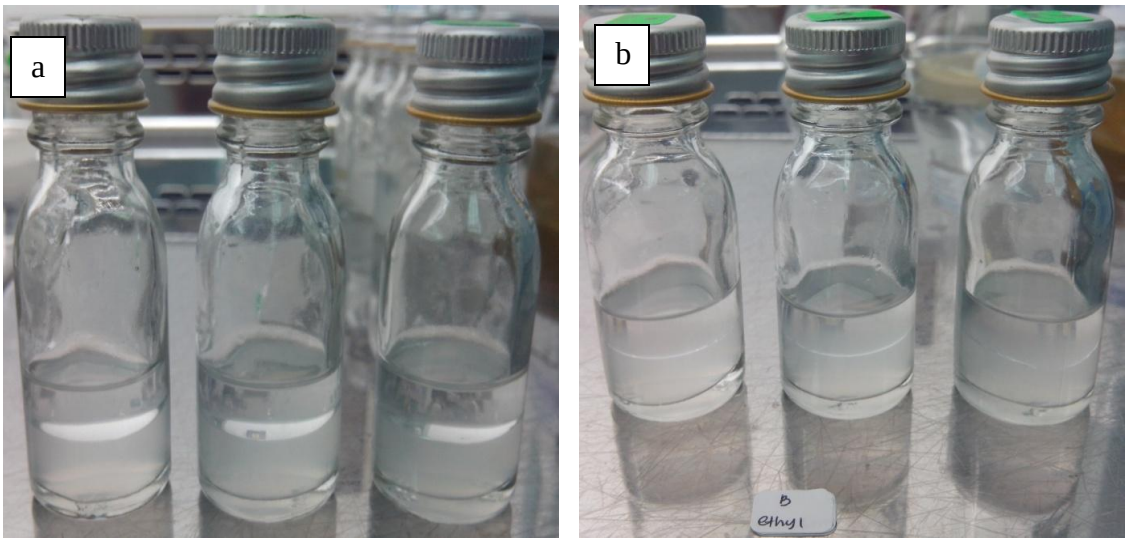


Figure 4. 4: (a) *E.coli* suspension into ethyl acetate (b) *B.subtilis* suspension into ethyl acetate at 66 hour fermentation

Table 4. 3: Summarized characteristics

	E. coli		***B. subtilis***	
Types	Gram-negative		Gram-positive	
	Exponential phase	Death phase	Exponential phase	Death phase
Size µm	1.23±0.07	1.59±0.09	1.69±1.76	2.00±0.06
Shape	Rod-shape		Rod-shape	
Hydrophobicity	Hydrophilic		Hydrophobic	

4.2 Growth curve of Escherichia coli and Bacillus subtilis

The purpose of the growth curve was to observe the pattern of growth for both *E. coli* and *B. subtilis* Bacterial growth is the division of one piece of bacteria into two daughter cells in a process called binary fission. Both of the bacteria growths were shaken at 180 rpm in order to increase the number of bacteria and fasten the reproduction process. The curve in graph form shows the change in the number of cells (or single-celled organisms) in an experimental culture at different times. The cell density of *E. coli* and *B. subtilis* was measure using UV-Vis Spectrophotometer where the live bacteria will be counted. But after 2 and 4 hours there is slightly growth of bacteria. At this stage the bacteria did not grows actively because the bacteria need to adapt to the environment. At this stage is called lag phase whereby the length of the lag phase is apparently dependent on a wide variety of factors including the size of the inoculums which is time necessary to recover from physical damage or shock in the transfer from Figure 4.5 bacteria have shown a rapid growth from 2 hours until 12 hours. During the exponential phase is a period where growth was characterized by cell doubling and number of new bacteria appearing per unit time is proportional to the present population. If growth is not limited,(e.g by medium or oxygen request) doubling will continue at a constant rate so both the number of cells and the rate of population increase doubles with each consecutive time period. In batch system, exponential growth cannot continue indefinitely, because of depletion of nutrients and enriched with wastes.

The stationary phase for *E. coli* occurred after 12 hours until 16 hours. While *B. subtilis* start to enter stationary phase after 12 until 16 hours. Population growth is limited by one of three factors which are exhaustion of available nutrients, accumulation of inhibitory metabolites or end products, exhaustion of space (Neidhert, 2005). During the stationary phase, if viable cells are being counted, it cannot be determined whether some cells are dying and an equal number of cells are dividing, or the population of cells has simply stopped growing and dividing. The stationary phase, like the lag phase, is not necessarily a period of quiescence (Ingraham, 2005).

After 16 hour for *E. coli* and 18 hour for *B. subtilis* the graph in figure 4.5shows a fluctuation of cell density of bacteria which can interpreted as the bacteria has undergo death phase. The population reaches, a death phase in which the viable cell have declines. During the death phase, the number of viable cells decreases geometrically (exponentially), essentially the reverse of growth during the log phase. It shows that the bacteria were running out of nutrients (Neidhart,2005).

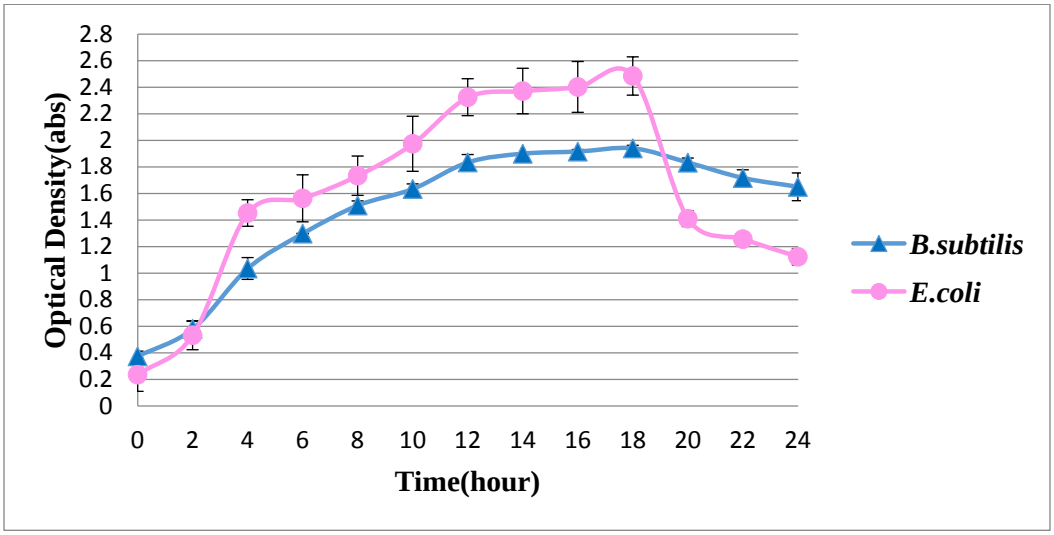


Figure 4. 5: Graph of *E. coli* and *B. subtilis* growth at 37^{o}C and 180 rpm for 24 hours.

