ASSESSING STORAGE STABILITY AND MERCURY REDUCTION OF FREEZE-DRIED *PSEUDOMONAS PUTIDA* WITHIN DIFFERENT TYPES OF LYOPROTECTANT

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Thesis submitted in partial fulfilment of the requirements
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SUPERVISOR'S DECLARATION

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

Signature	:
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Date	: 6 JULY 2015

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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Dedication

To my parents for their endless love, support and encouragement,
Sri Mulyanta and Nanik Sunarni

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This thesis is only a beginning of my journey. Hopefully this thesis can give quite a contribution to chemical engineering (Biothechnology) research in Malaysia and Indonesia.

ABSTRACT

Pseudomonas putida is a potential strain in biological treatment to remove mercury contained in the effluent of petrochemical industry due to its mercury reductase enzyme that able to reduce ionic mercury to elementary mercury. Freeze-dried *P. putida* allows easy, inexpensive shipping, handling and high stability of the product. This study was aimed to freeze dry *P. putida* cells with addition of lyoprotectant. Lyoprotectant was added into the cells suspension prior to freezing. Dried *P. putida* obtained was then mixed with synthetic mercury. Viability of recovery *P. putida* after freeze dry was significantly influenced by the type of lyoprotectant. Among the lyoprotectants, tween 80 / sucrose was found to be the best lyoprotectant. Sucrose able to recover more than 78% (6.2E+09 CFU/ml) of the original cells (7.90E+09CFU/ml) after freeze dry and able to retain 5.40E+05 viable cells after 4 weeks storage in 4^{o}C without vacuum. PEG pre-treated freeze dry cells and broth pre-treated freeze dry cells after freeze-dry recovered more than 64% (5.0 E+09CFU/ml) and >0.1% (5.60E+07CFU/ml). Freeze-dried *P. putida* cells in PEG and broth cannot survive after 4 weeks storage. Freeze dry also does not really change the pattern of growth *P. putida* but extension of lag time was found 1 hour after 3 weeks of storage. Additional time was required for freeze-dried *P. putida* cells to recover before introduce freeze-dried cells to more complicated condition such as mercury solution. The maximum mercury reduction of PEG pre-treated freeze-dried cells after freeze dry and after storage 3 weeks was 56.78% and 17.91 %.The maximum of mercury reduction of tween 80 / sucrose pre-treated freeze-dried cells after freeze dry and after storage 3 weeks were 26.35% and 25.03%. Freeze dried *P .putida* was found to have lower mercury reduction compare to the fresh *P. putida* that has been growth in agar. Result from this study may be beneficial and useful as initial reference before commercialize freeze-dried *P. putida*.

ABSTRAK

Pseudomonas putida ialah rawatan biologi yang berpotensi untuk menghapuskan merkuri yang terkandung dalam efluen industri petrokimia kerana enzim merkuri reductase yang dapat mengurangkan merkuri ionik kepada merkuri asas. Beku-kering *P. putida* membolehkan, penghantaran yang tidak mahal, cara pengendalian dan kestabilan produk adalah tinggi. Kajian ini bertujuan untuk membeku keringkan sel *P. putida* dengan penambahan lyoprotectant. Lyoprotectant telah ditambah ke dalam penggantungan sel sebelum beku. Kering *P. putida* diperolehi kemudiannya dicampur dengan merkuri sintetik. Daya maju pemulihan *P. putida* selepas pembekuan kering nyata dipengaruhi oleh jenis lyoprotectant. Antara lyoprotectants, tween 80 / sukrosa didapati lyoprotectant yang terbaik. Sukrosa dapat memulihkan lebih daripada 78% (6.2E+09CFU/ml) sel-sel asal (7.90E+09CFU/ml) selepas pembekuan kering dan dapat mengekalkan 5.40E + 05 sel-sel yang berdaya maju selepas penyimpanan 4 minggu di 4^{o}C tanpa vakum. Sel kering PEG pra-dirawat dan sel pembekuan kering sup pra-dirawat pembekuan selepas beku-kering pulih lebih daripada 64% (5.0E+09CFU/ml) dan > 0.1% (5.60E+07CFU/ml). Beku-kering sel *P. putida* dalam PEG dan broth tidak boleh hidup selepas penyimpanan 4 minggu. Freeze kering juga tidak benar-benar mengubah corak pertumbuhan *P. putida* tetapi lanjutan masa lag didapati 1 jam selepas 3 minggu penyimpanan. Masa tambahan diperlukan untuk sel pembekuan kering *P. putida* pulih sebelum sel beku kering diperkenalkan kepada keadaan yang lebih rumit seperti campuran merkuri. Pengurangan maksimum merkuri PEG pra-dirawat sel-sel beku-kering selepas pembekuan kering dan selepas penyimpanan 3 minggu adalah 56.78% dan 17.91% . Pengurangan maksimum merkuri daripada tween 80 pra-dirawat sel-sel beku-kering / sukrosa selepas pembekuan kering dan selepas penyimpanan 3 minggu ialah 26.35% dan 25.03%. Beku kering *P .putida* didapati mempunyai pengurangan merkuri yang rendah dibandingkan dengan *P. putida* segar yang telah ditumguhkan di dalm agar. Keputusan daripada kajian ini boleh memberi manfaat dan berguna sebagai rujukan awal sebelum mengkomersialkan beku-kering *P. putida*.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
NCTC	National Collection of Type Cultures
CFU	colony forming unit
FBD	fluidized bed drying
PEG	polyethylene glycol
OD	optical density
DI	deionized water
ppb	part per billion

Subscripts

spp	plural form species
Hg	mercury
ODo	initial optical density
h	hours

1. INTRODUCTION

1.1 Motivation and statement of problem

Freeze drying of bacteria has been widely used in pharmaceutical, food industry and other application that related to bio-preservation process. Attention has been given to the method of freeze-drying of certain bacteria due to the beneficial effect on the stability after long storage period, appreciable number of rehydrate cells and the transportable product (Morgan *et al*., 2006).

Dried bacteria is constantly used as the initial cells for culturing the bacteria, utilized for industrial purpose as either product, agent for bioremediation, addictive and culture collection purpose (Keivani *et al*., 2014). The type of bacteria that undergo freeze drying process is often selected based on the utility and interest.

Frequently, valuable bacteria that have substantial advantages and can be applied for industrial application caught tremendous interest by the industrial player due to the promising future of biological application. Bacteria also become one of green solution to overcome many industrial problems and enhance the performance of the process in industry.

Wastewater is often become an issue for petrochemical industry when it regards to high level of heavy metal contain such as mercury (Azoddein, 2013). High concern has been emerged from the effluent of wastewater in petrochemical industry. High mercury content is often detected from wastewater in industry related to the chemical manufacture such as chloralkali plant (Castein *et al*., 1999 and Mortazavi *et al*., 2005).

High mobility of the mercury in environment and its toxic effect has been a major concern. Hence, both reduction and elimination of mercury concentration in effluent is highly appreciable and demanding.

Asia is the largest contributors of mercury emission by account about 40% of the total global emission source in the world (UNEP, 2013) and expected to increase 47% by 2050 (Sloss, 2012). In 2010, coal industry generates 475 tons of mercury primary accumulated from power generation and industrial use and mainly the source is come

from industrial activity related to charcoal combustion, mining, production of cement and incineration (UNEP, 2013). The mercury release in the environment and accumulated in soil, water and air. Human get exposed by mercury mainly from the fish that has been contaminated with mercury in mercury–polluted area.

In Malaysia, several wastewater plants contain mercury in the amount that excess the permission level by Environmental Quality Act 2011 for standard A and B (Azoddein, 2013). Standard A for effluent mercury is at 5 ppb and standard B at 50 ppb.

Currently, chemical and physical treatment has been used in industry to treat the effluent of wastewater contain heavy metal but environmental and economic issue are raised as the effect of the sludge formation and expensive operation.

Practically, physico-chemical methods are not environmental friendly, costly and less effective. Therefore, biological treatment takes the lead as the alternative solution to alleviate the drawback of those methods.

Biological treatment is known to have a low cost on the process than any available methods, also often no environmental threat as there is no secondary pollutant produced. Importantly, it can be performed in situ at the site interest (Vijayaraghavan and Yun, 2008).

Bioremediation using bacteria is considered as one of potential method that can worldly applicable and acceptable in the near future because of its excellent ability to remove heavy metal such as mercury efficiently without causing significant harm to environment (Shafeeq *et al*., 2012).

In recent decade, biological treatment using bacteria is considered as the alternative solution to eliminate the needs of costly process. Particular bacteria that is selected for this purpose must capable to overcome the environmental stress and suitable for treating the mercury.

Pseudomonas putida is a gram-negative bacterium that has been extensively study for its ability to reduce the mercury ion from Hg^{2+} to Hg^{0} (Castein *et al.*, 1999; Mortazavi *et al.*, 2005 and Azoddein, 2013). *P. putida* draws many interests because of its broad application in bioremediation, in biotechnology and in supporting of plant growth.

P. putida can handle environmental stress better and can grow even at low temperature. *P. putida* can grow at 30^{o}C which is claimed as the optimum growth condition of this strain (Fonseca *et al*., 2011).

However, recent study also found that 37^{o}C as the best temperature to maximize the growth of *P. putida* (Azoddein, 2013). The use of *P. putida* is considerably safer than other bacteria such as *Escherichia coli* and *Pseudomonas Aeruginosa*. The reason behind this lies in non-pathogenic property of *P. putida*, hence the use of the bacteria pronounces less negative effect for both living creature and environment.

In Industry, it is often use bacteria culture as a starter culture or bio-agent. Dried bacteria offer convenient way to distributing the bacteria. Unlike other liquid preservation bacteria such as glycerol that must be stored at very low temperature for the cells to keep viable, dried cells reduce the damage of the cell and offer more stable product by maintaining higher viable cells (Wenfeng *et al*., 2013).

The use of *P. putida* in industry requires suitable form in which the bacteria can be more easily to transporting and must easy to handle. Furthermore, when there is a need to store a large number of cells, availability and cost for storage the frozen suspension become problematic.

Distributing the bacteria from one to other place is one obvious challenge. In the laboratory scale, glycerol is often given to the culture as cryoprotectant to avoid cell damage resulted from thawing and freezing.

Bacteria such as *Pseudomonas aeruginosa* and *Escherica coli* were found stay viable for at least 5 month by addition of 15% glycerol in the broth and stored below -70^{o}C (Howard, 1956). This preservation method has a drawback in which the culture must maintain at the temperature below -70^{o}C. Hence, it is not transportable and the risk of thawing is higher which lead to the danger of cell damage.

Desiccation method is one method to alleviate the drawback of temperature dependent of glycerol solution. For more than a decade, desiccation method has been used particularly for long term storage of many cultures. This method has been used to conserve a vast diversity of cultures.

In industry, drying form of culture is seems more preferable compare with the one stored in glycerol solution. This is because the cells culture in drying form is more stable and transportable even at the room temperature (Morgan *et al.*, 2006).

In convenient way, pure *P. putida* can be obtained in dried form. Dried cells allow storage for long period due to the minimal moisture content around the cells inside the container (Poddar *et al*., 2014). Drying lead *P. putida* to enter dormant state and allows the cells to build strong defense characteristic against environmental stress (Garcia *et al*., 2000).

Conventional drying methods often damage the cells during heating and drying process and yield lower viable cells after rehydration (Fu and Chen, 2011). Gram-negative bacteria often show lower survival rates than gram-positive bacteria (Miyamoto *et al*., 2000; Morgan and Vesey, 2009). Hence, gram-negative *P. putida* is one of species bacteria that are very likely to suffer of huge loss in number of rehydrating cells after drying and storage.

Freeze drying take a lead as most effective method compared to other method due to the process of drying that using sublimation process to remove water. Sublimation process reduces the damaging effect of the cell especially for sensitive bacteria by avoid treatment using high temperature and high pressure. Hence, viability and stability of most bacteria is high.

Freeze drying is preservation technique that is used by American Type Culture Collection (ATCC) and the National Collection of Type Cultures (NCTC). Freeze drying also suggested as the most suitable method for drying susceptible bacteria (Schoug, 2009).

The future of freeze drying is promising due to the high demand of freeze-dried biological matter. Forecast of freeze drying product was predicted to be dominated by biopharmaceutical and biotechnology industry. In 2014, global freeze drying market was $ 1,969.7 million and predicted to be increase at $2, 6559 million in 2019 (Marketandmarkets, 2014).

The forecast of freeze drying market up to 2020 is $35.81 billion in 2020 in which the application of freeze drying are mainly centered in Asia-Pacific region and followed by

North America and Europe region (Grandviewresearch, 2014). This may due to the higher market of freeze drying are come from Asia region.

The purpose of this study is to understand the parameters that effecting freeze drying tolerance of *P. putida*. This work also aims to find the maximum survival rate of dried *P. putida* using freeze drying technique and obtain transportable dried *P. putida* for laboratory used. The stability of *P. putida* towards freeze drying is studied for future used. This works also will provides the data for secondary consumers of freeze dried bacteria that want to preserve small number of bacteria in laboratory.

1.2 Objectives

The following are the objectives of this research:

- to obtain dried *P. putida* using freeze drying method
- to investigate the viability rate of freeze-dried *P. putida* using different lyoprotectant
- to study the storage stability of freeze dried *P. putida* in different lyoprotectant
- to compare the performance between freeze-dried *P. putida* and fresh *P. putida* to reduce mercury

1.3 Scope of this research

The following are the scope of this research:

- Determine the growth curve of freeze-dried of *P. putida* using sucrose, tween 80, polyethylene glycol 1000 and analyse using UV-Vis Spectrophotometer at wavelength of 600 nm
- Determine the survival rate of freeze-dried *P. putida* strain after freeze dry using spread-plate method and calculate colony forming unit (CFU)
- Study of storage stability of freeze dried *P. putida* strain by storing dried *P .putida* obtain after storage to 4 weeks at -4^{o}C in chiller and using spread-plate method to calculate colony forming unit (CFU)
- Find the residual moisture content in the freeze-dried *P. putida* in different lyoprotectant using Moisture Analyser
- Find the mercury reduction from freeze-dried *P. putida* and fresh *P. putida* using Mercury Analyser

1.4 Rational and Significant of Research

The following are the contributions from this study:

i. In an attempt to obtain dried *P. putida* using suitable lyoprotectant to freeze dry *P. putida* using freeze dryer in laboratory, whereas for the method could present viable cells after recovery

ii. Focus of stability study for bacteria towards freeze drying often on variation of type bacteria in general and obtain from the isolation in nature, the stability study of freeze drying for specific strain of *P. putida* from American Type Culture Collection is very rare to find

1.5 Organisation of this Thesis

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

Chapter 2 contains the overview of bacteria preservation, freeze drying, *Pseudomonas putida* strain and mercury. The general information of freeze drying such as principle, stability and contamination in freeze drying are briefly presented. This chapter also presents the general information of *Pseudomonas* strain. Characteristic and ability of *Pseudomonas putida* was discussed.

Chapter 3 presents the experimental ring set up for this study. Equipment, apparatus and chemicals required were listed. The detail of method to conduct the experiment based on the previous study from literature was present as well.

Chapter 4 describes the result obtained from experiment. The findings from the experiment were then compared to earlier study from the literature and journal. The findings, deviation between the result and literature were explained in details with supported data.

