ADHESION OF BACTERIAL ON METAL SURFACES: EFFECT OF SURFACE ROUGHNESS

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ADHESION OF BACTERIAL ON METAL SURFACES: EFFECT OF SURFACE ROUGHNESS

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Thesis submitted in partial fulfilment of the requirements
for the award of the degree of
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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 (Hons) 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.

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Date : JANUARY 2015

Dedication

Dedicated To

My Parents;

Chik Bin Yom

and

Che Mah Binti Hasan

for being so supportive

Brothers;

Abdul Rahim Bin Chik

Amiruddin Bin Chik

Sisters;

Nor Hayati Binti Chik

Nur Haliza Binti Chik

Nor Hayani Binti Chik

for being understanding

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ABSTRACT

This research reviews the adhesion of bacterial on metal surfaces and the effect of metal's surface roughness to the bacterial adhesion. The objectives of this research are, to study the adhesion of bacterial on metal surfaces (Stainless Steel (N690), Stainless Steel (AV220SC) and Titanium) used in medical application and the effect of surface roughness on the wettability thus adhesion. Three types of bacteria were used for this research which was *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus* attached on the individual metal which has eight different ranges of surface roughness, achieved by fabricating using electro depositing machine (E) and electropolishing (P) techniques. The adhesion test was done for 4 hours for each bacterium with different types of metal and surface roughness. After the adhesion test, the attached bacteria on metal surfaces were dyed using fluorescence dye, SYTO9 and examined under the fluorescence microscope. The number of attached cells was counted and results were displayed as number attached per square area. Besides that, the absorbance and the colony forming unit (CFU) were also measured and the surface which gives highest optical density (OD) was identified. Finally, metal with highest attachment of bacterial, were analyzed under Scanning Electron Microscopy (SEM) to observe the bacterial cells attachments. Results obtained indicated that *E. coli* was a Gram negative bacterium while both *B. subtilis* and *S. aureus* were Gram positive bacteria. After the adhesion test, the OD reading for *E. coli* shows the highest reduction which is in the range of 6% to 45.7% compared to others bacterial solution. CFU plating for *E. coli* at dilution factor 10^2 also showed the lowest among others after the adhesion test. Besides that, the adhesion of bacterial on Stainless Steel (N690) recorded that, the highest attachment of all bacterial were on smooth surface *(Ra* = 0.163 µm) and rough surface (*Ra* = 2.910) with the total adhesion were 0.1833/ μm^2 and 0.1755/ μm^2 respectively. Meanwhile, adhesion on Stainless Steel (AV220SC) and Titanium shows similar trend where both *B. subtilis* and *S. aureus* provide the highest adhesion at the roughness 0.110 µm and 0.104 (smooth surface), respectively. On the other hand, the adhesion of bacterial on different metal with the same roughness (*Ra* = ~0.15 µm) showed that *B. subtilis* like to adhere to Titanium surface with the adhesion value 0.9601/μm^2, while *S. aureus* and *E. coli* were adhered at Stainless Steel (N690) and Stainless Steel (AV220SC), respectively with the total adhesion 0.0679/μm^2 and 0.0704/μm^2 each.

ABSTRAK

Kajian ini adalah tentang kelekatan bakteria pada permukaan logam dan kesan kekasaran permukaan logam terhadap kelekatan bakteria. Objektif kajian ini adalah untuk mengkaji kelekatan bakteria pada permukaan logam (Stainless Steel (N690), Stainless Steel (AV220SC) dan Titanium) yang digunakan dalam aplikasi perubatan dan kesan kekasaran permukaan pada kebolehbasahan lekatan. Tiga jenis bakteria telah digunakan untuk kajian ini iaitu *Escherichia coli*, *Bacillus subtilis*, dan *Staphylococcus aureus* yang melekat pada logam individu dan mempunyai lapan jenis kekasaran permukaan yang berbeza. Jenis kekasaran permukaan logam tersebut tercapai selepas melalui mesin electrodepositing (E) dan teknik menggilap (P). Ujian kelekatan telah dilakukan selama 4 jam untuk setiap jenis bakteria dengan pelbagai jenis logam dan kekasaran permukaan. Selepas ujian lekatan, bakteria yang telah melekat pada permukaan logam telah dicelup menggunakan pewarna pendarfluor, SYTO9 dan diperiksa di bawah mikroskop pendarfluor itu. Bilangan sel-sel yang melekat di logam telah dikira dan keputusan telah dipaparkan dalam bentuk bilangan kelekatan di setiap kawasan persegi. Selain itu, keserapan dan pembentukan unit koloni (CFU) juga diukur dan permukaan yang memberikan ketumpatan optik (OD) tertinggi telah dikenal pasti. Akhir sekali, logam yang memmpunyai kelekatan bakteria tertinggi, dianalisis di bawah Mikroskopi Elektron Imbasan (SEM) untuk melihat bentuk kelekatan sel bakteria. Keputusan yang diperolehi menunjukkan bahawa *E. coli* adalah bakteria Gram negatif manakala, kedua-dua *B. subtilis* dan *S. aureus* adalah bakteria jenis Gram positif. Selepas ujian kelekatan dilakukan, OD untuk *E. coli* menunjukkan penurunan tertinggi iaitu diantara kadar 6% ke 45.7% berbanding dengan bakteria yang lain. CFU untuk *E. coli* pada faktor pencairan 10^2 juga menunjukkan yang paling rendah antara yang lain selepas ujian tersebut. Di samping itu, kelekatan bakteria pada Stainless Steel (N690) mencatatkan bahawa, lampiran tertinggi semua bakteria berada di permukaan licin (*Ra* = 0.163 μm) dan permukaan kasar (Ra = 2,910) dengan jumlah lekatan masing-masing adalah 0.1833 / μm^2 dan 0.1755 / μm^2. Sementara itu, keleketan bakteria pada Stainless Steel (AV220SC) dan Titanium menunjukkan trend yang sama di mana kedua-dua *B. subtilis* dan *S. aureus* menunjukkan lekatan yang paling tinggi pada kekasaran 0.110 μm dan 0.104 μm (permukaan licin). Sebaliknya, kelekatan bakteria pada logam yang berbeza tetapi dengan kekasaran yang sama (Ra = ~ 0.15 μm) menunjukkan bahawa *B. subtilis* suka melekat pada permukaan Titanium dengan jumlah kelekatan 0.9601/μm^2, manakala *S. aureus* dan *E. coli* masing-masing suka melekat di Stainless Steel (N690) dan Stainless Steel (AV220SC) dengan jumlah kelekatan untuk setiap satu adalah 0.0679/μm^2 and 0.0704/μm^2.

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LIST OF ABBREVIATIONS

E. coli	*Escherichia coli*
B. subtilis	*Bacillus subtilis*
B. cereus	*Bacillus cereus*
CFU	Colony Forming Unit
CO_2	Carbon Dioxide
EPS	Extracellular Polymeric Substance
Fe^{2+}	Iron
FKKSA	Fakulti Kejuruteraan Kimia dan Sumber Asli
HCl	Hydrochloric acid
KCl	Potassium Chloride
K_2HPO_4	Dipotassium Phosphate
KH_2PO_4	Monopotassium Phosphate
$MgSO_4$	Magnesium Chloride
NaOH	Sodium Hydroxide
NaCl	Sodium Chloride
OD	Optical Density
PIA	Polysaccharide Intercellular Adhesion
PBS	Phosphate Buffer Solution
Ra	Roughness average
Rq	Root mean square roughness
Rsk	Skewness
Rku	Kurtosis
S. aureus	*Staphylococcus aureus*
SEM	Scanning Electron Micoscope

SE	Staphylococcal Enterotoxin
US	United States
UMP	Universiti Malaysia Pahang

1 INTRODUCTION

1.1 Background of Study

Bacteria can attach to a variety surfaces, ranging from surfaces in the human body, plants and clays, to plastic and metals. The attachment of the bacterial colonies to the surfaces is termed as adhesion (Trevor *et al.*, 2008) which often developed biofilm. Biofilm is an irreversible of microbial cells such as bacteria on abiotic or biotic (Kokare, 2008). They adhere to exposed surfaces and subsequently cause problems ranging from contamination in medical devices to biofouling of industrial equipment (Xiaoxia *et al.*, 2007). In the medical application, biofilms or bacterial adhesion remain as major infection to the long term use of implanted or intravascular devices such as joints prosthese, heart valves, vascular cathters, contact lenses and dentures (Katsikogianni and Missirlis, 2004). These are due to the massive matters or biofilms of infectious bacteria which can form over the metal surface, especially roughened surface areas (Murr *et al.*, 2012). Most infections are hospital acquired and the only solution for an infected implanted device is by undergoing surgical removal.

Besides that, based on the data taken from the National Nosocomial Infections Surveillance System showed that, nosocomial infections affect approximately 10% of all in patients, delay discharge by average of 11 days and direct cause 5000 deaths/year in England (Katsikogianni and Missirlis, 2004). In U.S the various infections strike roughly 2 million people annually, 0.05% caused death (Murr *et al.*, 2012). Then, according to Rethman *et al*. (2011), orthopedic implant infection rates range from 0.3% to 8.3% which occurred during surgery, infection from a local source through blood transport, or the recurrence of sepsis in a previously infected joint. With roughly 400,000 primary hip arthroplasties and nearly 1 million total knee anthroplasties in the U.S. in 2012, the market exceeds $20 billion.

On the other hand, biofilms also are well known in the usefulness of bioremediation. The use of the organisms to remove contaminants like metals and radio nuclides (Barkay and Schaefer, 2001), oil spills, nitrogen compounds and for the purification of industrial waste water (Trevor *et al.*, 2008) is now common place. During this process, biofilms are grown on rocks or pieces of plastic to clean wastes out of water. Meanwhile, Edward and

Kjellerup (2013) also found that indigenous bacterial communities are capable of metabolizing persistent organic pollutants and oxidizing heavy metal contaminants. The durability and structure of biofilms together with the diverse array of structural and metabolic characteristics make these communities attractive actors in biofilm-mediated remediation solutions and ecosystem monitoring. However, their low abundance and activity in the environment, difficulties accessing the contaminants or nutrient limitations in the environment all prevent the processes from occurring as quickly as desired and thus reaching the proposed clean up goals (Edward and Kjellerup, 2013).

1.2 Motivation and statement of problem

Bacteria properties and surface properties play a significant role in the adhesion process. The properties of bacteria imposed a significant effect on the adhesion of bacterial on the metal surfaces. Bacteria can be classified based on their gram's types which are Gram-negative or Gram-positive. During adhesion process, a Gram-negative bacterium will be more attracted to a positively charged surface and vice versa (Faisal *et al.*, 2012). Besides that, surface properties are also one of the factors which influenced the adhesion of bacteria on metal surface include surface roughness, chemical composition, polarization and oxides coverage (Faisal *et al.*, 2012). However in this study, the focus was more on the surface roughness which is the main factor in the adhesion of bacteria on metal surfaces. In term of polarization, the hydropobicity and the surface charge of metal surface promoted the adhesion of bacteria (Baikun and Bruce, 2004).

The adhesion of bacteria on metal surfaces is very specific mechanism and contributed by many factors like surface properties, bacteria properties and also the condition of the process which is the environment. It is still largely unknown the main precursor of the mechanism, where the available literature discussing on that matter are very scarce and the molecular and physical interactions that govern bacterial adhesion to metal surfaces also have not been understood in detail (Katsikogianni and Missirlis, 2004). Besides that, statistic of bacterial infection occurred in the medical application was increasing nowadays and no permanent solution have been found. Therefore, this research can be used as one the reference to study the reason of bacterial adhesion as it was focussing on the factors that contributing to the adhesion of bacteria on metal surfaces used in medical application especially in the effect of surface roughness.

1.3 Objectives

The following are the objectives of this research:

- To study the adhesion of various bacterial on several types of metal surfaces used in medical application.
- To study the effect of surface roughness on the wettability and adhesion.

1.4 Scopes of this research

In this research study, the scopes function as a guideline to achieve the objectives. The study has been divided into several scopes in order to achieve the objectives which are:

i) To study the adhesion of various types of bacteria on metal surfaces. There are three types of bacteria that will be observed which are *Escherichia coli, Bacillus subtilis* and *Staphylococcus aureus.*

ii) To study the bacterial adhesion on various type of metal surfaces used in medical application. The examples of metals in medical application are titanium, AV220 stainless steel and N690 stainless steel.

iii) To study the effect of surface roughness on the wettability and bacterial adhesion. Eight different type of metal's surface roughness (*Ra*) will be tested in order to study the adhesion behaviour of bacterial on these metals.

1.5 Organization of this thesis

The structure of the reminder of the thesis is outlined as follow:

- **Chapter 2** provides a description on literature review about the types of bacteria, types of metal and factors that contributed to the adhesion of bacterial on metal surfaces.

- **Chapter 3** details all the general and repetitive materials and methods that were carried out throughout the study, including the adhesion test, SEM analysis, and fluorescence microscope analysis.

- **Chapter 4** discusses about the adhesion test results which consist of CFU, OD, SEM analysis and bacterial count.

- **Chapter 5** summarized the results, problems and contributions of this study. The conclusions were derived and some recommendations had been highlighted for future improvement.

2 LITERATURE REVIEW

2.1 Overview

This chapter will cover about all the important parts that related to the study of adhesion of bacterial on metal surfaces used in medical application with the effect of surface roughness.

2.2 Introduction

The important parts consist of types of bacteria used in this study, types of metals, biofilm formation and also the factors that contributed to the bacterial adhesion on metal surfaces. There were three types of bacteria involved in this study which were *Escherichia coli, Bacillus subtilis* and *Staphylococcus aureus* and metals used were titanium and stainless steel. The details about the biofilm formation and also the factors of bacterial adhesion will be explained in this chapter later.

2.3 Microorganisms

2.3.1 Escherichia coli

Escherichia coli is a gram negative bacteria, with a rod shaped, non-spore forming, motile with peritrichous flagella or nonmotile, and grow on MacConkey agar (colonies are 2 to 3 mm in diameter and red or colourless) (Farmer *et al.*, 2007). They can grow under aerobic and anaerobic conditions and do not produce enterotoxins (Wilson 2001). Besides that, *Escherichia coli* are also the name of a type of bacteria that lives in our intestines. Most strains of *E. coli* are harmless and are a part of the normal intestinal microflora. These strains serve a useful function in the body by suppressing the growth of harmful bacteria and by synthesizing appreciable amounts of vitamins (Bennett *et al*., 1987). However, some types of *E. coli* can make human sick and cause diarrheal. The worst type of *E. coli* causes bloody diarrheal, and sometimes cause kidney failure and even death. These problems are most likely to occur in children and adults with weak immune systems. There are four categories or strains of *E. coli* that can cause diarrheal illnesses or disease which are *enteropathogenic E. coli*, *enteroinvasive E. coli*, *enterotoxigenic E. coli* and *enterohemorrhagic E. coli* (Dupon *et al*., 1971).

Enteropathogenic E. coli causes severe diarrhea in infants that can last for over 2 weeks and results in death if dehydration is severe. In adults, the illness is characterized by severe diarrhea, nausea, vomiting, abdominal cramps, headache, fever, and chills. The time for onset of the illness is 17 to 72 hours; the duration of the illness is 6 hours to 3 days. This strain has caused illness to develop in people when it was transmitted in fecally contaminated water and a coffee substitute. Besides that, *enteroinvasive E. coli* is similar to shigellosis and is caused by bacterial penetration and destruction of intestinal mucosa. Symptoms include: chills, fever, headache, muscle pain, abdominal cramps, and profuse diarrhea. The illness occurs 8 to 24 hours after ingestion of food or water containing this organism. The ingestion of a large number of cells (104 to 105 cells) is required to cause the illness. These strains are biochemically and culturally different from other strains of *E. coli.*

Enterotoxigenic E. coli include strains that produce enterotoxins when the organisms multiply in the intestine. These strains are commonly responsible for "traveler's diarrhea". They have been responsible for illness in India, in U.S., soldiers in Vietnam, and in travellers in Mexico. This is a problem for travellers from developed countries with good hygiene who visit countries with poor hygiene standards. The illness is characterized by severe diarrhea, which may lead to dehydration. The diarrhea may last up to 19 days. Usually there is no fever. The onset of the illness can occur 8 to 44 hours after ingestion. Infective dose, as determined by a human study, is 108 to 1010 microorganisms. On the other hand, *enterohemorrhagic E. coli* is characterized by severe abdominal cramps usually, but not always, followed by bloody diarrhea (hemorrhagic colitis). Some individuals exhibit only watery diarrhea. Vomiting may occur but there is usually little or no fever. The incubation period is usually about 3 to 9 days. Figure 2-1 shows the general structure of *E. coli* under coloured scanning electron microscope.