4.3 Colony Forming Unit (CFU)

Microbial counting is useful in the basic sciences and is used determine the number of bacteria present for physiological or biochemical studies. The purpose of plate counting is to estimate the number of cells present based on their ability to give rise to colonies under specific conditions of nutrient medium, temperature and time. Theoretically, one viable cell (viable defined as able to multiply via binary fission under the controlled conditions) can give rise to a colony through multiplication. Scientists use a number of different methods to determine the number of microorganisms that are present in a given population. This can be accomplished by using the spectrophotometer to measure the optical density of the population, by directly counting the microorganisms using serial diluting the bacteria and plating the diluted bacteria on media that supports the growth of the microorganisms.

Both bacteria were harvested for 24 hours and the samples of bacteria were taken every two hours to observe the growth of the bacteria in more accurate way. To measure the growth bacteria using spectrophotometric analysis might not be accurate because of the light transmitted measure the cell numbers including die cells. The colony forming unit was used to measure the live bacteria. For this study, wide series of dilutions is normally plated because the exact number of bacteria is usually unknown.

From Table 4.4 and 4.5 it shows that there was there was pattern for the growth of bacteria where the count of bacteria on the plate keep increasing as it enter exponential phase. As it enters the exponential phase, the cell was actively reproduced and the colony formed also increase. The count of bacteria decrease as it enter stationary phase and death phase.

From the result shown the number of *B.* subtilis was greater *than E. coli*. *B. subtilis* grows better than *Escherichia coli* due to the nutrient supply where *E. coli* was grows better with Luria Bertani broth compared to nutrient broth supplied as the nutrient.

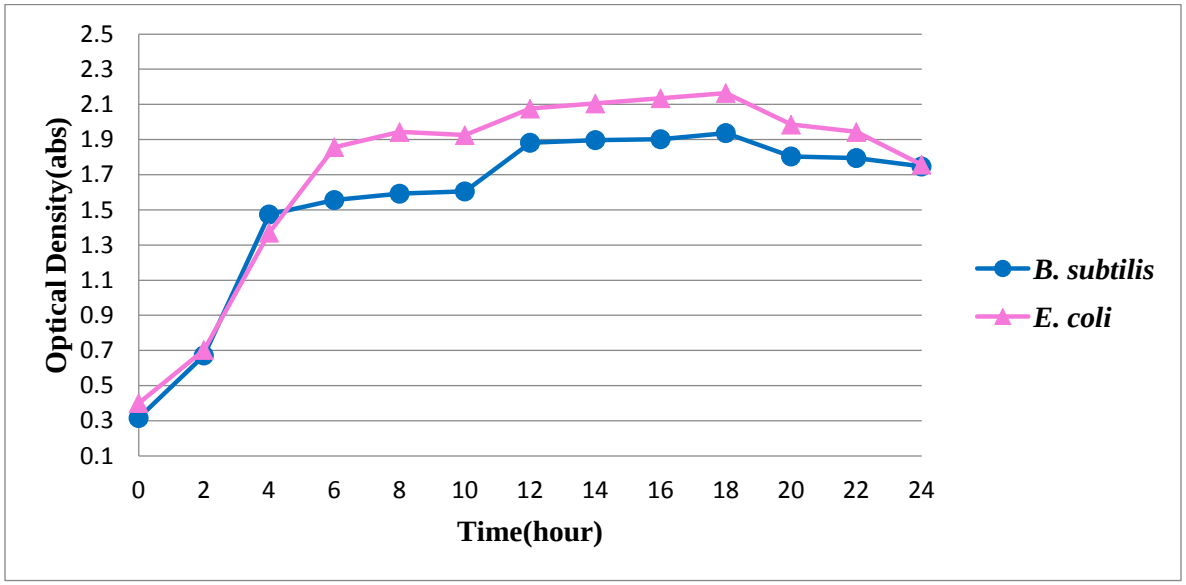


Figure 4. 6: Growth curve based on optical density (abs) for *B. subtilis* and *E. coli* in 24 hour of fermentation

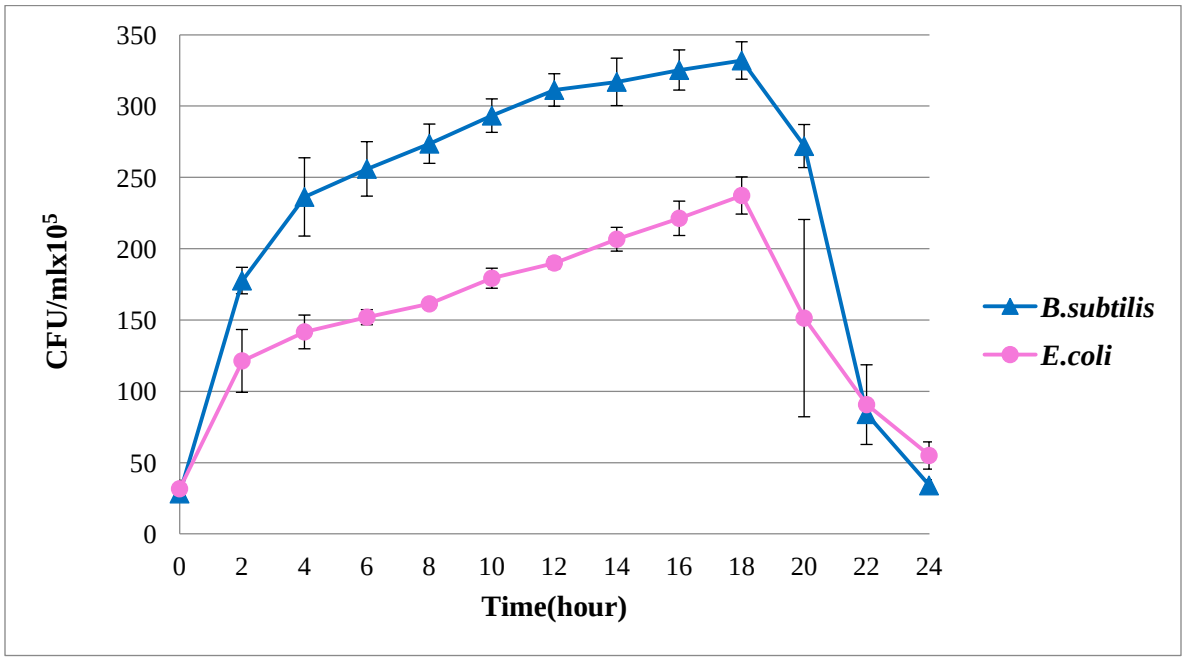


Figure 4. 7: Growth curve based on CFU for *B. subtilis* and *E. coli* in 24 hour of fermentation

