Pertanika J. Trop. Agric. Sc. 41 (3): 1003 - 1020 (2018)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Characterisation and Effect of Protectants on Preservation of *Bacillus methylotrophicus* UPMC 1166 Isolated from Liquid Biofertiliser

Musliyana Mansor[1], Tan Geok Hun[1,2*], Nor Umaira Abu Asan[1] and Raha Abdul Rahim[1,3]

[1]Microbial Culture Collection Unit, Institute of Bioscience, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia
[2]Faculty of Agriculture, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia
[3]Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia (UPM), 43400 Serdang, Selangor, Malaysia

ABSTRACT

UPMC 1166 bacterial strain was isolated from SG1 liquid biofertiliser and has proven to have an ability to produce indole acetic acid (IAA) and fixed atmosphere nitrogen. The objectives of this research were to characterise UPMC 1166 isolate, to determine the growth kinetics, and effect of different protectants for preservation. UPMC 1166 were characterised phenotypically and genotypically. The growth kinetics was determined using viable cell count and optical density methods. The effect of different protectants on the viability of UPMC 1166, subjected to freeze–drying and freezing at −80°C, was studied. UPMC 1166 belonged to Gram-positive bacteria (with the size of 0.49 – 0.52 × 1.56 – 2.34 µm), catalase positive, rod-shaped with the arrangement of single or paired bacilli, endospore forming and creamy white pigmentation colonies. Based on API biochemical test kit confirmed that UPMC 1166 was under the *Bacillus* genus. From BLAST, UPMC 1166 showed pairwise sequence similarity range of 99.0% and is closely related to *Bacillus siamensis*, *Bacillus amyloliquefaciens, Bacillus vallismortis*, *Bacillus subtilis, Bacillus mojavensis*, and *Bacillus atrophaeus.* 16S rRNA gene sequence used for phylogenetic tree analysis suggested that UPMC 1166 is *Bacillus methylotrophicus*. To obtain the maximum viability after preservation, it is important to harvest cells during the late logarithmic phase of growth and to choose a suitable protective agent. UPMC 1166 needs approximately 16

ARTICLE INFO

Article history:
Received: 26 October 2017
Accepted: 08 February 2018
Published: 29 August 2018

E-mail addresses:
musliyana@upm.edu.my/musliyana@gmail.com (Musliyana Mansor)
geok_hun@upm.edu.my (Tan Geok Hun)
nor_umaira@upm.edu.my (Nor Umaira Abu Asan)
raha@upm.edu.my (Raha Abdul Rahim)
* Corresponding author

ISSN: 1511-3701
e-ISSN: 2231-8542

h to reach the end of the logarithmic phase; consisting of a lag phase up to 2 h and the logarithmic growth that lasted up to 14 h before entering the stationary phase. During freeze–drying, the maximum protection for UPMC 1166 was achieved by using 10% skimmed milk with 1% sodium glutamate, and 5% trehalose. Maximum protection of cells during −80°C cryopreservation was achieved with 10% dimethyl sulfoxide (DMSO). A suitable selection of protectant seemed to be important to acquire maximum cells viability for long-term preservation. Resistance potential of bacterial strains toward preservation procedures is useful from a research and commercial point of view.

Keywords: Bacillus methylotrophicus, biofertiliser, freeze–drying, freezing, gram-positive bacteria, growth kinetics, logarithmic phase, preservation

INTRODUCTION

Farmers used organic fertilizers as nutrient supply before the introduction of chemical fertilizers. Due to the enhancement and rapid productivity of plant growth, farmers have depended on chemical fertilizers since World War II (Ward, 2009). Besides the important role of sustaining yields and ensuring level of adequate profit, a long-term application of chemical fertilizer unfortunately causes food safety issues, human health, economical factor, and environmental damages such as pollution (water, soil, and air), soil infertility, and disease outbreak (Othman & Jafari, 2014; Patil, Patil, & Pathade, 2012). Recently, the awareness regarding the safety issue of utilising chemical fertilizers has increased. Biofertiliser consists of living beneficial microorganisms that enhance plant growth by supplying phytohormones or increasing primary nutrients, and also acting as a complementary source for chemical fertilizers (Naher, Panhwar, Othman, Ismail, & Berahim, 2016). With the presence of natural microorganism, biofertiliser provides the supplements required by plants and serves to increase soil quality (Vessey, 2003).

Plant growth promoting rhizobacteria (PGPR) are characterized as a growth enhancer and plant roots colonist (Zahir, Arshad, & Frankenberge, 2003). The bacteria species of *Bacillus, Enterobacter, Pseudomonas*, and *Erwinia* are the largest group of PGPR (Grobelak, Napora, & Kacprzak, 2015). *Azospirillium, Azotobacter, Burkolderia, Rhizobium*, and *Serratia* are some other bacteria species that have successfully been commercialized (Chauhan, Bagyaraj, Selvakumar, & Sundaram, 2015). Other types of beneficial microorganisms used frequently as biofertiliser element are nitrogen fixers, and phosphorus solubiliser and potassium solubiliser, or a combination of moulds or fungi (Mohammadi & Sohrabi, 2012).

Methylotropic is the characteristic of bacteria that enables plants to grow in C1 compounds (such as methanol, methane, methylated amines). *Bacillus methylotrophicus* CBMB205^T is categorised as a methylotropic bacterium and has the ability to use methanol as a carbon source (Madhaiyan, Poonguzhali, Kwon, & Sa,

2010). Patent deposit strain, called *Bacillus methylotrophicus* UTM401 (CGMCC 5927), was isolated from sewage sludge (Anonymous 1, 2012). The strain was inoculated into an organic waste, fermented and used as biofertiliser that is environmental friendly, stable in viability, incurred low-cost production and showed significant yield on Chinese cabbage (Anonymous 1, 2012).

In microbiology, preservation techniques (whether it is short- or long-term preservation methods) are imperative. Different type of microorganisms was applied with varying methods of preservation to ensure the guarantee of optimal viability, survival, purity and storage of the strains (WFCC Guidelines, 2010). Usually, two different methods should be implemented for each strain for storage to minimise the possibility of strain loss. Some methods of storage to help decrease the risks of genetic change are: (1) freeze–drying; (2) ultra-low temperature (liquid nitrogen); (3) freezers (temperature of −140°C or lower) (WFCC Guidelines, 2010). During freezing, storage and thawing process of preservation, cryoprotectants (protective compound) act as a protection to microorganisms that could remove most of the multiple damage factors (Tedeschi & De Paoli, 2011; Uzunova-Doneva & Donev, 2005).