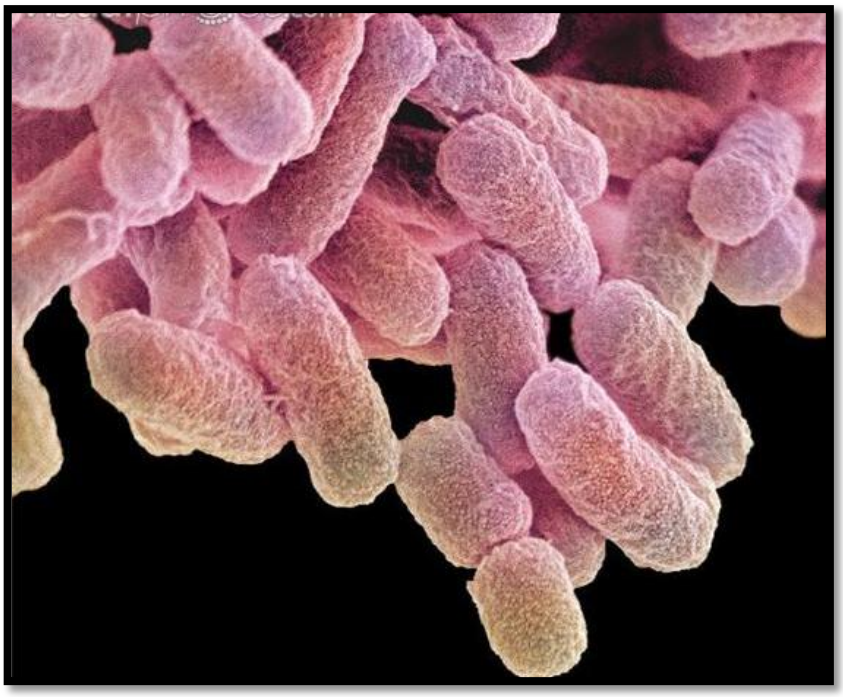


Figure 2-1: General structure of *E-coli* under coloured scanning electron microscope (Steve, 2013)

2.3.2 Bacillus subtilis

Bacillus subtilis is a gram positive with a rod-shaped bacterium that commonly found in soil. *B. subtilis* is an endospore forming bacteria. The endospore that it forms allows it to withstand extreme temperatures as well as dry environments. It also considered as an obligate aerobe, but can also function anaerobicly when in the presence of nitrates or glucose. It has a flagellum which makes motility faster and secretes numerous enzymes to degrade a variety of substances during metabolism (Western *et al.*, 2004). In addition to being a cell factory for pharmaceutical proteins, *B. subtilis* has many industrial and environmental applications. For instance, biosurfactant production from *B. subtilis* bioconversions has created great potential for biotechnological and pharmaceutical application for recent years (Kuo-Jen and Chung-Yi, 2012).

Besides that, *B. subtilis* appears to have a low degree of virulence to humans. It does not produce significant quantities of extracellular enzymes or possess other virulence factors that would predispose it to cause infection (Edberg, 1991). There are a number of reports where *B. subtilis* has been isolated from human infections. Earlier literature contains references to infections caused by *B. subtilis*. However, as previously stated,the term *B. subtilis* was synonymous for any aerobic sporeforming bacilli, and quite possibly,

many of these infections were associated with *B. cereus*. Reviews of *B. Subtilis* infections from several major hospitals suggest that *B. subtilis* is an organism with low virulence. Idhe and Armstrong (1973), reported that *B. subtilis* infections were encountered only twelve times over a 6-1/2 year period. Moreover, *B. subtilis* has also been implicated in several cases of food poisoning (Logan, 1988). There have been a number of cases of allergic or hypersensitivity reactions, including dermatitis and respiratory distress after the use of these laundry products (Norris *et al*., 1981). The general structure of *B. Subtilis* can be seen at Figure 2-2.

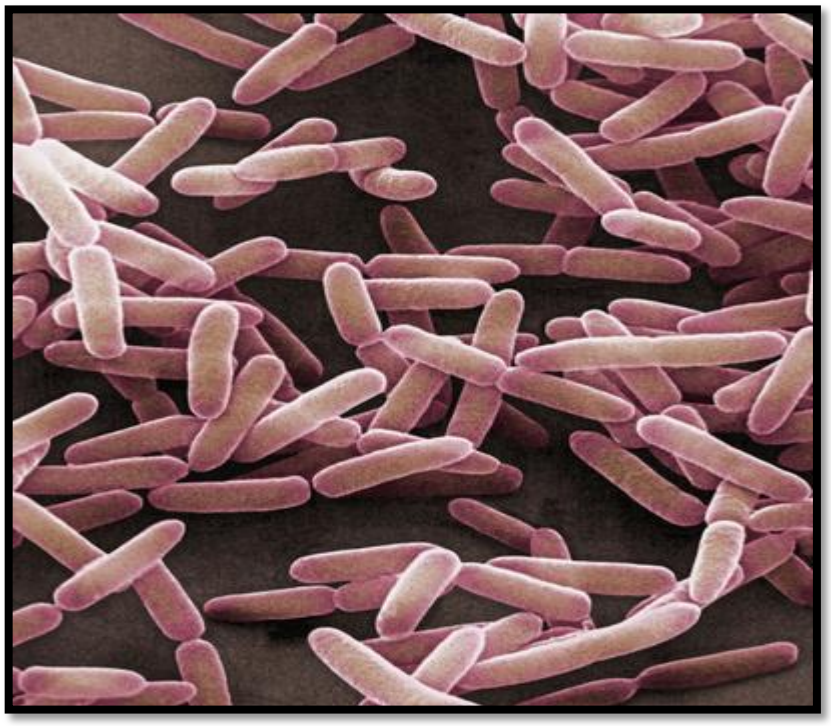


Figure 2-2: General structure of *B. subtilis* under coloured scanning electron microscope (David, 2014)

2.3.3 Staphylococcus aureus

S. aureus is a gram-positive, spherical bacterium (coccus) with a diameter of 1-1.3 µm. When viewed microscopically, *S. aureus* appears in clusters, like bunches of grapes. Growing in food, some strains can produce toxins which cause acute gastro-intestinal diseases if ingested. The enterotoxin produced by *S. aureus* is a heat-stable protein, which survives heating at 100 °C for 30 – 700 minutes. *S. aureus* can grow both aerobically and anaerobically in various foods. It is characteristic that staphylococci can grow at low water activity (~ 0.86), corresponding with a salt content of about 14%. The main reservoirs of *S.*

aureus are humans and animals. Healthy people carry the organism in their nose and throat (50 %), on their hands (5-30 %), and in wounds. *S. aureus* can also colonise food contact surfaces, and it can become a persistent organism in slaughterhouses.

S. aureus produces staphylococcal enterotoxin (SE) and is responsible for almost all staphylococcal food poisoning (Montville and Matthews, 2008). Staphylococcal food poisoning is an intoxication that is caused by the ingestion of food containing pre-formed SE (Argudin *et al*. 2010). SE is produced during the exponential phase of *S. aureus* growth, with the quantity being strain dependent. Staphylococcal food poisoning symptoms generally have a rapid onset, appearing around 3 hours after ingestion (range 1–6 hours). Common symptoms include nausea, vomiting, abdominal cramps and diarrhoea. Individuals may not demonstrate all the symptoms associated with the illness. In severe cases, headache, muscle cramping and transient changes in blood pressure and pulse rate may occur. Recovery is usually between 1–3 days (Stewart, 2003). Fatalities are rare (0.03% for the general public) but are occasionally reported in young children and the elderly (4.4% fatality rate) (Montville and Matthews 2008). *S. aureus* can cause various non-food related health issues such as skin inflammations (e.g. boils and styes), mastitis, respiratory infections, wound sepsis and toxic shock syndrome (Stewart, 2003; Montville and Matthews, 2008). Figure 2-3 displays the general structure of *S. aureus.*

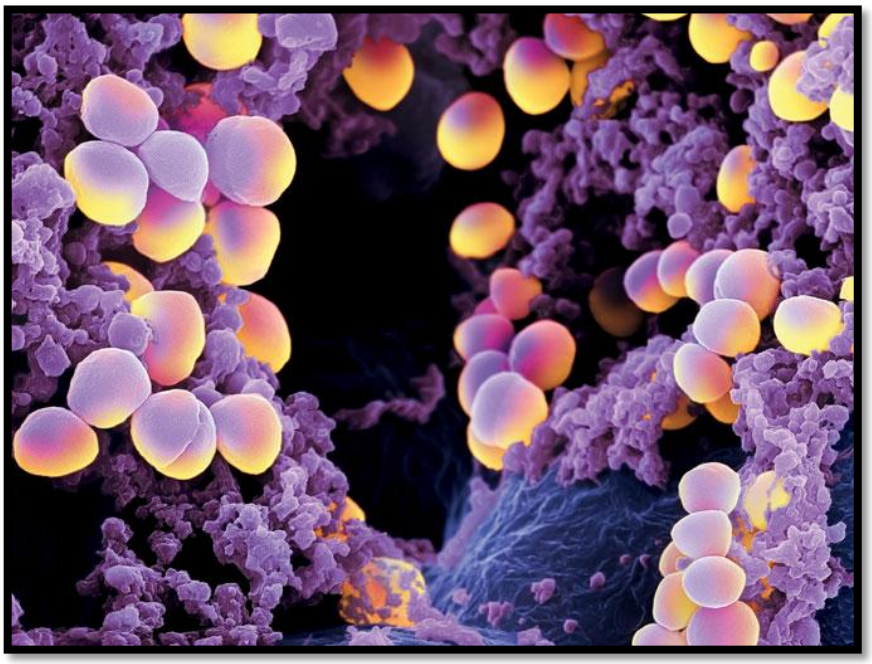


Figure 2-3**:** General structure of *S. aureus* (Martin, 2013)

2.4 Metals

Titanium and stainless steel are the types of metals used in this study. Below is the explanation about the details of the metals.

2.4.1 Titanium

Titanium is a reactive metal with a standard potential of -1.63 volts, roughly four times more negative than the negative potential of iron. Yet this very ignoble metal behaves in a very noble way in that the titanium dioxide skin provides such excellent protection. Titanium is so reactive that a titanium oxide skin forms spontaneously in contact with air, without the presence of water (Ko, 2008). Table 2-1 displays the details about the physical properties of titanium.

Table 2–1: Physical properties of titanium (Ko, 2008)

Physical Properties	**Data**
Melting Point	1660 °C
Density	4.51 g/cm^3
Co-efficient of expansion	8.9 x 10^{-6} / °C
Electrical resistivity at 20 °C	48.2 μΩ/ cm
Standard Potential	-1.63 volts
Elastic modulus	105, 000 N/mm^2

Recently in medical application, titanium is one of the metallic biomaterials which currently applied in orthopaedic surgery such as in intramedullar nails and total hip prostheses (Thomas *et al.*, 2006) and it becomes one of the metals which involved in adherence of bacteria. Figure 2-4 shows the filamentous of bacteria covered the titanium surface which means that bacteria like to attach on the titanium surface.

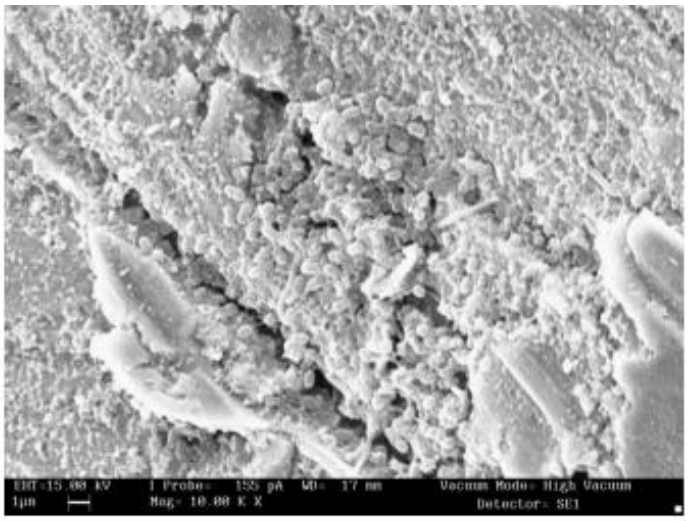


Figure 2-4: Filamentous bacteria cover titanium surface (Antonio *et al*., 2004)

2.4.2 Stainless Steel

Stainless steel is not a single specific material, but the name given to a group of corrosion-resistant steels. Stainless steels are those steels that have a chromium content of at least 11%. Varying additions of nickel, molybdenum, nitrogen, copper, manganese, wolfram, titanium, niobium, cerium, carbon, phosphorus, sulfur and other elements may also be present (Castle, 2007). The main requirement for stainless steels is that they should be corrosion resistant for a specified application or environment. The selection of a particular "type" and "grade" of stainless steel must initially meet the corrosion resistance requirements. Additional mechanical or physical properties may also need to be considered to achieve the overall service performance requirements. They are five different types of stainless steel are created by adding different levels of various alloys such as chromium or nickel during the manufacturing process. The types of stainless steel in common use, their properties and composition, are as follows:

2.4.2.1 Austenitic

Austenitic stainless basic make up is 18% chromium and 8% nickel and was boosted by the addition of elements such as manganese and nitrogen. It is highly resistant to corrosion and is easily drawn into wires or hammered into thin sheets. The versatility of this type of steel is demonstrated by the fact that it accounts for more than 70% of all steel production. Austenitic steel boasts superb hygiene properties and is good at working in both low and high temperatures. Common uses for this type of steel include food processing equipment, kitchen sinks and chemical equipment and plant (Castle, 2007).

2.4.2.2 Martensitic

This type of steel was actually the first to be commercially developed, and in those initial stages it was used mainly to make cutlery. It has a carbon content which is higher than most other stainless steels at between 0.1 and 1.2%, whilst also boasting 18% chromium. Additional materials found in martensitic stainless steel include the likes of molybdenum and nickel. The application of high temperature to this steel makes it harder and it also has some magnetic properties. Whilst able to resist corrosion brought about by environmental factors, it is still somewhat less durable than austenitic steel. The most common uses for this type of stainless steel are the manufacture of things such as spindles, pins, knife blades, shafts and surgical instruments (Castle, 2007).

2.4.2.3 Ferritic

Ferritic steel, together with martensitic steel is known, collectively, as the 400 series. It features carbon levels of 10.5% and as much as 27% chromium. Amongst the properties which ferritic steel can boast are the following; it is magnetic, is not as ductile as martensitic and austenitic steel and does not, unlike other types of steel, become harder after intense heating. The fact that it is very highly resistant to corrosion means that it can safely be used in sea water, and this is despite the fact that it is generally actually less durable than austenitic steel. This ability to resist corrosion means that it is also the material of choice when manufacturing the likes of boilers and washing machines. It is also extremely useful when making things such as car trim and exhaust systems (Castle, 2007).

2.4.2.4 Duplex

Duplex stainless steel is made, to put it simply, by mixing together the basic components of austenitic and ferritic steel. The two types of steel are combined in equal measure and the resulting steel contains a higher level of chromium and an amount of nickel which is lower. The fact that it is a mix of two different steels means that it brings the best of both types to bear, being more resistant than any other type of steel to corrosion as well as being able to deal with stress and, on occasion, displaying some magnetic properties. As well as this it is easy to work with, being simple to weld and to form into specific shapes. The very best quality stainless steel is actually known as ‘super duplex’. The particular qualities of duplex and super duplex mean that it is highly suited to use in tools or machinery that are to be employed in marine conditions (Castle, 2007).

2.4.2.5 Precipitation Hardening

Initially, this type of stainless steel is austenitic in nature and is then changed by the addition of other elements. Once altered, it becomes extremely tough, durable and hard wearing. One of its' other chief advantages is the way in which its shape can be altered once it has been heated to a sufficiently high temperature. Whilst being tougher than austenitic steel, it is equally as resistant to corrosion and this feature makes it especially useful in the manufacturing of aircraft parts as well as the creation of shafts and pumps (Castle, 2007). Summarization of stainless steel properties can be referred at Table 2-2.

Greater ductility (3-fold better percentage of elongation at fracture) of stainless steel relative to titanium and Co-Cr makes stainless steel ideal for fixation cables used in total-knee arthroplasty procedures. However, stainless steel also can be adhered by bacteria. Bacterial adhesion on stainless steel may cause problems such as microbially induced corrosion or represent a chronic source of microbial contamination and also human's infections (Marta *et al.,* 2012). Figure 2-5 shows the image of *S. epidermis* cells adherent to stainless steel plate under scanning electron microscope.

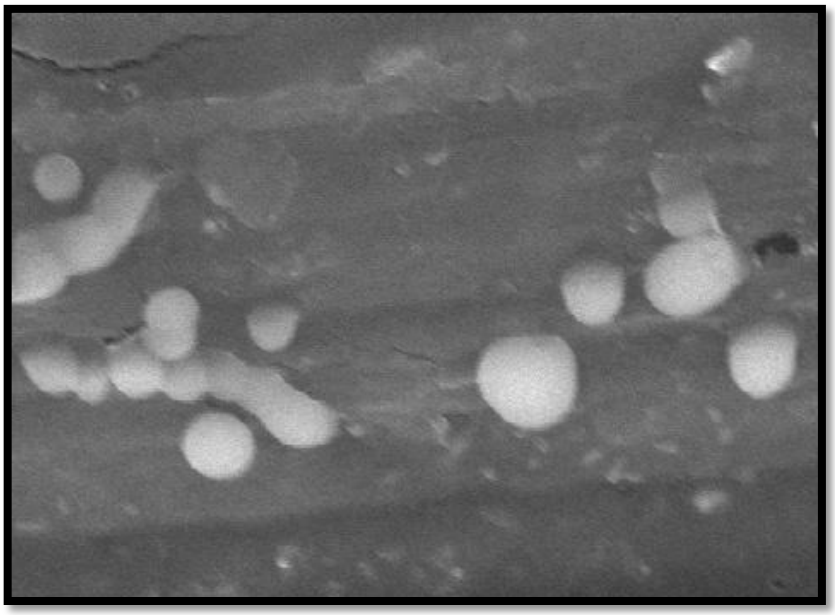


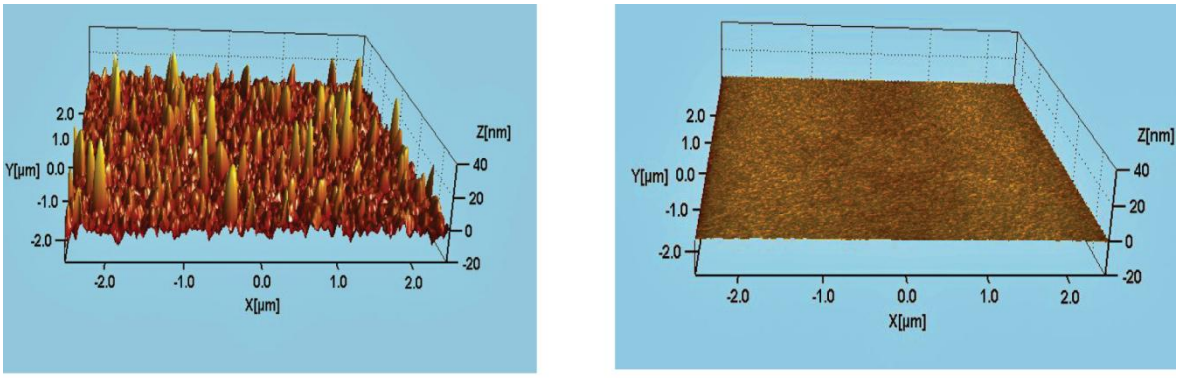
Figure 2-5: Image of *S. epidermis* cells adherent to stainless steel plate under scanning electron microscope (Ortega *et al*., 2008)

Table 2–2: Properties of stainless steel (Tverberg, 2000)

Alloy Group	**Magnetic Response**[1]	**Work Hardening Rate**	**Corrosion Resistance**[2]	**Hardenable**
Austenitic	Generally No	Very High	High	By Cold Work
Duplex	Yes	Medium	Very High	No
Ferritic	Yes	Medium	Medium	No
Martensitic	Yes	Medium	Medium	Quench and Temper
Precipitation Hardening	Yes	Medium	Medium	Age Harden

2.4.3 Metal Roughness

Each metal has different types of roughness which can affect the amount of bacterial adhesion on that metal. Roughness consists of surface irregularities which results from the various machining process (polishing, grinding, etc.). These irregularities combine to form surface structure ((Katsikogianni and Missirlis, 2004). Figure 2-6 demonstrated the surface structure of metal roughness.