Table 4. 4: Colony of *B. subtilis* count for 24 hour fermentation

Time (hour)	Optical Density (abs)	CFU/ml x 10^5
0	0.116	28.33±0.76
2	0.672	177.67±4.65
4	1.474	236.33±13.71
6	1.556	256.00±9.54
8	1.592	273.67±6.89
10	1.605	293.33±5.86
12	1.719	311.33±5.69
14	1.897	317.00±8.32
16	1.902	325.33±7.02
18	1.997	332.00±6.56
20	2.004	272.00±7.55
22	1.995	84.00±3.00
24	1.947	34.00±2.00

Table 4. 5: Colony of *E. coli* count for 24 hour fermentation

Time (hour)	**Optical Density (abs)**	**CFU/mlx10^5**
0	0.401	31.67±2.93
2	0.703	121.33±11.02
4	1.37	141.67±5.92
6	1.857	152.00±2.65
8	1.943	161.33±1.53
10	1.925	179.33±3.52
12	2.076	190.00±2.01
14	2.105	206.67±4.16
16	2.135	221.33±6.03
18	2.165	237.33±6.51
20	1.985	151.33±34.59
22	1.943	90.67±13.97
24	1.757	55.00±4.77

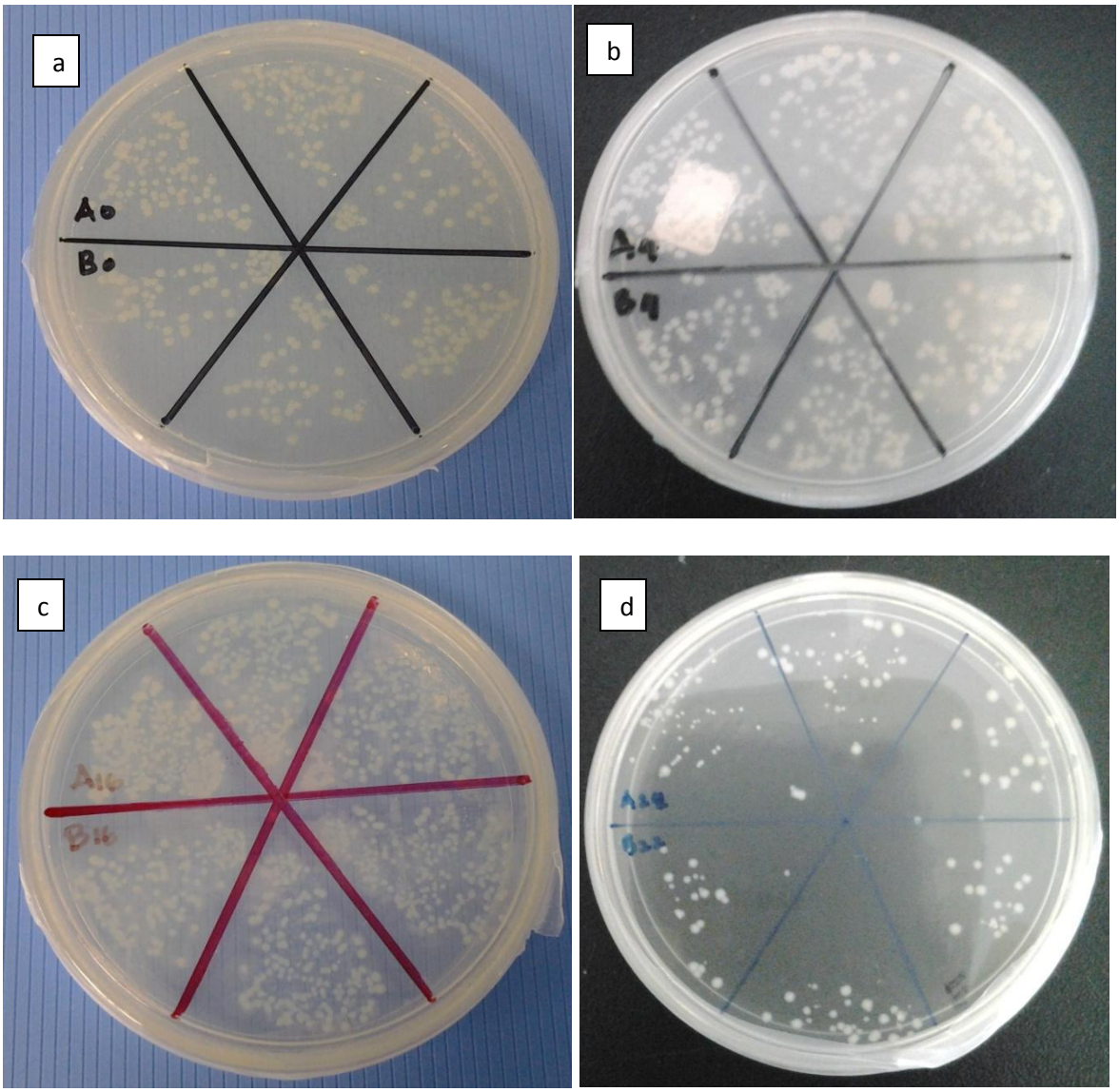
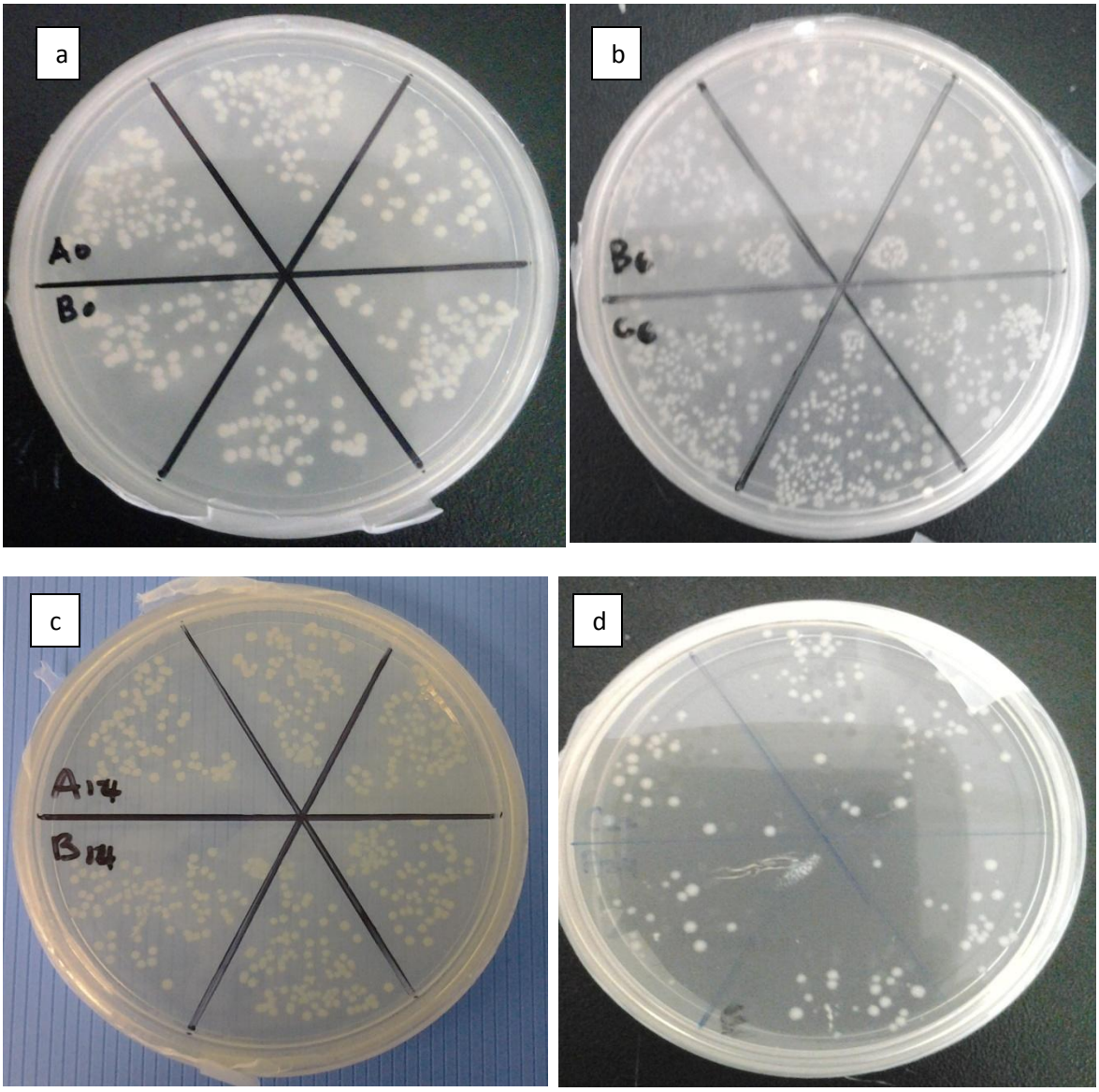