This experiment was carried out to characterize UPMC 1166 isolate, to determine the growth kinetics, and effect of different protectants for preservation. UPMC 1166 was isolated from a liquid biofertiliser product that was proven to have the ability as a nitrogen fixer and produce indole acetic acid (IAA) (Nazaruddin, 2014). Gram stain and API biochemical test kit were applied for characterisation process phenotypically. 16S ribosomal ribonucleic acid (rRNA) gene sequencing and construction of phylogenetic tree were used in order to identify and confirm the species of the strain genotypically. Pattern estimation of growth was obtained from the growth kinetics. The bacterial cells were in its most ideal state to be harvested during the late logarithmic phase before preservation, to acquire optimal cell viability upon recovery (ATCC Bacterial Culture Guide, 2015). The effect of various protectants on viability of UPMC 1166, subjected to freeze–drying and cryopreservation at −80°C, was studied.

METHODS

Phenotypic Characterisation

Bacterial isolate UPMC 1166 was isolated from SG1 liquid biofertiliser from Agricultural Department of Kelantan and the isolate was chosen for further study based on the ability to produce IAA and fixed nitrogen (Nazaruddin, 2014). During the experiments, the isolate was incubated for 18 to 24 hours at 30°C.

The colony morphology was studied by culturing the isolate on nutrient agar (Merck, Germany) and incubated at 30°C. Gram stain was performed by using a Gram staining kit (Merck, Germany) and the images were observed using light microscope (Leica, Germany). The observation of colony morphology and Gram stain was done after 24, 48, and 72 h of incubation. The

biochemical characteristics of the *Bacillus* genus were tested by using combination of API 50CH and API 20E system test kit (BioMérieux SA, France). The catalase activity was determined by dropping 40 to 50 µL of 3% hydrogen peroxide (H_2O_2) (Merck, Germany) onto the cultures and assessing bubble production was observed.

Identification of Bacterial Isolate using 16S rRNA Gene Sequencing

UPMC 1166 was cultured overnight in nutrient broth (Merck, Germany) for 18 to 24 h at 30°C and the genomic DNA was extracted using standard protocols with some modifications (Sambrook, Russell, & Maniatis, 2001). The 16S rRNA gene sequence was amplified from the genomic DNA by polymerase chain reaction (PCR) using thermal cycler (peqSTAR, Germany). The PCR for total volume of 25 µL per reaction was carried out as follows: deionized water (14 µL); 10× reaction buffer containing 15 mM MgCl2 (Lucigen, USA) (2.5 µL); 2.5 mM dNTP mix PCR Grade (Lucigen, USA)(2.0 µL); 100 pmol/µL forward universal primers (0.25 µL); 100 pmol/µL reverse universal primers (0.25 µL); 5 U/mL Taq Polymerase (Lucigen, USA) (0.5 µL) and genomic DNA as template (50 to 200 ng)(5 µL). The PCR was carried out using forward primer 8F (5′ GAG TTT GAT CCT GCT CAG 3′) and reverse primer 1492R (5′ GTT ACC TTG TTA CGA CTT 3′) (Tan & Nazaruddin, 2015). The thermal cycling conditions involved preheating at 94°C for 2 min, followed by denaturation (35 cycles at 94°C for 30 s); annealing (52°C for 30 s); extension (72°C for 1 min), and final elongation (72°C for 10 min). The PCR product was purified using HiYield™ Gel/PCR DNA Mini Kit (Real Biotech, Taiwan) and sent for sequencing (1st BASE Laboratories Sdn. Bhd.). Same forward and reverse primers were used for sequencing.

The sequences were aligned using BioEdit 7.2.4 (Hall, 1999) and ClustalW (Thompson, Higgins, & Gibson, 1994) software. The aligned partial 16S rRNA gene sequence (1403 bp) was compared with the genes from Basic Local Alignment Search Tool (BLAST), NCBI Genbank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The multiple alignments and phylogenetic tree were done using Mega 7.0 (Kumar Stecher, & Tamura, 2016). The phylogenetic tree was constructed with the following setting: analysis by standard neighbour-joining method (Saitou & Nei, 1987) and bootstraps of 1,000 replicates (Felsenstein, 1985).

Growth Kinetics

The growth kinetics were obtained by viable cell counting in colony forming unit (CFU) and optical density (OD) measurement of liquid culture at 600 nm (OD_{600}). The experiments were done using protocols by Pepper and Gerba (2004) with some modifications.

A loop of UPMC 1166 bacterial strain was aseptically transferred to 10-mL nutrient broth. The culture was incubated overnight at 30°C (Infors HT, Switzerland). Sixty microliters of the overnight culture was

inoculated in the flask containing 60 mL of nutrient broth. Experiment was started from 0 h with 1 mL of culture was used for the measurement of OD of the bacteria at 600 nm using spectrophotometer (CE 1021 Cecil, UK), and another 100 µL was removed aseptically for serial dilution (viable cell counting). The flask was then incubated with constant shaking at 200 rpm at 30°C. Every 2 h interval and up to 20 h, 100 µL of the culture suspension was removed for viable cell counting (CFU/mL) and 1 mL of culture was used for OD_{600}. As a control, 60 mL of nutrient broth without inoculation was also incubated.

For serial dilution, sterilized water was used in this study (Miyamoto-Shinohara, Sukenobe, Imaizumi, & Nakahara, 2008). Hundred microliters of bacterial suspension was transferred to 900 µL of sterilized water to obtain a dilution of 10^{-1}. Serial dilution was made starting from 10^{-1} to 10^{-6}, mixed well and 100 µL of each dilution was spread using hockey stick on the nutrient agar (Merck, Germany) in triplicates. All the nutrient agar plates were incubated at 30°C for overnight before counting. Regarded as being statistically reliable, the colonies between a range of 30 and 300 on the agar plate were counted (Hogg, 2005). By knowing the number of colonies, dilution factor and volume plated, the calculation of CFU/mL was done using the following equation (Ravishankar, 2004):

$$\text{CFU/mL} = \frac{\text{Number of colonies}}{\text{Dilution factor} \times \text{Volume plated}}$$

Protectants

The protectants used for this experiment can be referred in Table 1. Nutrient broth (Merck, Germany) was used as a control. Thirty percentage of stock solutions were prepared for all protectants [except for 10% (w/v) skimmed milk with 1% (w/v) sodium glutamate] and later were diluted with nutrient broth to obtain the required working concentration.

Table 1
Protectants used in this experiment

Group of Protectants	Protectants
Sugar	Trehalose (Sigma, USA), sucrose (Fisher Chemical, UK), glucose (Merck, Germany) and fructose (Sigma, USA). 5%, 10%, and 15% (w/v) of sugar solution were prepared for each
Combined protectants	10% (w/v) skimmed milk (Merck, Germany) added with 1% (w/v) sodium glutamate (Merck, Germany)
Triols	10% and 15% (v/v) glycerol (Fisher Chemical, UK)
Sulphoxides	5% and 10% (v/v) dimethylsulphoxide (DMSO) (QReC, New Zealand)

Sample Preparation

Sample preparation was carried out according to manual by ATCC Bacterial Culture Guide (2015) with some modifications. UPMC 1166 was grown in 60 mL nutrient broth at 30°C and harvested after 16 h of incubation. Three milliliters of culture suspensions were transferred into Falcon tubes (Fisher Scientific, USA) and centrifuged at 4000

rpm for 15 min at 10°C (5415 R Centrifuge, Eppendorf, Germany). Supernatants were discarded, and the pellet was suspended with the protectants.