Chapter 5 contains of summary from the present study and recommendation for future work.

2 LITERATURE REVIEW

2.1 Overview

This chapter discussed about general introduction of preservation of bacteria, freeze drying and *Pseudomonas putida*. Discussion from literature was also presented including the characteristic and ability of *Pseudomonas putida.*

2.2 Introduction

Biotechnology is term that explains the practice of biological science that utilizes biological matter and their product. The rapid growing of biotechnology practice leads to the requirement for preservation of bacteria. The need to preserve valuable bacteria is increase as the merit to utilize of bacteria raised over the time (Alex and Tan, 2003).

2.3 Preservation of Bacteria

Many of preservation of bacteria have been intensively studied. Basic principles for preserving bacteria can be divided as prevention of slow-drying and rapid desiccation (Morton and Pulaski, 1937). The important factors that need to be considered when choosing the drying method are the viability of the cells and the necessary to maintain its structure and its functional properties (Keivani *et al*., 2014).

One method that pioneers the preservation bacteria was by using the tube sealed the with glass stoppers or paraffin that has been filled with bacteria and stored at pH 7.2 room temperature in the dark (Morton and Pulaski, 1937). This method was found lack of cells survive.

The advance method of preservation bacteria is known as cryopreservation. Cryopreservation is named for any preservation method that utilized temperature below freezing point to slowing the chemical and biological reaction. Often cryoprotectant agent is added prior freezing. The common solute agents used for laboratory used are glycerol, sucrose and serum (Hubalek, 2003).

The challenging part of preservation using this method are lowering the temperature, maintaining the low temperature until the time the matter use and increase the

temperature to the room temperature. The method becomes problematic in term of maintenance of low temperature. The backup refrigeration system is costly and requires rigid operational maintenance. Refrigerant system such as liquid nitrogen can maintain the temperature to -196^{o}C but the personal safety is such a huge concern.

Another method which is rapid desiccant can be defined as drying method. The advantage of this method is the stability of the culture can be maintained and easiness in transporting the culture. This method has been used widely in food and pharmaceutical industry. Because of its broad application, study on its optimum condition is highly appreciable.

Stationary phase is known as optimum phase for drying method because this phase trigger the stress state of the cells due to limited carbon environment and favour the survival defences for the cells (Morgan *et al*., 2006). Drying method that commonly used in industry are spray drying, fluidized bed drying, and freeze drying.

Spray drying is a method to obtained powdery cells by using high temperature inlet air. The air used for this process can be varied but highest air temperature is needed to lowering the moisture content. Corcoran *et al*. (2004) used temperature at 170^{o}C with short residence time to dry *Lactobacillus rhamnosus GG*. The result showed that at store at 4^{o}C and 15^{o}C resulted in poor viability for 8 weeks storage. This result proved that spray drying method has low stability for storage.

Spray drying has gain success in preservation bacteria of *Lactobacillus plantarum com, L actobacillus paracasei A13* and *Lactobacillus acidophilus A9* without causing loss in number of cells after drying (Paéz *et al*., 2012). *Escherichia coli K12* and *Lactobacillus acidophilus* were reported to survive after spray dying but had lower viability than using freeze drying (Pispan, Hewitt and Stapley, 2013).

This method is considered 10 times more economical than freeze drying method but there was a risk that the powdery dried cells adhered on the chamber wall and resulted in lower cells obtained (Schuck *et al*., 2013). The use of spray drying may suitable for bacteria that have higher tolerance to temperature and drying process.

Strain such as *P. putida* that is susceptible to dryness (Antheunisse, Bruin-Tol, and Pol-Van Soest, 1981) considered unsuitable to undergo spray dying due to the high

temperature inside the chamber. Furthermore, there has not been reported successful spray drying method for *P. putida*.

Another drying method that has been recognized as alternative method to overcome log time needed for drying, higher operational temperature and lower storage stability of spray drying is fluidized bed drying method (FBD) (Morgan *et al*., 2006).

FBD requires hot air that moving up flow passing the sample. The main purpose of this method is to fluidizing the sample so then advance drying can occur and less time needed for drying. According to Keivani *et al*. (2014), FBD method resulted significant damage on the cells and lower the viability of the cells.

Lactobacillus casei CRL 431 that was drying using FBD and exposed to osmotic stress was found very stable for long period at ambient temperature. *L. casei CRL 431* was found to have high viability after 1 year at 25^{o}C of storage condition (Nag and Shantanu, 2013). The result showed that fluidized bed drying can provide better rehydrate cells compared to spray drying.

Despite of all satisfy result from many of previous experiment, application in industry of this method have to wait until the feasibility of this method is truly clarified (Morgan *et al*., 2006) and successful drying bacteria seems highly dependent with the type of the strain.

One common method of rapid desiccant is freeze drying. Freeze drying bacteria is a method for preserving susceptible bacteria (Schoug, 2009). It also named as lyophilisation in which the cells are cooled, freeze and dried in sequence to remove the water by means of sublimation process so it can stored for long period (Avis and Wagner, 1999).

Bacteria need to undergo cooling and freezing procedure before drying to prevent the cell damage unless the bacteria are susceptible with cold temperature or have a molecule complexity (Adams, 2007).

The main advantage of lyophilisation lies in the moisture content. Moisture content is low enough that the matter is safe to store at room temperature after proper sealing (Wenfeng *et al*., 2013). The simplicity of storing and the ability of the technique to maintain the stability in long period lead freeze drying as the ideal method to preserve

most of bacteria. Miyamoto e*t al.* (2000) succeed to produce freeze-dried *Pseudomonas* strain that able to survive 10 years.

2.4 Freeze Drying

Freeze drying is one of desiccant technique that has been used for preserving a diverse culture of bacteria and always related to long time storage of bacteria culture. Freeze drying has been used commercially in food, pharmaceuticals and biotechnology industries (Tsinontides *et al*., 2004). Freeze drying is suitable drying method to preserve susceptible bacteria including lactic acid bacteria such as *Lactobacillus delbrueckii subsp. bulgaricus* CFL1 (Passot *et al*., 2011)

Freeze drying has been used for preservation of bacteria for long period due to its convenient storing, transporting and easy rehydration process. This method is broadly accepted and has been used for culture bank collection including American Type Culture Collection (ATCC) and National Type Culture Collection NCTC (Morgan and Vesey, 2009).

Study on this method mostly focused on providing tolerable amount of rehydrate cells after freeze drying and storage (Palmfedt, Radstrom and Hahn-Hagerdal, 2003; Wenfeng *et al*., 2013). Freeze drying produce stable product at ambient temperature, shippable and easy to handle. It has been reported that freeze dried bacteria can survive more than 20 years (Miyamoto *et al*., 2005).

Freeze dried material allows easy, inexpensive shipping and handling due to higher stability of freeze dried product that can be stored in moderate temperature , hence eliminate the necessary to provide freezing temperature for storing and distribution.

2.4.1 Principle of Freeze Drying

Freeze drying is a method to preserve the biological matter by removing the water. The water is removed by sublimation process from freezing state. The basic procedures of freeze drying consist of three steps, which are freezing, primary drying and secondary drying.

Freezing can use either in drying chamber, cooled rack or freezing before drying such as drop in liquid nitrogen (-196^{o}C) prior entering drying. The difficult part of freezing

outside from the drying chamber is the risk of thawing that can damage the cells (Zhao *et al*., 2003).

The freezing temperature before drying influences greatly the successful rate of drying product. In the case of drying bacteria, the freezing temperature will affect the viability of the bacteria after drying (Zhao and Zhang, 2005).

The solution contains cells and solute is freezing below the temperature of surrounding water and lead to the change of concentration of the solute. As the time proceeds for cooling and water turn into ice, the water is separated from the solute. The solute becomes more concentrated (Morgan *et al*., 2006).

The process also can turn another way in which the suspension become more viscous and freeze at glass transition. It is important to ensure that the water, solvent and solute are perfectly frozen before drying. The existence of unfrozen part in the suspension can influence the stability of the final product (Morgan *et al*., 2006).

Drying process can spend to almost 20 hours at pressure below 0.1 bar (Miyamoto *et al.*, 2006). The time for drying is depend on the type of bacteria and often is based on the desired percentage of moisture content in the final product.

Drying up to 48 hours has been used for drying prebiotic bacteria at the pressure below 0.08 mbar (Savini *et al.*, 2010). The process include two main processes consist of 6 hours primary drying and the rest is for secondary drying (Palmfedt, Radstrom and Hahn-Hagerdal, 2003).

Primary drying is process where frozen of unbound water is sublimated into water vapour at reduced pressure below ice vapour pressure. It is common to use vacuum pressure below 0.1 Pa (Miyamoto *et al.*, 2000).

Secondary draying is used to remove bound water. Bound water exists on the matrix of the product (Morgan *et al*., 2006). Removal of this water takes longer time than removing frozen water.

The rule of thumb of freeze dried product is to keep the product at 50^{o}C below the Tg of the product in order to retain the viability of the bacteria and the quality of the product (Schoug, 2009). Often the freeze dried product is stored at temperature below 5^{o}C.

Commonly, bacteria obtain from American Type Culture Collection (ATCC) is stored at temperature range between 2^{o}C to 8^{o}C. The most suitable condition for storage is sealing under vacuum or insert inert gas often nitrogen to prevent moisture and oxygen to have a contact with dried sample (Miyamoto *et a*l., 2006).

Temperature and pressure plays important role in the drying process. The water sublimes from high pressure area to lower pressure area. The suspension must be warmer compared to cold trap condenser temperature. Often the suspension is frozen below glass transition temperature and continues to rise below critical temperature as the pressure is reduced (Passot *et al*., 2013).

The sublimation of frozen water to water vapour occurs due to force from vapour pressure of the sample. The movement of the water is due to the difference of vapour pressure between the suspension and the condenser (Passot *et al*., 2013). Hence, it is necessary to have lower condenser temperature than temperature of the suspension.

Freezing and drying process damage the cell and reduce sharply the number of viable cell in the final product. This is due to the physical changes on the membrane lipids and denaturation of protein or damage of protein that very sensitive in the cell (Barner and Viernstein, 2006).

This lyoprotectant is often added into the solution prior freeze drying process to protect the cells from freezing and drying (Palmfedt *et al*., 2003). The viability of the cells is improved by addition of protective medium as matrix in the solution (Barner and Viernstein, 2006).

2.4.2 Stability of Freeze Dried Product

In order to have strong stability of the dried product, several factors need to be considered. By nature, both freeze and drying are harmful for the cell and can damage the cell (Zayed and Roos, 2004).

It always desired to have very low moisture and oxygen in the final product. It has been studied that higher water activity in the sample after freeze drying tend to give lower storage stability of the final product (Barner and Viernstein, 2006).

The stability of the bacteria is higher after freeze drying due to lower water activity. Water activity is reduced by remove the frozen water and able to reduce the structural damage of the bacteria (Jagannath *et al*., 2010).

According to earlier study, the stability and viability of *Lactobacillus paracasei* was high with low amount of moisture content in the final drying powder (Poddar *et al*., 2014). The results suggest that the viability and stability of prebiotic bacteria was affected by water contained as it affects the mobility of the cells in the matrix.

The amount of moisture left in the final product is dependent with the extent of secondary drying and nature of the sample. It is suggested water content in range of 2.8% to 5.6 % can improved the survival rate for long term storage (Zayed and Roos, 2004).

The stability of final product is also dependent with storage condition. Higher storage temperature reduces the stability by decrease the viability of the bacteria. It is often to store the final product at the temperature of 4^{o}C. Store at temperature at 8^{o}C cause the viability to decrease about 75% after 50 days even with the addition of lyoprotectant (Palmfedt *et al.*, 2003). Perform storage of freeze died bacteria at 4^{o}C showed better viability than store at 16^{o}C under both aerobic and anaerobic storage condition (Song *et al*., 2014).

The stability of the product also depends on the type of freeze dried sample. In case of bacteria, gram- positive bacteria have better stability than gram negative bacteria. The viability of gram- positive bacteria after drying is higher than gram-negative bacteria.

According to Miyamoto *et al*. (2006), stability of *Pseudomonas putida* after freeze drying is about 50% and decreasing over 5 years before stabilized in the next 20 years. After 20 years of storage under vacuum condition, the viability of *P. putida* reduce to 33.5%. Gram- positive bacteria such as *Lacobaciullus acidophilus* have viability of 62.5% after freeze drying with survival rate about 96 % per year for storage test stability.

Lyoprotectant also provide significant contribution on stability of bacteria by providing protection towards freezing and drying. Freeze drying without protectant cause detrimental effect on the cell. Freeze drying of *Lactobacilus salivarius* without lyoprotectant and only used distilled water as solvent cause 99% of the bacteria to loss viability (Zayed and Roos, 2004).

However, it is not all of the lyoprotectant to be successfully provide good stability of the freeze dried product. Common freeze drying medium used that mostly give a success in providing good stability is skimmed milk and disaccharides (Hamoudi, Goulet and Ratti, 2007).

In freeze drying *Pseudomonas chlororaphis* using variant medium, sucrose is the most suitable solute that can provide optimum viable number of cell (Palmfedt *et al*., 2003). A mixture of trehalose, sucrose in addition and skim milk was the found to be the most effective protective medium used for freeze drying as it gives more than 83% of the bacteria can survive after freeze drying as well as improve the stability for storage (Zayed and Roos, 2004).

2.4.3 Contamination in Freeze Drying System

Freeze drying system has a drawback that the surface of the equipment may contaminated by the freeze dried bacteria. The area that very likely to be contaminated in the equipment is on the condenser and vial. The dried bacteria may flow through out of the system into the condenser trough vapour steam. The prevention that can be taken to reduce the change of contamination is by installed protective filter such as bacteriological filter (Labconco, 2010).

Contamination by the bacteria risk the operator depends on the type of the bacteria. The risk of damaging the equipment comes from the corrosive sample. Commonly, freeze drying system is non-corrosive design. But care must always be taken to avoid substantial damage on the equipment (Labconco, 2010).

2.5 Bacteria Overview

Pseudomonas spp. is a bacterium that was found in the sediments at mercury-polluted area of Minamata Bay. It had been identified that about 6.7 % of the overall bacteria in Minamata Bay was *Pseudomonas spp.* (Nakamura *et al.*, 1990).

Pseudomonas strain is known as mercury-resistant bacteria and exists in soil, water, animals and plant (Kavanaugh and Ribbeck, 2012). *Pseudomonas spp*. also has been proved to enhance the growth of plant root hair and encourage the plant growth (Zamioudis *et al*., 2013).

Pseudomonas able to retain higher viability at most temperature, both aerobic to less oxygen soil, water and at modest nutrient contain in the medium (Timmis, 2002). *Pseudomonas* strain also often found to has high resistance to disinfectants, detergents, antibiotics, heavy metal and organic solvents (Palleroni *et al*., 2006).

Most of bacteria with genus *Pseudomonas* is aerobic, gram-negative bacteria with rod-shape the cell and has one or more polar flagella (Clarke, 1982). The type of *Pseudomonas spp.* is very diverse. *Pseudomonas aeruginosa, Pseudomonas fluorescents* and *Pseudomonas putida* are a few of *Pseudomonas* genus that often studied for its ability.