Figure 4.8: Single colony of *Escherichia coli* at (a) T_0 (b) T_4 (c) T_{16} (d) T_{22} of fermentation



(c) (d)

Figure 4.9: Colony of *B. subtilis* form at (a) T_0; (b) T_6; (c) T_{14};

(d) T_{24} of fermentation

4.5 Effect of exposure time and cell concentration on bacteria adhesion

For adhesion of *E .coli* and *B. subtilis* on the glass substrata, both of these bacteria were suspended in Phosphate Buffer Saline solution (PBS) with bacteria concentration 1.0 abs. Both of these bacteria were left suspended in the PBS solution for 24 hour with rpm 100 and 30^{o}C. From the Figure 4.10, it shows a decreasing of cell density for both bacteria after 12 hours for *E. coli* and 18 hours for *B. subtilis* of incubation. PBS solution was used because it is isotonic and non-toxic to cells and sodium chloride provides osmotic protection of microbial cells. In addition, phosphates provide a stable physiological pH (7.0) value which is also important for the maintenance of cell viability. When *E. coli* and *B. subtilis* suspended in the PBS solution, it will inhibit the bacteria to grow. From the decreasing of the cell density of the bacteria it has proven that bacteria have adhered to the glass slide. The amount of bacteria adhered on the glass slide at slow agitation increasing as the time increase.

Other researchers have concluded that electrostatic interaction between the bacterium and surface is the main factor affecting bacterial-surface adhesion, with hydrophobic interactions and polymer bridging playing only minor roles. Glass surface is hydrophilic. Bacteria and other microorganism have evolved many different ways to use hydrophobic effect in order to adhered to substrata (Doyle, 2002). Both *E. coli* and *B. subtilis* have different hydrophobic effect in order to adhere to the glass surface. This is why percentage of from Table 4.6 it shows the percentage of *B. subtilis* adhered on the glass surface increase as the time increase. While in Figure 4.6(b) it shows that *E. coli* has reached stationary point at 12 hour of incubation. From Table 4.8, adhesion of *B. subtilis* to the glass at cell concentration 0.8 abs have shown the greatest percentage by 46.88% after 24 hours suspended in PBS solution. *B. subtilis* able to adhere the most at this concentration due to less concentrated of cell suspended in PBS solution. When bacteria have less concentrated it make the bacteria less agglomerate and make it easier to attach to the glass surface. Different with the *E. coli*, the attachment on the glass is the best when the cell concentration at 1.0 abs with 29.8 %. *E. coli* reach it stationary phase at 12 hour. *E. coli* has stop attach to the glass slide after 12 hour. *E. coli* poorly attach to uncoated glass

surface (Cooke, 2008). From the research that has been done by Cooke, when the glass was coated with collagen, laminin and fibronected, the result appear *E. coli* adhere more to the surface. *E. coli* able to adhere to the glass due to the hyrdophobicities effect and nutrient availability. With the presence of protein, *E. coli* more to attracter to the glass surface . For this study the glass slide used was uncoated with any protein. Besides that, due to the repulsive effect of hydrophilic *E. coli* and hydrophilic glass surface reduce the attachment of *E. coli* on the glass surface (Johnson, 2000)

Table 4. 6 :Adhesion of *B.subtilis* on glass slide suspended in PBS solution with optical density 1.0 (abs)

Time (hour)	**Optical Density (abs)**	**Percentage of *B.Subtilis* adhesion %**	**Total of adhesion of *B.subtilis* per area,cm^2**	**CFU Count per ml x 10^{-9}**
T_0	1.051	0.00	0±0	72.75±1.086
T_4	0.964	8.27	30.34±5.67	58.52±1.01
T_8	0.884	8.29	67.89±10.16	50.45±1.01
T_{12}	0.805	8.93	89.17±5.46	40.36±1.00
T_{24}	0.673	16.39	104.12±112.31	2 7.91±1.05

Table 4. 7: Adhesion of *E. coli* on glass slide suspended in PBS solution with optical density 1.0 (abs)

Time (hour)	**Optical Density (abs)**	**Percentage of *E.coli* adhesion %**	**Total of adhesion of *E.coli* per area,cm^2**	**CFU Count per ml x 10^6**
T_0	1.001	0.00	0±0	82.75±1.07
T_4	0.958	4.29	59.38±18.76	73.32±0.58
T_8	0.867	9.49	96.50±4.40	65.23±1.54
T_{12}	0.781	9.91	96.38±21.92	55.15±0.76
T_{24}	0.703	9.98	28.86±10.71	45.40±1.00