Freeze–drying Technique

Freeze-drying was carried out according to protocol by Microbial Culture Collection Unit UPM (2009) and freeze-dryer (Virtis, USA) manufacturer's manual with some modifications. Two-hundred microliters of bacterial suspensions were placed into 1 mL pre-scored ampoules (Wheaton, USA). The ampoules were transferred to −20°C freezer (Liebherr, Germany) for 1 h and then to −80°C freezer (Sanyo, Japan) for another hour. The primary and secondary freeze–drying using freeze-dryer was carried out with a condenser at a temperature of −50°C and vacuum condition at 100 mTorr. The ampoules were sealed to maintain the vacuum condition. Tesla coil spark tester (Electro-Technic Products Inc, USA) was used to check the ampoules for vacuum leakage. The preserved cultures were recovered after 2 weeks of preservation by adding 0.2 mL of nutrient broth. Mixed well and let it stand for 15 min at room temperature to equilibrate. The suspensions were serially diluted with sterilized water and spread on nutrient agar plates.

Freezing at −80°C Technique

Cryopreservation technique was performed according to a manual by ATCC Bacterial Culture Guide (2015) with some modifications**.** Eight-hundred microliters of bacterial suspensions were placed into 1.8 mL cryotubes (Nalgene, USA). Caps were sealed with Parafilm and incubated at room temperature to equilibrate in the protectants for a minimum period of 15 min but no longer than 40 min. The cryotubes were frozen at −20°C freezer for 1 h before transferring to −80°C deep freezers. The recovery was made after 2 weeks of preservation. The cryotubes were warmed as quick as possible by thawing at water bath (30°C) for approximately 2 min or until all samples were melted. The suspensions were serially diluted with sterilized water and spread on nutrient agar plates.

Survival Rate Determination

Bacterial viable cell count was conducted for bacterial suspensions before and after preservation. Change in viability after preservation is expressed as survival rate. The survival rate was calculated as follows (Peiren et al., 2015):

$$\text{Survival rate (\%)} = \frac{\text{Log (CFU/mL) after preservation}}{\text{Log (CFU/mL) before preservation}} \times 100$$

RESULTS AND DISCUSSION

Phenotypic Characterisation

UPMC 1166 strain was deposited at Microbial Culture Collection Unit (UNiCC), Institute of Bioscience, Universiti Putra Malaysia. The morphology of UPMC 1166 was observed after 24, 48, and 72 h of incubation at 30°C. UPMC 1166 is a

Gram-positive bacterium with the size of 0.49 – 0.52 × 1.56 – 2.34 µm, rod-shaped with single or in pair bacilli arrangement (Figure 1). After 24 h of incubation, the size of the colonies were 3 to 5 mm. However, it expanded to 5 to 7 mm after 48 h of incubation. The isolate showed the formation of endospores during the 48 h observation. The colonies of UPMC 1166 had creamy white pigmentation, raised elevation, irregular form, bumpy and shiny appearance, undulate margin, translucent and gummy structure. UPMC 1166 showed production of bubbles during application of H_2O_2 indicating catalase positive. Morphological characteristics of UPMC 1166 such as Gram-positive bacterium, rod-shaped cell, catalase positive and exhibited with one endospore in one cell, showed similarities with characteristics of *Bacillus* species (Logan & Vos, 2015). The morphology of colonies for both within and between *Bacillus* species, surely with strong effect of medium component and other incubation environment, show a very broad range (Logan & Vos, 2015).

Biochemical test results from API 50 CHB and API 20E kit obtained from APIWEB (https://apiweb.biomerieux.com) showed a very good identification result up to *Bacillus* genus. UPMC 1166 was identified (closest matched species) as *Bacillus subtilis/amyloliquefaciens* with 99.9% similarity. Some of the biochemical test results for UPMC 1166 were compared with *Bacillus methylotrophicus* CBMB205^T (Madhaiyan et al., 2010) in Table 2.

Molecular Identification based on 16S rRNA Gene Sequence

From the BLAST results, UPMC 1166 was closely related to *Bacillus siamensis*, *Bacillus amyloliquefaciens, Bacillus vallismortis*, *Bacillus subtilis, Bacillus*

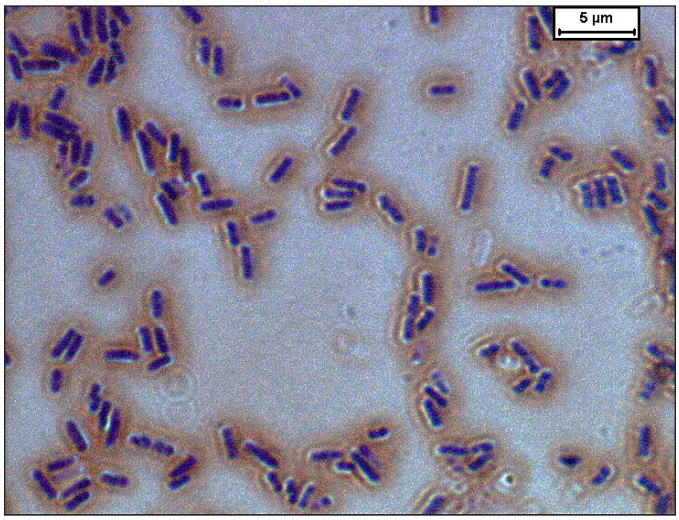

Figure 1. Gram-stain image of UPMC 1166. 24 h of incubation. 100× magnifications. Gram-positive, small size (0.49 – 0.52 × 1.56 – 2.34 µm), rod shape with single or in pair bacilli arrangement

mojavensis, and *Bacillus atrophaeus* showing pairwise sequence similarities up to 99.0%. UPMC 1166 results also revealed that the isolate was phylogenetically related to the genus *Bacillus* and showed a close relationship with *Bacillus methylotrophicus* (Figure 2). UPMC 1166 was grouped together and monophyletic with *Bacillus methylotrophicus* CBMB205^T and *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42^T with bootstrap value of 55%. *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42^T was recategorised as *Bacillus methylotrophicus* by Dunlap, Kim, Kwon and Rooney (2015) because results of phenotypic and genotypic analyses indicated that the strain showed highly similarity with *Bacillus methylotrophicus* CBMB205^T.