In general, the strain is commercially used as biosurfactant, plant growth promotion, biopolymers, recombined protein and biocatalyst. Furthermore, *Pseudomonas spp*. is potentially used as bioremediation, biotransformation and chiral compound (Flickinger, 2010). *Pseudomonas* is bacteria that resistance to most of antibiotics, disinfectant, detergent and heavy metal (Ramos *et al*., 2002).

2.5.1 Characteristic of Pseudomonas putida

Pseudomonas putida is a gram-negative, non-pathogenic bacterium that able to use more than 100 different carbon sources (Timmis, 2002). *Pseudomonas putida* has a rod shape, flagella, fluorescent gram-negative bacterium that can be found in environment (Clarke, 1982).

P. putida cells are often found to have five and seven flagella that emerge from the same one base end of the cell with length varies from two to three wavelengths (Harwood, Fosnaugh and Dispensa, 1989).

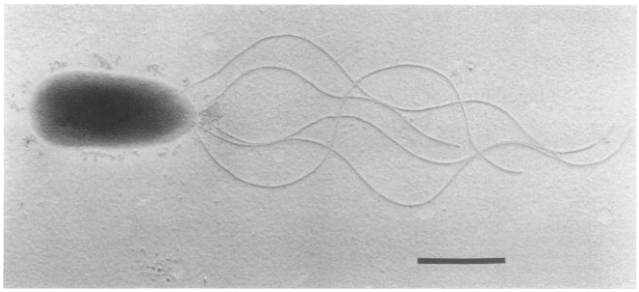


Figure 2-1: *Pseudomonas putida* cell

(**Source:** Harwood, Fosnaugh and Dispensa, 1989)

Taxonomic classification of *P. putida* is identified as below (BacDive, 1895).

Domain : Bacteria

Phylum : Proteobacteria

Class : Gammaproteobacteria

Order : Pseudomonadales

Family : Pseudomonadaceae

Genus : *Pseudomonas*

Species : *Pseudomonas putida*

P. putida is ubiquitous bacteria and often isolated from polluted soil such as from wastewater chloralkali plant (Castein *et al.*, 1999 and Mortazavi *et al.*, 2005), rhizosphere (Sarkar, Seenivasan and Asir, 2010; Ahemad and Khan, 2012) and water. Optimum condition to grow the *P. putida* bacteria in laboratory scale was at 37^{o}C, pH 7 and 180 rpm of vigorous shaking with acclimation 1 day (Azoddein, 2013).

The growth of *P. putida* has 4 distinguish phases mainly identified as lag phase, log phase, stationary phases and death phase. In each phase, there are different mechanism responses inside the bacteria cells.

Stationary phase is the state in which the bacteria are no longer multiple due to the nutrient depleted and limited sources of carbon (Lee, 2003). This state also thought as the state that the cell starts to produce various metabolites (higher saturation fatty acid

and increase membrane fluidity) that enhance the cell protection towards various stress like drying and freezing (Morgan *et al.*, 2006).

Azoddein (2013) constructed the graph that showed the concentration of bacteria over the time at optimum growth condition. Stationary phase is represent by slighly straight line in the growth curve. Six hours after lag phase is required for the bacteria *P. putida* to reach stationary phase (Azoddein, 2013).

2.5.2 Ability of Pseudomonas putida

Pseudomonas strain has broad application. There were many studies had been conducted on *P. putida* strain. *P. putida* BIRD-1 is suggested as rhizobacterium that effectively support and improve the plant growth (Roca *et al*., 2013). *P. putida* PCI2 also can enhance the growth of tomato plants by solubilized phosphate in the medium (Pastor *et al.*, 2014). *P. putida* strain has high potential used in agronomic practice.

P. putida SP1 also had been studied for its ability to shift both organic and inorganic mercury to metallic mercury. *P. putida* SP1 was proved resistant to mercury and other metal such as $CdCl_2$, $CoCl_2$, $CrCl_3$, $CuCl_2$, $PbCl_2$, $ZnSO_4$ and total of almost 100% mercury was able to volatile by the strain (Zhang *et al*., 2012).

P. putida strain also high resistance towards mercury. Another study showed that *P. putida* ATTC 49128 was able to remove more than 90% mercury. The studies imply that the strain has high functionality to be used as bioremediation.

In another study *P. putida* V1 was used to remove 89% methylmercury with the addition of 10% NaCl and lead (Cabral *et al*, 2014). *P. putida* V1 had been used to remove methylmercury in a wide range of pH (4.0 -8.0) and temperature (10 ^{o}C –35^{o}C) (Cabral *et al*, 2013). It implies that *P. putida* able to overcome the environmental stress caused by changes in temperature and pH.

The ability of *Pseudomonas* strain in remove mercury is related to enzyme of mercury reductase. This enzyme catalyses the reduction process from toxic mercury ion (Hg^{2+}) to elemental mercury (Hg^{0}). The enzyme of *mer T*, *merP* and *merA* are involved in mercury reduction for gram-negative bacteria (Wong and Chu, 2003).

P. putida can use toluene solvent as source of carbon, mineralize toluene, able to growth in toluene, styrene and p-xylene (Ramos *et al*., 2002) that mostly toxic for most of organism (Krell *et al*., 2012).

In another study, *P. putida* cell was reported to embed into solid matrix and forming a swarm to construct biofilms (Ramos and Filloux, 2007). *P. putida* had been reported to infect the fish by growth over the kidney, spleen, skin and liver of the fish and cause 45% mortality of rainbow trout fish (Altinok *et al*., 2006). But evidence of baneful effect of *P. putida* in nature has not been recorded.

2.6 Summary

Freeze dry biological matter such as bacteria allows easy, inexpensive shipping, handling and high stability of the product. Many bacteria have been successfully preserved in dried form using freeze dry technique. *Pseudomonas putida* is mercury-resistant bacteria that have a potential to remove toxic mercury. *P. putida* was suggested can be preserve in dried form using freeze drying technique.

3 MATERIALS AND METHODS

3.1 Overview

The study focused on the freeze drying method in which *P. putida* strain is freeze and dry subsequently. Freezing process used freezer at -80^{o}C and drying used freeze dryer in the laboratory of University Malaysia Pahang including all the apparatus, equipment and chemicals required in the study.

3.2 Introduction

This study focused on the methods of freeze drying in which *P. putida* is tested for its stability at variance of freeze drying condition. This section presents preparation method to growth *P. putida*, freeze drying method of *P. putida*, CFU determination, and mercury preparation.

3.3 Bacteria

Bacteria *P. putida* was obtained from BIOREV SDN BHD and has been growth by previous student in agar medium. The type of bacteria in this study was *P. putida* ATTC49128. Before undergo the experiment, it is necessary to ensure that the bacteria are pure *P. putida* and do not other bacteria or contaminated with other bacteria. The identification process was done using visual determination using Microscope (ZEISS) in University Malaysia Pahang.

3.4 Chemicals

Nutrient Agar (BD 213020) and Nutrient Broth (BD234000) were purchased from Merck (Germany). Mercury (II) chloride was obtained from Merck. Sucrose, Tween 80, Polyethylene glycol (PEG) 1000 and other chemical in list below were obtained from Faculty of Chemical Engineering (FKKSA) laboratory.

List of Chemical:

i. Distilled water
ii. Ethanol 70%
iv. Nutrient Agar (BD 213020)
v. Nutrient Broth (BD234000)

vi. Mercury (II) Chloride

vii. Sucrose

x. Tween 80

xi. Polyethylene glycol (PEG) 1000

3.5 Equipment

The equipment used in this study was provided by Faculty of Chemical Engineering, UMP. Mercury Analyser was provided by Central Lab in UMP.

i. Freeze Dryer (BIOTRON/CLEANVAC)

ii. Freezer (THERMO SCIENTIFIC)

iii. Autoclaved (HIRAYAMA)

iv. Colony counters (FUNKE GERBER)

v. Centrifuged (EPPENDORF 5810 R)

vi. Oven (HAERAEUS)

vii. Microbiological Incubator (MEMMERT)

viii. Mercury Analyser (MILESTONE DMA-80)

ix.. UV-Vis. Spectrophotometer (U-1800)

x. Laminar flow cabinet (ISOCIDE)

xi. Microscope (ZEISS)

xii. Incubator shaker (GRAUN)

3.6 Methods of Medium Preparation

The aseptic technique was used during handling the experiment to reduce the risk of contamination of unwanted microbes. The experiment was carried out in laminar flow for optimal controllable work. The surface work and gloved was disinfectant with 70% ethanol and wiping clean (Cote, 1998).

3.6.1 Preparation of Growth Medium

The study media for growing the bacteria consist of nutrient broth and nutrient agar. Nutrient broth (RDU 1103102) and nutrient agar (RDU1103102) were obtained from MERCK KGaA. The medium contains 10% peptone, 5 %meat extract, and 15 % agar or broth ingredient. The broth media was prepared according the instruction given in the bottle.

3.6.2 Preparation of Solid Medium

Nutrient agar was prepared by suspend 20 gram of pellet in one litre of demineralized water as recommended by MERCK KGaA (2013). Heat was given to boil the water and continue with sterilization process by autoclave at 121^{o}C for 15 minute. After autoclaved process finished, agar solution was poured into plates. The agar can be used as medium after it become solid. The edge of the plate was bind with parafilm after it hardened for later use. The solid agar was stored in chillier at 4^{o}C until the time to use.

3.6.3 Preparation of Liquid Medium

Nutrient broth was prepared according the instruction on the medium bottle by MERCK KGaA (2013). Eight grams of pellets of nutrient broth was suspended in 1 litre of demineralized water. Schott bottle was used to dispense the solution. The solution was autoclaved for 15 min at 121^{o}C to sterilize the solution. The lid of the bottle was loosened enough and was wrapped with aluminium foil before autoclaved. Autoclave tape was adhered in the bottle as indicator. After autoclave, black strips appear as indicator that the bottle has been autoclaved. Solution that has been autoclaved was leave for 15 minute or until it cooled down and store in 4^{o}C until time to use.

3.7 Growth P. putida for Analysis the Growth Curve

10 ml of nutrient broth formulated as describe above was drawn and added in the test tube. A full loop of *P. putida* bacteria was transferred from agar plate to nutrient broth using inoculating loop. The ratio suggested for the culture to medium broth was 1: 9 (Azoddein, 2013). Inoculating loop was flamed and allowed to cool before touch the bacteria. The cover of agar plate was removed partly and used as shield to protect the sample from airborne contamination while the bacteria were taken with inoculating loop.

The loop was then withdrawn and the plate cover was closed. The tube neck containing nutrient broth was flamed. The tube was keep tilted while the loop containing bacteria was submerged in the broth. The tube neck was flamed again before replace the cap. Inoculating loop was flamed after used to sterilize it from bacteria. Inoculation loop and tube neck were always flamed before and after transfer the bacteria in order to sterilize

the equipment. This way can reduce the contamination of the bacteria to working place and researcher (Chess, 2009).

The culture in broth medium was incubated for 1 day. The colony was transferred into 90 ml nutrient broth. The culture was shaken at 180 rpm and 37^{o}C. This condition has been studied as the most optimum condition for *P. putida* to growth (Azoddein, 2013). The sample was drawn every 1 hour and analysed its concentration using UV-Vis Spectrophotometer at the wavelength of 600 nm to determine the growth curve (Kim *et al.*, 2005).

3.8 Determination of Cell Dry Weight

The method to obtain cell dry weight was based on Azoddein (2013). The sample with the same amount withdrawn as above was incubated at 100^{o}C for 20 minute. The pellet was then collected by centrifuge the sample at 5000 rpm for 3 minute. Supernatant was decanted to obtain the cell pellet. The cell was then filtered onto 0.2 μm cellulose nitrate filter and subsequently washed twice with DI water. The sample was then dried in oven at 95^{o}C for 24 h.

The dried cell was then measured. The blank was measured using fresh medium and use the same method as the cell sample. One ml of dried cell sample was centrifuge at 5000 rpm for 5 minute in micro centrifuge. The supernatant was decanted and the cell pellet washed twice using saline solution. The sample was then dried in vacuum drier and dried in oven at 80^{o}C until its weight constant. The data was record in term of weight and OD.

3.9 Method of Freeze Drying

There are several methods to determine the survival rate of *P. putida* after freeze drying and determine the stability *P. putida* during storage. Survival rate after freeze drying was determined based on different types of lyoprotectant for the freeze dried the bacteria. The stability was determined by using storage test. Different interval storage time was used to test the viability of *P. putida* cells after storage. The method used in this study was based on the previous study on freeze drying of *Pseudomonas putida* by Weng *et al*. (2013).

The stability of *P. putida* towards freeze drying was investigated by using different lyoprotectant. Lyoprotctants that were tested consist of glutamate polyethylene glycol (PEG) 1000 and sucrose/tween 80. The lyoprotectants were suspended in sterile DI water. The protectants solution was then exposed in UV light for 15 minutes before mix it with the bacteria.

3.9.1 Freeze-dried cells preparation by using PEG

100 millilitres of 2.5 g/l PEG 1000 solution was prepared using sterile DI water ad boiled for 5 min. The PEG solution was cooled down to room temperature prior to mixing it with 50 ml of cells (A600 nm, 2.5). After mixed, the solution was left to stand to ensure that the lyoprotectant media had sufficient time (Approximately 10 min) to toughly permeate the cells of the suspension (Wenfeng *et al*., 2013). After standing, 2 ml of the mixture was added into each vial (15 ml). The mouth of the vials was filled with sterile cotton to avoid the dry cells to escape from the vial. The cells were freeze inside the freeze dryer (BIOTRON/CLEANVAC) to let the slow freezing occur. The frozen cells were then freeze-dried for 24 h in freeze dryer. All the dry samples were stored in 4^{o}C.

3.9.2 Freeze-dried cells preparation using tween 80/sucrose

A 500 ml of 50% sucrose stock solution was prepared using DI water and was sterilised by passing the solution through a 0.22-µm filter. The sucrose stock solution was exposed to UV light for 5 min. Twenty five millilitres of 50% sucrose and 0.2 ml of tween 80 were mixed with 80 ml of cells (A600 nm, 2.5). This solution was left to stand to ensure that the lyoprotectant media had sufficient time (Approximately 10 min) to toughly permeate the cells of the suspension (Wenfeng *et al.,* 2013). After standing, 2

ml of the mixture was added into each vials vial (15 ml). The mouth of the vials was filled with sterile cotton to avoid the dry cells to escape from the vial. The cells were freeze inside the freeze dryer (BIOTRON/CLEANVAC) to let the slow freezing occur. The frozen cells were then freeze-dried for 24 h in freeze dryer. All the dry samples were stored in 4^{o}C.

3.9.3 Recovery of Freeze-dried Cells

Freeze-dried cells were rehydrated by shaking the freeze-dried *P. putida* in 20 ml broth at 180 rpm in 37^{o}C incubator for 24 h. This solution was mixed with 180 ml of fresh broth and shaking at 180 rpm in 37^{o}C incubator for 24 h.

3.10 Spread-Plate Method

Spread-plate method was used to obtain a pure culture of *P. putida* and determined the colony forming unit (CFU). A single colony was then immobilized a top of a solid agar growth media. A sterile glass rod with L-shape was used to spread the cells over the surface of the agar plate. Relatively few cells in the culture will be physically separated from one another and will grow into isolated colonies. The number of colonies was then counted to determine the CFU number.