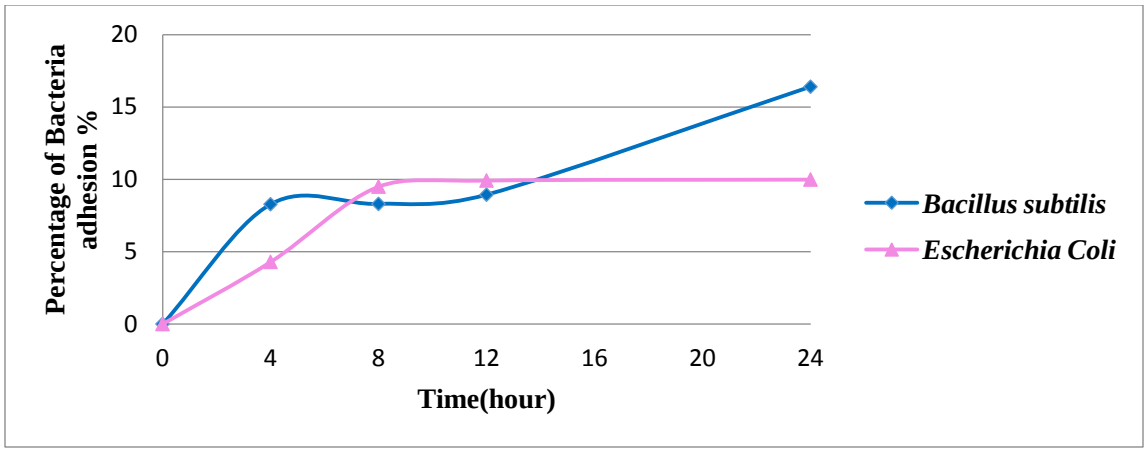


Figure 4.10: Graph of percentage adhesion of *E. coli* and *B. subtilis* on glass slide at optical density 1.0 (abs)

Table 4. 8: Adhesion test of *E .coli* and *B. subtilis* on glass surface at different cell concentration.

Bacteria	**Cell concentration (abs)**	**Time (hour)**	**Optical Density (abs)**	**Percentage of Bacteria adhesion %**	**Total of adhesion of Bacteria per area,cm2**	**CFU Count per ml x 10^6**
Bacillus subtilis	0.8	T_0	0.802	0	0±0	53.44±1.26
		T_{24}	0.426	46.88	100.88±5.43	25.56±2.27
Escherichia coli		T_0	0.821	0	0±0	57.94±1.54
		T_{12}	0.765	6.82	77.13±8.76	22.41±1.13
Bacillus subtilis	1.2	T_0	1.201	0	0±0	89.80±4.48
		T_{24}	0.787	34.47	89.90±4.48	45.73±0.77
Escherichia coli		T_0	1.207	0	0±0	110.99±1.00
		T_{12}	1.071	11.27	69.25±11.83	56.50±1.00

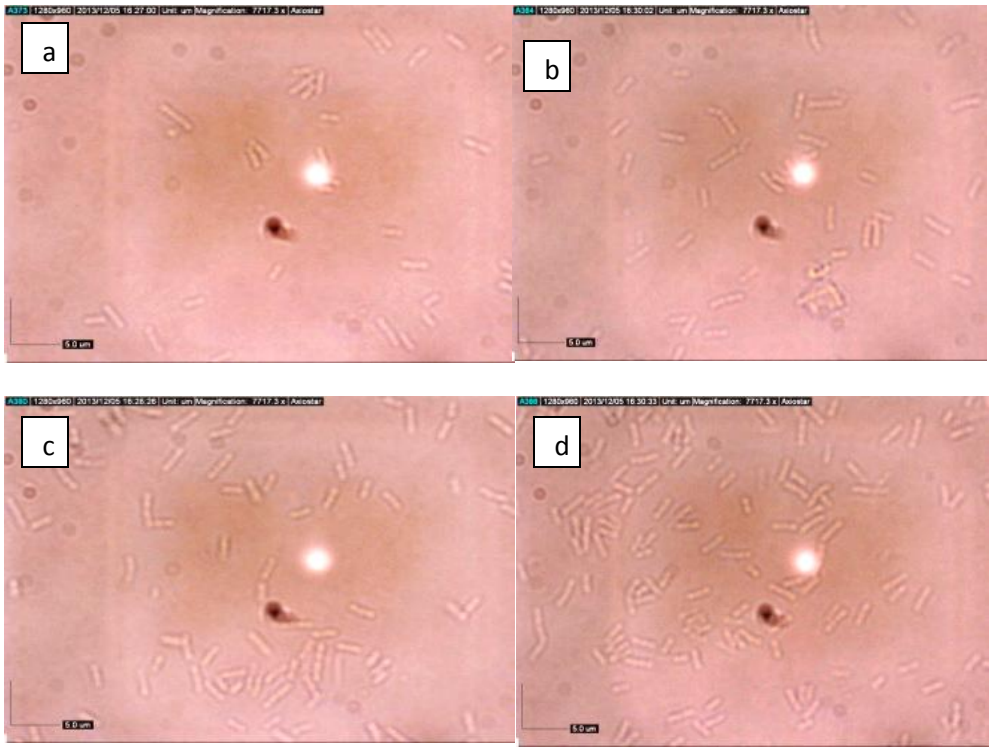


Figure 4.11: Sequence of *B. subtilis* adhere to glass surface as the time increase (T_4, T_8, T_{12}, T_{24}) at cell concentration 0.8 abs.