In this study, combination of API 50 CHB and API 20E biochemical test kit did not give the same species outcomes as the phylogenetic tree analysis, but produced good results up to the *Bacillus* genus level. This exhibited that the API system was not

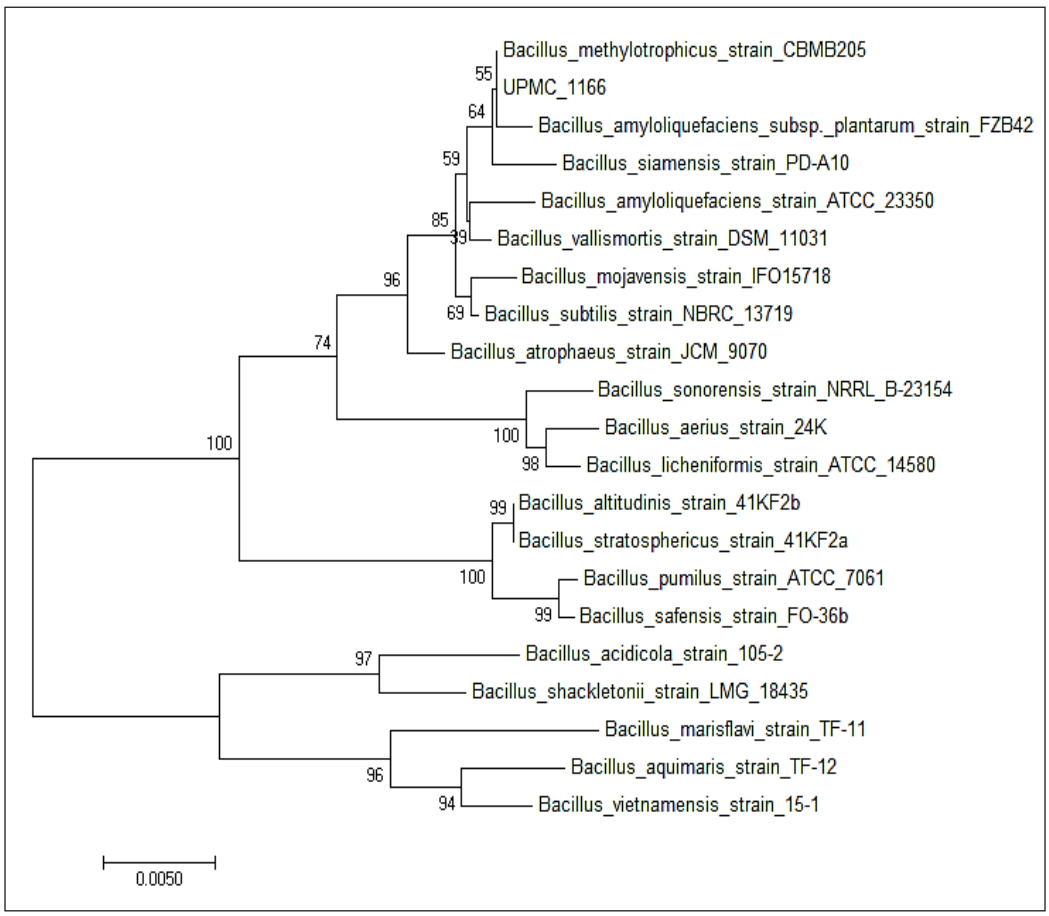

Figure 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequence comparison for UPMC 1166 using Mega 7.0. Bar is 0.005 nucleotide substitution rate per site

a dependable test for characterisation up to species level. The commercial test kit databases that carried out for identification of bacterial species was limited, relying on the number of strains in the database and also the variety of phenotype of strains tested (Janda & Abbott, 2002). 16S rRNA gene sequencing has different advantages compared to phenotypic characterisation procedures such as results are not limited to a particular group of bacteria because public online database comprises the whole spectrum of phylogenetic diversity and novel species can be allocated to a group of related species (Bosshard et al., 2006).

Characteristics Comparison of Strain UPMC 1166 and *Bacillus methylotrophicus* CBMB205^T

The differential characteristic of strain UPMC 1166 and *Bacillus methylotrophicus* CBMB205^T are summarized in Table 2. From the previous research, the species of *Bacillus methylotrophicus* CBMB205^T was reported by Madhaiyan et al. (2010) and isolated from rice rhizoplane. UPMC 1166 and *Bacillus methylotrophicus* CBMB205^T showed similarity in some of the characteristics such as Gram-positive bacteria, arrangement that single or in-pair, endospore-forming, colonies with creamy

Table 2
Differential characteristics of isolate UPMC 1166 and Bacillus methylotrophicus *CBMB205^T*

Characteristic	UPMC 1166	*Bacillus methylotrophicus* CBMB205^T (Madhaiyan et al., 2010)
Gram results	Positive	Positive
Shape	Rods (0.49 – 0.52 × 1.56 – 2.34 μm)	Rods (0.63 - 0.64 × 1.8 – 2.7 μM)
Arrangement	Singly or in pairs	Singly or in pairs
Endospore-forming	Yes	Yes
Pigmentation	Creamy white	Creamy white
Motility	nd	Yes
Some biochemical test results:		
L-Arabinose	–	+
D-Mannose	–	+
L-Rhamnose	–	+
D-Sorbitol	–	+
N-Acetylglucosamine	–	+
Potassium gluconate	–	+
Trisodium citrate	–	+
Nitrate reduction to nitrite	–	+

Note. Both isolates were positive for catalase production. In biochemical tests, both taxa were positive for glycerol, D-ribose, D-glucose, inositol, D-mannitol, esculin ferric citrate, D-cellobiose, glycogen and gelatin. Both taxa were negative for L-fucose, potassium 2-ketogluconate, potassium 5-ketogluconate, arginine dihydrolase, urease and indole production. Data of UPMC 1166 were obtained from this research. +, growth; -ve, no growth; nd, no data.

white pigmentation, catalase positive and producing IAA. However, *Bacillus methylotrophicus* CBMB205^T produced a negligible amount of IAA (Madhaiyan et al., 2010).

Future work can be done to reconfirm the characteristic of the strains and obtain further information on other abilities such as morphology observation on motility, methanol-utilising ability, antagonistic activity, and so on.

Growth Kinetics

The growth kinetics results for UPMC 1166 were compared between viable cell quantification and OD_{600}. Both methods yielded similarity growth kinetics (Figure 3). The growth kinetics showed the lag phase up to 2 h, and the logarithmic growth lasted up to 14 h, which followed by entering the stationary phase. UPMC 1166 has the tendency to form clumps at 10th hour of incubation. Bacterial cells harvested during the late logarithmic phase were important to acquire optimal cell viability consequent to recovery after preservation process (ATCC Bacterial Culture Guide, 2015). During late logarithmic phase and entering early stationary phase, older cells grown in this phase would activate a starvation response that was an initiation of internal protection mechanism system against stress and develop an ability to survive under extremely critical environment (Morgan, Herman, White, & Vesey, 2006; Pletnev, Osterman, Sergiev, Bogdanov, & Dontsova, 2015).