(a) (b)

Figure 2-6: Image of surface roughness a) Crystalline Aluminium b) Amorphous Metal (Laurie, 2014)

Roughness of metals can be measured based on a few parameters which are Roughness average (*Ra*), Root means square roughness (*Rq*), Skewness (*Rsk*) and also Kurtosis (*Rku*) (Jim, 2014). *Ra* is the average of the individual height (asperities) and depths from the arithmetic mean elevation of the profile while *Rq* is the square root of the sum of the squares of the individual height and depths from the mean line. Besides that, *Rsk* is a measure of the average of the first derivative of the surface (the departure of the surface from symmetry). A negative value of *Rsk* indicates that the surface is made up of valleys, whereas a surface with positive skewness is said to contain mainly peaks and asperities. Meanwhile, *Rku* is a measure of sharpness of profile peaks (Jim, 2014). However, for this study only one parameter has been used which was *Ra*. The roughness used in this study has undergone electropolishing technique and also electro deposition technique and further explanation will be discussed in the discussion part later. The image of how *Ra* been measured can be seen in the Figure 2-7.

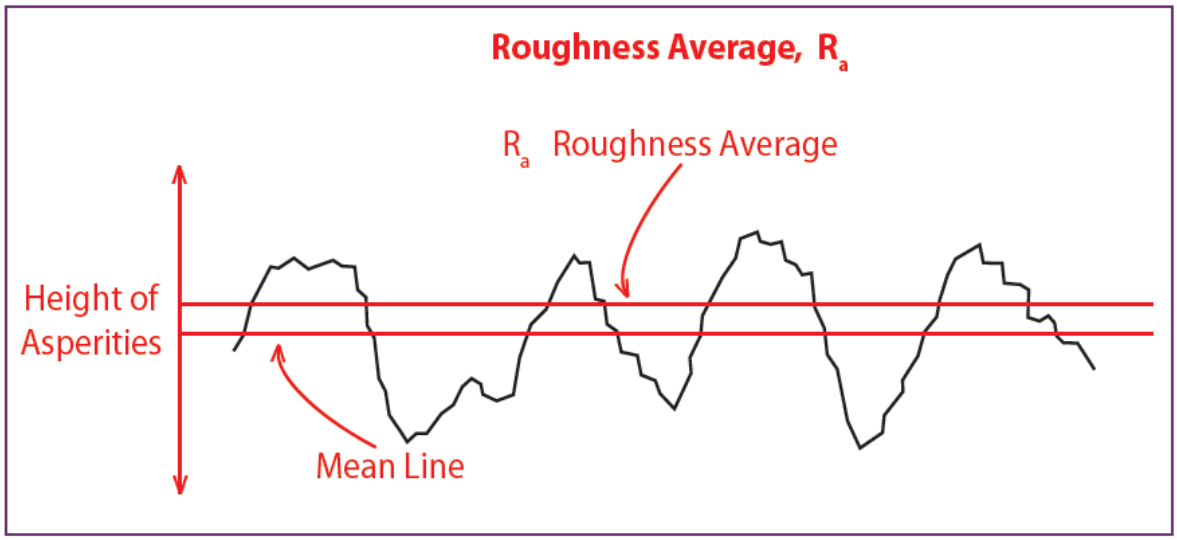


Figure 2-7: Image of Roughness average (Ra) been measured (Jim, 2014)

2.5 Biofilm formation

Biofilm can exist on all types of surfaces such as plastics, metal, glass, soil particles, wood, medical implant materials, tissue and food product. Bacterial attachment is mediated by fimbriae, pilli, flagella and extracellular polymeric substance (EPS) that act to form a bridge between bacteria ant the conditioning film (Kokare *et al.*, 2008). Biofilm formation occurs step by step, such as formation of conditioning layer, bacterial adhesion, bacterial growth (Figure 2-8) and biofilm expansion (Kokare *et al.*, 2008).

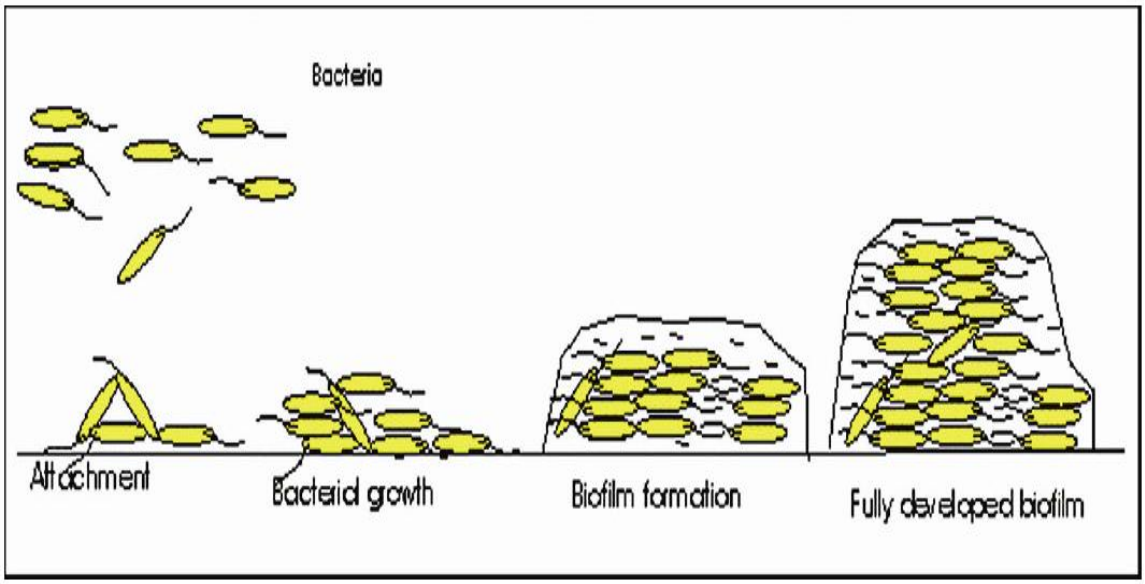


Figure 2-8: Biofilm formation (Kokare *et al.*, 2008)

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 (Trevor *et al.,* 2008). Then, microbial cells are transported from bulk liquid to the conditioned surface either by physically forces or bacterial appendages such as flagella. Factors such as, available energy, surface functionality, bacterial orientation, temperature and pressure condition are local environment variables which contribute to bacterial adhesion (Trevor *et al*., 2008).

After the initial lag phase, a rapid increase in population is observed where the bacterial were started to grow. 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 substrate. At this stage, the physical and chemical contribution to the initial attachment ends and the biological process begin to dominate (Trevor *et al.,* 2008) Excretion of polysaccharide intercellular adhesion (PIA) polymers and the presence of divalent cations interact to form stronger bonding between cells (Dunne, 2002).

The next stage, the biofilms started to form and develop. 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 signalling mechanisms are employed by the biofilm, and this is collectively termed quorum sensing (Bassler, 1999). Quorum sensing describes 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. Simultaneously, the operons coding for flagella proteins are up regulated so that the organisms have the apparatus for motility, and the genes coding for a number of porins are down-regulated,thus completing a genetic cycle for biofilm adhesion and cohesion (Trevor *et al.,* 2008).

2.6 Factors Adhesion of Bacterial on Metal Surfaces

The adhesion of microbial cells to metal surfaces in aqueous media is an important phenomenon in both natural environment and engineering system (Xiaoxia *et al.,* 2007). In food industry, the adhesion of microbial to equipment surfaces especially on metal surfaces has become one of the serious issues since it can cause cross-contamination which leads to food spoilage, and transmission of disease (Melba *et al.,* 2008). This issue also has been faced in medical industry which was lead to the human body infection. The adhesion process of bacterial on metal surface was influenced by many factors, including bacterial properties, the material surface characteristics, the medium characteristics (Faisal *et al.,* 2012), the environmental factors, such as the presence of serum proteins and the associated flow conditions (Katsikogianni and Missirlis, 2004).

2.6.1 Bacterial properties factor

Bacterial adhesion to surfaces consists of the initial attraction of the cells to the surface followed by adsorption and attachment (Rijnaarts *et al*., 1995). Bacteria move to or are move to a material surface through and by the effect of physical forces, such as Brownian motion, van der Waals attraction forces, gravitational forces, the effect of surface electrostatic charge and hydrophobic interaction (Katsikogianni and Missirlis, 2004). Bacteria can be classified into Gram-negative or Gram-positive. The differences between them related to the cell wall configuration, and the great majority of microbial cells tend to be Gram-negative (Faisal *et al*., 2012).

During adhesion, negatively charged bacteria will attach to positive charge surface and positively charged bacteria will attach to negative charge surface (Baikun Li *et al*., 2004). Besides that, proteinaceous appendages including pili and flagella also initiate the bacterial adhesion by establishing bridges between surface and cell. Bacteria in aqueos suspension are almost negatively charged. Bacterial surface charge are varies according to the types of bacteria and are influenced by the growth medium, the pH and the ionic strength of the suspending buffer, bacterial age, and bacterial surface structure (Katsikogianni and Missirlis, 2004). The electrostatic interaction between bacterial cell and substratum greatly influenced the adhesion force by controlling the electrostatic interaction and resulted in stronger repulsive forces in the cell-metal surface interaction (Faisal *et al*., 2012).

2.6.1.1 Bacterial Hydrophobicity

Generally, bacteria with hydrophobic properties prefer hydrophobic material surfaces; the ones with hydrophilic characteristics prefer hydrophilic surfaces. Vacheethasanee *et al. (*1998) showed that more hydrophobic *S. epidermidis* adhered to a greater extent than the less hydrophobic *S. epidermidis* for shear stresses between 0-8 dyn/cm^2 in Phosphate Buffer Solution (PBS), whereas the differences in adhesion for high and low hydrophobic bacteria decreased with increasing shear stresses. The correlation between bacterial surface hydrophobicity and adhesion disappeared at shear stress higher than 15 dyn/cm^2. Positive correlation between bacterial surface hydrophobicity and adhesion was at 0 dyn/cm^2. However it has been shown that material surface hydrophobicity plays a more important role in bacterial adhesion than bacterial surface hydrophobicity even for shear stresses up to 65 dyn/cm^2 (Katsikogianni and Missirlis, 2004).

2.6.1.2 Bacteria surface charge

Most particles acquire a surface electric charge in aqueous suspension due to the ionization of their surface groups. Bacteria in aqueous suspension are almost always negatively charged. The surface charge of bacteria varies according to bacterial species and is influenced by the growth medium, the pH and the ionic strength of the suspending buffer, bacterial age, and bacterial surface structure (Katsikogianni and Missirlis, 2004).. The surface charges on the metal substrates and the bacteria have a significant effect on the bacterial-metal adhesion. It is greatly influence the adhesion force by controlling the electrostatic interaction (Xiaoxia *et al*., 2008).

2.6.2 Surface Roughness Factor

Other factors that influenced the bacterial adhesion to the metal surfaces are physical configuration and the surface roughness (Scheurman *et al*., 1998). Roughness is defined as the pattern or texture of surface irregularities that are introduced by manufacturing process (Faisal *et al*., 2012). It has been found that, irregularities surfaces can increase the adhesion of bacterial and the deposition of biofilm while for the smooth surface, does not favour bacterial adhesion and biofilm deposition (Scheurman *et al*., 1998). This is because, rough surface have a greater surface area and the depressions in the roughened surface provide more favourable site for colonization (Katsikogianni and Missirlis, 2004).

Boyd *et al.* (2002) also showed that the increase in the surface roughness of stainless steel will increased the adhesion of bacterial since roughest surface increases surface area at the micorganism-materials interface that may then lead to more film attachment by providing more contact point. This statement also has been supported by Wu *et al.* (2011), who said that, surface roughness encourages the adhesion of bacteria. Besides that, the hydrophobicity and surface charges on the metal substrate and the bacteria charge also have greatly effect on bacterial adhesion. The surface charge influences the adhesion force by controlling the electrostatic interaction while bacterial adhesion forces are enhanced by increasing surface hyrophobicity (Xiaoxia *et al.*, 2008). Furthermore, it has been found that implant site infection rates are different between porous and dense material with porous material having a much higher rate. This implies bacteria adhere and colonize the porous surface preferentially (Katsikogianni and Missirlis, 2004). Moreover, bacteria adhere more to grooved and braided materials compared to flat ones (Scheurman *et al.*, 1998; Bos *et al*., 2000; Medilanski *et al.*, 2002). However, some literature said that bacterial also tend to adhere on smooth surfaces based on the condition that suits the adhesion process.

2.6.3 Medium characteristics factor

Medium concentration, pH, and total organic and inorganic strength can influence the microbial settlement potential (Mansfeld, 2007; Little and Lee, 2007; Javaherdashti, 2008; Javaherdashti, 2010). Based Xiaoxia *et al*. (2008), the bacteria–metal adhesion force was the highest when the pH of the solution was near the isoelectric point of the bacteria, i.e., at the zero point charge. The adhesion forces at pH 9 were higher than those at pH 7 due to the increase in the attraction between the irons (Fe^{2+}) and the negative carboxylate groups.

2.6.4 Environmental factors

Certain factors in the general environment, such as temperature, pH, time of exposure, bacterial concentration, the presence of antibiotics and the associated flow conditions affect bacterial adhesion. In this research we will explain about the effect of pH and temperature on bacterial adhesion.

2.6.4.1 Effect of pH

Changes in pH can have a marked effect on bacterial growth and as such is frequently exploited in the production of detergents and disinfectants used to kill bacteria. Bacteria possess membrane-bound proton pumps which extrude protons from the cytoplasm to generate a transmembrane electrochemical gradient, like the proton motor force (Rowland, 2003). The passive influx of protons in response to the proton motive force can be a problem for cells attempting to regulate their cytoplasmic pH. Large variations in external pH can overwhelm such mechanisms and have a biocidal effect on the microorganisms. Bacteria respond to changes in internal and external pH by adjusting the activity and synthesis of proteins associate with many different cellular processes (Olsen, 1993). Studies have shown that a gradual increase in acidity increases the chances of cell survival in comparison to a sudden increase by rapid addition of Hydrochloric acid(HCl). This suggests that bacteria contain mechanisms in place which allow the bacterial population to adapt to small environmental changes in pH. However, there are cellular processes which do not adapt to pH fluctuations so easily. One such process is the excretion of exopolymeric substances (polysaccharides). Optimum pH for polysaccharide production depends on the individual species, but it is around pH 7 for most bacteria.

2.6.4.2 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 dependant 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 so has a bearing on the development of the cells. Optimum temperatures result in the healthy growth of bacterial populations. Conversely, temperatures away from the optimum reduce bacterial growth efficiency. This is due to a reduction in bacterial enzyme reaction rates.

In addition to enzymes, environmental temperature affects the physical properties of the compounds within and surrounding the cells. Fletcher, (1977) reported the effect of temperature on attachment of stationary phase cells. Findings showed that a decrease in temperature reduced the adhesive properties of a marine Pseudomonad. 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 °C cells were shown to have a single flagellum whilst at 21 °C they had two to three flagella and at 10 °C, cells exhibited several 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 more uniform properties of polysaccharides at lower temperatures increase the possibility of biofilm adhesion, because 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 (Nisbet, 1984).

2.7 Summary

The details about all the important things in this study, have been discussed in this chapter. This chapter covered the explanation about the types of bacterial and metals used, biofilm formation and also factors adhesion of bacterial on metal surfaces.

3 MATERIALS AND METHODS

3.1 Overview

This chapter will discuss more details about the procedures involved in this research which including the material used, methods conducted during the experiment and also analysis that have been done after the experiment.

3.2 Introduction

Materials used in this research consisted of bacteria, chemicals and metals. Meanwhile, the methods involved were preservation of stock culture, media preparation, culture preparation, and also cell – surface adhesion experiment. The analysis parts were divided into a few category which were, cell concentration measurement (Optical Density, OD), Colony Forming Unit (CFU), staining process (Fluorescence dye-SYTO9), quantifying attached cell using fluorescence microscope, fixing and preparation of samples for Scanning Electron Microscopy (SEM) analysis and lastly counting and morphological observation of adhered microorganism using SEM. The overall process involved in this experiment has been summarized in Figure 3-1.