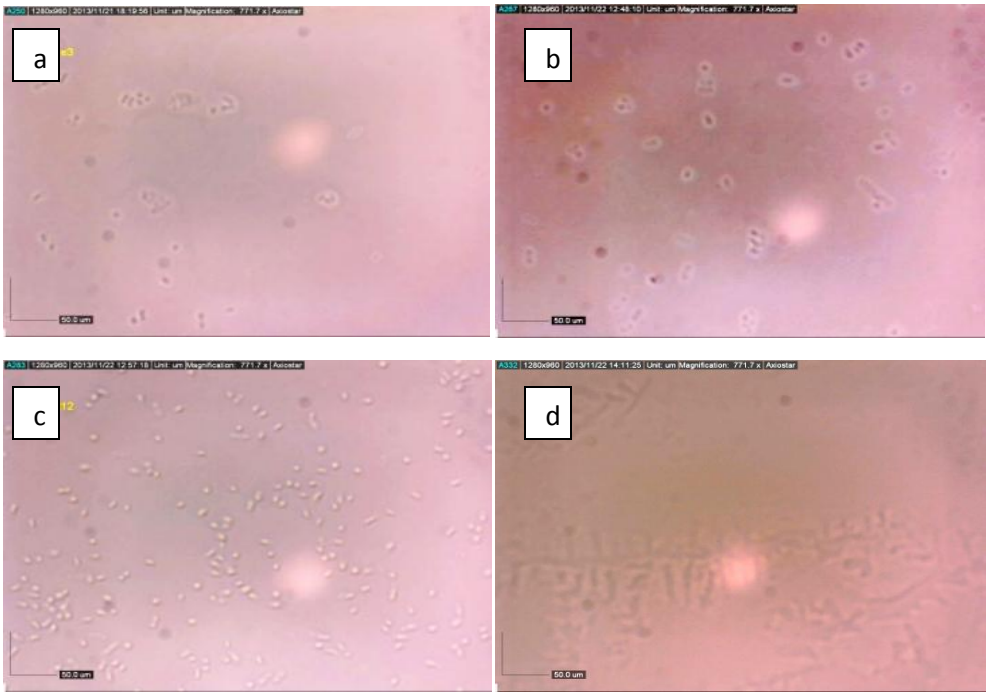


Figure 4.12: Sequence of *E. coli* adhere to glass surface as the time increase (T_4, T_8, T_{12},$T_{24)}$ at cell concentration 1.0 abs.

4.6 Effect of culture age

For culture age, both of *E. coli* and *B .subtilis* able to adhere to glass surface at exponential and death phase. Table shows that percentage of bacteria adhesion at different incubation time has increase. The percentage of bacteria was measured by reading the value of optical density. The cell concentration decrease as the time of incubation increase. To ensure the bacteria adhesion to the glass the glass slide that suspended in the PBS solution was observed under light microscope which is more accurate compared to the optical density of the cell concentration. *E. coli* less adhere to the glass due to the hydrophobicities where the finding obtains is in the agreement with the report by Donlan (2002) and Sinde and Carballo in which they reported that glass is hydrophilic materials. Table 4.8 shows that the percentage of *E. coli* adhered on the glass surface at exponential was 37.27% while percentage of *B. subtilis* was 46.02%. From figure 4.13(a) and 4.13(b) is shows that there are slightly decrease of bacteria adhesion when the bacteria were incubates at 66 hour. Besides that, even though both of the bacteria has entered the death phase where both bacteria unable to survive due to starvation but both these two bacteria able attach to the glass surface. At stationary phase the growth of both bacteria has decline and reduce the attractive force to the glass surface.

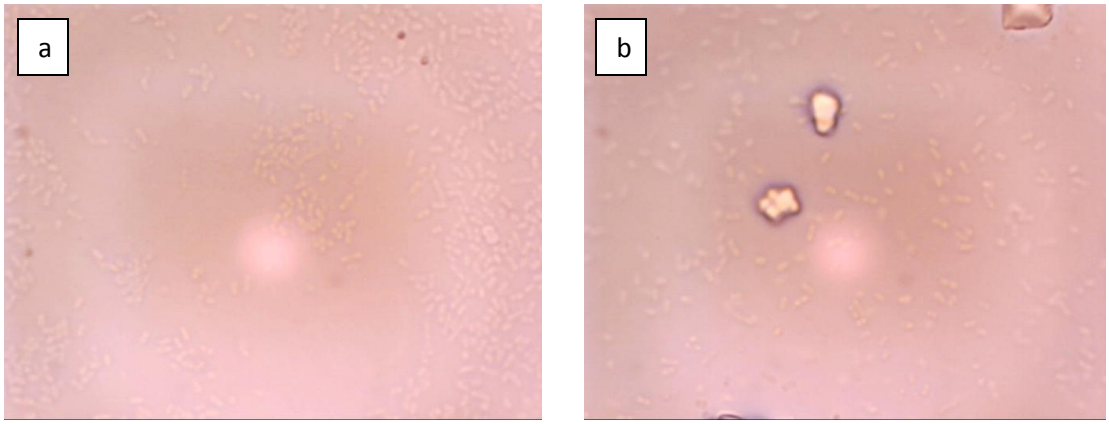


Figure 4.13 (a): (a) Adhesion of *E. coli* at 16 hour (b) Adhesion of *E. coli* at 66 hour

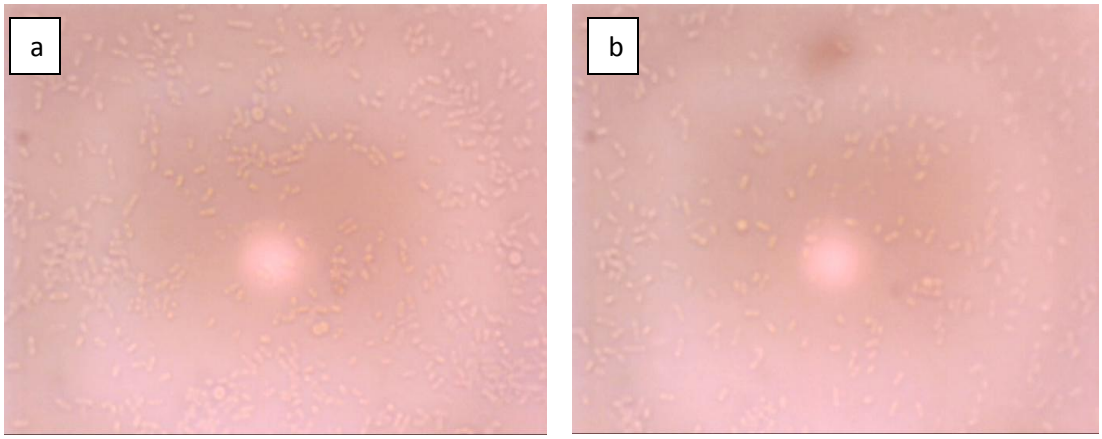


Figure 4.13 (b): Adhesion of *B.subtilis* at 16 hour (b) Adhesion of *B.subtilis* at 66 hour

Table 4. 9: Cell adhesion to glass at exponential phase and stationary phase

Bacteria	Time (hour)	Optical Density (abs)	Percentage of Bacteria Adhesion %	Total of Bacteria Adhesion per area/cm2
Escherichia coli	T_{16}	0.884	37.27	85.00±7.57
	T_{66}	0.667	17.46	71.25±4.87
Bacillus subtilis	T_{16}	0.768	46.02	83.75±7.58
	T_{66}	0.57	27.27	71.25±4.86

5 CONCLUSION

In order to prevent the bacteria adhesion on the glass surfaces factors of microbial cell number, bacteria concentration, exposure time and culture age was studied to identify the maximum of bacteria adhesion on the glass. This is important to prevent the bacteria adhesion on the glass surface.

