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

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**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°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 kering 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°C tanpa vakum. Sel kering PEG pra-dirawat dan sel pembekuan kering sup pra-dirawat pembeukan 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 pembeukan 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 pembeukan 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 mengomersialkan beku-kering *P. putida*. 
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUPERVISOR’S DECLARATION</td>
<td>IV</td>
</tr>
<tr>
<td>STUDENT’S DECLARATION</td>
<td>V</td>
</tr>
<tr>
<td>Dedication</td>
<td>VI</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td>VII</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>VIII</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>IX</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>X</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>XII</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>XIII</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>XIV</td>
</tr>
<tr>
<td>1 INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Motivation and statement of problem</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Objectives</td>
<td>5</td>
</tr>
<tr>
<td>1.3 Scope of this research</td>
<td>5</td>
</tr>
<tr>
<td>1.4 Main contribution of this work</td>
<td>6</td>
</tr>
<tr>
<td>1.5 Organisation of this thesis</td>
<td>6</td>
</tr>
<tr>
<td>2 LITERATURE REVIEW</td>
<td>7</td>
</tr>
<tr>
<td>2.1 Overview</td>
<td>7</td>
</tr>
<tr>
<td>2.2 Introduction</td>
<td>7</td>
</tr>
<tr>
<td>2.3 Preservation of Bacteria</td>
<td>7</td>
</tr>
<tr>
<td>2.4 Freeze Drying</td>
<td>10</td>
</tr>
<tr>
<td>2.4.1 Principle of Freeze Drying</td>
<td>10</td>
</tr>
<tr>
<td>2.4.2 Stability of Freeze Dried Product</td>
<td>12</td>
</tr>
<tr>
<td>2.4.3 Contamination in Freeze Drying System</td>
<td>14</td>
</tr>
<tr>
<td>2.5 Bacteria Overview</td>
<td>15</td>
</tr>
<tr>
<td>2.5.1 Characteristic of Pseudomonas putida</td>
<td>15</td>
</tr>
<tr>
<td>2.5.2 Ability of Pseudomonas putida</td>
<td>17</td>
</tr>
<tr>
<td>2.6 Summary</td>
<td>18</td>
</tr>
<tr>
<td>3 MATERIALS AND METHODS</td>
<td>19</td>
</tr>
<tr>
<td>3.1 Overview</td>
<td>19</td>
</tr>
<tr>
<td>3.2 Introduction</td>
<td>19</td>
</tr>
<tr>
<td>3.3 Bacteria</td>
<td>19</td>
</tr>
<tr>
<td>3.4 Chemicals</td>
<td>19</td>
</tr>
<tr>
<td>3.5 Equipment</td>
<td>20</td>
</tr>
<tr>
<td>3.6 Methods of Medium Preparation</td>
<td>20</td>
</tr>
<tr>
<td>3.6.1 Preparation of Growth Medium</td>
<td>20</td>
</tr>
<tr>
<td>3.6.2 Preparation of Solid Medium</td>
<td>21</td>
</tr>
<tr>
<td>3.6.3 Preparation of Liquid Medium</td>
<td>21</td>
</tr>
<tr>
<td>3.7 Growth P. putida for Analysis Growth Curve</td>
<td>21</td>
</tr>
<tr>
<td>3.8 Determination of Cell Dry Weight</td>
<td>22</td>
</tr>
<tr>
<td>3.9 Method of Freeze drying</td>
<td>23</td>
</tr>
<tr>
<td>3.9.1 Freeze-dried Cells Preparation Using PEG</td>
<td>23</td>
</tr>
<tr>
<td>3.9.2 Freeze-dried Cells Preparation Using Tween 80 / Sucrose</td>
<td>23</td>
</tr>
<tr>
<td>3.9.3 Recovery of Freeze-dried Cells</td>
<td>24</td>
</tr>
<tr>
<td>3.10 Spread-Plate Method</td>
<td>24</td>
</tr>
<tr>
<td>3.11 Viable Plate Count</td>
<td>24</td>
</tr>
</tbody>
</table>
3.12 Determination of Residual Moisture Content ........................................ 25
3.13 Methods of Mercury Preparation .......................................................... 25
3.14 Determination of Cell Viability using Viable Plate Count ...................... 26
3.15 Determination of Mercury Reduction .................................................... 26
3.16 Experimental Ring .................................................................................. 27
3.17 Summary .................................................................................................... 29