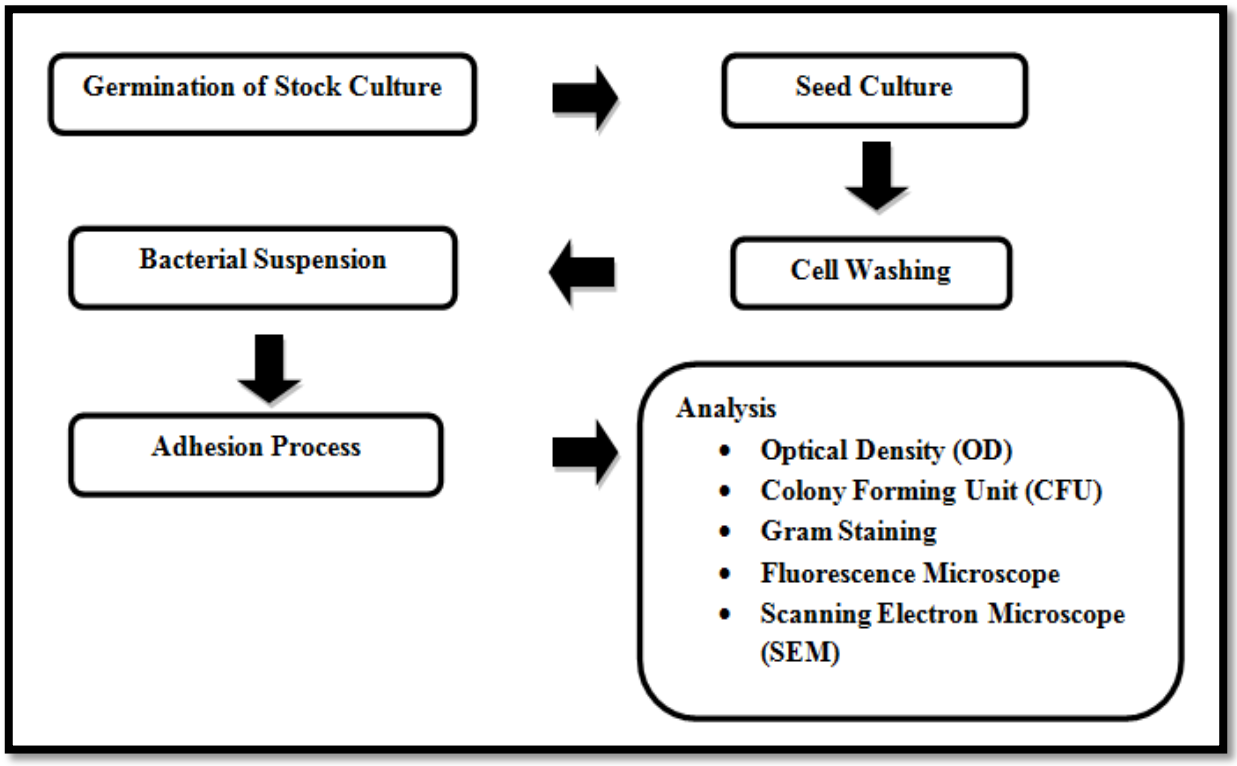


Figure 3-1: Flow chart for adhesion process

3.3 Materials

3.3.1 Bacteria

Three types of bacterial were used in this study which were *Escherichia coli, Bacillus subtilis* and *Staphylococcus aureus.* All the bacterial were obtained from the Centre Laboratory of Universiti Malaysia Pahang and kept in the FKKSA laboratory chiller at 4-6 °C before handling the experiment.

3.3.2 Chemicals

Glucose, bactopeptone, yeast extract, agar powder, sulphuric acid, peptone from caseine, ethanol, NaOH, K_2HPO_4, KH_2PO_4, KCl, $MgSO_4$, NaCl and glutaraldehyde were obtained from FKKSA Laboratory, UMP. All the chemicals were purchased from Sigma-Aldrich, Malaysia and were of biological grade.

3.3.3 Metals

There were three types of metals used in this study which were Stainless Steel (N690), Stainless Steel (AV220SC) and Titanium. Each of the metal was consisted eight different types of roughness. All the metals were put in the bacterial solution and undergo adhesion test for 4 hours. Below is the image (Figure 3-2) of metals used in this study. The symbols P1, P2, P3, P4, E1, E2, E3 and E4 denoted the types of metals surface roughness used in this study and at each of surface roughness contained three different types of metals as shown in the Figure 3-2.

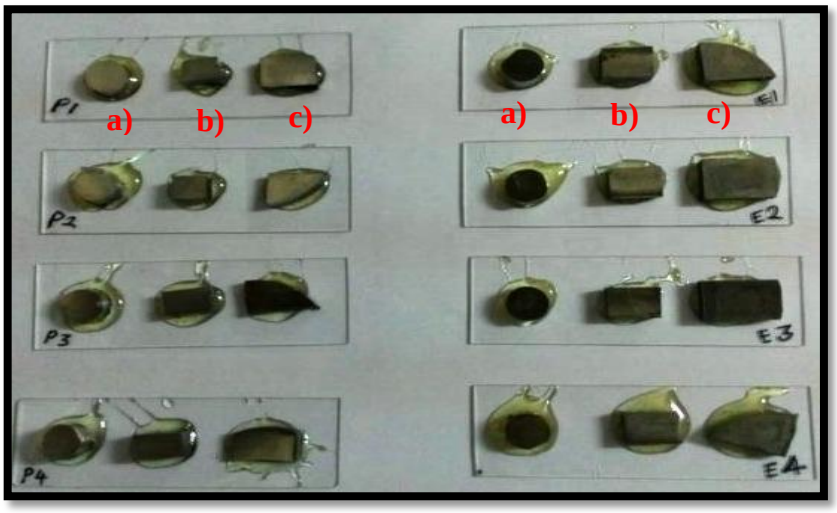


Figure 3-2: Images of the metals used a) Stainless Steel (N690) b) Stainless Steel (AV220SC) c) Titanium

3.4 Methods

3.4.1 Preservation of Stock Culture

Escherichia coli, Bacillus subtilis and *Staphylococcus aureus* were obtained from the Centre Laboratory of University Malaysia Pahang. For long term preservation, the culture was kept in 20% (v/v) glycerol, in a freezer at -80 °C (Zain, 2013). For use in subsequent microbial work, *E. coli, B. subtilis* and *S. aureus* stock were stored in the chiller at 4 - 6 ^{0}C, transferred to an agar plate and incubated for 24 hours at 30 °C.

3.4.2 Media Preparation

3.4.2.1 Preparation of Nutrient Broth

8 g nutrient broth powder made up of 20 g/L glucose, 20 g/L bactopeptone and 10 g/L yeast extract were weighted and adjusted to pH 5.5 by using 0.1 M sulphuric acid and 0.1 M NaOH solution. The powder then added to 1 L of distilled or deionized water in a 1 L Schott bottle. The powder was dissolved in the water and heated for 15 minutes by using hot magnetic plate to make sure it was dissolve completely and finally autoclaved at 121 °C for 20 minutes.

3.4.2.2 Preparation of Nutrient Agar

20 g of nutrient agar powder containing 20 g/L glucose, 23 g/L agar powder, 20 g/L bactopeptone and 10 g/L yeast extract were measured and adjusted to pH 5.5 by using 0.1 M sulphuric acid and 0.1 M NaOH solution. The powder then added to 1 L of distilled and heated for 15 minutes using hot magnetic plate to makes sure it dissolved completely. The solution was autoclaved at 121 °C for 20 minutes and allowed to cool to 50 °C before pouring into the petri dish. The agar was kept in 4 °C freezer until further use.

3.4.2.3 Preparation of Agar Plates

15-20 mL of a warm sterile nutrient agar was poured per petri plate. The nutrient agar then allowed to solidify at room temperature in sterile environment and kept in 4 °C until further use. Figure 3-3 showed agar plates that have been prepared.

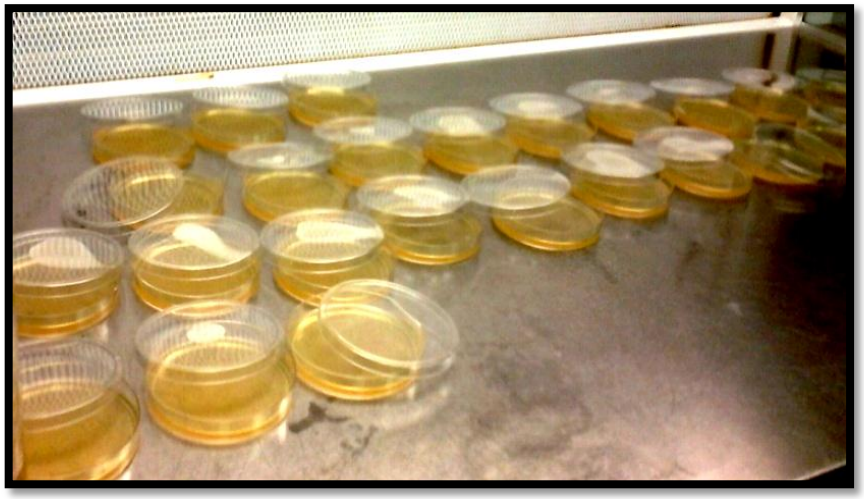


Figure 3-3: Figure of agar plates that have been prepared

3.4.2.4 Preparation of Luria Bertani (LB) Broth

10 g of peptone from caseine, 5 g of yeast extract, and 10 g of NaCl were measured and adjusted to pH 7.0 with 1.0 mL of NaOH. The powder was dissolved in 1 L of distilled water and heated for 15 minutes using hot magnetic plate to make sure it dissolved completely. The solution was autoclaved at 121 °C for 20 minutes.

3.4.2.5 Preparation of Saline Solution

8.5 g of NaCl was measured and added to 1 L of distilled water in a 1 L Schott bottle and stirred using magnetic stirrer to ensure it dissolved completely. The solution was autoclaved at 121 °C for 20 minutes.

3.4.2.6 Preparation of Phosphate Buffer Solution (PBS)

PBS solution of was prepared according to the composition listed in Table 3-1 below. The solution was adjusted to pH 7.4 with NaOH (1M) and autoclaved at 121 °C for 20 minutes. Then, it was stored at room temperature for further use.

Table 3–1: Composition of PBS solution

Chemicals	**Amount**
1M KH_2PO_4	2.4 g
1M Na_2HPO_4-$7H_2O$	26.8 g
5M KCl	2 g
Distilled water	97.0 ml
NaCl	80 g

3.4.3 Culture Preparation

3.4.3.1 Germination of Stock Culture and Inoculum

A loopful of refrigerated stock culture was transferred onto a petri dish containing medium agar and incubated at 30 °C. After 24 hours of incubation, a colony of germinated cells was transferred to a 250 mL shake flask containing 50 mL of Luria Bertani (LB) broth, then allowed to be incubated in incubator shaker at 100 rpm for 18 hours. The cells were then centrifuged at 5000 rpm for 5 minutes, washed twice with 0.85% (w/v) NaCl, and re-centrifuged for 3 minutes (Jamai *et al*., 2001). The supernatant was discarded and the pellet was put into 100 mL of PBS solution for bacterial suspension process until the absorbance achieved approximately ~1.0 before adhesion test is started. The absorbance was adjusted by spectrophotometer at 600 nm. Figure of bacterial inoculums can be seen in Figure 3-4.

Figure 3-4: Figure of bacterial inoculum

3.4.4 Cell – Surface Adhesion Experiment

3.4.4.1 Adhesion Test

The adhesion test was carried out in a glass container containing a baby cradle-like holder for holding the metal slide in the upright-vertical position. The metals slide has 4 different types of surface roughness and was suspended in the bacterial solution and shake at 70 rpm for 4 hours. Samples were taken after 4th hours and were dried in the incubator at 30 °C overnight. Then, the samples were examined under the fluorescence microscope to determine the numbers of cell attached per square area. Besides that, once the adhesion test was finished the absorbance and the colony forming unit (CFU) was also measured.

The steps were repeated for different types of bacteria with different types of metals and surface roughness. The sample of glass container and metals was shown in Figure 3-5.

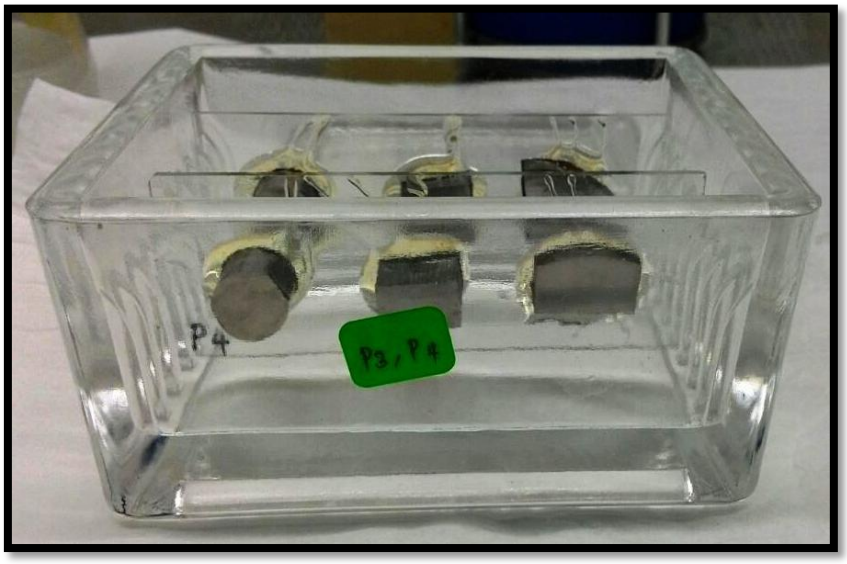


Figure 3-5: Sample of glass container and metals used during the adhesion test

3.5 Analysis

3.5.1 Optical Density (OD)

200 mL of the seed culture was taken out and centrifuged at 5000 rpm for 5 minutes. Then, the supernatant was discarded and the cell pellets were washed by NaCl solution. Next, the cell pellets was added to 100 mL of fresh PBS buffer and the optical density was checked at 600 nm. The cell solution must be was adjusted until it reach at ~1.0 absorbance. The same procedure was carried out for preparation of different types of bacteria with different types of metals and surface roughness. After the desired absorbance was obtained, the mixture was poured into a glass container until all the metal slides were fully immersed in the bacterial solution. The experimental rig was shake at 100 rpm for 4 hours and samplings were done after 4th hours. 1 mL of sample was taken for OD reading while 10 μL for CFU procedure.

3.5.2 Colony Forming Unit (CFU)

10 µL of samples were diluted to 10^2 dilutions using sterilized distilled water. 10 µL of aliquots were transferred to an agar plate and incubated at 30 ^oC for 24 hours. The number of colonies formed on the agar surface were counted and measured as CFU/µL. The image of Colony Forming Unit (CFU) can be seen in Figure 3-6.

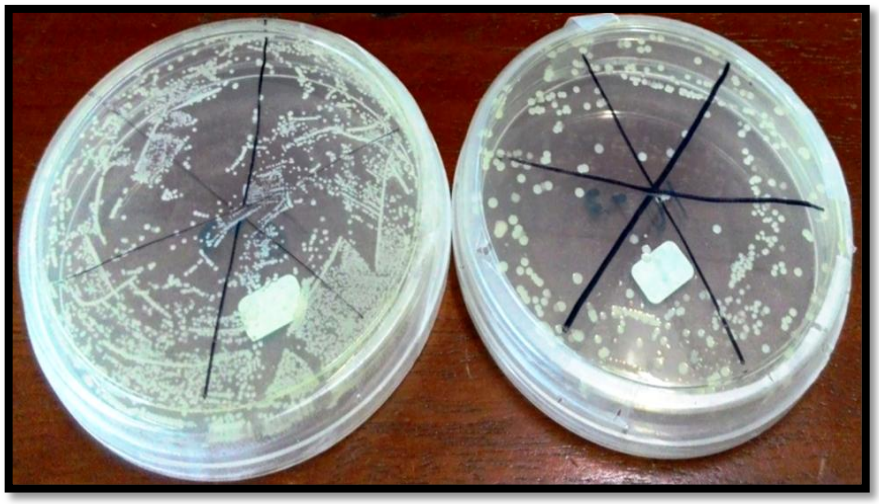


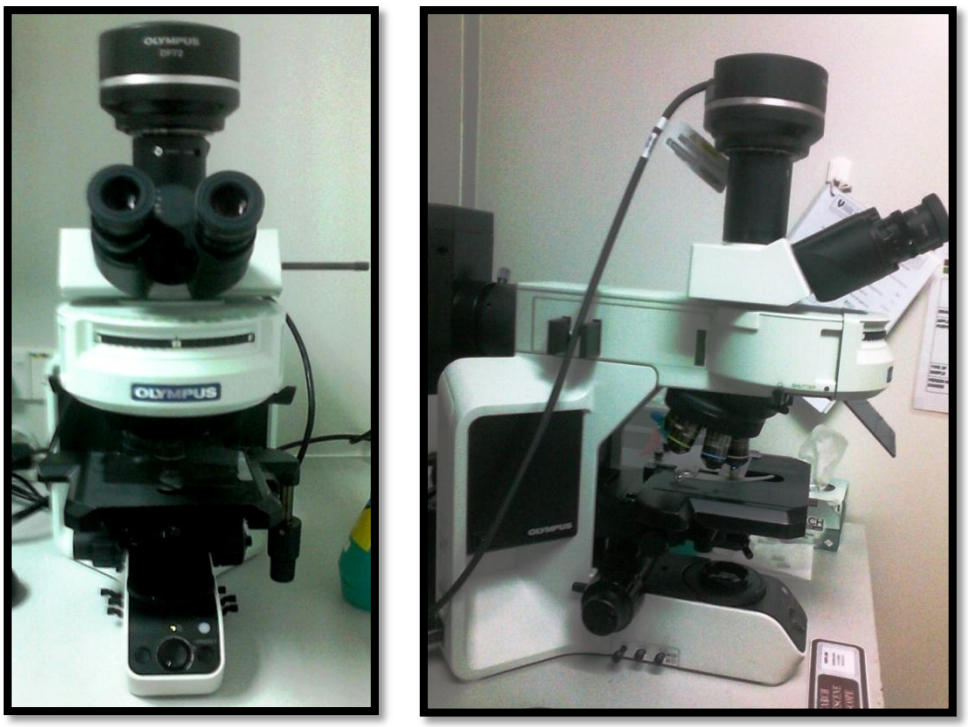
Figure 3-6: Image of Colony Forming Unit (CFU)

3.5.3 Gram Staining

A smear of bacterial culture was prepared and fixed on the surface of clean glass slide and allowed to dry. The smear was covered with crystal violet for 1 minute and then with Gram's iodine also for 1 minute after being washed with distilled water. Next, 95% of ethyl alcohol was used to decolorize the smear until no large amounts of purple wash out, but do not over decolorize the smear. After that, safranin was added for 1 minute and then washed with distilled water. The stained slide then was examined under light microscope with 100x magnification level using immersion oil. All the procedures were done for three types of bacteria which were *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus.*

3.5.4 Fluorescence Microscope

Each of metal substrate was examined under Olympus, BX53 Fluorescence Microscope using the software Olympus FluoView Ver. 1.3 in order to determine the amount of attached bacterial per square area. The step involved including preparing a 1/1000 dilution of the dye. For this 2 µL of SYTO9 (3.34 mM) dye was dissolved in 2 mL of PBS solution. Eight spots of 10 µL drops of dissolved dye solution were placed on top of each sample along with glass slide to spread the liquid. The samples then, were examined under Fluorescence Microscope with the level of magnification were 20x and 100x and emulsion oil was used for 100x magnification. Three different locations of samples were observed for each level of magnification. The image of fluorescence microscope can be seen at Figure 3-7 below.