3.11 Viable Plate Count

The viable plate count was used to estimate the number of cells in a liquid sample. Serial dilutions were made to produce several samples with decreasing cell densities, in which each dilution was represented by dilution factor. A small amount of these samples were then plated onto the agar media. The samples were then incubated for 24 h at 30^{o}C. The number of colonies arising after incubation was used to determine the approximate number of cells in the original sample. This method was used to calculate the CFU because only viable living cells were counted. CFU allows sidestepping the problem of exactly how many cells were involved in the formation of a single colony by grouping together pairs, tetrads, clusters and chains under a common term.

3.12 Determination of Residual Moisture Content

The residual moisture was estimated using moisture analyser provided by FKKSA, UMP. The freeze dried cultures were weight and dried above 100^{o}C until no further change in weight was observed. The result was obtained was in percentage.

3.13 Method of Mercury Preparation

Sterilized DI water is used to dilute mercury stock solution to concentration of 1 ppm at room temperature. Twenty ml of freeze-dried *P. putida* that has been growth in broth for 24 hours was mixed with 180 ml of synthetic mercury. This solution was then shaking at 180 rpm in 37^{o}C as suggested by Azoddein (2013). After 24 hours, 30 ml of this solution was drawn and used for analyse in Mercury Analyser (MILESTONE DMA-80).

3.14 Determination of Cell Viability using Viable Plate Count

Each test with new batch of trial sample *P. putida* was repeated in triplicate. The viability of *P. putida* was determined as CFU (Colony Forming Unit). The number of colony forming unit (CFU) was calculated according the following equation (Chess, 2009) :

$$CFU = \frac{Colony\ on\ the\ plate}{Volume\ of\ sample\ plated\ x\ dilution\ factor}$$

Formula to determine the stability after freeze drying is stated as below (Wenfeng *et al*., 2013; Palmfedt, Radstrom and Hahn-Hagerdal, 2003).

$$Stability\ after\ freeze\ drying = \frac{\frac{CFU}{ml} after\ freeze\ drying}{\frac{CFU}{ml} before\ freezing}$$

2.15 Determination of Mercury Reduction

Mercury analysis was performed using Mercury analyser. The efficiency of mercury removal was calculated according the following equation (Azoddein, 2013):

$$\%\ Mercury\ Removal = \frac{(Inital\ -\ Final\ )concentration\ of\ mercury}{Initial\ concentration\ of\ mercury}\ x100\%$$

Where, the initial concentration of mercury is 1000 ppb. The amount of moisture content was determined using Moisture Analyser.

3.16 *Experimental Ring*

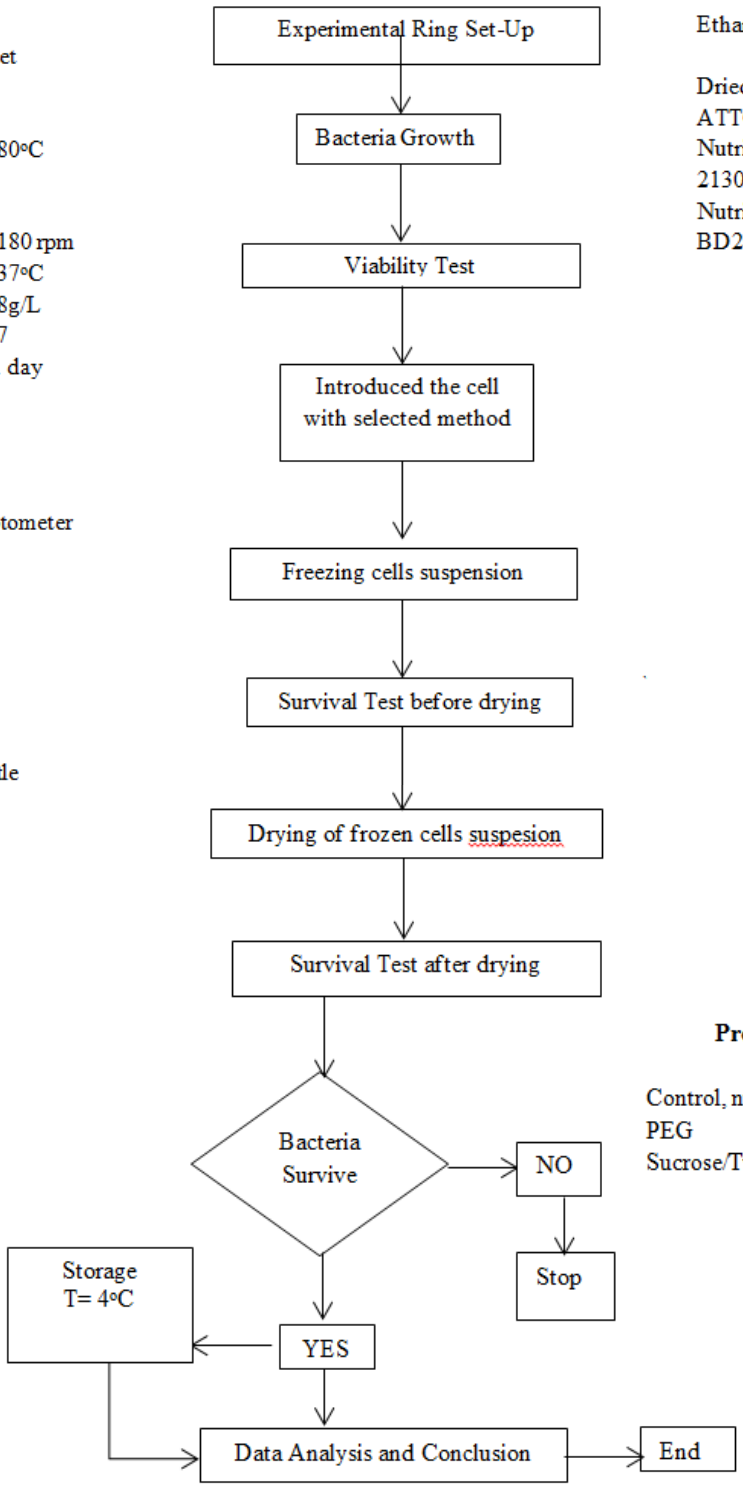
Equipment

-Autoclave
-Hotplate
-Laminar Flow Cabinet
-Incubator
-Chiller
T= 8°C, 4°C, -40°C, -80°C
-Analytical Balance
-Rotary shaker
Speed =180 rpm
T =37°C
C of nutrient =8g/L
pH =7
Acclimation time=1 day
(Azoddein, 2013)
-Freeze dryer
-Oven
-Centrifuge
- UV-Vis Spectrophotometer
-Autoclave
- Incubator Shaker

Apparatus

- 1000 ml Beaker
- 1000 ml Schott Bottle
-250 ml conical flask
- Agar Plate
-Universal Test Tube
-Vials
-Pipet
-Aluminum foil
-Spatula
-Weighing boat
-Parafilm
-Centrifuge tube
-Cuvette
-Inoculating loop
-Bunsen burner

Process Flow



Material

Ethanol 70%

Dried Cells
ATTC49128
Nutrient Agar BD 213020
Nutrient Broth BD234000

Protective Medium

Control, nutrient broth
PEG
Sucrose/Tween 80

Figure 3-1: Process flow diagram of freeze drying process of *Pseudomonas putida*

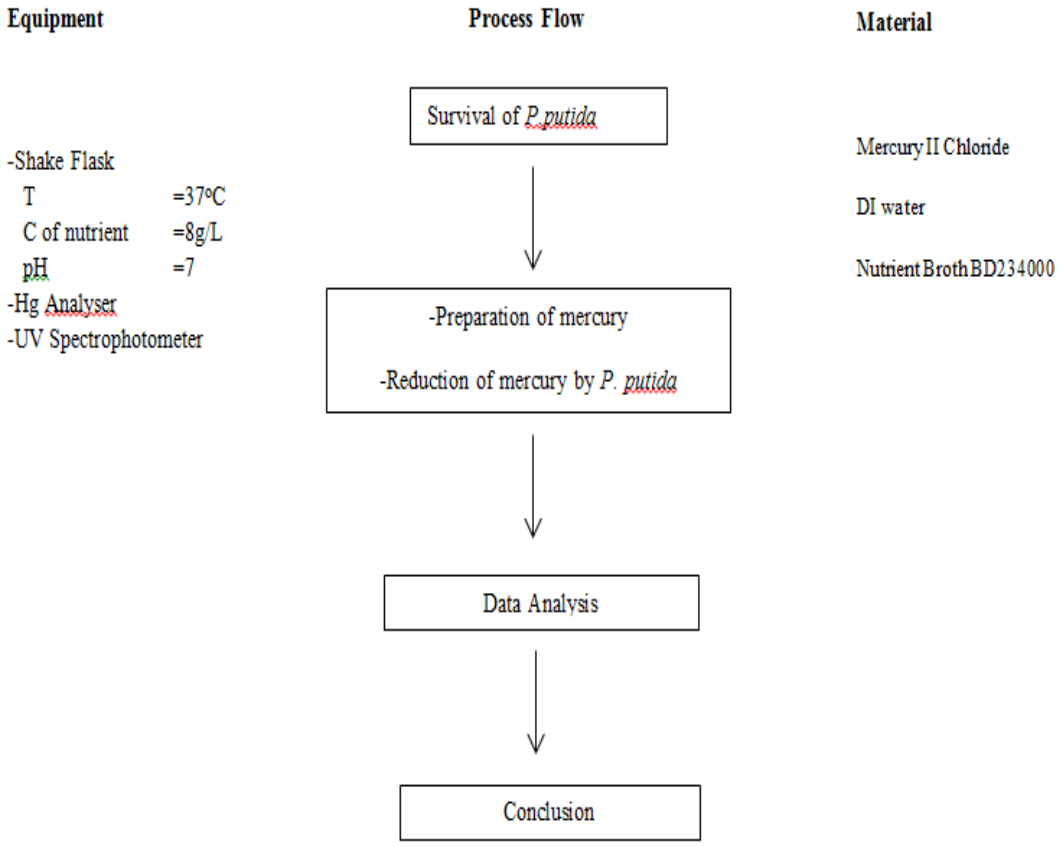


Figure 3-2: Process flow of mercury treatment using *Pseudomonas putida*

3.17 Summary

All the equipment except Mercury Analyser was provided by Faculty of Chemical Engineering (FKKSA). Analyses of mercury were done in Central Lab, UMP. The method to freeze dry and mercury reduction were based on literature. It was estimated that a single drying cycle requires one about one week to finish.

4 FREEZE DRYING OF *PSEUDOMONAS PUTIDA* AND MERCURY REMOVAL

4.1 Overview

This paper presents a numerical study of viable cells in term of colonies forming unit (CFU) before and after freeze drying using standard spread-plate method by Chess (2009). Performance of freeze-dried *P. putida* cells in removing mercury and moisture content after freeze-dried were also determined. The effect of freeze dry in *P. putida* growth was modelled as growth curve. The result in CFU, moisture content and percentage of mercury reduction were analyses and compared to literature.

4.2 Introduction

The effects of freeze drying and storage condition on freeze-dried *P. putida* were evaluated using spread-plate method during 0, 1, 2 and 3 weeks of storage. Three of rehydrated freeze-dried *P. putida* for each lyoprotectant were streaked into agar plate and incubated for 24 h. Each experiment was done in triplicate.

4.3 Effect of lyoprotectant on the storage stability of freeze-dried P. putida

In this study, PEG and tween 80/sucrose were tested with respect to their ability to protect bacterial cells against freezing and drying. The shelf life of the both lyoprotectants was investigated at refrigerated storage of 4^{o}C and at normal pressure for time interval of 4 weeks.

All the lyoprotectants were found to be effectively in protecting *P. putida* cells during freeze dry. In general, there was no significant loss of the initial viability for both lyoprotectants. At 4^{o}C, high viability of the initial cells demonstrated by PEG and tween 80/sucrose. The highest viability after freeze dry (without storage) was found in sucrose pre-treated freeze-dried *P. putida* cells. More than 78% (6.2E+09 CFU/ml) of freeze-dried *P. putida* cells were able to survive during freeze dry.

Rehydrate of PEG solution pre-treated freeze-dried *P. putida* cells after freeze-dry were also found higher but lower than tween 80 / sucrose pre-treated cells. The viability of

PEG pre-treated freeze dry cells after freeze-dry were higher than 64% (5.0 E+09 CFU/ml).

After lyophilisation or freeze drying, cell viabilities of *P. putida* in the absent of protective agents were significantly lower than with the addition of any lyoprotectants. Without the addition of any lyoprotectants, freeze dry was introduced more damage to the cells, resulting in relatively low viabilities of 5.60E+07 CFU/ml (>0.1%). Broth seemed does not provide sufficient protection during storage. No viable cells were observed after third week.

Viability of the freeze-dried *P. putida* after 1, 2, 3 and 4 weeks were also observed to determine the storage stability of freeze-dried *P. putida*. Freeze dry using tween 80/sucrose solution decrease to 5.60E+08 CFU/ml after one week of storage at 4^{o}C and at normal pressure. The viability of freeze-dried *P. putida* in tween 80/sucrose pre-suspended was reduced in the following week to 2.00E+07 CFU/ml.

Tween 80/sucrose was seemed able to stabilize the cells from the first week. The CFU number of tween 80/sucrose were found to stabilized by 1.80E+06 and 5.40E+05 during storage at three and four weeks storage at 4^{o}C. The pattern of cell reduction in freeze-dried *P. putida* cells were found by the value of log 10 per week.

Damage of the *P. putida* cells was lesser by the formulation of tween 80/sucrose than PEG. The viability was greatly reduced to 8.80E+05CFU/ml after one week storage by formulation freeze dry using PEG and greatly decline to 6.90E+03 CFU/ml in the second week of storage.

Using PEG as lyoprotectant caused a significant loss in viability after 3 and 4 weeks. More than half of the freeze-dried cells were not able to survive during storage, resulted in 4.10E+01 during the first 3 weeks. PEG were not able to provide protection for the cells during storage and cause membrane damage to the cells, resulted in no viable recovery cells after 4 weeks storage at 4^{o}C.

Table 4-1: Viability *P. putida* using slow freezing

WEEK	CFU/ml		
	PEG 1000	**Tween 80/ Sucrose**	**Broth**
0	5.00E+09	6.20E+09	5.60E+07
1	8.80E+05	5.60E+08	7.20E+04
2	6.90E+03	2.00E+07	1.80E+02
3	4.10E+01	1.80E+06	0.00E+00
4	0.00E+00	5.40E+05	0.00E+00
Fresh Culture		7.90E+09	

The result from this experiment in table 1 showed that the highest viability without storage (time 0) was obtained from tween 80 / sucrose pre-treated freeze-dried *P. putida* cells. The result indicate that lyoprotectant tween 80/sucrose could give an effective protection for frozen cells when undergoing freeze-dry process (Hubalek, 2003., Song, Ma and Wei, 2014).

PEG is polyhydric alcohol and one among the most commonly used and effective cryoprotectants. PEG 1000 is low molecular weight polymers and categorized as cryoprotective addictive that penetrating the cell wall but not cytoplasmic membrane as well as sucrose (Hubalek, 2003).