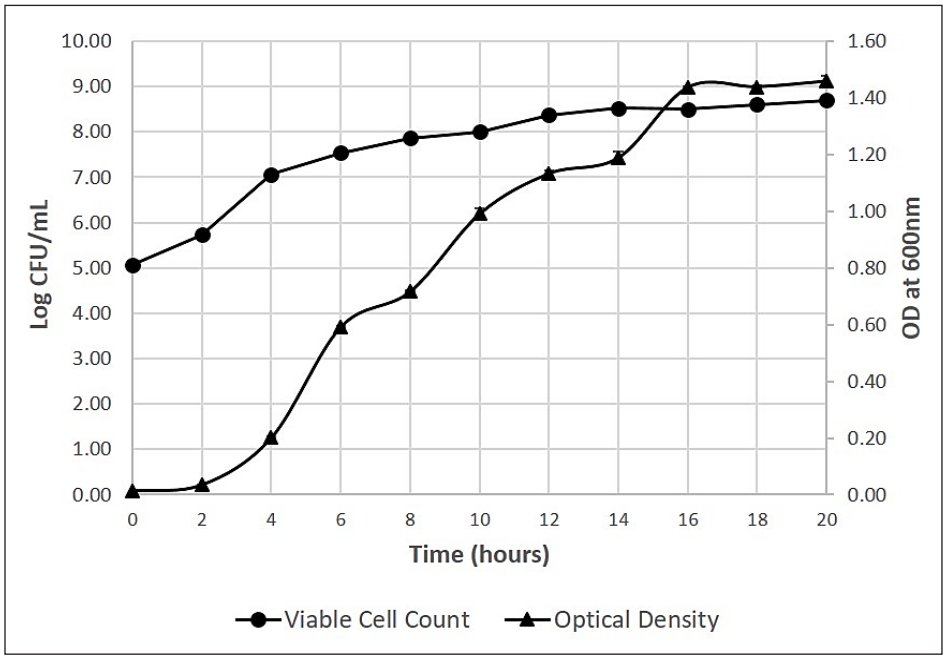

Figure 3. Bacillus methylotrophicus UPMC 1166 growth kinetics determined by viable cell count and optical density. The test was carried out using three replicates

Viable Cell Count before Preservation Process

For freeze–drying and freezing of UPMC 1166, 17 various protectants with different concentrations were used in this study. During the late logarithmic phase, the cell viability was approximately at 1×10^8 CFU/mL (Figure 3). Before preservation, the viable cell counts were carried out to obtain the results of cells' viability of the suspended cultures (Figures 4 and 5). The control (nutrient broth) showed the result of 8.31 log CFU/mL. All protectants showed cell viability of more than 8.0 log CFU/mL except for 5% (w/v) glucose, 15% (w/v) glucose, 5% (w/v) fructose, 10% (w/v) fructose, and 15% (w/v) fructose. Lowest cell viability with 7.19 log CFU/mL was obtained by 15% (w/v) fructose, while 15% (v/v) glycerol displayed the highest cell viability of 8.32 log CFU/mL. Before preservation, the cells recovery was greater if the initial number of cells is higher (Tedeschi & Paoli, 2011). Most of UPMC 1166 with mixture of protectants obtained viability of more than 8.0 log CFU/mL before preservation was possibly related to the ability of bacterial cell wall adapting to the protectants.

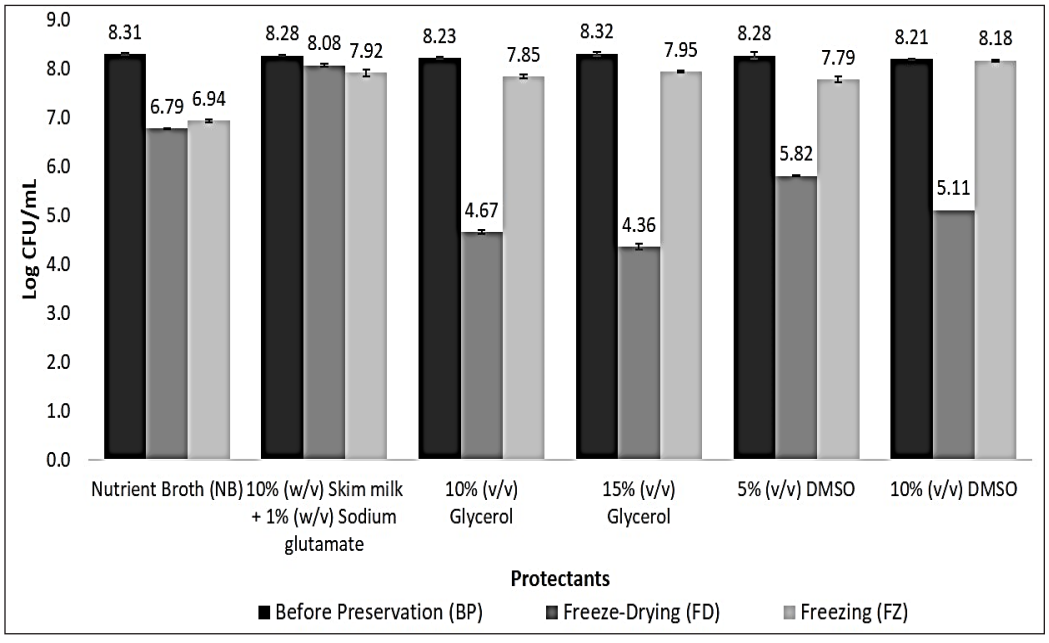

Figure 4. Effect of various protectants [10% (w/v) skimmed milk with 1% (w/v) sodium glutamate, glycerol and DMSO] on viability of *Bacillus methylotrophicus* UPMC 1166 preserved by freeze-drying and freezing technique. The test was done using three replicates

