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Assessing Storage of 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 4oC without vacuum. Polyethylene glycol (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*.

Keywords*—Pseudomonas putida*, freeze-dry, PEG, Tween80/Sucrose, mercury, cell viability.

I. Introduction

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 [1].

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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 [2], [3] and petrochemical industry [4]. 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. 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 [4].

Bacteria become one of green solution to overcome many industrial problems and enhance the performance of the process in industry. 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 [5]. 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 [6].

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} [2]-[4]. *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 [7]. The use of *P. putida* is considerably safer than other bacteria such as *Escherichia coli* and *Pseudomonas Aeruginosa.* The reason of that is non-pathogenic property of *P. putida*, hence the use of the bacteria pronounces less negative effect for both living creature and environment.

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 [8]. 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.

Conveniently, 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 [9]. Gram-negative bacteria often show lower survival rates than gram-positive bacteria [10], [11]. 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 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 is suitable for drying susceptible bacteria [12].

There are fewer studies about the parameter that may influence *P. putida* tolerance in freeze drying. The purpose of this study is to understand the parameters that effecting freeze drying tolerance of *P. putida*. This work aims to maximize the survival rate of dried *P. putida* using freeze drying technique and obtain transportable dried *P. putida* in laboratory scale. The stability of *P. putida* towards freeze drying is studied for future used. This works also will provides the data for second consumers of freeze dried bacteria that want to preserve the bacteria.

II. Materials and Methods

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 [13].

A. Bacteria

Bacteria *P. putida* was obtained from BIOREV SDN BHD and has been growth by previous student in agar medium. The type of bacteria used in this study is *P. putida*. Before undergo the experiment, it is necessary to ensure that the bacteria are pure *P. putida*. Microscope was used to identify the type of bacteria in agar. Only pure *P. putida* was used in this experiment.

B. Chemicals

Nutrient Agar and Nutrient Broth were purchased from Merck (Germany). Sucrose, Tween 80, and Polyethylene glycol (PEG) 1000 were obtained from FKKSA laboratory. Mercury (II) chloride was obtained from Merck.

C. Solution Preparation

Distilled water (DI) was used to prepare the agar and broth solution at room temperature and followed by autoclave at 121°C for 15 minutes. DI water was used to dissolve sucrose, tween 80 and PEG. Sterilized DI water is used to dilute mercury stock solution to concentration of 1 ppm at room temperature.

D. Microbial Culture Preparation

The cell culture was prepared by inoculating a single colony from agar plate into 20 ml broth in universal bottle. The cells were incubated for 24 h, 180 rpm in 30°C. The cells were removed into 180 ml of fresh broth for another 16 h at the same condition.

Cell were harvested by centrifuge (EPPENDORF 5810 R) at 5000xg for 5 minute at room temperature and then washed three times with phosphate buffer. The cells concentrations were adjusted to absorbance near 2.3 (A600nm) [14].

E. Freeze-Dried Cells Preparation by Using PEG

One hundred milliliters 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 [8]. 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°C.

F. Freeze-Dried Cells Preparation using Tween 80/sucrose

A 500 ml of 50% sucrose stock solution was prepared using DI water and was sterilized by passing the solution through a 0.22-µm filter. The sucrose stock solution was exposed to UV light for 5 min. Twenty five milliliters 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 [8]. 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°C.

G. Cell Viability Test

Freeze dried were rehydrate by shaking dried cells with broth in incubator at 180 rpm, 30°C for 24 h. After 24 h, the cells were shaking with 180 ml of broth at 180 rpm, 30°C for 24 h. Cell viability was evaluated based on the petri–dish plate count standard method. A drop of cells and a drop of sterile DI water were spread into an agar plate. Serial dilution was performed to ensure that cells were growth in colony, therefore the number of colony in the plate were able to read.

H.Data Analysis

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 to [15]:

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

Mercury analysis was performed using mercury analyzer. The efficiency of mercury removal was calculated according to [4]:

$$\%\ Mercury\ Removal = \frac{(Initial - Final) concentration\ of\ mercury}{Initial\ concentration\ of\ mercury}\ x\ 100\%$$

where, the initial concentration of mercury is 1000 ppb. The amount of moisture content was determined using moisture analyzer.

III. Results and Discussion

A. Cell Viability Study

The effects of freeze drying and storage condition in *P. putida* were evaluated using plate counts 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.

The result in Table I reveals that the highest viability immediately after freeze drying (time 0) was obtained from PEG pre-treated freeze-dried *P. putida* cells. The result indicates that lyoprotectant PEG could give an effective protection for frozen cells when undergoing freeze-dried process [16], [17]. 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 [8]. In contrast, freeze-dried *P. putida* cells prepared in Tween 80/Sucrose were able to maintain low decrement along the time during storage in 4ºC. Tween80/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 4ºC [8], [18]. Sucrose allows the cells to survive during the storage time mainly because sucrose is not toxic compared to PEG [19]. Sucrose was proved as a good cryoprotectant and even suitable for human oocyte cryopreservation [20].