PEG was suggested as an effective agent for many mechanical injuries in which membrane disruption was implicated. PEG able to repairs mechanically injured cells such as membrane damage by two ways, which are resealing of the disrupted plasma membrane and direct protection of mitochondria (Shi, 2013).

Although there was no significant different CFU number between PEG pre-treated freeze-dried cells and Tween80 / sucrose pre-treated freeze-dried cells at time 0, but a rapid lost was exhibited from rehydrate cells prepared in PEG after storage (time 1, 2 and 3). PEG pre-treated cells seemed to be more susceptible to storage after freeze

drying due to the higher toxicity level (Wenfeng *et al*., 2013). During storage, death and sublethal injury are very high during initial period of 1 week and decrease during further storage.

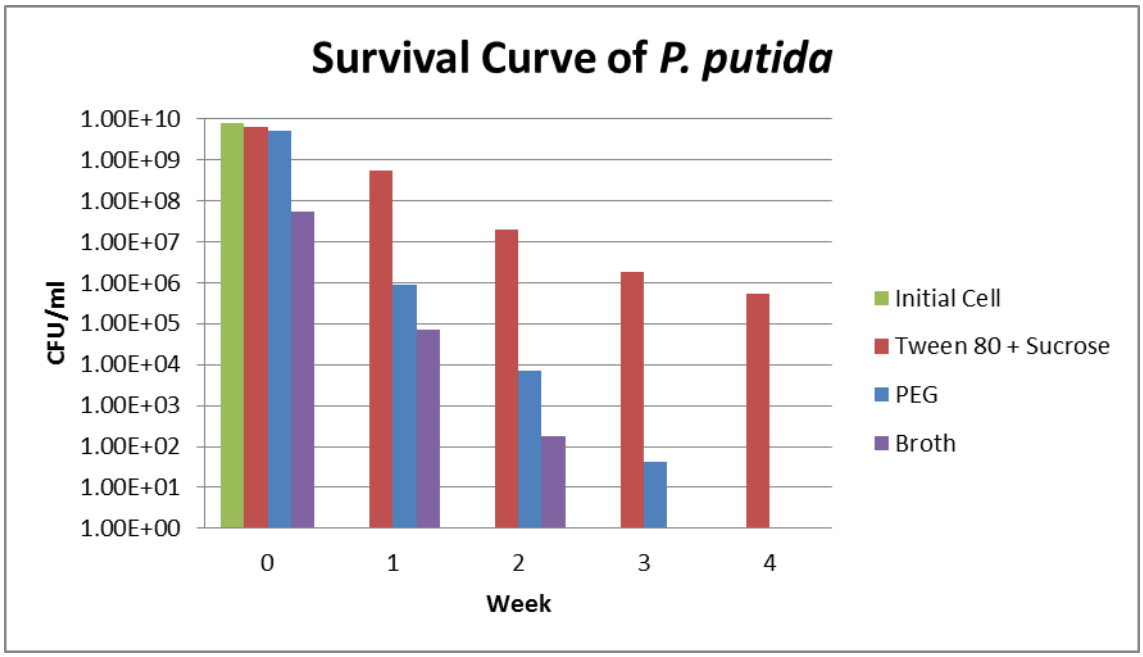


Figure 4- 1: Viability rate of *P. putida*

In contrast, freeze-dried *P. putida* cells prepared in Tween 80/sucrose were able to maintain low decrement along the time during storage in 4^{o}C. Tween 80/sucrose pre-treated freeze-dried cells were able to give an effective protection for the freeze-dried cells when undergoing freeze-dry and during storage at atmospheric pressure and 4^{o}C (Wenfeng *et al*., 2013; Santivarangkna *et al*., 2008).

Sucrose allows the cells to survive during the storage time mainly because sucrose is not toxic compared to PEG (Kuleshova *et al*., 1999). Sucrose was also suggested as a good cryoprotectant and even suitable for human oocyte cryopreservation (Chen *et al*., 2004). The best result for storage condition was obtained from sucrose solution as the result of the reaction between carbonyl compound and cellular compound.

Sucrose able to enhance the protection of the cell during desiccation by replacing the water around polar residue within macromolecular structure. Sucrose forming hydrogen bonding, maintaining the lipid in the liquid crystalline phase at room temperature and lowering the temperature of the membrane phase transition (Leslie *et al*.,1995). It protects the structure and avoids denaturation of protein in the cells during drying.

In addition, sucrose able to forming a glass state in dry tissue, avoids the cells to burst and deterioration (Berner and Viernstein, 2006). Sugar replace structural water in membrane after dehydration and prevent unfolding and aggregation of protein by hydrogen bonding with polar groups of proteins.

The viability of the cells was improved by addition of tween 80 in the solution. Tween 80 solution yielded a gel-like residue and insoluble in water. It has very little anti-bacteria activity and prevents damage to cytoplasmic (Wenfeng *et al*., 2013).

Lyoprotectant that made from disaccharide based such as sucrose and lactose was a good protective agent and has been widely used in the freeze dry in the lab scale. Sucrose form glasses in the dry state. Glasses have several properties that may relevant to anhydrobiosis. Anhydrobiosis is an adaptive strategy of organisms to survive in the dry state or absence of water.

Glasses are expected to have lower water vapour pressure than the corresponding crystalline solid and thereby may add resistance to further dehydration. Glasses are exceedingly viscous and the reduced molecular diffusion must stop essentially all chemical reactions and may ensure complete dormancy and stability over the time (Storey and Storey, 2000).

In view of the simplicity of the biosynthetic pathway of sucrose, that is a two-step one, involving a synthase and phosphatase, it was suggested that the engineering of sucrose synthesis in the cells that do not normally synthesis these molecules, might provide a means to manipulate desiccation tolerance (Crowe, *et al*., 1997).

In general, the viability obtained from this experiment was lower than the earlier study. Wenfeng *et al.* (2013) obtained more than 90 % viability of tween 80/sucrose pre-treated freeze-dried *P. putida* during recovery after freeze dry and more than 40% viability after storage for 3 months.

The result for tween 80/sucrose pre-treated freeze-dried *P. putida* cells were lower than the literature may due to the different strain type used. In this study, strain *P. putida* used was *Pseudomonas putida* ATTC 49128. Meanwhile, Wenfeng *et al*. (2013) used *Pseudomonas putida* ESR Collection 852 CDC KC1074 for study. The difference of the survival pattern between the present study and earlier study point out that the behavior

of given strain should not be generalized to other strains of the same species. *Pseudomonas putida* ATTC 49128 was found quite sensitive to freeze-drying than other strain with the same species.

The same case also has been studied in earlier study. Briggs *et al*. (1955) has studied more than 452 different strains of Lactobacilli and found that different strain resulted in different viability towards freeze drying. Different *P*. *putida* strains also have been suggested develop different strategies for changing the composition of phospholipid head groups to maintain the barrier function of cellular membranes to overcome damage produced by organic solvent (Ruhl *et al*., 2012).

The apparatus used might have been responsible for some variations in the data obtained. Further comparison was made between present study and Wenfeng *et al*. (2013) based on the type of freeze dryer used in which Wengeng *et al*. (2013) used 24-well plates (Coasta 3524) and Labconco freeze drier.

Even though different type of apparatus were used like plating and centrifugation, but overall the optimum performance of the freeze dryer equipment in this study was not meet the requirement resulted in not perfectly representative for the real freeze drying process. Consequently, the data becomes insignificant and become more variance among the same sample.

The initial freezing has been known as the earlier important stages for the successful freeze drying. This stage must have taken into consideration as freezing also may be a source of most injury will occur. In this study, to decrease the injury from the drying process, slow freezing was used to freeze the cells. The use of slow freezing reduces the duration of freeze drying process.

In addition, the cost is significantly reduce by the lesser drying time. The significant reducing time is due to the structure of the ice crystal created during the freezing. Slow freezing rate allows the growth of larger ice crystal than the rapid freezing.

The greatest resistance of vapor transport occurs across the dried product layer where water molecules have to pass the pores and the channels, depending on the sizes of the ice crystal. Thus the slow freezing treatment increase the ice means size, improving the

water vapor mass transfer, gives higher permeability of the dried layer and consequently reducing the primary drying times.

The samples were first cooled subsequently to 4^{o}C, -20^{o}C and finally to -80^{o}C to induce larger ice crystal, lead to the larger pores at the beginning of the freeze drying and lowering the final reside of final moisture content. But at -20^{o}C most of the cells will have lethal or sub-lethal injury. The main source of freezing damage was from the ice crystal. Damage and death of the cells were more pronounce at slow freezing than quick freezing. This effect was even more pronounced in gram negative bacteria such as *Pseudomonas aeruginosa* (Venugopal, 2006).

Moreover, *Pseudomonas* species has the maximum viability reduction in the first 24 h after freezing (Thampuran and Gopakumar, 1993). This explained the reason for the low viability for sucrose-based lyoprotectant after freeze drying. In this study, the frozen *P. putida* cells were drying after overnight freezing in refrigerator at -80^{o}C. The amount of this freezing time was sufficient enough to damage the cell up to the maximum reduction.

Even after short period of primary drying, damage by the initial freezing may cause the most lethal damage to the cell. Slow freezing cause more shrinkage and damage to the cell compared with fast freezing due to the extracellular ice that formed gradually which increases the time needed to reach the eutectic point thus allowing more dehydration and shrinkage (Zhao and Zhang, 2005).

It has been reported that freeze-dried *P. putida* can survive more than 10 years under strict storage condition, such as vacuum condition (Miyamoto-Shinohara rt al , 2000). Miyamoto *et al*. (2005) suggested that survival during storage was strongly influenced by the degree of vacuum under which the vial was sealed. Higher survival rate after freeze-drying of different species might be attribute to the high level of desiccation and to sealing under vacuum.

In this study, atmospheric pressure in the vial for long term storage also may contribute to higher depletion of freeze-dried *P. putida.* Engineered *Pseudomonas* strain was more sensitive to air (oxygen) after freeze-dry than their wild type parental strain (Israeli *et a.l*, 1993). The present study proved that an exposure to oxygen after freeze-dried cause lethal damage to the cell membrane of *P. putida*.

The death rate of the freeze-dried *P. putida* was higher can be attribute to the additional energy needed to maintain the plasmid. In this study, open air factor after freeze dry and the present of oxygen in the vial during storage has been suggested as the main factor that cause enhancement of dry-state *P. putida* death. This result was in agreement with earlier study of freeze dry *Pseudomonas* strain (Israeli *et a*l., 1993).

Great decrease of freeze dried *P. putida* after 1 week and the following week for both lyoprotectants can be easily explained by the presence of air or oxygen in the vial. The effect of oxygen was very lethal to most of freeze-dried bacteria (Lion and Bergmann, 1961). Store under vacuum or inert gases can be used to replace the air or oxygen and demonstrated to improve the viability during storage.

The result demonstrated that store dried *Pseudomonas* in air or presence of oxygen cause rapid die when the condition of the cells was very dry or very moist. Earlier studies have been conducted to determine the effect of various gases towards the storage freeze-dried *Pseudomonas* species. Storage in vacuum with inert nitrogen was suggested better than air and inert gas such as argon has been reported to give the best survival when the *Pseudomonas* were dried in broth (Marshall *et al*., 1973).

4.4 The effect of freeze drying in growth of P. putida

Comparison between freeze dried *P. putida* and fresh *P. putida* in term of growth curve were made. From figure 4-4 to figure 4-9, it showed that the optical density (OD) of the freeze-dried cells was greatly reduce compare to the fresh *P. putida*. The maximum ln (OD /ODo) found for fresh *P. putida* was 7.1. In contrast, the maximum ln (OD /ODo) found after storage for 1 week were 3.92 and 3.95 for PEG and tween 80/sucrose based.

After storage for the following week, maximum ln (OD/ODo) fall to 3.94 and 3.8 for tween 80/sucrose based lyoprotectant. PEG based lyoprotectant also reduced to 3.82 and 3.88 after subsequent storage. This maximum ln (OD/ODo) was higher when tween 80/ sucrose was used as lyoprotectant compare to PEG freeze dry may related to the initial ODo in which initial ODo for sucrose was higher than PEG.

This may related to the ability of sugar to penetrate trough the cell wall. Hence, even the cells were washed in buffer solution before rehydration, but some sugar compound was still attached into the cell and caused the initial OD to be higher. Mager *et al*. (1956) showed that the optical densities of live bacterial suspensions in osmotically

active solutions were greater than in distilled water, and interpreted this 'optical effect' as an increase in the refractive index of the cytoplasm consequent on its adjustment to the osmotic pressure of the environment.

Higher molecular weight of PEG cannot penetrate trough the cell wall, hence it is easily washed during recovery and did not influence initial OD as much as sucrose did. The result also showed freeze dry does not really change the pattern of growth *P. putida.*