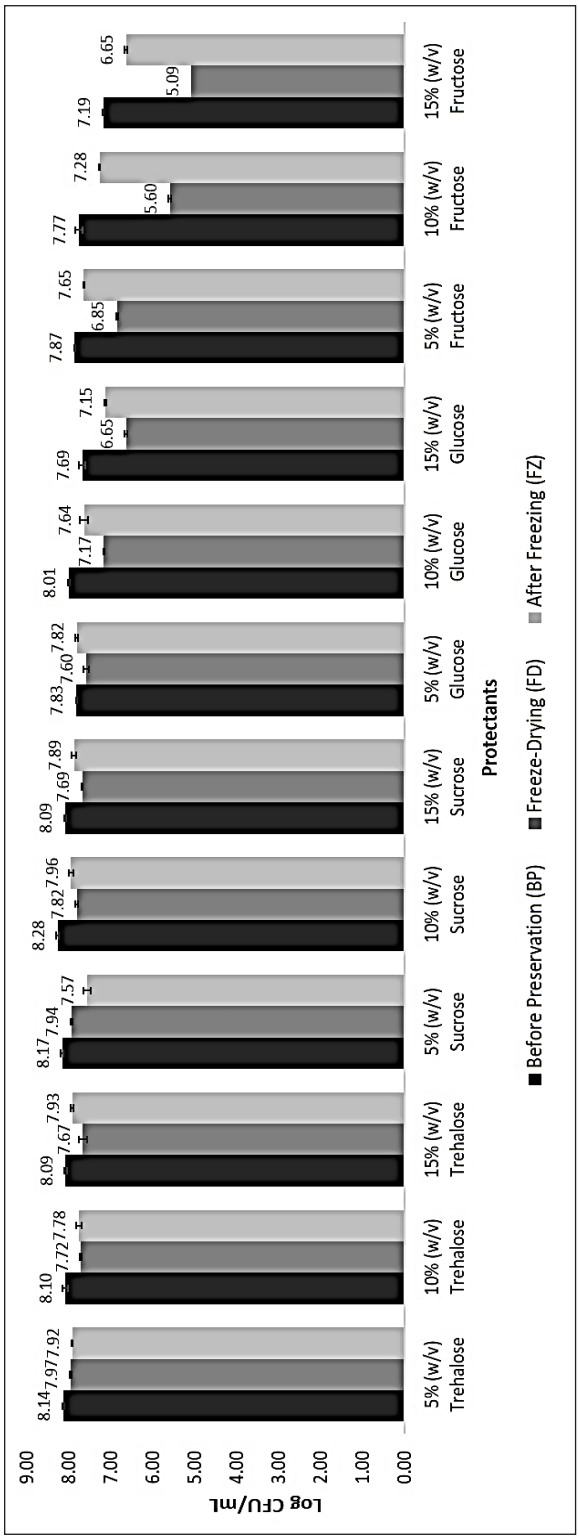

Figure 5. Effect of various protectants (sugar) on viability of *Bacillus methylotrophicus* UPMC 1166 preserved by freeze-drying and freezing technique. The test was done using three replicates

Effects of the Protectants on *Bacillus methylotrophicus* UPMC 1166 Viability Preserved by Freeze-drying and Freezing Method

Figures 6 and 7 showed the survival rate of UPMC 1166 toward various protectants after freeze–drying and freezing process. From this study, a maximum protection of UPMC 1166 during freeze–drying was achieved with 10% (w/v) skimmed milk with 1% (w/v) sodium glutamate and 5% (w/v) trehalose. Maximum protection of UPMC 1166 during freezing at −80°C was achieved with 10% (v/v) DMSO. DMSO at 10% (v/v) was proposed to be utilised as protectant in the future work for freezing process because it could maintain the viability of 8.0 log CFU/mL before freezing and gave 100% survival rate after freezing. Although 5% (w/v) glucose achieved 100% viability during freezing, it did not showed the viability of more than 8.0 log CFU/mL before preservation. The summary results of protectants' effect before and after preservation were obtained in the percentage of survival rate and log CFU/mL (refer Table 3).

After freeze–drying process for UPMC 1166, disaccharides (trehalose and sucrose) provided better viability than monosaccarides (glucose and fructose). Trehalose and sucrose were very good protective agents toward proteins and membranes in bacteria during freeze–drying (Leslie et al., 1995). When the cell wall was protected and preserved by the protective agent and also with its ability of intracellular trehalose uptake, critical intracellular macromolecules could possibly be protected from inside against injuries during the freezing and freeze–drying procedure (Peiren et al., 2015).

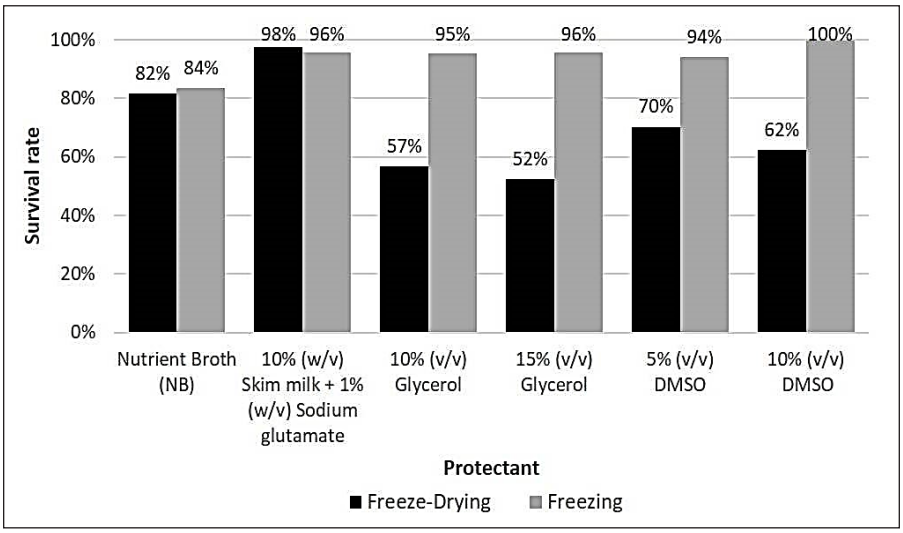

Figure 6. Effect of various protectants [10% (w/v) skimmed milk with 1% (w/v) sodium glutamate, glycerol and DMSO] on the survival rate of *Bacillus methylotrophicus* UPMC 1166 preserved by freeze–drying and freezing technique. The test was done using three replicates.