4 FREEZE DRYING OF P. PUTIDA AND MERCURY REMOVAL ................. 30
  4.1 Overview .................................................................................................. 30
  4.2 Introduction ............................................................................................... 30
  4.3 Effect of lyoprotectant on the storage stability of freeze-dried P. putida .... 30
  4.4 Effect of freeze drying in growth of P. putida ........................................... 37
  4.5 Effect of Lyoprotectant in Residual Moisture Content ............................ 42
  4.6 Effect Freeze Drying in Mercury Reduction .......................................... 46
  4.7 Summary .................................................................................................... 49

5 CONCLUSION ............................................................................................... 50
  5.1 Conclusion ................................................................................................ 50
  5.2 Future work ............................................................................................... 50

REFERENCES .................................................................................................. 51
APPENDICES ..................................................................................................... 59
LIST OF FIGURES

Figure 2-1: *Pseudomonas putida* cell................................................................. 16
Figure 3-1: Process flow diagram of freeze drying process of

*Pseudomonas putida* ............................................................................................. 27
Figure 3-2: Process flow of mercury treatment using freeze-dried *P. putida* .......... 28
Figure 4-1: Viability rate of *P. putida* ................................................................. 33
Figure 4-2: Typical growth curve *P. putida* (1) ..................................................... 38
Figure 4-3: Typical growth curve *P. putida* (2) ..................................................... 38
Figure 4-4: Typical growth curve freeze-dried *P. putida* after 1 week (1) .......... 39
Figure 4-5: Typical growth curve freeze-dried *P. putida* after 1 week (2) .......... 39
Figure 4-6: Typical growth curve freeze-dried *P. putida* after 2 week (1) .......... 39
Figure 4-7: Typical growth curve freeze-dried *P. putida* after 2 week (2) .......... 39
Figure 4-8: Typical growth curve freeze-dried *P. putida* after 3 week (1) .......... 40
Figure 4-9: Typical growth curve freeze-dried *P. putida* after 3 week (2) .......... 41
Figure 4-10: Freeze dried *P. putida* in PEG ......................................................... 44
Figure 4-11: Freeze dried *P. putida* in Tween 80/Sucrose .................................... 45
LIST OF TABLES

Table 4-1: Viability *P. putida* using slow freezing ................................. 32
Table 4-2: Moisture contents of dry *P. putida* cell after 3 weeks storage ............ 43
Table 4-3: Mercury reduction using freeze-dried *P. putida* cells at time 0 .............. 46
Table 4-4: Mercury reduction using freeze-dried *P. putida* cells at time 3 weeks ...... 46
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>NCTC</td>
<td>National Collection of Type Cultures</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
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<tr>
<td>FBD</td>
<td>fluidized bed drying</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>DI</td>
<td>deionized water</td>
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<tr>
<td>ppb</td>
<td>part per billion</td>
</tr>
</tbody>
</table>

*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, additive 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 exceed 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°C which is claimed as the optimum growth condition of this strain (Fonseca *et al.*, 2011).

However, recent study also found that 37°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°C (Howard, 1956). This preservation method has a drawback in which the culture must maintain at the temperature below -70°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 \textit{P. putida}. This work also aims to find the maximum survival rate of dried \textit{P. putida} using freeze drying technique and obtain transportable dried \textit{P. putida} for laboratory used. The stability of \textit{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 \textbf{Objectives}

The following are the objectives of this research:

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

1.3 \textbf{Scope of this research}

The following are the scope of this research:

- Determine the growth curve of freeze-dried of \textit{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 \textit{P. putida} strain after freeze dry using spread-plate method and calculate colony forming unit (CFU)
- Study of storage stability of freeze dried \textit{P. putida} strain by storing dried \textit{P. putida} obtain after storage to 4 weeks at -4°C in chiller and using spread-plate method to calculate colony forming unit (CFU)
- Find the residual moisture content in the freeze-dried \textit{P. putida} in different lyoprotectant using Moisture Analyser
- Find the mercury reduction from freeze-dried \textit{P. putida} and fresh \textit{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°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°C with short residence time to dry Lactobacillus rhamnosus GG. The result showed that at store at 4°C and 15°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, Lactobacillus 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 drying 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°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 et 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 (Palmfедt, 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°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°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°C.

Commonly, bacteria obtain from American Type Culture Collection (ATCC) is stored at temperature range between 2°C to 8°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 al., 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°C. Store at temperature at 8°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 dried bacteria at 4°C showed better viability than store at 16°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 (Palmfiedt 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, recombinde 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).

![Image of Pseudomonas putida cell](image)

**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°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