TABLE I
VIABILITY *P. PUTIDA* USING SLOW FREEZING

WEEK	CFU	
	Quantity	Tween 80/Sucrose
0	6.48E+09	6.24E+09
1	8.80E+05	2.80E+06
2	6.90E+03	2.00E+06
3	4.10E+01	1.80E+05
Control	7.90E+09	

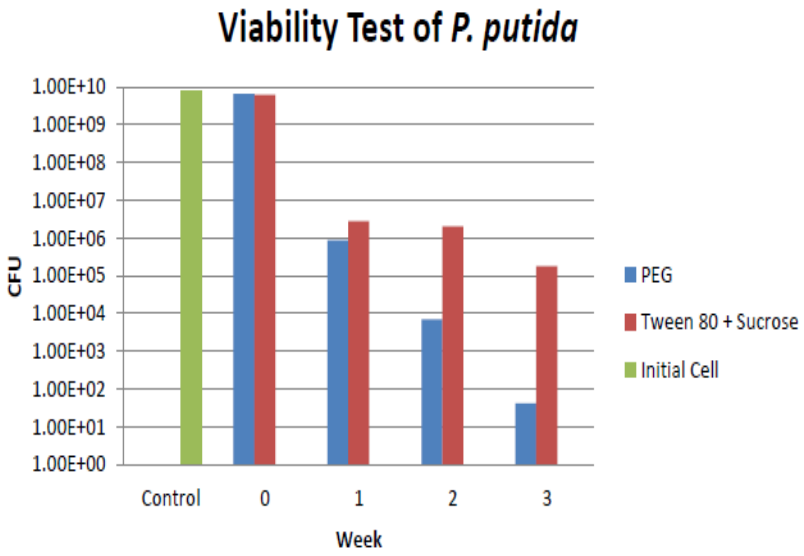


Fig. 1 Viability test of *P. putida*

Sucrose is able to enhance the protection of the cell during desiccation by replacing the water around polar residue within macromolecular structure. When sucrose forming hydrogen bonding, it maintain the lipid in the liquid crystalline phase at room temperature and lowering the temperature of the membrane phase transition [21]. 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 [22]. The viability of the cells was improved by addition of tween 80 in the solution. is Tween 80 solution yielded a gel-like residue and insoluble

in water It has very little anti-bacteria activity and prevents damage to cytoplasmic [8].

B. Moisture Content

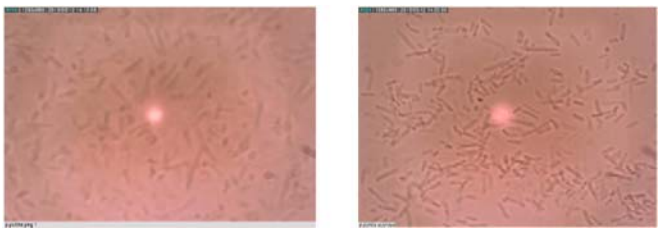
In this experiment, all the samples were run at the same time in one freeze drying machine. The difference of moisture content shows that moisture content is depending on the type of lyoprotectant used [21].

TABLE II
MOISTURE CONTENTS OF DRY *P. PUTIDA*

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

The results in Table II prove that the water activity 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. Based on the result, lowering the moisture content had increased 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 [23].

Under the microscope, it seemed that there is no visual difference between PEG pre-treated freeze-dried *P. putida* cells and Tween80/Sucrose pre-treated freeze-dried *P. putida* cells as shown in Fig. 2. Without any rehydration process the cells movement were monitored. For both samples, *P. putida* was not shown any physical movement, but a few of the cells were less active movement after thawing to room temperature.



(a) (b)

Fig. 2 (a) Freeze dried *P. putida* in PEG and (b) in Tween 80/Sucrose (right)

C. Mercury Reduction

Mercury reduction testing was performed using freeze-dried *P. putida* cells taken from the sample that has been stored for 3 weeks to relate the effect of lyoprotectant with the performance of *P. putida* in removing mercury as shown in Table III. Both PEG and Tween80/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 were higher during 3 weeks storage.

TABLE III
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	0.0001 ppb	99.99 %

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 *Pseudomona*s 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 merT, merP and merA are involved in mercury reduction for gram-negative bacteria [24]. 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 [25].

IV. CONCLUSION

A good protectant attribute to the high CFU. It also depends on the drying purpose which is high CFU immediately after freeze drying or high CFU after storage time. The result shows that *P. putida* freeze dry in tween80/sucrose solution was quite resistant to freeze drying which is useful from commercial viewpoint. The moisture content in tween 80/sucrose solution was the lowest. The maximum viability is essential for mercury reduction. The highest mercury reduction (25.03%) after 3 weeks storage was from tween80/sucrose freeze-dried *P. putida* cells. In contrast, 56.78% mercury reduction was obtained from PEG pre-suspended cells after immediate rehydration. PEG freeze-dried *P. putida* cells have higher moisture content. Hence, has a low cell viability and low mercury reduction after storage time. This study proved that Tween80/sucrose is a suitable freeze dry lyoprotectant for *P. putida*.

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