(a) (b)

Figure 3-7: Diagram of fluorescence microscope (a) Front view (b) Side view

3.5.5 Scanning Electron Microscope (SEM)

The metal samples were observed under Carl Zeiss, SEM EVO 50 Scanning Electron Microscope for observing the morphological of adhered microorganism on the metals surface. Before being observed, the samples were fixed in 2% of gluteraldehyde/phosphate buffer saline solution (PBS) and stored at 4 °C until required. Prior to SEM analysis, the samples were dehydrated in an ethanol series; 30%, 50%, 70%, 80%, 90%, 100% (each step for 10 minutes excepting 100% ethanol treatment was for 30 minutes). The dehydrated sample in 100% ethanol was critical-point dried with liquid CO_2 prior to viewing with SEM and then, the samples were kept in dessicator to remove extra moisture. Observation of the attachment of bacterial cells on metal surface was carried out using scanning electron microscopy at the end of incubation time for each experiment. The image of scanning electron microscope is as shown in Figure 3-8.

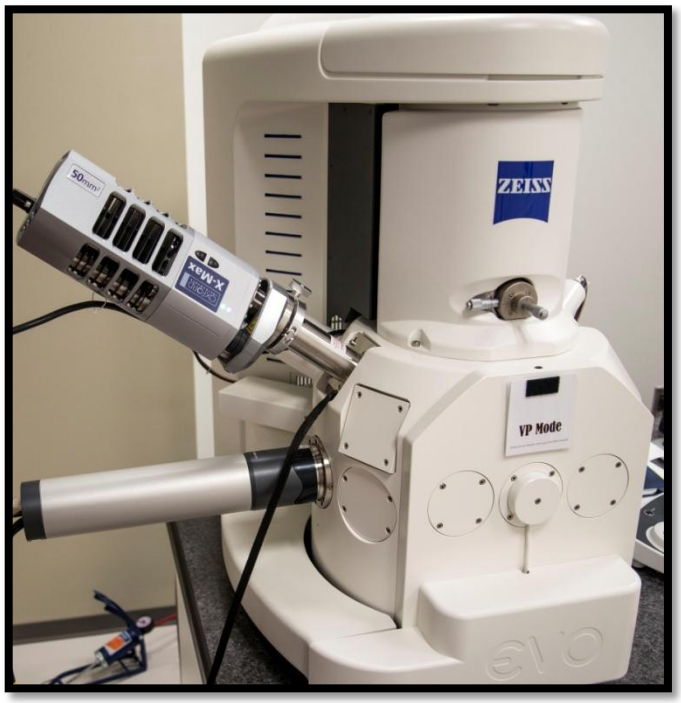


Figure 3-8: Diagram of Scanning Electron Microscope (SEM)

3.6 Summary

All the methods and analysis that involved in this study were successfully done for the research of adhesion of bacterial on metal surfaces with the effect of surface roughness.

4 RESULTS AND DISCUSSIONS

4.1 Overview

This chapter will cover the results and discussions obtained after the experiment. The results and discussions were regarding the characteristics of *Escherichia coli, Staphylococcus aureus* and *Bacillus subtilis*, Optical Density (OD) and Colony Forming Unit (CFU) obtained after the adhesion test, adhesion of bacterial on metal surfaces used in medical application and also the effect of surface roughness on the adhesion of bacterial on metal surfaces per square area.

4.2 Introduction

Characteristics of *Escherichia coli, Staphylococcus aureus* and *Bacillus subtilis* were discussed based on the image obtained from the light microscope and scanning electron microscope (SEM). Meanwhile, the results for OD and CFU were obtained after 4 hour of adhesion test and discussion regarding the analysis will be discussed later in this chapter. The adhesion of bacterial on three different types of metals with eight different surface roughnesses each also will be explained after this including the effect of surface roughness on the adhesion of bacterial onto metal surfaces.

4.3 Characteristics of Escherichia coli, Staphylococcus aureus and Bacillus subtilis

The image of gram stained of *Escherichia coli, Staphylococcus aureus* and *Bacillus subtilis* under light microscope with 100x magnification level using immersion oil was shown in Figure 4-1. This gram staining method was done in order to distinguish bacterial species into two large groups which are Gram positive bacteria and Gram negative bacteria by colouring the cells red or violet (purple). Bacteria that retain the initial crystal violet stain which is purple are said to be Gram positive while for bacteria stained red are said to be Gram negative (Beveridge, 2001).

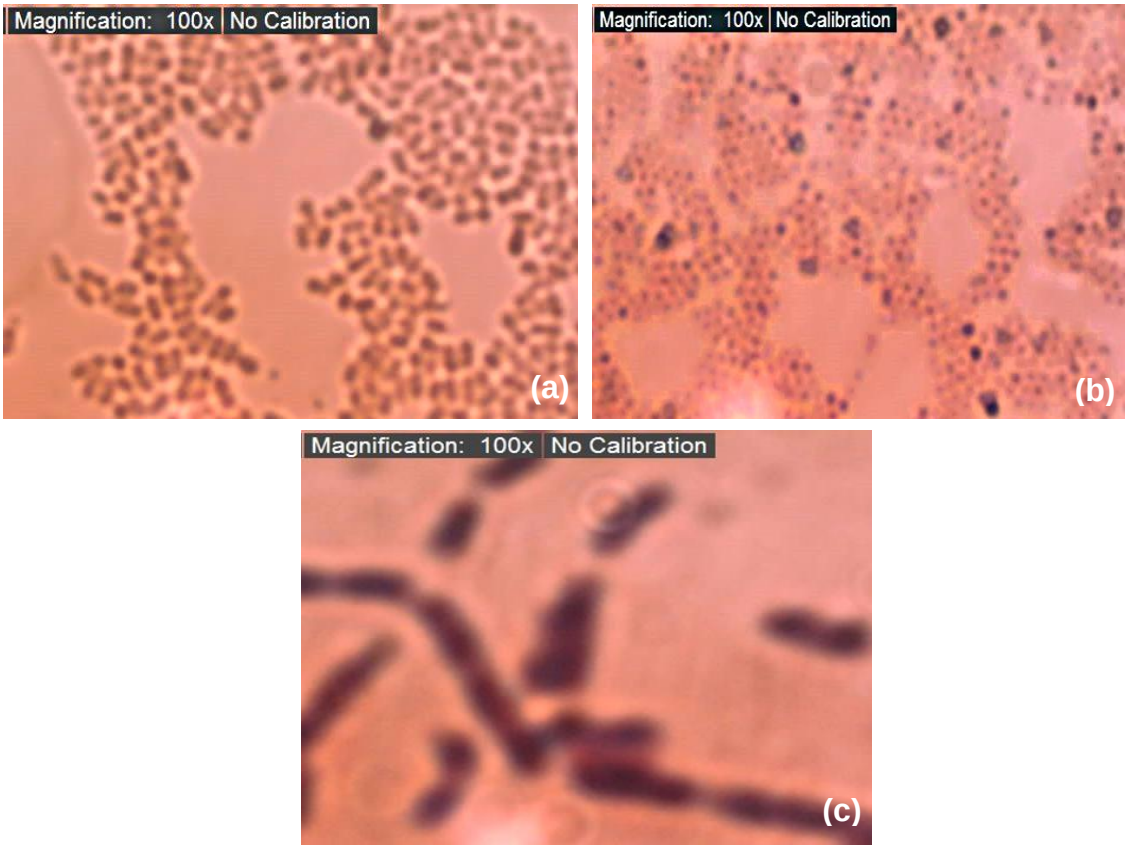


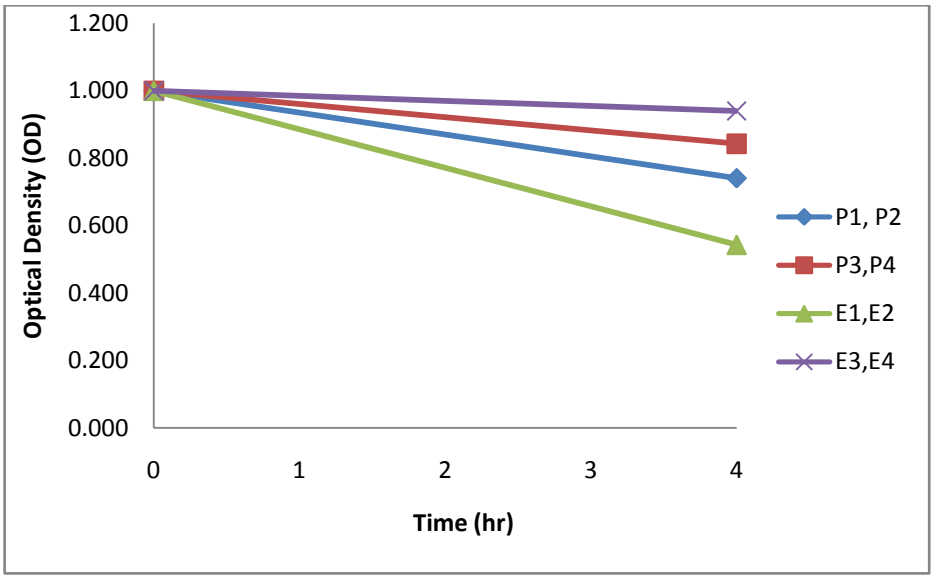
Figure 4-1: Image of gram stained (a) *E. coli* (b) *S. aureus* (c) *B. Subtilis*

Therefore based on the image, *E. coli* can be categorized as Gram negative bacteria as it was stained as red while for *S. aureus* and *B. subtilis* both were Gram positive bacteria since the stained colour was purple. This staining response is based on the chemical and structural makeup of the cell wall of bacteria. Gram positive bacteria which are *S. aureus* and *B. subtilis* have a thick, relatively impermeable wall that resist decolourization during staining process and composed of thick peptidoglycan and secondary polymers (Beveridge, 2001). Meanwhile, a Gram negative bacterium which is *E. coli* has a thin peptidoglycan layer plus the outer membrane, which can be easily disrupted by decolourization (Beveridge, 2001). Besides that, the image also shows the shape of each bacterium where *E. coli* and *B. subtilis* were in rod shape and *S. aureus* was in spherical shape.

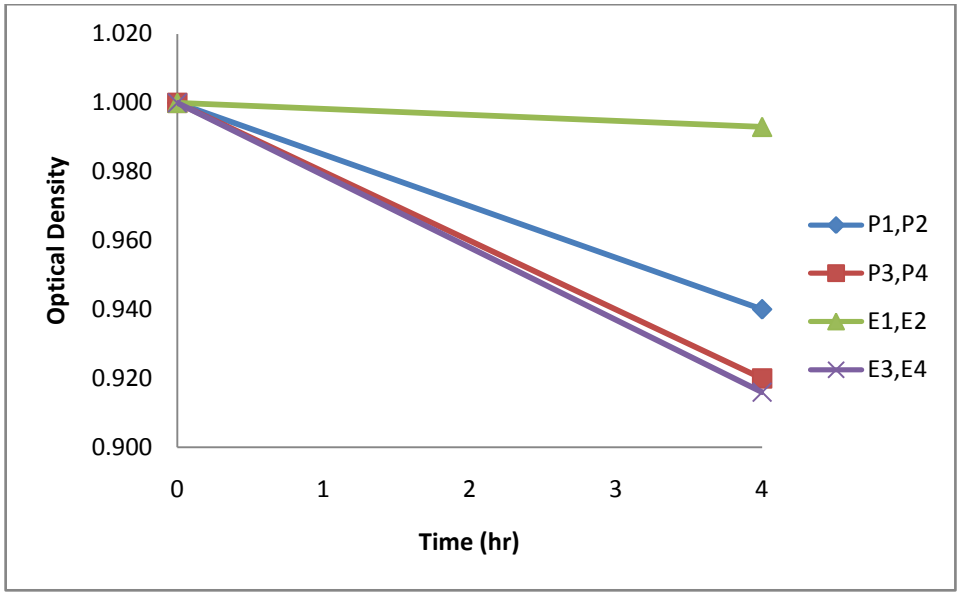
4.4 Optical Density (OD) and Colony Forming Unit (CFU) of Bacterial after Adhesion Test

The initial absorbance of all bacterial solution was adjusted to ~1.0 before started the adhesion test. Figure 4-2 are the graphs that show the OD of all bacterial solution after 4 hours adhesion and Table 4-1 shows the data comparison between reduction of OD and CFU at different types of metals and roughness also after 4 hours of adhesion test. Based on the table, after the adhesion test, solution that contained *E. coli* showed the highest reduction of absorbance which is in the range of 6% to 45.7% compared to others bacterial solution and this shows that *E. coli* was highly attracted to the metal surfaces. Based on Faisal *et al*., 2012 during adhesion process, a Gram negative bacterium will be more attracted to a positively charged surface while Gram positive bacterium attracted to a negatively charged surface.

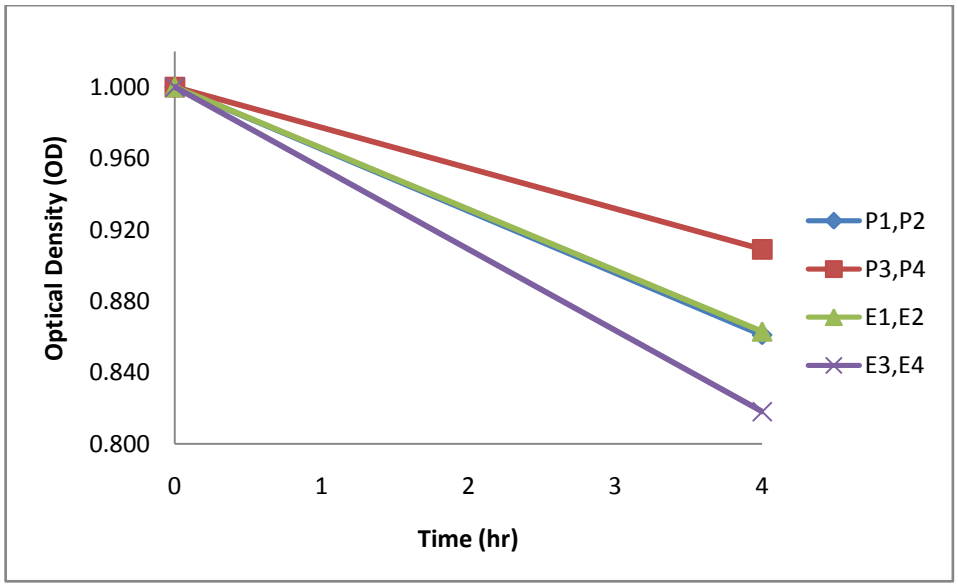
Therefore, the metals used in this study were high possibility have positively charged surface since *E.coli* was a Gram negative bacteria while *S. aureus* and *B. subtilis* both were gram positive bacteria. This reason was also supported by Xiaoxia *et al.* (2008) where stated that stainless steel is composed of a layer of oxides which creates a high positively charged region. The metals used in this study composed of two types of stainless steel thus, it contributed to the reduction of absorbance in *E. coli* solution after the adhesion test due to the higher attachment of *E. coli* onto the positive charged metal surfaces. Besides that, CFU plating for *E. coli* at dilution factor 10^2 also showed the lowest among others after the adhesion test (Table 4-1). However, some of the CFU measurements were slightly misleading because of the long storage period.