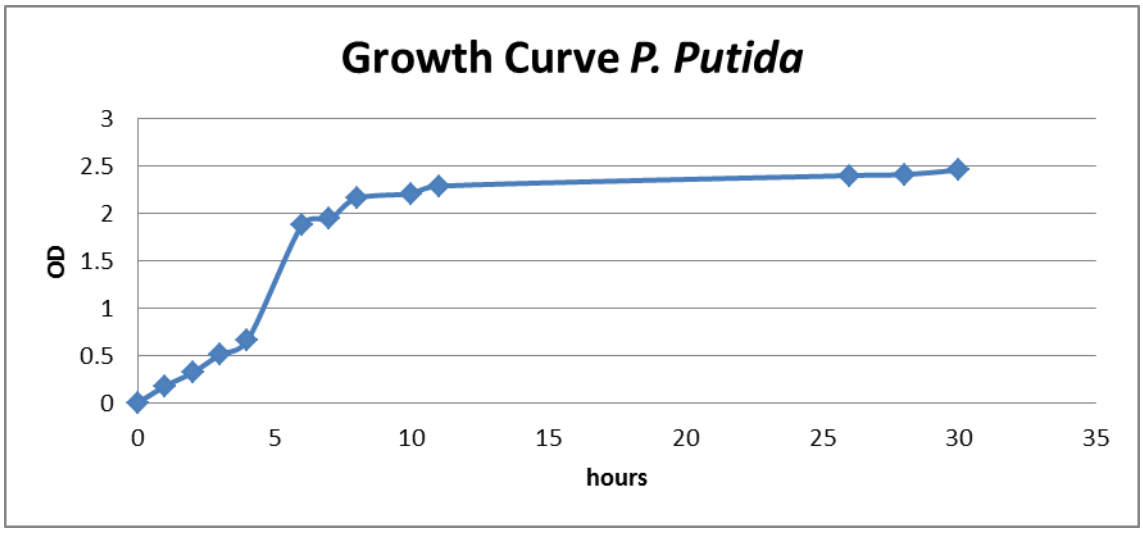


Figure 4-2: typical growth curve *P. putida*

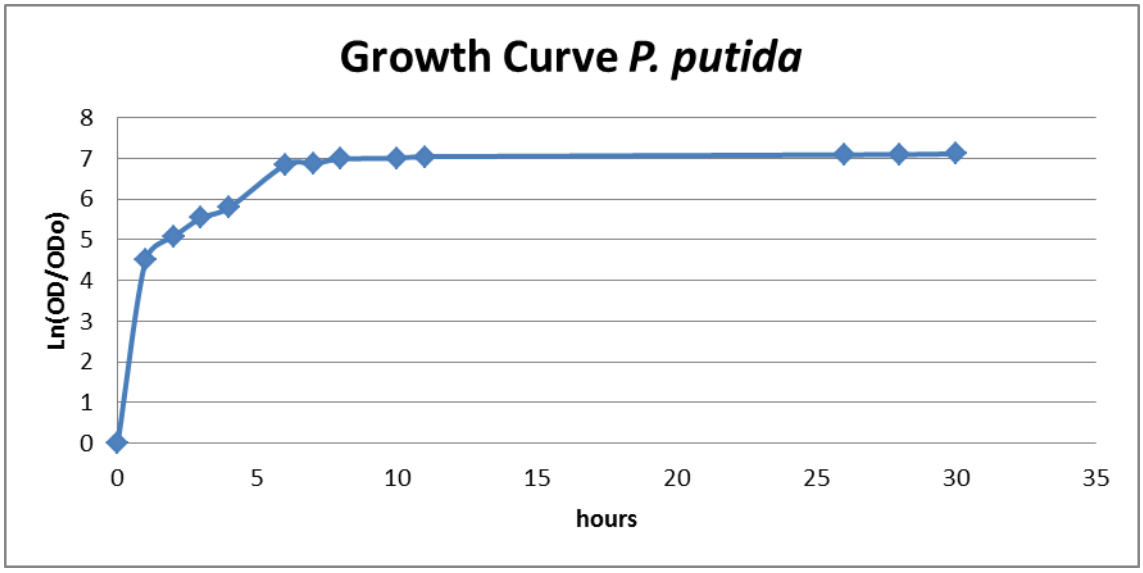


Figure 4-3: Typical growth curve *P. putida*

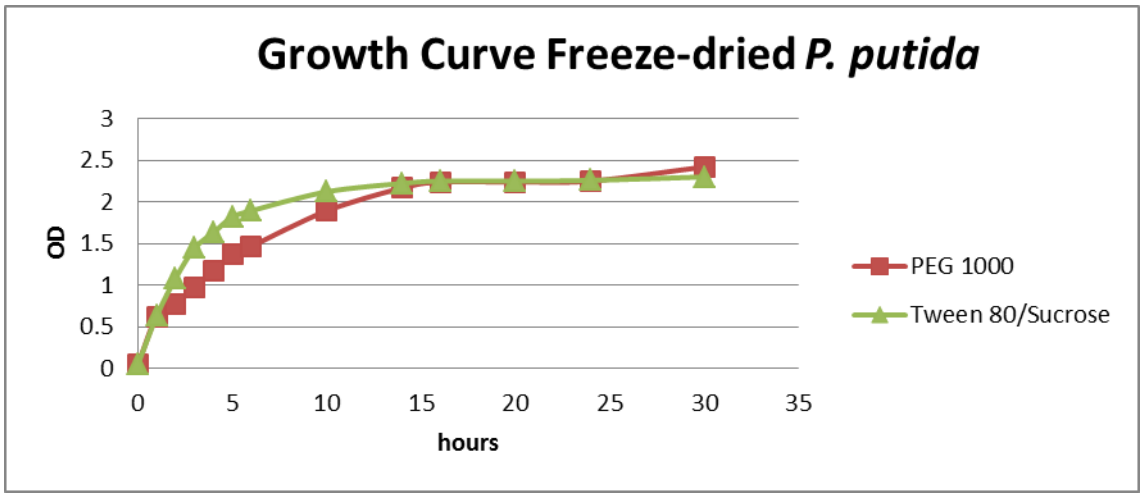


Figure 4-4: Typical growth curve freeze-dried P. putida after 1 week storage

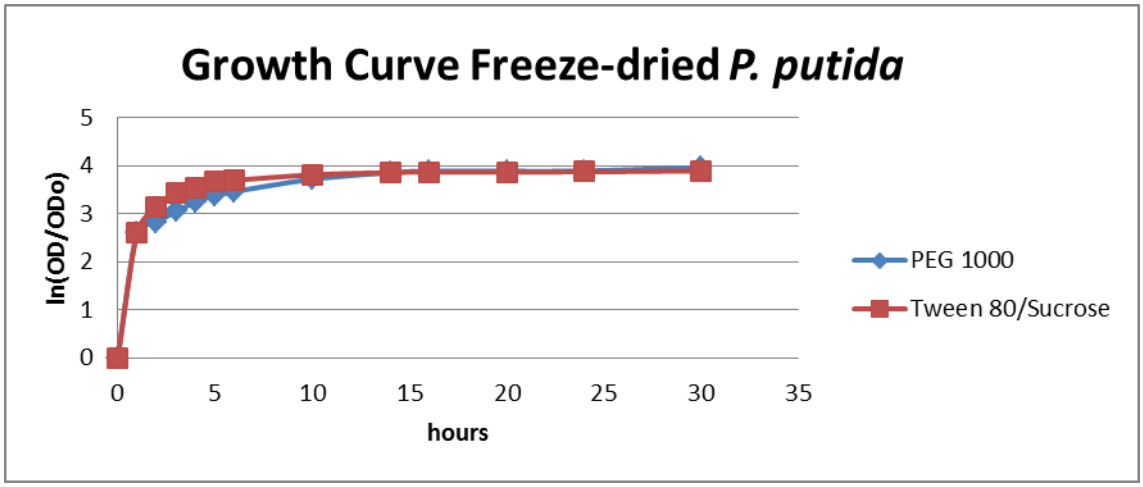


Figure 4-5: Typical growth curve freeze-dried P. putida after 1 week storage

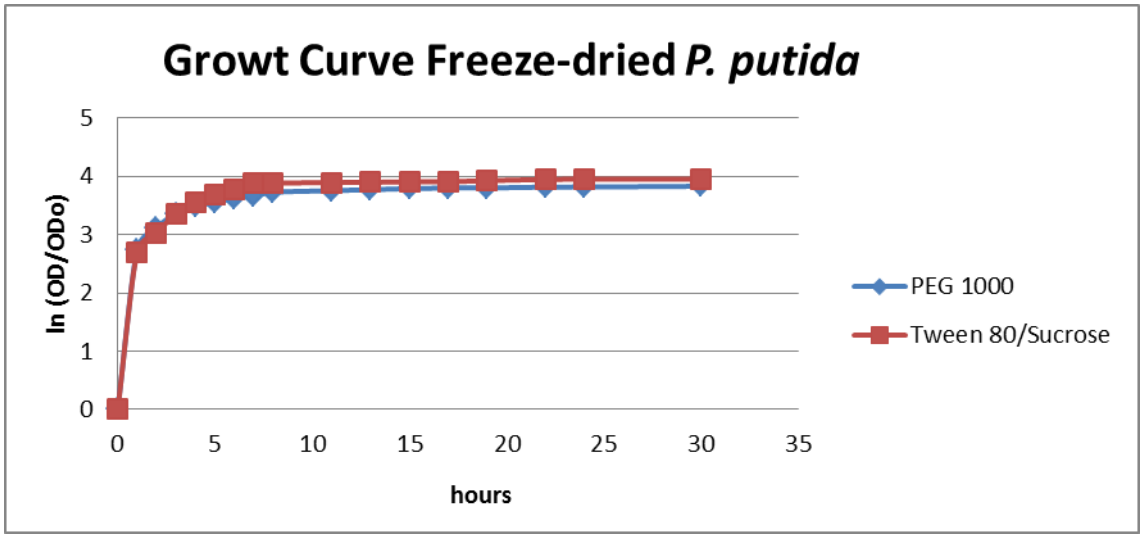


Figure 4-6: Typical growth curve f reeze-dried P. putida after 2 week storage

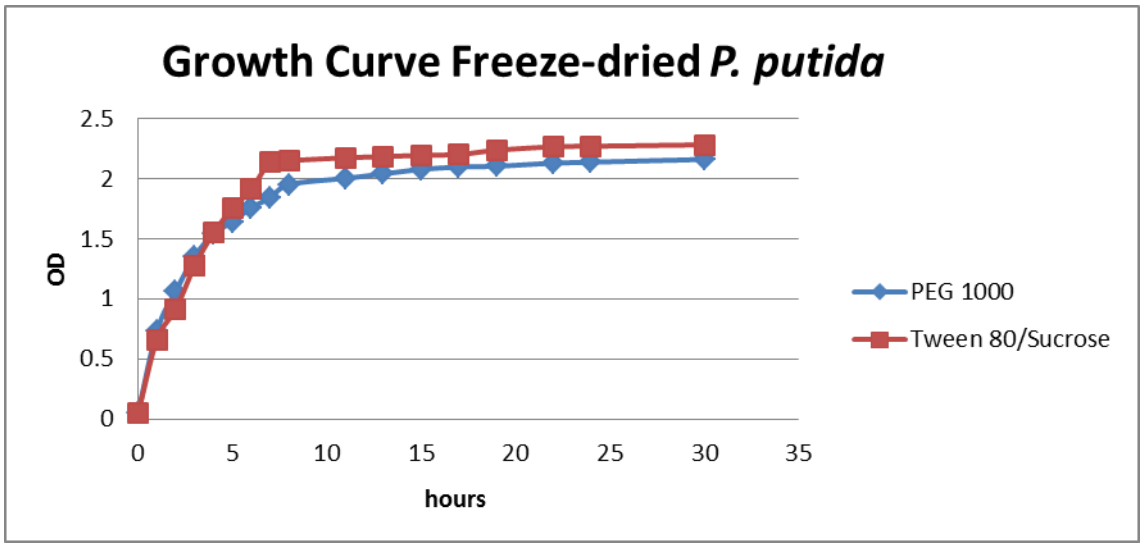


Figure 4-7: Typical gr owth curve f reeze-dried P. putida after 2 week storage

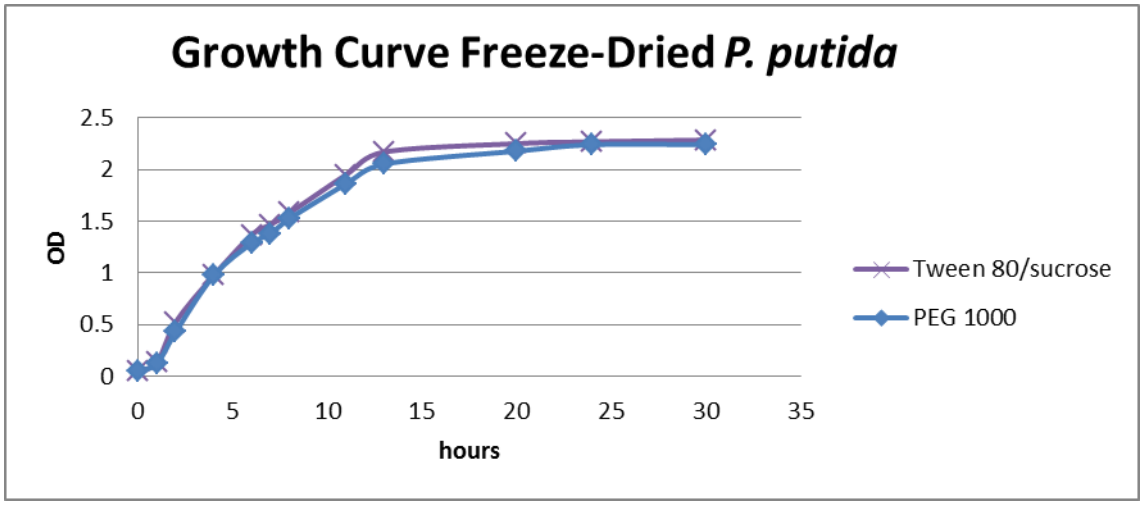


Figure 4-8: Typical growth curve f reeze-dried P. putida after 3 week storage

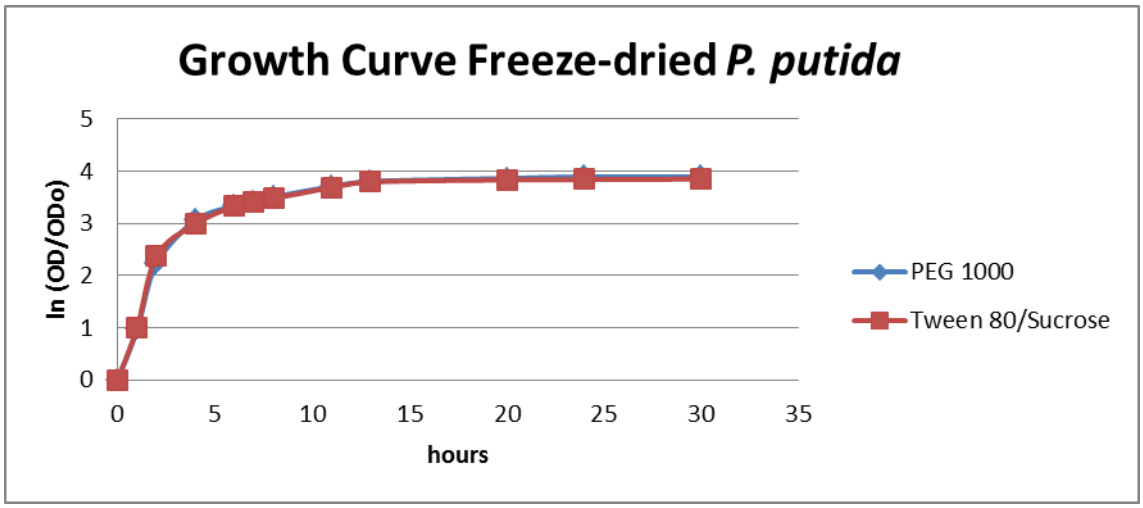


Figure 4-9: Typical growth curve f reeze-dried P. putida after 3 week storage

The optical density (OD) of PEG pre-treated freeze-dried *P. putida* cells were found lesser than tween 80 / sucrose pre-treated freeze-dried *P. putida* after freeze drying and after storage. It may identify that the volume of viable cells per unit volume of the rehydrate solution of tween 80/sucrose pre-treated freeze-dried *P. putida* cells were higher than PEG pre-treated freeze-dried *P. putida* cells.

It is suggested that PEG with high molecular weight solute does not penetrate the bacteria cell wall cause rapid reduction in pellet volume. Meanwhile, sucrose readily permeates the cell wall but not the plasma membrane. Sucrose able to induced plasmolysis resulted in an increased volume between the cell wall and plasma membrane. This space between cell wall and plasma will be occupied by sugar solution of the same concentration as that outside the cell (Record *et al*., 1962).

Compare to the control (figure 4-2), an extended time was observed in the lag phase in figure 9. The lag phase for control *P. putida* was 24 h and does not shown in the graph. The growth curve after freeze drying and after one week storage and the following week has no significant difference with the control but an additional time of lag phase was observed about 1 h after 3 weeks storage. This additional time can be figured at the first 1 hour of the growth curve.

According to Robinson (2002), starter cultures preserved by freeze-drying tend to have a prolonged lag phase and they are mainly used as inoculants for the propagation of mother culture. Larger quantities are needed for direct inoculation of the bulk starter and an extended incubation time may be required.