Bacteria at exponential phase and death phase showed different in size where the size of E. coli at exponential phase was 1.23±0.07 while at death phase was 1.59±0.09 with 22.64% increase in size. For *B. subtilis* the size at exponential phase was 1.69±0.17 while at death phase was 2.00±0.06 with 15.50% increase in size. There were slightly different with theoretical size of bacteria at different phase.

To identify the hydrophobicity of the bacteria, three different solvent with different characteristics was used. The types solvent used were hexane, dodecane and ethyl acetate. *E. coli* showed a decreasing percentage from exponential phase to stationary state by using dodecane and ethyl acetate but showed an increasing in percentage using hexane. *B. subtilis* showed a decreasing percentage from exponential phase to stationary phase using hexane and dodecane and showed increasing in percentage using ethyl acetate. From the test *E. coli* was proved as hydrophilic bacteria and *B. subtilis* as hydrophobic bacteria. The hydrophobicity effect the adherence of bacteria to the surface.

Growth curve of *E .coli* and *B. subtilis* were studied by colony forming unit (CFU) and the absorbance culture bacteria. The optical density and CFU of the cultured bacteria shows the growth pattern of the bacteria. The absorbance of bacteria was measured using UV-vis spechtrophotometer to read the turbidity or optical density of the bacteria by the light that emitted and penetrates the culture bacteria. To enhance the growth pattern of the bacteria CFU test were used by inoculate the culture bacteria on the nutrient agar. The growth of bacteria was measured by counting the colony form after 24 hour incubation in microbial incubator. The result shows the absorbance and colony form increase as it enter exponential phase and decrease as it enter the decline phase.

The adherence of bacteria to the surface was measured by the incubation of bacteria in the phosphate buffer solution at different time and cell concentration. Adherence of both

bacteria was test every 4 hour until 24 hour at cell concentration 1.0 to identify the best time of bacteria attachment to the surface. Adherence of E. coli to the surface reaches stationary at 12 hour with 29.77% while *B. subtilis* adhere the best at 24 hour with 35.96% at cell concentration 1.0 abs. adherence then test with different cell concentration at 1.2 and 0.8 abs. *B. subtilis* attach to the surface better at 0.8 abs with 46.88% and *E. coli* adhere the best at 1.0 abs.

For the culture age test, both bacteria were incubated at 16 and 66 hour. Adherence of bacteria was the best at 16 hour. Eventhough, both bacteria were incubated until 66 hour both bacteria able to adhere to the glass surface.

6 RECOMMENDATION

In order to get better result in the future, *E. coli* must be grow in Luria bertani agar and broth to enhance the growth of *E. coli* in this study . This is because *E. coli* barely consumed glucose as contain in nutrient broth. Luria bertani contains a low concentration of sugars (<0.1 mM), meaning that several amino acids, including serine, proline, leucine, alanine, arginine, and lysine, are the principal carbon sources (Sezonov G,2007). Besides that, adherence of bacteria to the surface should be varied with temperature to obtain optimum adherence condition on the surface. Yet, the agitation of the incubation of bacteria should be varied to study the effect of shear stress on the adherence of bacteria to the surface. Shear stress plays a crucial role in deposition of bacteria from the surface.

7 REFERENCES

Allison, (2003), The biofilm matrix. Biofouling, 19, 139–150.

Anonymous, 1993, Hygienic Design Of Closed Equipment For The Processing Of Liquid Food. Trends in Food Science &Technology, 4:375-9.

Barker J, and Bloomfield S.F, 2000, Survival of Salmonella in bathrooms and toilets in domestic homes following salmonellosis, J Appl Microbiol, 89:137–44.

Barnes *et al*., 1999, Effect Of Milk Proteins On Adhesion Of Bacteria To Stainless Steel Surfaces. Applied and Environmental Microbiology, 65:4543-8.

Brant and Childress, (2002), Membrane Science, Budny , 2002, Compositions For Treating Biofilm, Vol 203, 257.

Busscher H.J,and Weerkamp A.H, (1987), Specific and Non-specific Interaction on Bacterial Adhesion to Solid Substrata, FEMS Micobiology Review, 46, 165.

Busscher H.J, Sjollema J and Van Der Mei H.C, 1990, Microbial Cell Surface Hydrophobicity, American Society of Microbiology, 335.

Bellon-Fontaine and Rault C.J, 1996, Microbial adhesion to solvents: a novel method to determine the electron-donor/electron-acceptor or Lewis acid-base properties of microbial cells, Colloids and Surfaces B: Biointerfaces 71,147-53

Bott, 1998, Techniques for reducing the amount of biocide necessary to counteract the effects of biofilm growth in cooling water systems, Appl Therm Eng, 18:1059–66.

Breyers *et al*., (2004). Bioinspired implant materials befuddle bacteria. ASM News, 70, 232–237.

Chye and Abdullah, 2004, Bacteriological quality and safety of raw milk in Malaysia. Food Microbiol, 21:535–41.

Chiffre, 1999, Industrial Survey On ISO Surface Texture Parameters. *Annals Of The CIRP*, 48:4.

Curtis V.A, Biran A, Deverell K, *et al*., 2003, Hygiene in the home: relating bugs and behavior. Social Sci-Med, 57:657–72.

Daeschel and Joseph M, (1998), Interrelationship between Protein Surface Adsorption and Bacterial Adhesion, Biotechnology and Genetic Reviews, 15.

Daniels R, Vanderleyden J, Michiels J , et al., (2004). Quorum sensing and swarming migration in bacteria. FEMS Microbiology Reviews, 28, 261–289.

Dankert, Hogt A.H, Feijen J, (1986), Biomedical polymers: bacterial adhesion, colonization, and infection, CRC Crit. Rev. Biocompat. 2 219–301.

Dexter SC, Sullivan JD, Williams J, *et al*, (1975), Influence of Substrate Wetability on Attachement of Marine Bacteria Various Surfaces, Application Microbial, 30, 298-308.

Donlan, (2002). Biofilms: microbial life on surfaces, Emerging Infectious Diseases, 8, 881–890.

Doyle and R.J, (2000), Contribution of the Hydrophobic effect to Microbial Infection, Microbe and Infection, 2, 391-400.

Duncan-Hewitt and W.C, (1990), Nature of the Hydrophobic Effect, In Microbial Infection Cell Surfaces, 39-73.

Drenkard E and Ausubel FM, (2002), *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation, Nature, 416, 740–743.

Eberhard A, Burlingame AL, Eberhard C,1981), Structural identification of autoinducer of Photobacterium fischeri luciferase. Biochemistry, 20, 2444–2449.

Fletcher, (1990), Method of Studying Adhesion and Attachment to Surfaces, Method Microbiol, 22, 251-280.