(a)



(b)



(c)

Figure 4-2: The optical density (OD) of bacterial after 4 hours adhesion a) *E. coli* b) *S. aureus* c) *B subtilis*

Table 4–1: Data comparison between Reduction of Optical Density (OD) and Colony Forming Unit (CFU)

Types of Roughness	***E. coli***		***S. aureus***		***B. subtilis***	
	*** Reduction of OD (%)**	**Colony Forming Unit (CFU/µL) x 10^2**	*** Reduction of OD (%)**	**Colony Forming Unit (CFU/µL) x 10^2**	*** Reduction of OD (%)**	**Colony Forming Unit (CFU/µL) x 10^2**
P1, P2	25.90	9.95±0.25	6.00	53.75±4.75	13.90	30.60±0.90
P3, P4	15.70	6.50±0.50	8.00	52.05±6.60	9.10	22.35±0.85
E1, E2	45.70	5.25±0.45	0.70	39.40±0.10	13.70	15.05±0.75
E3, E4	6.00	11.90±0.50	8.40	19.40±2.30	18.20	12.65±0.25

***Taken after 4 hour adhesion**

4.5 Adhesion of Bacterial on Metal Surfaces Used in Medical Application

The metals used in this study were consisted eight different types of roughness each (see Table 4-2). Some of the metals have been electropolished while some of them have undergone electro deposition process in order to reduce the roughness of the metals before being used in the adhesion test. Before being polished, the average roughness of the metals was in between 4.811 µm – 14.014 µm and was reduced to 0.097 µm – 0.232 µm after polished. While, for the metals that have undergone electro deposition technique, the average roughness of the metals was reduced from 4.010 µm – 14.014 µm to 2.470 µm – 3.880 µm. Then, all the metals were used in the adhesion test.

Table 4–2: Types of metals used in this study with different types of surface roughness

Types of Metals	Average Roughness *Ra* (µm)			
	P		E	
Stainless Steel (N690)	P-01	0.195	E-01	3.336
	P-02	0.188	E-02	2.501
	P-03	0.120	E-03	2.910
	P-04	0.162	E-04	2.470
Stainless Steel (AV220SC)	P-01	0.232	E-01	3.880
	P-02	0.143	E-02	3.604
	P-03	0.110	E-03	3.825
	P-04	0.097	E-04	3.752
Titanium	P-01	0.171	E-01	3.356
	P-02	0.165	E-02	3.299
	P-03	0.127	E-03	3.350
	P-04	0.104	E-04	3.255

P- Polishing Technique, 01/02/03/04 – Substrate number,
E – Electro Deposition Machine (EDM) Technique, 01/02/03/04 – Substrate number

After the adhesion test, bacterial that attached to the metal surfaces were stained using SYTO9 dye and subsequently analyzed with fluorescence microscope for observing how many bacterial attached per square area on each of the metals surfaces. SYTO9 dye is membrane-permeable which diffuses into the cells and fluoresces upon binding nucleic acids in green fluorescence (Xiaoxia *et al*., 2008). Figure 4-3 shows the examples of images of bacterial attached and counted on each of metals surfaces.

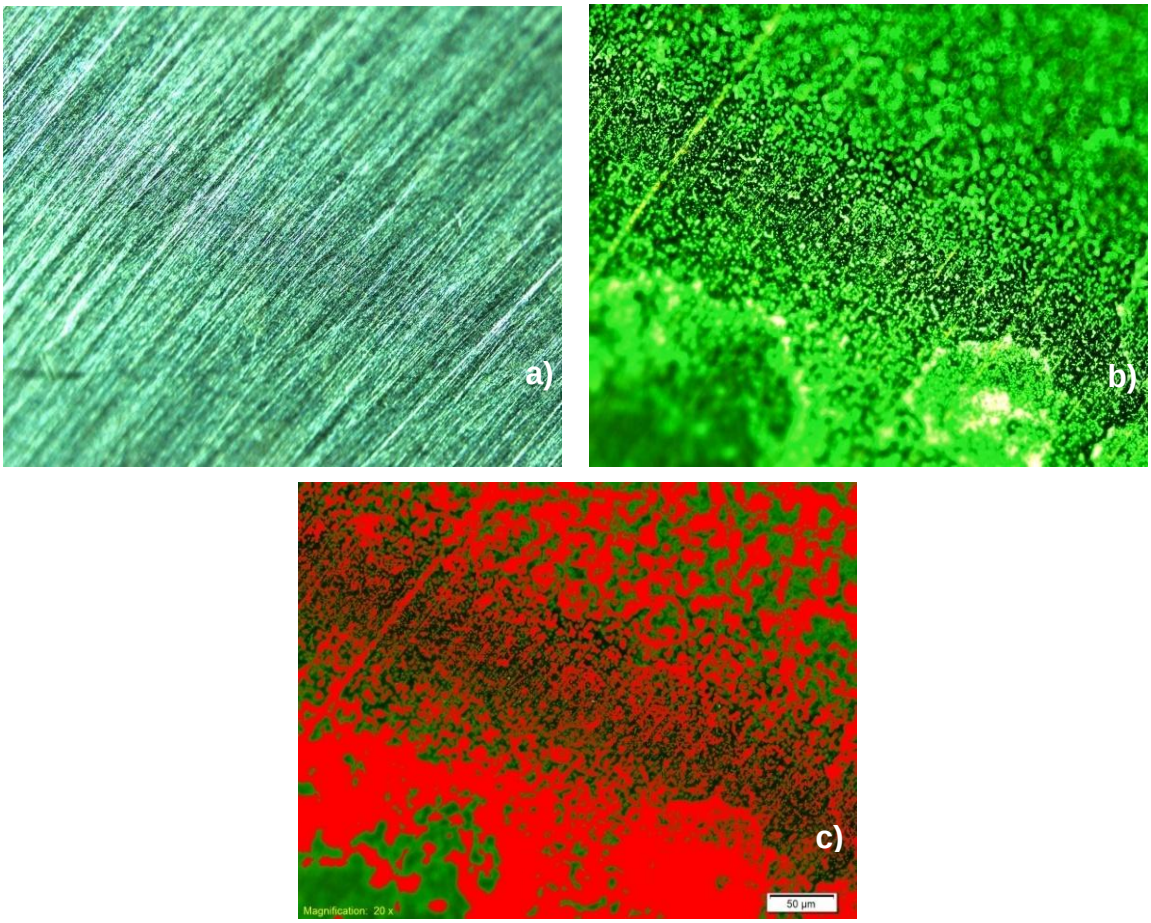
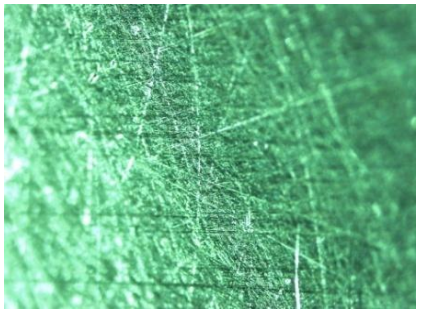
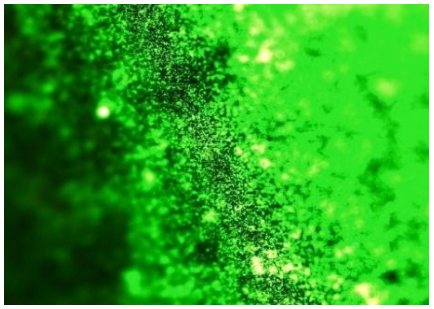
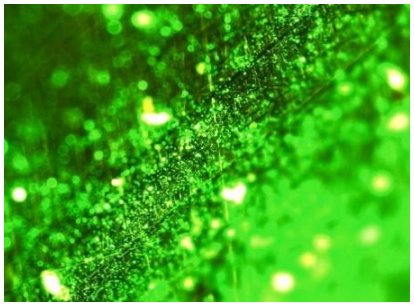
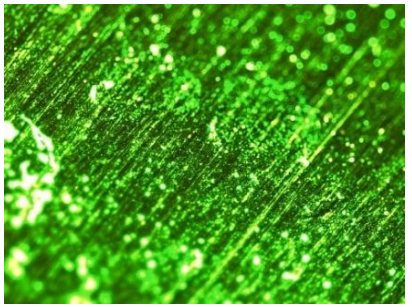
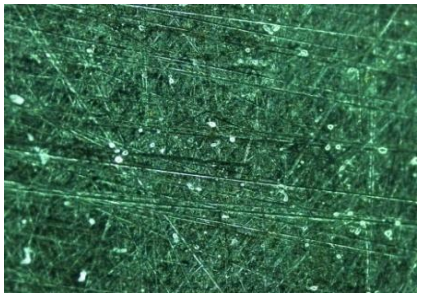
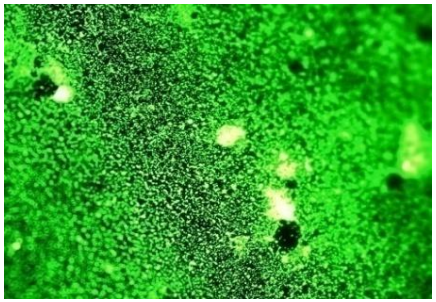
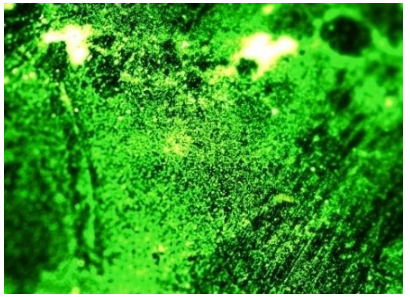
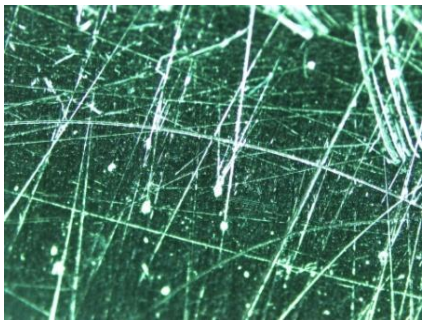
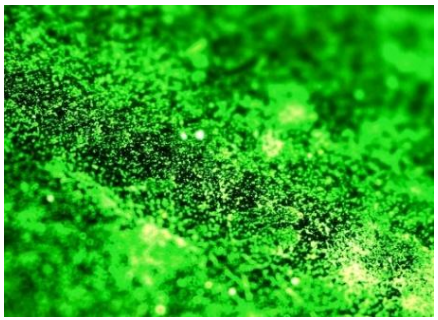
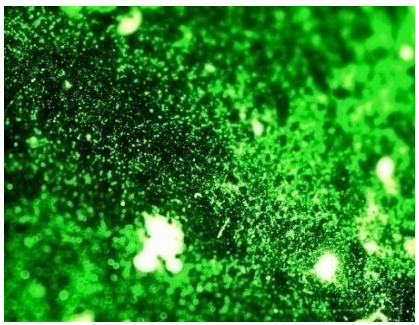
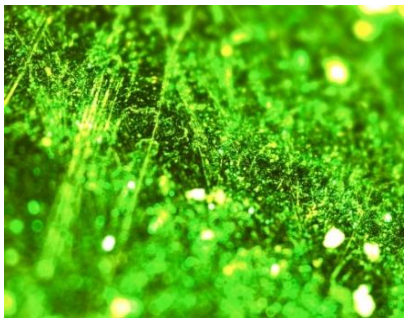


Figure 4-3: Images of *E. coli* under Fluorescence Microscope a) Image of Stainless Steel (N690) surface b) Image of stained *E. coli* on Stainless Steel (N690) c) Image of counted *E. coli*

The image (a) shows the surface of Stainless Steel (N690) before being viewed with the fluorescence light and image (b) is the image of stained nucleic acid of *E. coli* under the fluorescence light where green images was produced. While image (c), shows the image on how the attachment of *E. coli* on the Stainless Steel (N690) was counted per square area using the software as mentioned before. Meanwhile, Table 4-3 demonstrated the attachment of bacterial on different types of metals by using fluorescence microscope.

Table 4–3: Image of bacterial adhesion on different metals under fluorescence microscope

Types of Metals	Surface of Metals	*E. coli*	*S aureus*	*B. subtilis*
Stainless Steel (N690)				
Stainless steel (AV220)				
Titanium				

4.6 Effect of Surface Roughness on the Adhesion of Bacterial on Metal Surfaces

Based on the Figure 4-4, *S. aureus* recorded the highest attachment of bacterial at the roughness of 2.910 µm and 3.336 µm which were the rougher surfaces with the adhesion per square area were 0.0983/µm^2 and 0.0876/µm^2 (see Table 4-4) respectively. As reported by Scheurman *et al*. (1998), irregularities of polymeric surfaces promote bacterial adhesion and biofilm deposition, as rough surface has a greater surface area and the depressions in the roughened surfaces provide more favourable sites of colonization. Another explanation for the enhanced adhesion on the roughest surface may be entrapment of microbial cells in the crevices of the surface (Ortega *et al.,* 2008). However, the highest adhesion of all the bacterial on the Stainless Steel (N690) was on surface roughness 0.162 µm and 2.910 µm with the total adhesion 0.1833/µm^2 and 0.1755/µm^2 respectively. Therefore, this trend shows that the attachment of the bacterial on the metal surfaces were not influenced by the rough surfaces only but also on the smooth surfaces. Attachment of *E. coli* and *S. aureus* also showed the same trend where the minimal adhesions were observed at the middle roughness of 0.195 µm with the adhesion 0.0193/µm^2 and 0.0175/µm^2 respectively. Medilanskia *et al*. (2002) demonstrated that smoother and rougher surfaces enhance the bacterial attachment. They had tested four different bacterial strains on the surface of Stainless Steel 304 that had five different surfaces finishes with roughness values that ranged from 0.03 – 0.089 µm and minimal adhesion was observed at roughness 0.16 µm while both smoother and rougher surfaces showed more adhesion.

Table 4–4: Data of bacterial adhesion/ µm^2 on Stainless Steel (N690)

Types of Roughness (µm)	**Adhesion/ µm^2**		
	E. coli	***B. subtilis***	***S. aureus***
0.1200	0.0320 ± 0.0110	0.0416 ± 0.0100	0.0559 ± 0.0100
0.1620	0.0443 ± 0.0100	0.0711 ± 0.0100	0.0679 ± 0.0120
0.1880	0.0613 ± 0.0370	0.0291 ± 0.0040	0.0663 ± 0.0200
0.1950	0.0193 ± 0.0020	0.0175 ± 0.0003	0.0338 ± 0.0100
2.4700	0.0191 ± 0.0020	0.0299 ± 0.0100	0.0676 ± 0.0200
2.5010	0.0329 ± 0.0020	0.0323 ± 0.0100	0.0441 ± 0.0020
2.9100	0.0594 ± 0.0020	0.0178 ± 0.0020	0.0983 ± 0.0200
3.3360	0.0344 ± 0.0010	0.0376 ± 0.0100	0.0876 ± 0.0100

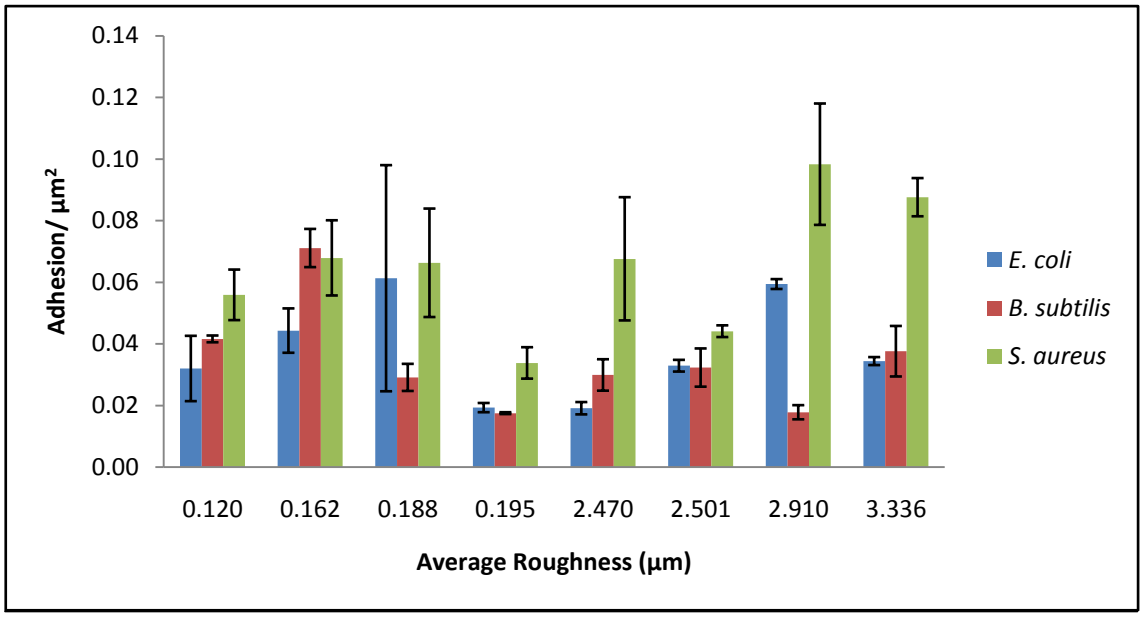


Figure 4-4: Adhesion of bacterial on Stainless Steel (N690) with different roughness

On the other hand, Figure 4-5 demonstrated the adhesion trend of bacterial on Stainless Steel (AV220SC). The difference between the Stainless Steel (N690) and Stainless Steel (AV220SC) is the chemical composition for both metals, which might be the reason for the discrepancies of adhesion. Stainless Steel (N690) contained of 0.05% Nitrogen, 0.08% Carbon, 0.015% Hydrogen, 0.40% Iron, 0.20 Oxygen, 6.75% Aluminium and 4.5% Vanadium while, Stainless Steel (AV220SC) has low carbon content which is 0.03% compared to Stainless Steel (N690). Based on Argelia *et al.* (2011), carbon surface has great biocompatibility properties and good resistance to microbial adhesion. Therefore, the amount of bacterial adhesion on Stainless Steel (N690) was lesser than Stainless Steel (AV220SC) due to the higher carbon content in that metal. Besides that, biocompatibility is define as, the ability of the material, intentionally in contact or implanted into the body tissues, to perform as designed without inducing any local effect in the cells or tissue or a systemic response that elicits an immunological reaction (Argelia *et al*., 2011). Hence, Stainless Steel (N690) can be suggested to be one of the metals that can be used in medical application as implanted devices because it has great biocompatibility.