Compared to the control *P. putida* cells that has been growth in the broth, freeze-dried cells exhibit an extended lag time. The extended lag phase was also observed in the study of other freeze-dry gram-negative bacteria of *E. coli* (Sinskey and Silverman, 1969).

It is also suggested that sucrose was effective carbon sources for lyoprotectant. In contrast, PEG 1000 was less effective for the storage and growth of freeze-dried cells. Additional of time was required for the initiation of the growth. It may also suggested that only after 4 weeks interval, normal protein and DNA synthesis began in freeze-dried *P. putida*.

4.5 Effect of Lyoprotectant in Residual Moisture Content

Cellular water plays an important role in freeze-drying and that the removal of unfreezable bound water affects cell survival in microorganisms. On the other hand, residual moisture content is closely related to the drying medium, and an appropriate selection of both the suspending medium and the protector seems to be essential, since variations in the drying medium may affect the survival of bacteria during the freeze-drying process as well as their viability during the period of storage and reconstruction (Graciela *et al*., 1985).

In this experiment, all the samples were run at the same time in one freeze drying machine. The difference of moisture content shows that moisture content is depending on the type of lyoprotectant used. This is with agreement with Graciela *et al*. (1985) who also utilized PEG 1000 to freeze *dry L. murinus* and found that samples were processed high survival cell after freeze drying but observed significant loss of viability within 48 to 72 h after freeze drying.

Erlier study also suggested that residual moisture content also was directly related to the type of drying medium (Strasser *et al*., 2008). In another study of freeze-dried of Lactic Acid Bacteria has residual moisture content for freeze dried samples varied to a higher extent from 1 to 4 % (Champagne & Gardner, 2001).

Meanwhile, Valdez *et al*. (1985) were able to achieve residual moisture content up to 0.01 in PEG 1000 pre-treated freeed-dried cells. The variation obtained in this study and

literature may due to the different capacity of the freeze dryer apparatus and to the higher extent of the type of medium used during drying.

Table 4-2 : Moisture contents of dry *P. putida* cell after 3 weeks storage

Lyoprotectant	Moisture content, w/w%
PEG	0.124-0.714
Tween 80/Sucrose	0.039-0.242

Since the residual moisture content of the final product may have a considerable impact on the survival rate after drying and during storage, it is conceivable that the protective properties found in the present study are at least partially due to indirect effects, caused by the fact that the addition of different types of carbohydrates results in different moisture contents after the drying process.

The results prove that the moisture content has significant effect on the storage stability of freeze-dried *P. putida*. Higher CFU indicates high viable cells after rehydration. The lower range of moisture content was found in tween80/sucrose pre-suspended cells.

The minimal water content or hydric potential for surviving desiccation-tolerant cells is extremely low. In preserving biological matter, the present of water is a factor that considerably provides a major control parameter in successful preservation (Poddar *et al*., 2014).

Based on the result, lowering the moisture content increase the stability of *P. putida* survival during storage. Freeze-dried pre-suspended in PEG has higher range of moisture content and greatly decrease the CFU along the time. Maintaining the minimum water contain in the cells of *P. putida* is essential to obtain tenacious preserved cells for storage (Adams, 2007).

The result from this study also suggested that over drying may cause the residue moisture content reduced to below the minimum allowable moisture content and tend to reduce viability of the cells for both lyoprotectant.

Maintaining the minimum water contain in the cells of *P. putida* is essential to obtain tenacious preserved cells for storage (Adams, 2007) but a certain amount of water must

remain in freeze-dried cells during storage to achieve the maximum survival rate (Zayed and Roos, 2004).

The high death rate shown in table 2 also indicates that over drying might injured the cell during freeze dry and cause significant reduction viability cell. High death rates may be related to the elimination of the three fraction of cellular water (free water, intermediate water and structured water), with lethal damage of cellular proteins (Mellor, 1978).

Under the microscope, it seemed that there is no visual difference between PEG pre-treated freeze-dried *P. putida* cells and tween 80/sucrose pre-treated freeze-dried *P. putida* ***cells.***

Without any rehydration process (incubation) the cells movement were monitored. For both samples, *P. putida* shown physical movement right after thawing to room temperature after freeze-dried *P. putida* stored under 4^{o}C for 3 weeks, but a few of the cells were less active after thawing to room temperature.

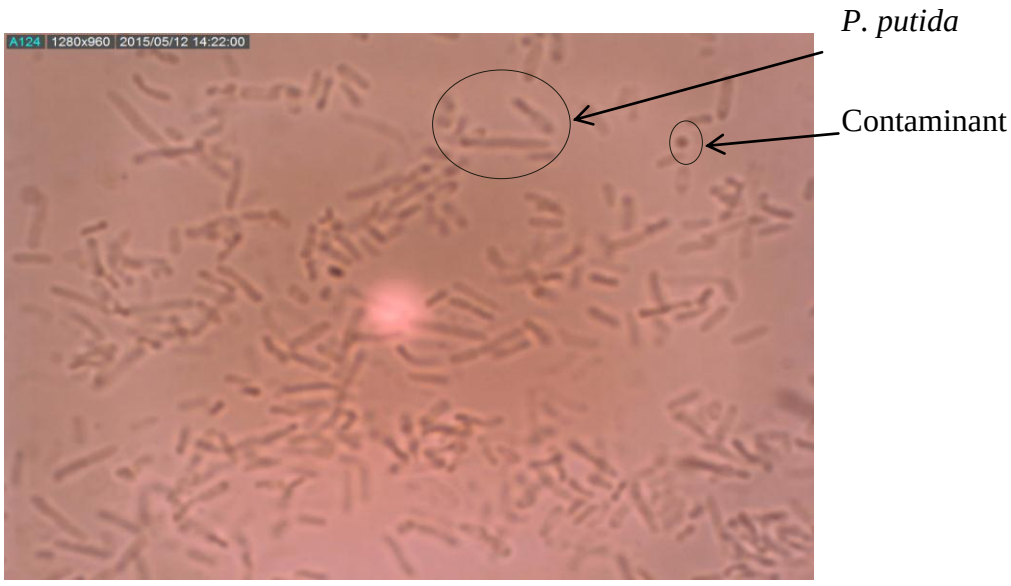


Figure 4-10: Freeze dried *P. putida* in Tween 80/Sucrose

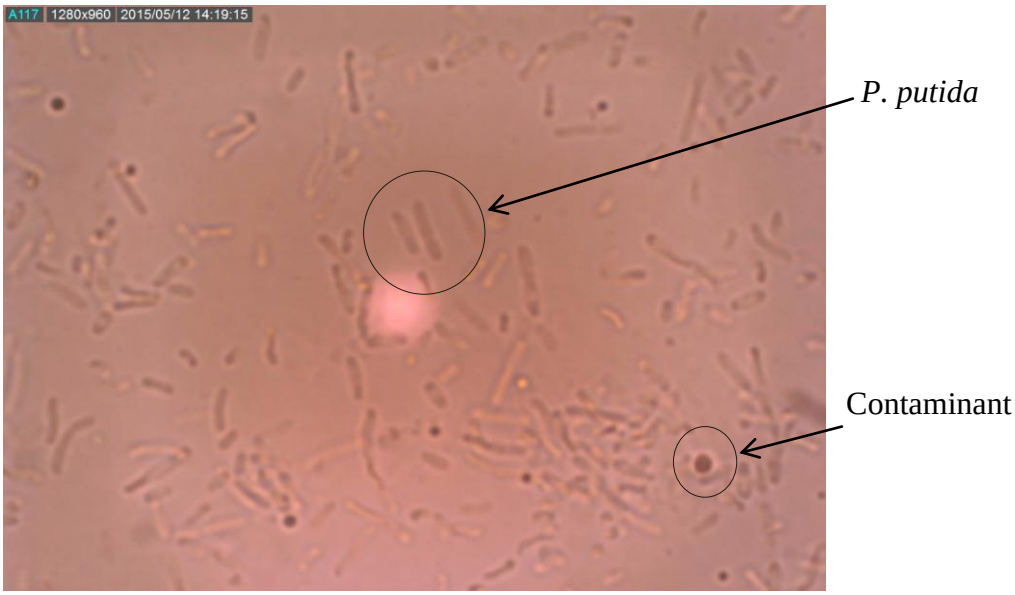


Figure 4-11: Freeze dried *P. putida* in PEG

Figure 4-10 and figure 4-11 were taken from the part of the cells that active cells were observed. It was shown that less *P. putida* cells in PEG pre-treated freeze-dried cells compared to tween 80/sucrose pre-treated freeze-dried cells.

Furthermore, the observation also found that there were PEG and tween 80 / sucrose freeze-dried *P. putida* cells that instantly active after thawing in the present of broth. A few of the cells were showed a little movement after have a contact with broth.

P. putida was reported to sustain the live and growth in low temperature of 4^{o}C. At lower temperature than 30^{o}C, *P. putida* growth at lower rate due to the different expression of 266 genes that directly related with energy metabolism (Fonseca *et al*., 2011).

In addition, it was observed the existence of contaminant in the PEG and tween 80 / sucrose pre-treated freeze-dried cells. It was difficult to maintain pure *P. putida* during freeze drying process because freeze drying was a complex process and involved with many process that take a longer time than any drying process.

In addition, freeze drying is preservation process that is very difficult to maintain uniform distribution of the product. Freezing process was the initial primary difficulties to control. From the observation, slow freezing cause the cell settling and local concentration occurs.

During the freezing, large volumes of suspension with large distance between the heat transfer surface has slow freezing velocity and the cells could rejected and become concentrated in the liquid phase. This is the main reason of the insignificant result obtain in this study that involved with the wide range of the data.

Overall residue water for both lyoprotectants freeze-dried was higher than the expected may due to the air or the presence of oxygen in the vial. Marshall *et al*. (1973) suggested that difference gases in vial for storage and residue water content influence the viability. The study suggested that the viability of *P. putida* was not good either the moisture content is low or high when air was in the vial during storage.

4.6 Effect Freeze Drying in Mercury Reduction

Mercury reduction testing was performed using freeze-dried *P. putida* cells taken from the sample that has been stored for 3 weeks and after freeze dry without storage to relate the effect of lyoprotectant with the performance of *P. putida* in removing mercury.

Table 4-3 : Mercury reduction using freeze-dried *P. putida* cells at time 0

Lyoprotectant	**Initial Mercury Concentration**	**Final Mercury Concentration**	**% Reduction**
PEG 1000	1000 ppb	432.19 ppb	56.78%
Tween 80/Sucrose	1000 ppb	736.46 ppb	26.35%
Fresh Culture	1000 ppb	0.0001ppb	99.99%

Table 4-4 : Mercury reduction using freeze-dried *P. putida* cells after 3 weeks storage

Lyoprotectant	**Initial Mercury Concentration**	**Final Mercury Concentration**	**% Reduction**
PEG 1000	1000 ppb	820.91 ppb	17.91 %
Tween 80/Sucrose	1000 ppb	749.64 ppb	25.03%
Fresh Culture	1000 ppb	0.0001ppb	99.99%

Both PEG and Tween 80/Sucrose pre-treated freeze-dried *P. putida* cells were able to reduce mercury. The overall total mercury reduction for both lyoprotectant was not higher may due to the less viable *P. putida* cells after rehydration. Mercury reduction of

PEG pre-treated freeze-dried *P. putida* cells was lower compared to tween80/sucrose pre-treated freeze-dried *P. putida* cells. It seems reasonable as the cell viability tween80/sucrose pre-treated freeze-*dried P. putida* cells was higher during 3 weeks storage.

The lower percentage reduction of mercury for both freeze—dried cells compared to the fresh culture may indicates that the cells suffer an internal damage due to the extreme freezing and drying process that affecting the ability of *P. putida* to remove mercury.

The ability of *Pseudomonas* strain in remove mercury is related to enzyme of mercury reductase. This enzyme catalyses the reduction process from toxic mercury ion (Hg^{2+}) to elemental mercury (Hg^{0}). The enzyme of mer T, merP and merA are involved in mercury reduction for gram-negative bacteria (Wong and Chu, 2003).

The cells were stress by the physical damage of the process and resulting in sublethally injured the cells. A sufficient amount of recovery time and appropriate environmental conditions may require repairing the metabolic system of the cells.

The types of stress that contribute in the cells damage were freeze injury cause by physical damage, exposure to concentrated solutes and osmotic stress that is cause by the move of water in or out from the cells. These factors lead to the membrane damage, protein and enzyme change (Wesche *et al*., 2009).

The performance of freeze-dried *P. putida* cells to reduce mercury also was performed at time 0 which was immediate rehydration after freeze drying. It was found that the highest reduction of mercury was performed by PEG-pre-treated freeze-dried cells. About 56.78% of mercury was able to remove by *P. putida* cells. In contrast, tween80/sucrose pre-treated cells only able to remove 26.35% of mercury. It may due to the contamination in the sample during the process.

The percentage of reduction of mercury using tween80/sucrose pre-suspended cells should be not significantly different with PEG pre-suspended cells. Freeze drying involved complex process that cause difficult to maintain the uniformity of the product in term of quality and quantity.

Contamination is also the factor that very difficult to control when dealing with many samples at once. In this study for example, too many samples were made might provide

a way for undetected contamination to occur in the samples, hence reducing the mercury reduction percentage.

Lower reduction of mercury for the samples after freeze dry and after storage compare to the control might because of the dissolve molecules of sucrose, tween 80 and PEG 1000 decrease the mercury penetrating ability. Freeze dried of *E. coli* was suggested to have reducing mercury penetrating ability or have some inhibitory effects and suppress growth of bacteria in media (Vasilenko, 2012).

Cells might grow slower or faster in the matrix due to the high nutrient content or on the other hand the dissolve macromolecules can prevent ion penetration trough the cell wall (Vasilenko, 2012).

Some ion may attach to the particle, so after addition of mercury an exchange mechanism occurs and this trapped fraction releases to the media. A complete disruption of mercury in the matter may prevent ions consumption (Vasilenko, 2012).

The extension of lag-phase time presence in the beginning-time of growth or exponential phase in the samples after storage 3 weeks was the sign that the cells needed additional time for penetration of ions trough the cell wall and also a time for adaptation of the cells to the complicated condition.

Due to the unavailability of the equipment (mercury analyser), all the samples were analyse by another parties that involved storage before analysis. Long-time of measurement can decrease the reliability of the data, because after an adaptation period , the cell number and so the signal intensity increase resulted in deviation of the result and do not reflect the actual metal concentrations (Vasilenko, 2012).

In addition, freeze-drying may have undesirable side effects that are not observed with preservation only by freezing. These include potential for genetic change during freeze-drying due to DNA strand breakage and selection mutants (Asada *et al*., 1980), denaturation of sensitive protein and decreased viability for many bacterial types.

It has been reported by Asada *et al*. (1980) that mutation was induced in freeze-dried other negative-strain bacteria, *E. coli* during repair of damage DNA after rehydration (Asada *et al*., 1980). The study found that *E. coli* B/r drying-resistant strains repaired the damaged DNA partially during post drying incubation in a growth medium but not

in phosphate buffer solution, while *E . coli* Bs-1 drying-sensitive strains could not at all and DNA was degrade due to the a lack of repair enzyme system. *E. coli* aslo found was mutants were found in the preserved cell after drying and storage at higher temperature above 5^{o}C.