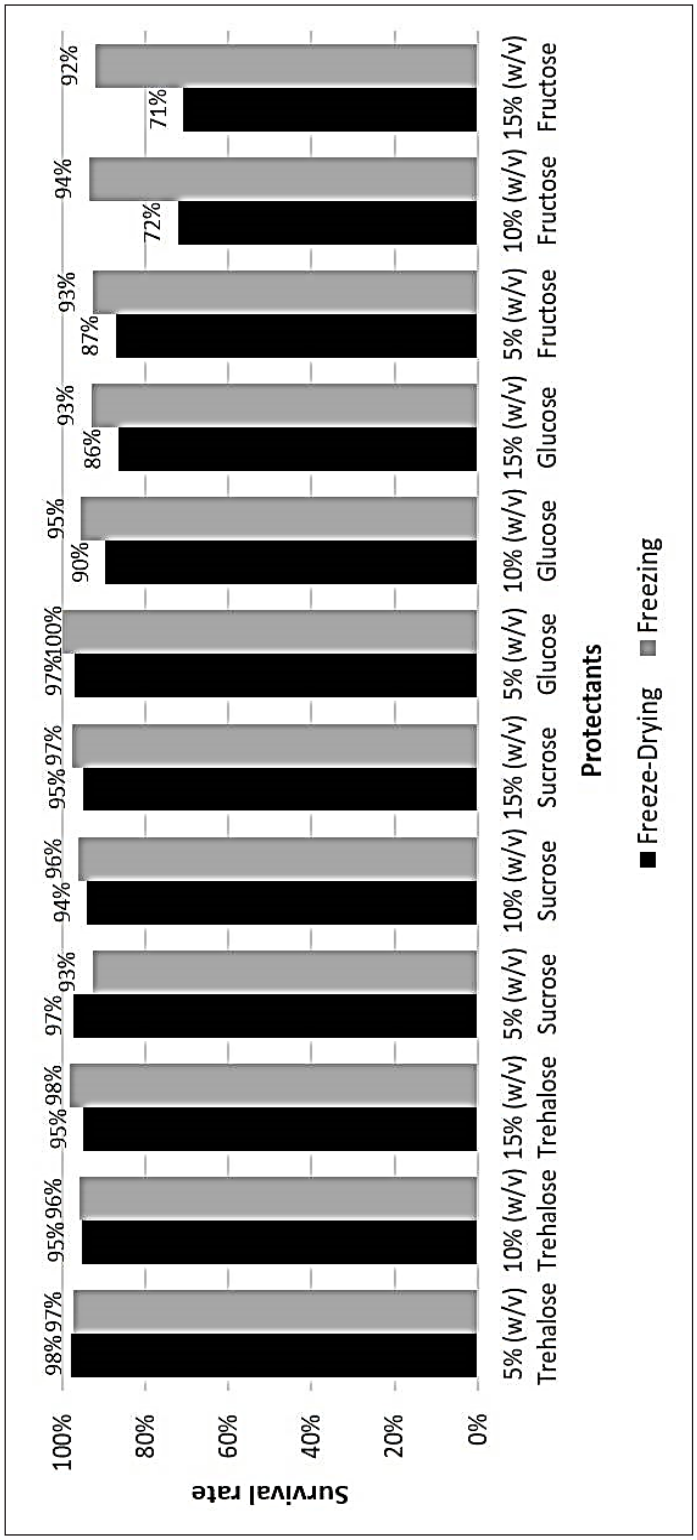

Figure 7. Effect of various protectants (sugar) on Survival Rate of *Bacillus methylotrophicus* UPMC 1166 preserved by freeze-drying and freezing technique. The test was carried out using three replicates

Table 3
Summary of preservation results for UPMC 1166

Observation	Results
Growth kinetics: Late logarithmic phase	16 hours
Before preservation:	
- Control (nutrient broth only)	8.31 log CFU/mL
- Protectants: Cell viability more than 8.0 log CFU/mL	All protectants except for except for 5% (w/v) glucose, 15% (w/v) glucose, 5% (w/v) fructose, 10% (w/v) fructose and 15% (w/v) fructose
- Protectants: Highest log CFU/mL	15% (v/v) glycerol (8.32 log CFU/mL)
- Protectants: Lowest log CFU/mL	15% (w/v) fructose (7.19 log CFU/mL)
Freeze-drying results:	
- Control (nutrient broth only)	82%
- Protectants: Survival rate more than 90%	All protectants except for 10% (v/v) glycerol, 15% (v/v) glycerol, 5% (v/v) DMSO, 10% (v/v) DMSO, 15% (w/v) glucose, 5% (w/v) fructose, 10% (w/v) fructose and 15% (w/v) fructose
- Protectants: Highest survival rate	10% (w/v) skimmed milk with 1% (w/v) sodium glutamate, and 5% (w/v) trehalose (98% survival rate)
- Protectants: Lowest survival rate	15% (v/v) glycerol (52% survival rate)
Freezing at −80°C results:	
- Survival rate of control (nutrient broth only)	84%
- Protectants: Survival rate more than 90%	All protectants showed survival rate of more than 90%
- Protectants: Highest survival rate	10% (v/v) DMSO and 5% (w/v) glucose (100% survival rate)
- Protectants: Lowest survival rate	15% (w/v) fructose (92% survival rate)

In this study, after the freeze–drying procedure, 10% (w/v) skimmed milk with 1% (w/v) sodium glutamate displayed a compact or small porous physical formation but DMSO and all sugars showed a large porous physical and glass-like formation. Both 10% and 15% (v/v) glycerol remained as a gel-like residue after freeze–drying procedure, which possibly influenced the viability of the cells as it gave the lowest survival rate after the freeze–drying procedure. The low survival rate of UPMC 1166 when glycerol was utilised as protectant was possibly due to freeze–thawed process before drying, or the cells were probably sensitive in the presence of unfrozen glycerol during the whole long procedure of primary and secondary drying. Ten percentage (v/v) and 15% (v/v) glycerol providing very good protection to freezing at −80°C but showed less protection to freeze–drying.

The connection between protectants's crystallization and their ability to protect bacterial cells during preservation was not investigated in this study. During the

initial freezing of the bacterial cell wall, ice crystals begin to develop and concentration of solute (osmotic stress) in the suspension increases (ATCC Bacterial Culture Guide, 2015). If excessive water remains inside the cell, injury happens due to ice crystal formation (Zhao & Zhang, 2005). Slow cooling rates were recommended as this will result in less formation of internal ice crystal, therefore letting for more efficient water sublimation from frozen sample (ATCC Bacterial Culture Guide, 2015). Survival of freeze–drying demonstrate the cells' ability to resist the rapid freezing and drying effect (Miyamoto et al., 2008).

CONCLUSION

From morphological observation, biochemical test and molecular identification concluded that bacterial isolate UPMC 1166 comes under the *Bacillus* group of species and suggested (closely related to known or identified species) to be *Bacillus methylotrophicus*. Identification of bacteria is essential to differentiate one type of bacteria from the others. *Bacillus methylotrophicus* UPMC 1166 would be also tested as a biofertiliser on crops to observe the effectiveness and yield for future work.

When subjected to freeze–drying and −80°C freezing, the recovery of cells for UPMC 1166, are dependent on the protectant agent used. To obtain maximum cells viability for long-term preservation, a suitable selection of protectants is important. To get the maximum viability for the strain, it is also important to ensure that the initial cell load should be at least 1×10^8 CFU/mL and the cells should be harvested during the late logarithmic phase of growth. Resistance potential of bacterial strains toward preservation procedures is functional from a research and commercial point of view.

ACKNOWLEDGEMENT

Authors would like to thank Microbial Culture Collection Unit, Institute of Bioscience, Universiti Putra Malaysia and All Cosmos Industries Sdn. Bhd. for providing facilities and funding for this project.

REFERENCES

Anonymous 1. (2012). *Bacillus methylotrophicus* UTM401 and applications thereof. (2012, October 3). Retrieved on September 10, 2017, from http://www.google.com/patents/CN102703363A

ATCC Bacterial Culture Guide (2015). Retrieved on August 23, 2017, from https://www.atcc.org/~/media/PDFs/Culture%20Guides/ATCC_Bacterial_Culture_Guide.ashx

Bosshard, P. P., Zbinden, R., Abels, S., Böddinghaus, B., Altwegg, M., & Böttger, E. C. (2006). 16S rRNA gene sequencing versus the API 20 NE system and the VITEK 2 ID-GNB card for identification of nonfermenting gram-negative bacteria in the clinical laboratory. *Journal of Clinical Microbiology, 44*(4), 1359–1366. doi:10.1128/JCM.44.4.1359-1366.2006

Chauhan, H., Bagyaraj, D. J., Selvakumar, G., & Sundaram, S. P. (2015). Novel plant growth promoting rhizobacteria - prospects and potential. *Applied Soil Ecology*, *95*, 38–53. doi:10.1016/j.apsoil.2015.05.011

Dunlap, C. A., Kim, S. J., Kwon, S. W., & Rooney, A. P. (2015). Phylogenomic analysis shows that *Bacillus amyloliquefacien*s subsp. *plantarum* is a later heterotypic synonym of *Bacillus methylotrophicus*. *International Journal of Systematic and Evolutionary Microbiology, 65*, 2104–2109. doi:10.1099/ijs.0.000226

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution, 39*(4), 783–791. doi:10.2307/2408678

Grobelak, A., Napora, A., & Kacprzak, M. (2015). Using plant growth-promoting rhizobacteria (PGPR) to improve plant growth. *Ecological Engineering*, *84*, 22–28. doi:10.1016/j.ecoleng.2015.07.019

Hall, T. A. (1999). *BioEdit*: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series, 41*, 95–98.