Table 4-5 shows the data adhesion of bacterial on Stainless Steel (AV220SC). Based on the Figure 4-5, *B. subtilis* and *S. aureus* has the highest adhesion at the roughness 0.110 µm which is the second smooth surface with the adhesion value 0.3687/µm^2 and 0.1264/µm^2 respectively. The results obtained, reinforced that the highest adhesion of bacterial were not only on the rougher surfaces but also on the smoother surfaces.

Table 4–5: Data of bacterial adhesion/ µm^2 on Stainless Steel (AV220SC)

Types of Roughness (µm)	**Adhesion/ µm^2**		
	E. coli	***B. subtilis***	***S. aureus***
0.0970	0.0362 ± 0.0020	0.0477 ± 0.0100	0.0819 ± 0.0200
0.1100	0.0461 ± 0.0100	0.3687 ± 0.1000	0.1264 ± 0.0300
0.1430	0.0704 ± 0.0110	0.0703 ± 0.0050	0.0606 ± 0.0100
0.2320	0.0343 ± 0.0040	0.0217 ± 0.0010	0.0305 ± 0.0030
3.6040	0.0745 ± 0.0100	0.0784 ± 0.0050	0.0933 ± 0.0050
3.7520	0.0752 ± 0.0100	0.0431 ± 0.0050	0.0629 ± 0.0200
3.8250	0.0281 ± 0.0100	0.0562 ± 0.0110	0.0644 ± 0.0110
3.8800	0.0182 ± 0.0100	0.0558 ± 0.0060	0.0915 ± 0.0120

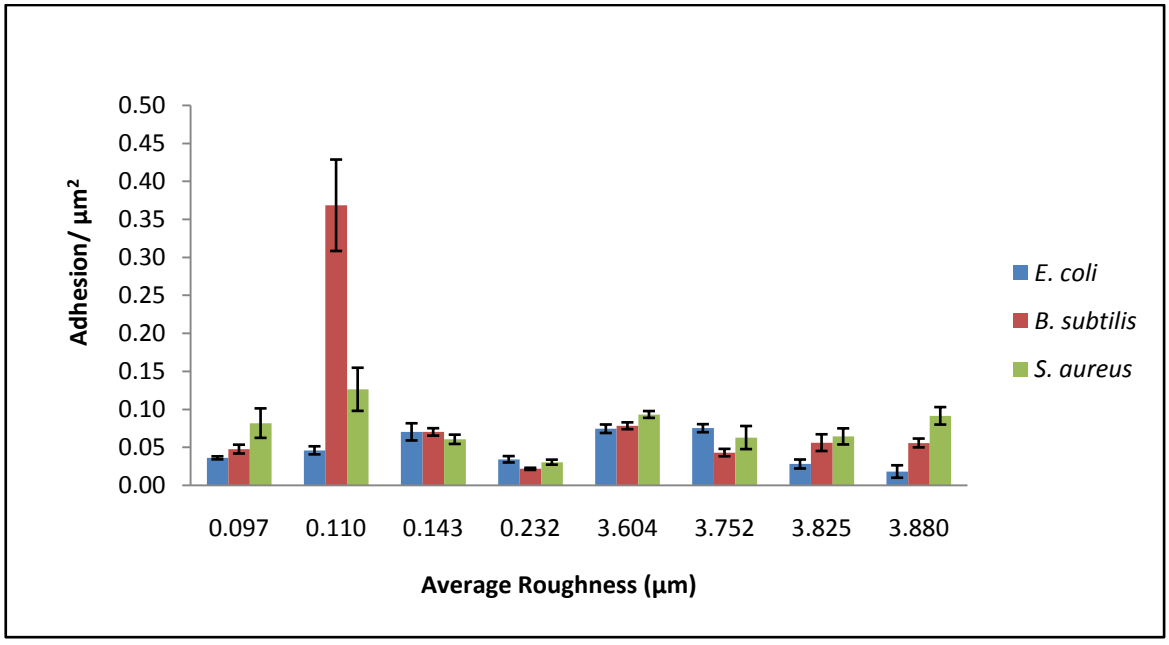


Figure 4-5: Adhesion of bacterial on Stainless Steel (AV220SC) with different roughness

Previous research already stated that highest adhesion of bacterial could be on the smooth surfaces, but the explanations were not given in details because the research is still ongoing. In medical application, bacteria have been found to colonize smooth surfaces, such as electropolished stainless steel (Woodling and Moraru, 2005). Hence, stainless steel at this roughness has been undergone electropolishing technique and this might be one of the reasons the smooth surfaces tend to be adhered by the bacterial. Literature also said that, surface roughness alone does not appear to be sufficient in predicting bacterial attachment, and surface topography need to be considered next (Woodling and Moraru, 2005). Besides that, based on the schematic diagram in Figure 4-6 shows that metals with smooth surfaces has high contact point with the bacteria thus, the bacteria is strongly attracted to the metals with smooth surfaces compared to the rough surfaces.

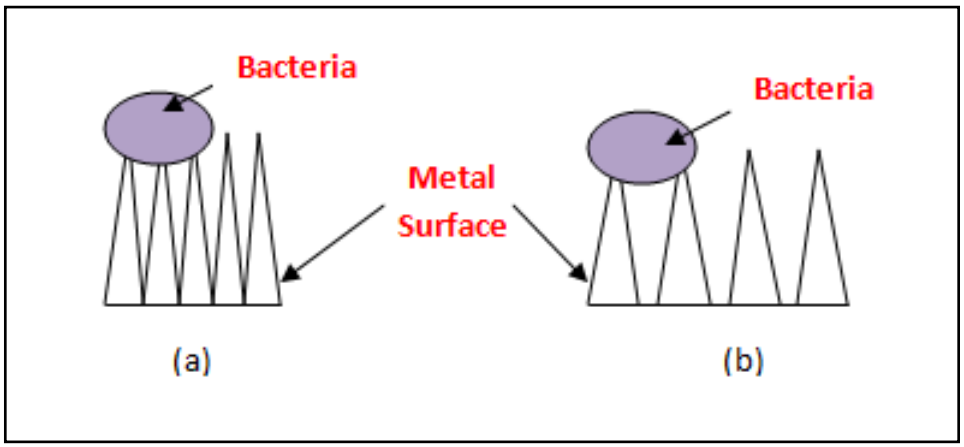


Figure 4-6: Schematic diagram adhesion of bacteria on a) smooth surface b) rough surface

Highest adhesions of bacterial on Titanium (refer Figure 4-7 and Table 4-6) also were recorded on the smoother surfaces which was at 0.104 µm where *B. subtilis* showed the highest attachment followed by *S. aureus* and *E. coli* with the adhesion value was 0.9601/µm^2, 0.1396/µm^2 and 0.0744/µm^2 respectively. Smooth surfaces still exhibit the highest attachment of bacterial and *B. subtilis* has the highest attachment than *E. coli* and *S. aureus* mybe due to the hydrophobicity interaction between the titanium and all the bacteria. *B. subtilis* can be considered as high hydrophobic bacteria since it showed highest attachment to all the materials discussed in this study. This is because, hydrophobic bacteria will attach to the hydrophobic surfaces. Stainless steel and titanium was a positively charged metal with high hydrophobic characteristics. Therefore, *B. subtilis* has high hydrophobic interaction between the metals compared to *S. aureus* and *E. coli*.

As discussed before, *E. coli* was a Gram negative bacterium and will be attracted the most to the high positively charged surfaces. But, Xiaoxia *et al*. (2007) stated that bacterial that less hydrophibic will exhibit lowest adhesion even though it has high electrostatic forces between the metal and the bacteria. Therefore, the *E. coli* cells maybe do not adhere to the metals but were dropped after the adhesion test which is during the process in between before being observed under fluorescence microscope.

Table 4–6: Data of bacterial adhesion/ μm^2 on Titanium

Types of Roughness (µm)	**Adhesion/ µm²**		
	E. coli	***B. subtilis***	***S. aureus***
0.1040	0.0744 ± 0.0110	0.9601 ± 0.0200	0.1396 ± 0.0100
0.1270	0.0238 ± 0.0010	0.0292 ± 0.0100	0.0544 ± 0.0130
0.1650	0.0187 ± 0.0040	0.1090 ± 0.0340	0.0378 ± 0.0100
0.1710	0.0406 ± 0.0100	0.0685 ± 0.0200	0.0552 ± 0.0110
3.2550	0.0377 ± 0.0020	0.0176 ± 0.0010	0.0424 ± 0.0030
3.2990	0.0518 ± 0.0100	0.0291 ± 0.0020	0.0384 ± 0.0030
3.3500	0.0241 ± 0.0004	0.0471 ± 0.0100	0.0434 ± 0.0060
3.3560	0.0502 ± 0.0100	0.0177 ± 0.0030	0.0344 ± 0.0020

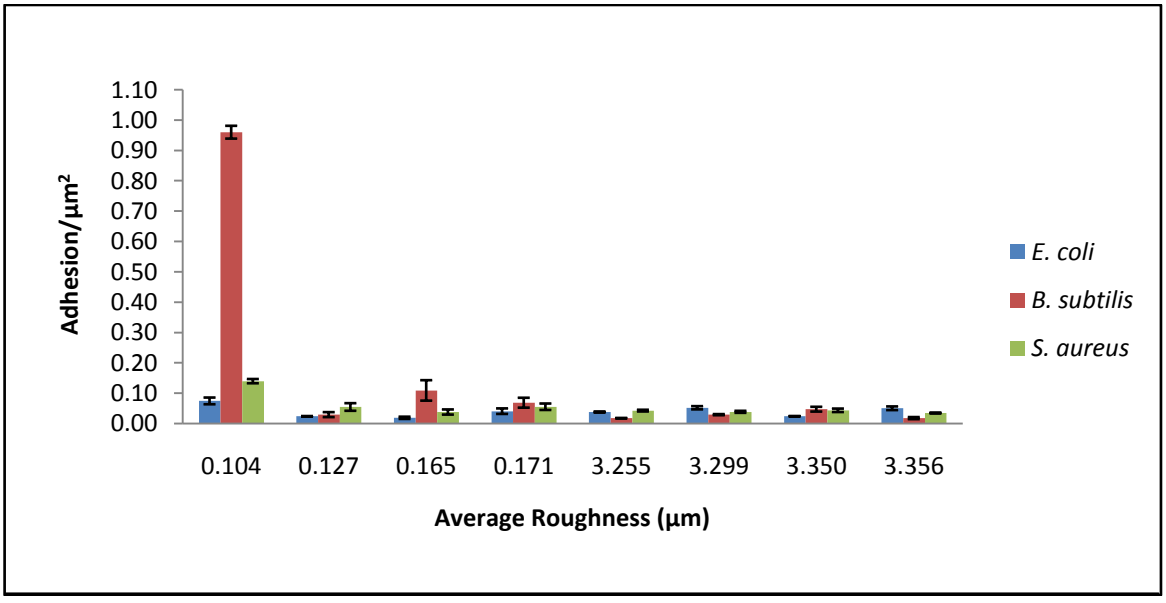


Figure 4-7: Adhesion of bacterial on Titanium with different roughness

The adhesion of bacterial on three different metals at the same roughness which was *Ra* = ~ 0.15 µm was shown in Figure 4-8. *B. subtilis* has high adhesion on all the metals due to the hydrophobic interaction between the metals and also maybe the dimension of the *B. subtilis* fitted the surface roughness used in this study. The highest adhesion of *B. subtilis* was on titanium with 0.9601/µm^2 while for *S. aureus* it adhered the most was on Stainless Steel (N690) with 0.0670/µm^2 which is only 0.0073/µm^2 high than adhesion on Stainless Steel (AV220SC). For *E. coli*, it adhered the most on Stainless Steel (AV220SC) with 0.0704/µm^2 (refer Table 4-7). Each of bacteria tend to be adhered at variety of metals, this is because adhesion factors was governed by many factors including surface roughness, electrostatic and hydrophobic forces between cell and metal.

Table 4–7: Data adhesion/ µm^2 on different metals

Types of Metals	**Adhesion /µm^2**		
	E. coli	***B.subtilis***	***S. aureus***
Stainless Steel (N690)	0.0443 ± 0.0100	0.0711 ± 0.0100	0.0679 ± 0.0120
Stainless Steel (AV220SC)	0.0704 ± 0.0110	0.0703 ± 0.0500	0.0606 ± 0.0100
Titanium	0.0132 ± 0.0040	0.9601 ± 0.0200	0.0298 ± 0.0090

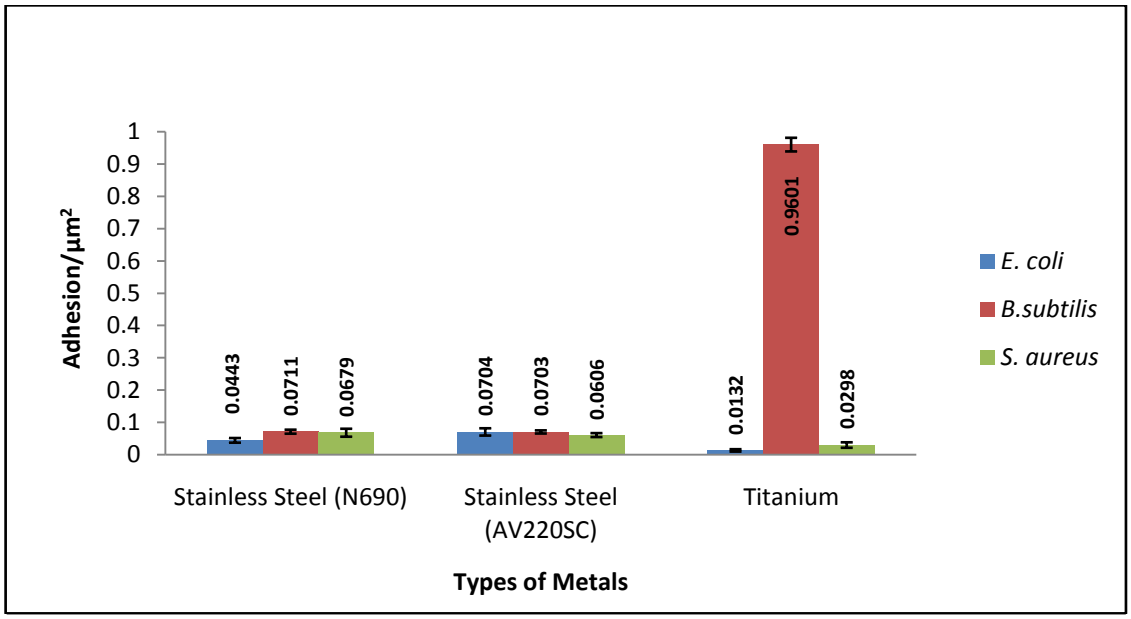
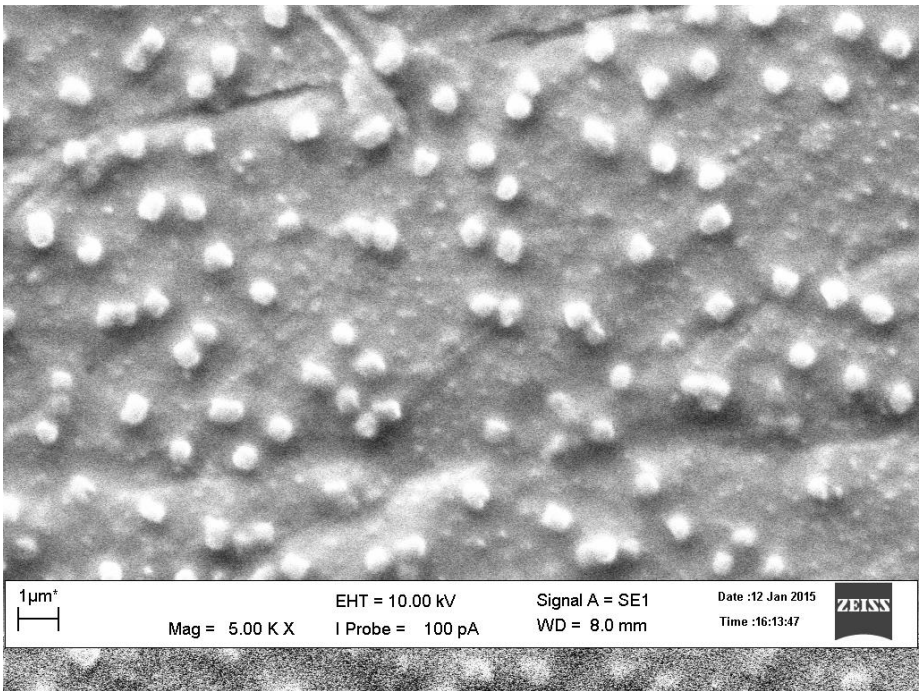
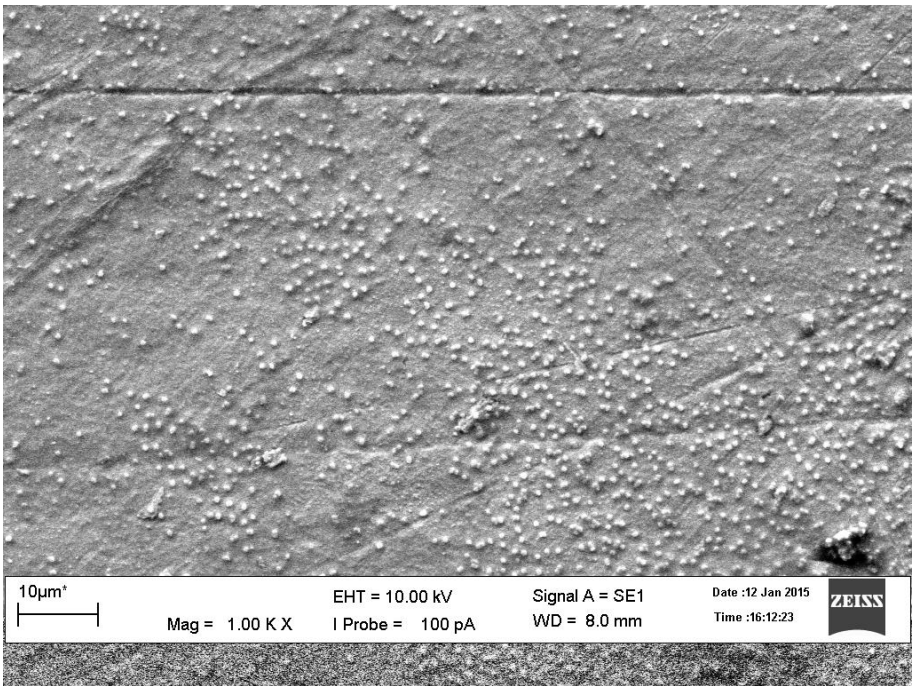