According to Tanaka *et al*. (1979) DNA damage was found as the result of either freeze-drying or drying, but repair damage of the DNA was found during rehydration and mutation occur after freeze drying may occur during rehydration when damage DNA was repaired by bacterial enzyms.

DNA damage might also be the caused of low reduction of freeze-dried *P. putida* during freezing and freeze dry. A treatment in broth or solid medium for *P. putida* cell or additional time may be require to improve the stability of *P. putida* before introduce freeze-dried cells to more complicated condition such as mercury treatment.

4.7 Summary

There were many factors that can influence viability rate of freeze-dried *P. putida*. Mainly, viability of recovery *P. putida* after freeze dry significantly influence by the type of lyoprotectant. Tween 80 / sucrose was found to be the best lyoprotectant than PEG. Sucrose able to recover more than 78% (6.2E+09 CFU/ml) of the original cells (7.90E+09) after freeze dry and able to retain 5.40E+05 viable cells after 4 weeks storage in 4^{o}C without vacuum. Freeze-dried *P. putida* cells in PEG and broth cannot survive after 4 weeks storage. The viability also suggested to dependent on the moisture content. The moisture content of PEG freeze-dried cells were fall in the range of 0.124-0.714 % and moisture content of sucrose freeze-dried cells was found in the range of 0.039-0.242%. Freeze dry also does not really change the pattern of growth *P. putida* but extension of lag time was found 1 hour after 3 weeks of storage. The result suggested that additional time was required for freeze-dried *P. putida* cells to recover before introduce freeze-dried cells to more complicated condition such as mercury solution. The maximum mercury reduction of PEG pre-treated freeze-dried cells after freeze dry and after storage 3 weeks was 56.78% and 17.91 %.The maximum of mercury reduction of tween 80/sucrose pre-treated freeze-dried cells after freeze dry and after storage 3 weeks was 26.35% and 25.03%. Freeze dried *P .putida* was found to have low mercury reduction compare to the fresh *P. putida* that has been growth in agar.

5 CONCLUSION

5.1 Conclusion

Dried *P. putida* can be obtained using freeze drying technique. Viability of recovery *P. putida* after freeze dry significantly influence by the type of lyoprotectant. Among the lyoprotectants, tween 80/sucrose was found to be the best lyoprotectant. Sucrose able to recover more than 78% (6.2E+09) of the original cells (7.90E+09) after freeze dry and able to retain 5.40E+05 viable cells after 4 weeks storage in 4^{o}C without vacuum. Freeze-dried *P. putida* cells in PEG and broth cannot survive after 4 weeks storage. Freeze dry also does not really change the pattern of growth *P. putida* but extension of lag time was found 1 hour after 3 weeks of storage. Additional time was required for freeze-dried *P. putida* cells to recover before introduce freeze-dried cells to more complicated condition such as mercury solution. The maximum mercury reduction of PEG pre-treated freeze-dried cells after freeze dry and after storage 3 weeks was 56.78% and 17.91 %.The maximum of mercury reduction of tween 80/sucrose pre-treated freeze-dried cells after freeze dry and after storage 3 weeks was 26.35% and 25.03%. Freeze dried *P .putida* was found to have low mercury reduction compare to the fresh *P. putida* that has been growth in agar.

5.2 Future work

It is suggested to find the optimum condition to freeze dry *P .putida* cells using good performance freeze dryer. The role of different concentration of lyoprotectant may also contribute to different viability cells. Study about the optimum concentration of lyoprotectant and optimum moisture content can be the key to obtain excellent freeze-dried *P. putida*. In addition, further study involved with the response of DNA, cell wall and inner membrane gene (mer T, merP and merA) of *P. putida* towards freeze drying also will provide significant useful data about performance freeze-dried *P. putida* for secondary user especially when the dried cells are used for industrial purpose.

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APPENDICES

Growth curve data after 1 week storage

h	Lycoprotectant			
	PEG		Tween 80/Sucrose	
	OD	Ln OD/Odo	OD	Ln OD/Odo
0	0.05	0.00	0.05	0.00
2	0.77	2.82	1.09	3.14
4	1.17	3.24	1.63	3.55
6	1.47	3.46	1.89	3.70
10	1.89	3.72	2.12	3.81
14	2.17	3.86	2.22	3.86
16	2.24	3.89	2.25	3.87
20	2.24	3.89	2.25	3.87
24	2.25	3.89	2.26	3.87
30	2.32	3.92	2.46	3.96

Growth curve data after 2 week storage

h	Lycoprotectant			
	PEG		Tween 80/Sucrose	
	OD	Ln OD/Odo	OD	Ln OD/Odo
0	0.05	0.00	0.04	0.00
2	1.07	3.12	0.92	3.03
4	1.54	3.49	1.55	3.56
6	1.76	3.62	1.91	3.77
10	2.00	3.75	2.18	3.90
14	2.04	3.77	2.18	3.90
16	2.08	3.79	2.20	3.91
20	2.11	3.80	2.24	3.92
24	2.14	3.82	2.27	3.94
30	2.16	3.83	2.28	3.94

Growth curve data after 3 week storage

h	Lycoprotectant			
	PEG		Tween 80/Sucrose	
	OD	Ln OD/Odo	OD	Ln OD/Odo
0	0.05	0.00	0.05	0.00
2	0.43	2.23	0.52	2.37
4	0.97	3.05	0.97	2.99
6	1.29	3.33	1.36	3.33
10	1.86	3.70	1.95	3.69
14	2.05	3.80	2.17	3.79
13	2.05	3.80	2.17	3.79
20	2.18	3.86	2.25	3.83
24	2.24	3.89	2.27	3.84
30	2.24	3.89	2.28	3.84

Growth curve data for fresh *P. putida*

time, h	OD	Exponential growth
		Ln OD/ODO
0	0.00	0.00
1	0.18	4.50
2	0.32	5.08
3	0.51	5.54
4	0.66	5.80
6	1.88	6.85
7	1.94	6.88
8	2.16	6.98
10	2.21	7.01
11	2.29	7.04
26	2.40	7.09
28	2.41	7.09
30	2.46	7.11

Moisture Content

Sample no.	Lyoprotectant	
	PEG 1000	Tween 80/Sucrose
sample 1	0.124	0.242
sample 2	0.714	0.039
sample 3	0.365	0.447
sample 4	0.623	0.831
sample 5	0.443	0.218

ANOVA

SUMMARY

Groups	Count	Sum	average
PEG	5	2.269	0.4538
Sucrose/tween80	5	2.777	0.5554

Source of Variation	
Between groups	0.025806
Within groups	1.562736
total	1.588542
F	0.132109

CFU Determination

Number of initial cell before freeze drying

Sample Initial cell	Volume of sample used (1)	Volume of diluent used (2)	Volume of sample (3)	Dilution factor of previous sample	Dilution factor for this sample	Number of colonies observed	CFU/ml
Fresh Bacteria	1 ml	0 ml	1 ml	-	1	TNTC	-
Tube A	1 ml	99 ml	100 ml	1	10^{-2}	TNTC	-
Tube B	1 ml	99 ml	100 ml	10^{-2}	10^{-4}	TNTC	-
Tube C	1 ml	99 ml	100 ml	10^{-4}	10^{-6}	TNTC	-
Tube D	1 ml	99 ml	100 ml	10^{-6}	10^{-8}	79	7.9×10^{9}

TNTC = too numerous to count

Number of colonies after drying in PEG treatment

Sample in PEG T=0	Volume of sample used (1)	Volume of diluent used (2)	Volume of sample (3)	Dilution factor of previous sample	Dilution factor for this sample	Number of colonies observed	CFU/ml
Dried Bacteria	1 ml	0 ml	1 ml	-	1	TNTC	-
Tube A	1 ml	99 ml	100 ml	1	10^{-2}	TNTC	-
Tube B	1 ml	99 ml	100 ml	10^{-2}	10^{-4}	TNTC	-
Tube C	1 ml	99 ml	100 ml	10^{-4}	10^{-6}	TNTC	-
Tube D	1 ml	99 ml	100 ml	10^{-6}	10^{-8}	50	5.0×10^{9}

Sample in PEG T=1 week	Volume of sample used (1)	Volume of diluent used (2)	Volume of sample (3)	Dilution factor of previous sample	Dilution factor for this sample	Number of colonies observed	CFU/ml
Dried Bacteria	1 ml	0 ml	1 ml	-	1	TNTC	-
Tube A	1 ml	99 ml	100 ml	1	10^{-2}	TNTC	-
Tube B	1 ml	99 ml	100 ml	10^{-2}	10^{-4}	88	8.8×10^{5}

Sample in PEG T=2 week	Volume of sample used (1)	Volume of diluent used (2)	Volume of sample (3)	Dilution factor of previous sample	Dilution factor for this sample	Number of colonies observed	CFU/ml
Dried Bacteria	1 ml	0 ml	1 ml	-	1	TNTC	-
Tube A	1 ml	99 ml	100 ml	1	10^{-2}	69	6.9×10^{3}
Tube B	1 ml	99 ml	100 ml	10^{-2}	10^{-4}	0	0

Sample in PEG T=3 week	Volume of sample used (1)	Volume of diluent used (2)	Volume of sample (3)	Dilution factor of previous sample	Dilution factor for this sample	Number of colonies observed	CFU/ml
Dried Bacteria	1 ml	0 ml	1 ml	-	1	41	4.1×10^{1}
Tube A	1 ml	99 ml	100 ml	1	10^{-2}	0	0
Tube B	1 ml	99 ml	100 ml	10^{-2}	10^{-4}	0	0

Sample in PEG T=4 week	Volume of sample used (1)	Volume of diluent used (2)	Volume of sample (3)	Dilution factor of previous sample	Dilution factor for this sample	Number of colonies observed	CFU/ml
Dried Bacteria	1 ml	0 ml	1 ml	-	1	0	0
Tube A	1 ml	99 ml	100 ml	1	10^{-2}	0	0
Tube B	1 ml	99 ml	100 ml	10^{-2}	10^{-4}	0	0

Number of colonies after drying in Tween 80/Sucrose treatment

Sample in Tween 80/Sucrose T=0	Volume of sample used (1)	Volume of diluent used (2)	Volume of sample (3)	Dilution factor of previous sample	Dilution factor for this sample	Number of colonies observed	CFU/ml
Dried Bacteria	1 ml	0 ml	1 ml	-	1	TNTC	-
Tube A	1 ml	99 ml	100 ml	1	10^{-2}	TNTC	-
Tube B	1 ml	99 ml	100 ml	10^{-2}	10^{-4}	TNTC	-
Tube C	1 ml	99 ml	100 ml	10^{-4}	10^{-6}	TNTC	-
Tube D	1 ml	99 ml	100 ml	10^{-6}	10^{-8}	62	$6.2x10^{9}$

TNTC = too numerous to count

Sample in Tween 80/Sucrose T=1 w	Volume of sample used (1)	Volume of diluent used (2)	Volume of sample (3)	Dilution factor of previous sample	Dilution factor for this sample	Number of colonies observed	CFU/ml
Dried Bacteria	1 ml	0 ml	1 ml	-	1	TNTC	-
Tube A	1 ml	99 ml	100 ml	1	10^{-2}	TNTC	-
Tube B	1 ml	99 ml	100 ml	10^{-2}	10^{-4}	TNTC	-
Tube C	1 ml	99 ml	100 ml	10^{-4}	10^{-6}	560	$6.2x10^{8}$

Sample in Tween 80/Sucrose T=2 w	Volume of sample used (1)	Volume of diluent used (2)	Volume of sample (3)	Dilution factor of previous sample	Dilution factor for this sample	Number of colonies observed	CFU/ml
Dried Bacteria	1 ml	0 ml	1 ml	-	1	TNTC	-
Tube A	1 ml	99 ml	100 ml	1	10^{-2}	TNTC	-
Tube B	1 ml	99 ml	100 ml	10^{-2}	10^{-4}	TNTC	-
Tube C	1 ml	99 ml	100 ml	10^{-4}	10^{-6}	20	$2.0x10^{7}$

Sample in Tween 80/Sucrose T=3 w	Volume of sample used (1)	Volume of diluent used (2)	Volume of sample (3)	Dilution factor of previous sample	Dilution factor for this sample	Number of colonies observed	CFU/ml
Dried Bacteria	1 ml	0 ml	1 ml	-	1	TNTC	-
Tube A	1 ml	99 ml	100 ml	1	10^{-2}	TNTC	-
Tube B	1 ml	99 ml	100 ml	10^{-2}	10^{-4}	180	$1.8x10^{6}$

Sample in Tween 80/Sucrose T=4 w	Volume of sample used (1)	Volume of diluent used (2)	Volume of sample (3)	Dilution factor of previous sample	Dilution factor for this sample	Number of colonies observed	CFU/ml
Dried Bacteria	1 ml	0 ml	1 ml	-	1	TNTC	-
Tube A	1 ml	99 ml	100 ml	1	10^{-2}	TNTC	-
Tube B	1 ml	99 ml	100 ml	10^{-2}	10^{-4}	54	$5.4x10^{5}$

Number of colonies after drying in Broth

Sample in Broth T=0	Volume of sample used (1)	Volume of diluent used (2)	Volume of sample (3)	Dilution factor of previous sample	Dilution factor for this sample	Number of colonies observed	CFU/ml
Dried Bacteria	1 ml	0 ml	1 ml	-	1	TNTC	-
Tube A	1 ml	99 ml	100 ml	1	10^{-2}	TNTC	-
Tube B	1 ml	99 ml	100 ml	10^{-2}	10^{-4}	TNTC	-
Tube C	1 ml	99 ml	100 ml	10^{-4}	10^{-6}	56	$5.6x10^{7}$

TNTC = too numerous to count

Sample in Broth T=1 w	Volume of sample used (1)	Volume of diluent used (2)	Volume of sample (3)	Dilution factor of previous sample	Dilution factor for this sample	Number of colonies observed	CFU/ml
Dried Bacteria	1 ml	0 ml	1 ml	-	1	TNTC	-
Tube A	1 ml	99 ml	100 ml	1	10^{-2}	720	$7.2x10^{4}$
Tube B	1 ml	99 ml	100 ml	10^{-2}	10^{-4}	0	0

Sample in Broth T=2 w	Volume of sample used (1)	Volume of diluent used (2)	Volume of sample (3)	Dilution factor of previous sample	Dilution factor for this sample	Number of colonies observed	CFU/ml
Dried Bacteria	1 ml	0 ml	1 ml	-	1	18	$1.8x10^{2}$
Tube A	1 ml	99 ml	100 ml	1	10^{-2}	0	0
Tube B	1 ml	99 ml	100 ml	10^{-2}	10^{-4}	0	0

Sample in Broth T=3 w	Volume of sample used (1)	Volume of diluent used (2)	Volume of sample (3)	Dilution factor of previous sample	Dilution factor for this sample	Number of colonies observed	CFU/ml
Dried Bacteria	1 ml	0 ml	1 ml	-	1	0	0
Tube A	1 ml	99 ml	100 ml	1	10^{-2}	0	0
Tube B	1 ml	99 ml	100 ml	10^{-2}	10^{-4}	0	0

Equipment

Incubator Shaker

Freeze Dryer

Centrifuge

UV-Vis Spectrophotometer

Laminar Flow

Microscope

Analytical Balance