Hogg, S. (2005). *Essential microbiology*. NJ, US: John Wiley & Sons Ltd. ISBN: 0471497533.

Janda, J. M., & Abbott, S. L. (2002). Bacterial identification for publication: When is enough enough? *Journal of Clinical Microbiology, 40*(6), 1887–1891. doi:10.1128/JCM.40.6.1887-1891.2002

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution, 33*(7), 1870–1874. doi:10.1093/molbev/msw054.

Leslie, S. B., Israeli, E., Lighthart, B., Crowe, J. H., & Crowe, L. M. (1995). Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. *Applied and Environmental Microbiology, 61*(10), 3592–3597.

Logan, N. A., & Vos, P. D. (2015). Bacillus. *Bergey's Manual of Systematics of Archaea and Bacteria*, 1-163.

Madhaiyan, M., Poonguzhali, S., Kwon, S.-W., & Sa, T.-M. (2010). *Bacillus methylotrophicus* sp. nov.: A methanol-utilising, plant-growth-promoting bacterium isolated from rice rhizosphere soil. *International Journal of Systematic and Evolutionary Microbiology, 60*(10), 2490–2495. doi:10.1099/ijs.0.015487-0

Microbial Culture Collection Unit UPM (2009). *Procedure of Management on Culture Collection*. Selangor, Malaysia: Microbial Culture Collection Unit (UNiCC), Institute of Bioscience, Universiti Putra Malaysia.

Mohammadi, K., & Sohrabi, Y. (2012). Bacterial biofertilisers for sustainable crop production: A review. *ARPN Journal of Agricultural and Biological Science, 7*(5), 307-316.

Morgan, C. A., Herman, N., White, P. A., & Vesey, G. (2006). Preservation of micro-organisms by drying; A review. *Journal of Microbiological Methods, 66(*2), 183–193. doi:10.1016/j.mimet.2006.02.017

Naher, U. A., Panhwar, Q. A., Othman, R., Ismail, M. R., & Berahim, Z. (2016). Biofertiliser as a supplement of chemical fertilizer for yield maximization of rice. *Journal of Agriculture Food and Development*, *2*(0), 16–22.

Nazaruddin, S. A. (2014). *Isolation and characterisation of effective microorganisms* (Undergraduate's thesis), Universiti Putra Malaysia, Malaysia.

Othman, J., & Jafari, Y. (2014). Research note: Selected research issues in the Malaysian agricultural sector. *Jurnal Ekonomi Malaysia*, *48(*2), 127–136.

Patil, V. S., Patil, S. V., & Pathade, G. R. (2012). Comparative use of chemical and biological fertilizers and pesticides and their health effects on farmers in Satara Tahsil, Maharashtra. *Universal Journal of Environmental Research and Technology, 2*(3), 174–178.

Peiren, J., Buyse, J., De Vos, P., Lang, E., Clermont, D., Hamon, S., & Arahal, D. R. (2015). Improving survival and storage stability of bacteria recalcitrant to freeze-drying: a coordinated study by European Culture Collections. *Applied Microbiology and Biotechnology*, *99*(8), 3559–3571. doi:10.1007/s00253-015-6476-6

Pepper, I., & Gerba, C. (2004). Basic Protocol. *Environmental Microbiology* (2nd ed.). Retrieved on February 7, 2017, from https://www.elsevier.com/books/environmental-microbiology/pepper/978-0-08-047051-1

Pletnev, P., Osterman, I., Sergiev, P., Bogdanov, A., & Dontsova, O. (2015). Survival guide: *Escherichia coli* in the stationary phase. *Acta Naturae, 7*(4), 22–33.

Ravishankar, S. (2004). Food microbiology: A laboratory manual. In A. E. Yousef & C. Carlstrom (Eds.), *Food Microbiology* (pp. 489). Singapore: Wiley-Interscience.doi:10.1016/j.fm.2004.01.008

Sambrook, J., Russell, D., & Maniatis, T. (2001). *Molecular cloning: A laboratory manual* (3rd ed.). New York, USA: Cold Spring Harbor Laboratory Press: 5.2-5.14.

Saitou, N., & Nei, M. (1987). The neighbour-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*(4), 406–425.

Tedeschi, R., & De Paoli, P. (2011). Collection and preservation of frozen microorganisms. In J. Dillner (Ed.), *Methods in biobanking* (pp. 313–326). New York, NY: Humana Press. doi:10.1007/978-1-59745-423-0_18

Tan, G. H., & Nazaruddin, S. A. (2015). Isolation, characterisation and identification of effective microorganisms from Malaysia biofertiliser products. *Direct Research Journal of Agriculture and Food Science, 3*(8), 157–161.

Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, *22*(22), 4673–4680.

Uzunova-Doneva, T., & Donev, T. (2005). Anabiosis and conservation of microorganisms. *Journal of Culture Collections*, *4*(1), 17–28.

Vessey, J. K. (2003). Plant growth promoting rhizobacteria as biofertilisers. *Plant and Soil*, *255*(2), 571–586. doi:10.1023/A:1026037216893

WFCC Guidelines. (2010). *World Federation of Culture Collections guidelines*. Third Edition. Retrieved on May 12, 2017, 2017, from http://www.wfcc.info/pdf/WFCC_Guidelines_Version_Third_Edition.pdf

Ward, M. H. (2009). Too much of a good thing? Nitrate from nitrogen fertilizers and cancer. *Reviews on Environmental Health*, *24*(4), 357–363.

Zahir, Z. A., Arshad, M., & Frankenberger Jr., W. T. (2003). Plant growth promoting rhizobacteria: Applications and perspectives in agriculture. *Advances in Agronomy*, *81*, 97–168. doi:10.1016/S0065-2113(03)81003-9

Zhao, G., & Zhang, G. (2005). Effect of protective agents, freezing temperature, rehydration media on viability of malolactic bacteria subjected to freeze-drying. *Journal of Applied Microbiology, 99*, 333–338. doi:10.1111/j.1365-2672.2005.02587.x