Figure 4-8: Adhesion of bacterial on different types of metals with similar roughness (*Ra* = ~0.15µm)

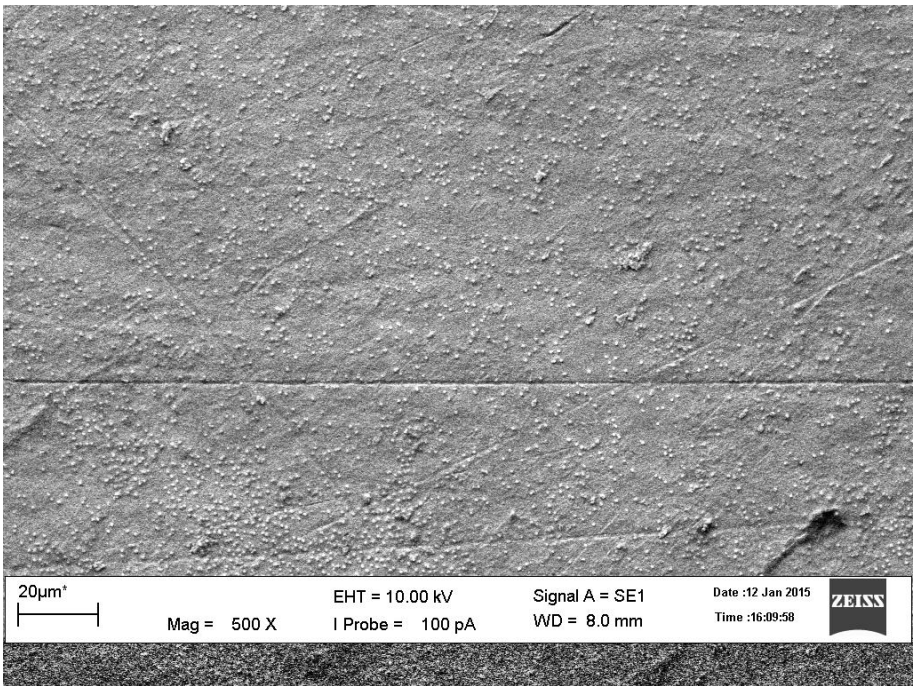
The image of bacteria attached on metal surface can be seen in the Figure 4-9. The figures showed the image of *E. coli* attached on Titanium with smooth surface (*Ra* = 0.104) under Scanning Electron Microscope at different magnification which were 5000x, 1000x, and 500x.



(a)



(b)



(c)

Figure 4-9: Image of *E. coli* on Titanium at different magnification a)5000x b) 1000x c) 500x

4.7 Summary

All the results and discussion have been explained in this chapter. Based on the discussion, the characteristics of the bacterial have been identified, the readings of OD and CFU also have been discussed and lastly the effect of metal surface roughness on the adhesion of bacterial also has been investigated. Therefore, based on the results and discussion the bacterial tend to be adhered on smooth surfaces compared to rough surfaces due to some factors that have been discussed before. Meanwhile, *B. subtilis* gave the highest attachment on Titanium surface while *S. aureus* and *E. coli* was on Stainless Steel (N690) and Stainless Steel (AV220SC) respectively.

5 CONCLUSION

5.1 Conclusion

It can be concluded that the adhesion of bacterial on metal surfaces was influenced by many factors. For this study, surface roughness, bacterial and metal surface properties play a very important role. The adhesion of bacterial on different metal with the same roughness showed that, *B. subtilis* like to adhere to titanium surface, while *S. aureus* and *E. coli* were adhered at Stainless Steel (N690) and Stainless Steel (AV220SC) respectively. On the other hand, during the adhesion of bacterial on Stainless Steel (N690), the highest attachment of all bacterial were on smooth surface *(Ra* = 0.163 µm) and rough surface (*Ra* = 2.910) with the total adhesion were 0.1833/µm^2 and 0.1755/µm^2 respectively. Meanwhile, adhesion on Stainless Steel (AV220SC) shows that *B. subtilis* and *S. aureus* has the highest adhesion at the roughness 0.110 µm (smooth surface) with adhesion value 0.3687/µm^2 and 0.1264/µm^2 respectively. Titanium also gave the same trend, where *B. subtilis* showed the highest attachment on the smoother surface followed by *S. aureus* and *E. coli* with the adhesion value was 0.9601/µm^2, 0.1396/µm^2 and 0.0744/µm^2 respectively. Therefore, bacterial tend to be adhered on smooth surfaces and both electrostatic and hydrophobic interaction between metals surfaces and bacterial gave high influenced to the adhesion of bacterial on metals surfaces used in the medical application. However, the best roughness that might contribute to lower adhesion should be in the range size of the bacteria, so that the bacteria has less area of contact with the surface, thus easy to flush out during the self cleaning surface.

5.2 Recommendations

There are a few recommendations that can be made in this study:

1) The CFU analysis must be done straight away after the adhesion test in order to avoid any inaccurate or misleading results. This is because if the adhesion samples were stored for a long period, a few cells in the sample might have died or contaminated.
2) The aseptic technique must be applied in a correct way because it can avoid contaminations to occur.
3) The fluorescence microscope must be handled correctly during the observation in order to get a good image and have a correct calculation on the adhesion of bacterial on metals surfaces per square area.
4) For future research, investigation about the adhesion of bacterial on surfaces by focussing on the effect of gibbs energy and surface energy for both bacteria and surfaces can be done.
5) Study more details about the factors that contributed to the adhesion of bacterial on smooth surfaces.

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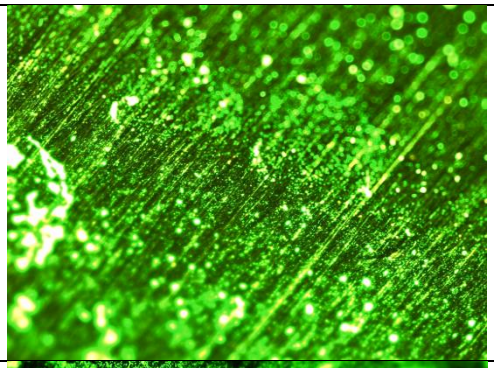
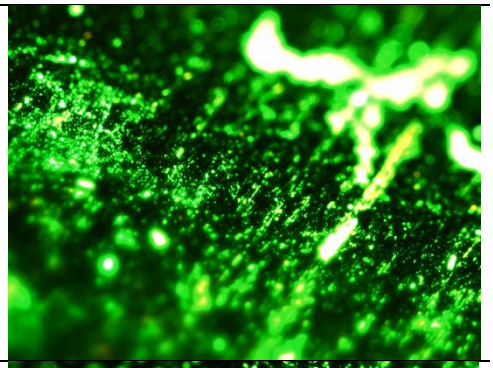
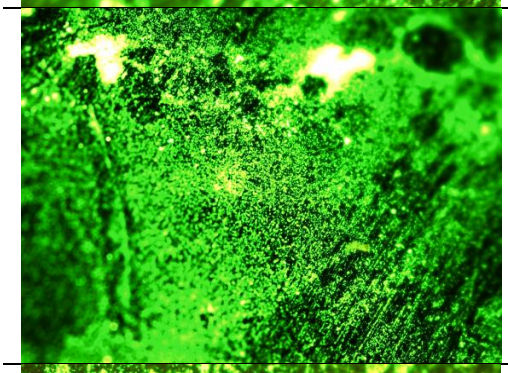
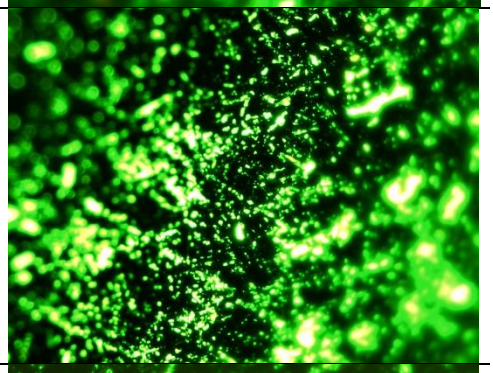
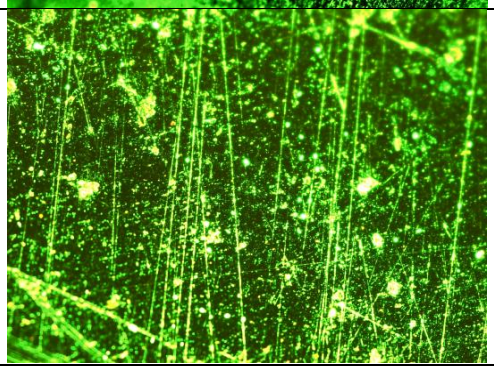
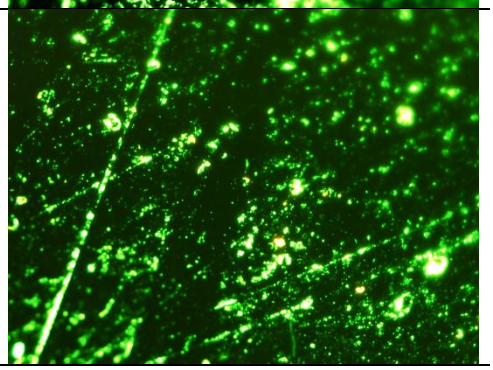
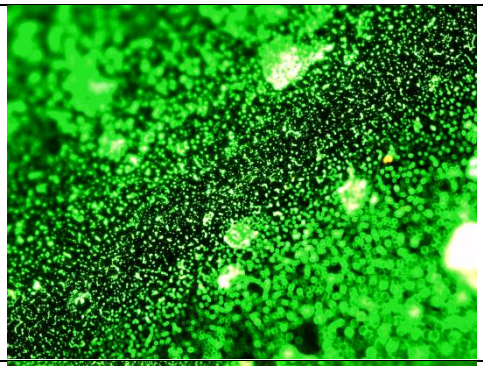
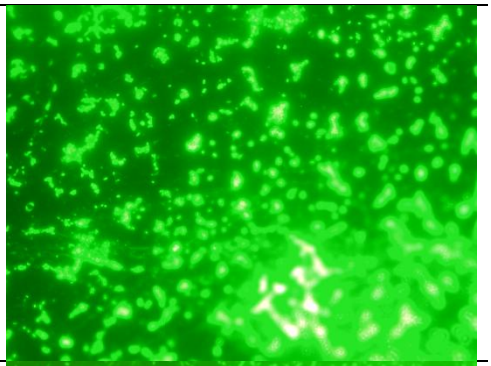
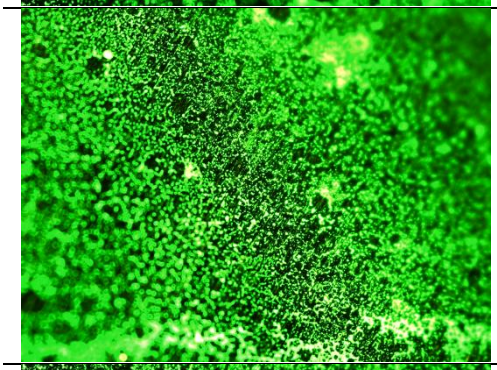
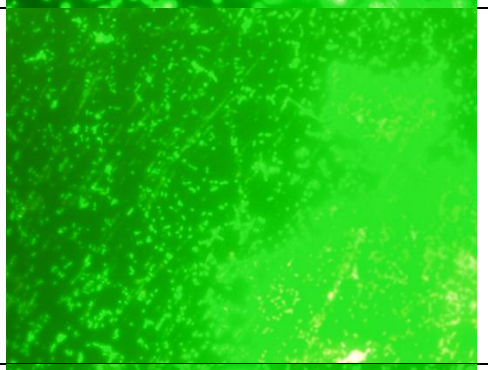
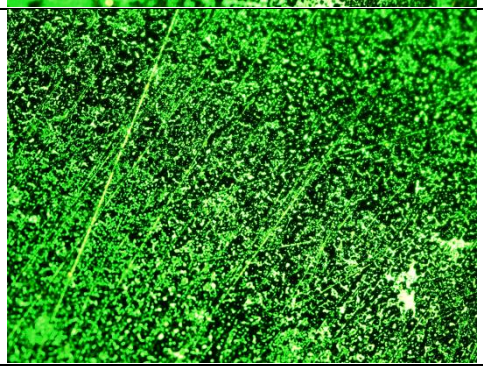
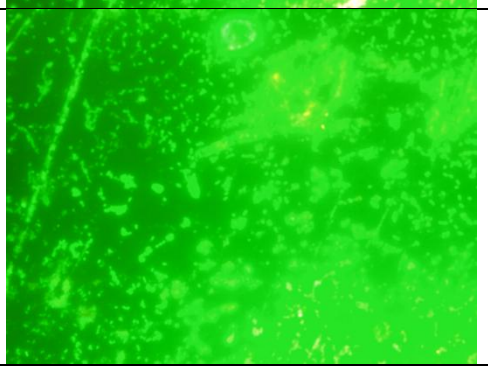
APPENDICES

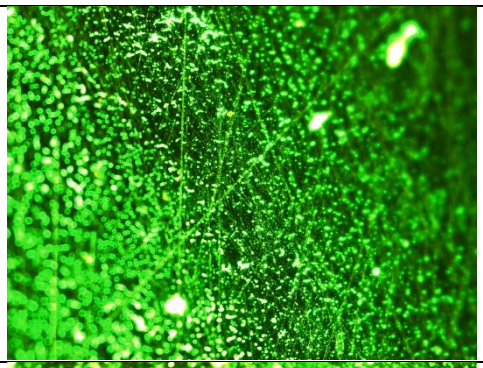
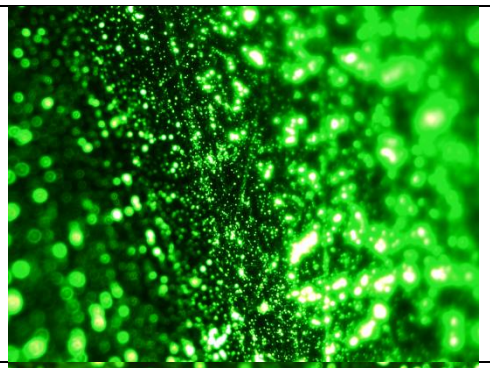
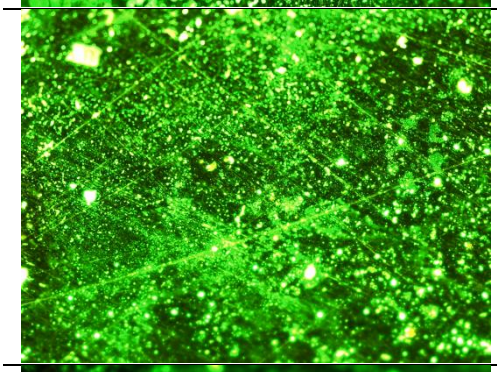
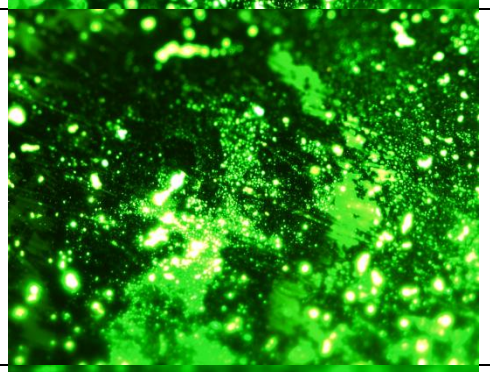
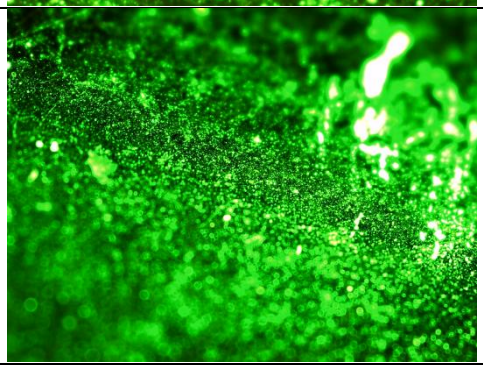
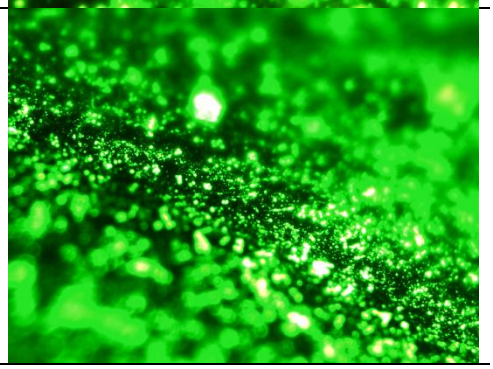
Table A1: Images of bacterial under fluorescence microscope at different magnification a) *B.subtilis* b) *E.coli* c) *S. aureus*

Types of Metals	*B. subtilis*	
	20x	100x
Stainless Steel (N690)		
Stainless Steel (AV220SC)		
Titanium		

(a)

Types of Metals	*E. coli*	
	20x	100x
Stainless Steel (N690)		
Stainless Steel (AV220SC)		
Titanium		

(b)

Types of Metals	*S. aureus*	
	20x	100x
Stainless Steel (N690)		
Stainless Steel (AV220SC)		
Titanium		

(c)