Flint S.H, Brooks J.D, Bremer P.J*, et al*., 2000, Properties of The Stainless Steel Substrate, Influencing The Adhesion Of Thermo-Resistant *Streptococci*, Journal of Food Engineering, 43,235-42.

Flint S.H, Bremer P.J, Brooks J.D. Biofilms In Dairy Manufacturing Plant - Description, Current Concerns And Methods Of Control, Biofouling, 1997,11,81-97.

Fuqua C and Greenberg E.P, (2002), Listening in on bacteria acyl-homoserine lactone signaling, Nature Reviews in Molecular Cell Biology, 3, 685–695.

Kumar C.G and Anand S.K, 1998, Significance of microbial biofilms in food industry a review, Int J Food Microbiol, 42,9–27.

Gorman R, Bloomfield S, Adley C.C 2002, A study of cross-contamination of food-borne pathogens in the domestic kitchen in the Republic of Ireland, Int J Food Microbiol, 76,143–50.

Hancock C, 1985, Microbial Cell Surface Analysis, Structural and Physicochemical Method, 23

Halabi M, Wiesholzer-Pittl M, Schöberl J, Mittermayer H, 2001, Non-touch fittings in hospitals: a possible source of *Pseudomonas aeruginosa* and *Legionella spp*. ,J Hospital Infect, 49,117–21.

Hall and Stoodley, (2002), Developmental regulation of microbial biofilms, Current Opinion in Biotechnology, 13, 228–233.

Harbron and Kent, (1988), Aspects of cell adhesion, In L. F. Melo, T. R. Bott, & C. A. Bernardo (Eds.), NATO ASI series, 145, 125–140.

Herbert, (1990), Method for Enumerating and Determining Biomass in Natural Environment, Method of Microbial, 22, 287-307.

Katsutoshi Hori and Shinya Matsumoto, (2010), Bacterial Adhesion: Mechanism to Control, Biochemical Engineering Journal, 48, 424-434.

Klahre and Flemming, 2000, Monitoring of biofouling in paper mill process waters. Water Res, 34:3657–65.

Lee, (2004), Adhesion to glass and metal oxide surfaces, *Colloids and Surfaces*, 81-90.

Liesegang Y, 1997, Contact lens related microbial keratitis. Part II: Pathophysiology, Cornea, 16,265–73.

Hua L, Michael N.H, and Dennis G.C, 2001, Cell density modulates acid adaption in Streptococcus mutans Bacteriol, 183, 75–84.

Mah T.F and O'Toole G.A, (2001), Mechanisms of biofilm resistance to antimicrobial agents, Trends in Microbiology, 9, 34–39.

Marshal K.C, Wicken A.J and Loeb G.I, 1985, Bacterial Adhesion, Mechanism and Physicochemical Significance, 133

Marotta M, Martino A , A De Rosa, *et al*., 2001 Degradation of dental plaque glucans and prevention of glucan formation using commercial enzymes, Process Biochem, 38, 101–8.

Mark E.S and Mader J.T, (2000), Molecular interactions in biofilms, Chem Biol , 9, 859–71

Mattick K, Durham K, Domingue G*, et al*., 2003, The survival of foodborne pathogens during domestic washing-up and subsequent transfer onto washing-up sponges, kitchen surfaces and food, Int J Food Microbiol, 85,213–26.

Nemati M, Jenneman G.E and Voordouw G, 2001, Mechanistic study of microbial control of hydrogen sulfide production on oil reservoirs, Biotechnol , 74,424–30.

Neu T.R, (1996), Significance of bacterial surface-active compounds in interaction of bacteria with interfaces, Microbiological Reviews, 60, 151–166.

Niels P.B, Willem N, Henry C.V and Henk J.B, (2008), Forces Involved in Bacterial Adhesion to Hydrophilic and Hydrophobic Surfaces, Microbiology, 154, 3122-3133.

Olsen, 1993, Influence of pH on bacterial gene expression, Mol Microbiol , 8,5–14

Parsek M.R and Greenberg E.P, 2005, The connection between quorum sensing and biofilm, Socimicrpbiology,13, 27-33

Peter and Eldere, 2010, Prevention of *Staphylococcus* Biofilm Formation.

Renner and Weibel, 2011, Physicochemical Regulation of Biofilm Formation, MRS Bulletin, 36,347-55.

Rowland BM, 2003, Bacterial contamination of dental unit waterlines: what is your dentist spraying into your mouth? Clin Microbiolm Newslett , 25, 73–7.

Ryan R.P and Dow J.M, (2008), Diffusive signals and interspecies communication in bacteria, Microbiology, 154, 1845–1858.

Sauer and Camper, (2001), Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. Journal of Bacteriology, 183, 6579–6589.

Simões M, Pereira M.O, Sillankorva S*, et al*., (2007), The effect of hydrodynamic conditions on the phenotype of Pseudomonas fluorescens biofilms, Biofouling, 24, 249–258.

Stout and Blunt, 1995, Nanometres To Micrometres: Three-Dimensional Surface Measurement In Bio-Engineering. Surface And Coatings Technology, 71, 69-81.

Truong V.K, Rundell S, Lapovok R, *et al*., 2009, Effect of Ultrafine-Grained Titanium Surfaces on Adhesion of Bacteria, Application of Microbial Biotechnology, 83, 925-37.

Stepanovic S, Dakic I, Opavskin, Jezek P and Ranin L, 2003, Influence of growth medium composition on biofilm formation by *Staphylococcus sciuri*, Annal of Microbiology, 53, 63-74

Sutherland, (2001), The biofilm matrix – an immobilized but dynamic microbial environment, Trends in Microbiology, 9, 222–227.

Sutherland I.W, Hughes K.A, Skillman L.C ,*et al*., (2004), The interaction of phage and biofilms, FEMS Microbiology Letters, 232, 1–6.

Tide, 1999, Influence of Welding Procedures on Bacterial Colonization of Stainless Steel

Verran and Jones, (2000), Industrial biofouling. New York: John Wiley and Sons Ltd.

Vishwakarma V, Josephine J, George RP , *et al*., (2009), Antibacterial Copper-Nickel Bilayers and Multilayer Coatings by Pulsed Laser Deposition On Titanium, Biofouling, 25,705-10.

Weldments, 2003, Journal of Food Engineering, 42,85-96.

Whitchurch et al., (2002), Extracellular DNA required for bacterial biofilm formation, Science, 295, 1487.

Wingender *et al*., 2004, Microbial extracellular polymeric substances – Characterization, structure and function (pp. 1–19).

Yuehuei and Richard Friedman, (1997), Laboratory Methods for Studies of Bacterial Adhesion, Journal of Microbial Methods, 30, 141-152.

Yoo and C.f, 2002, An emission pattern of a thermophilic bacteria attached to or imbedded in porous supports. Int J Food Microbiol, 73:11–21.