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Role of protective agents on the viability of probiotic *Lactobacillus plantarum* during freeze drying and subsequent storage

[1*]Savedboworn, W., [1]Kerdwan, N., [1]Sakorn, A., [2]Charoen, R., [2]Tipkanon, S. and [3]Pattayakorn, K.

[1]Department of Agro-Industry Technology and Management, Faculty of Agro-Industry, King Mongkut's University of Technology North Bangkok, Prachinburi, Thailand 25230
[2]Department of Innovation and Product Development Technology, Faculty of Agro-Industry, King Mongkut's University of Technology North Bangkok, Prachinburi, Thailand 25230
[3]Department of Food Technology and Nutritional, Faculty of Natural Resources and Agro-Industry, Kasetsart University, Chalemphrakiat Sakon Nakhon Provinces campus, Sakon Nakhon, Thailand 47000

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Abstract

The aim of this study was to determine the effect of various protective agents on the survival of probiotic *Lactobacillus plantarum* TISTR 2075 grown in Plai Ngahm Prachin Buri rice extract during freeze drying and subsequent storage. A combination of protein-trehalose (Prot + Tre) and protein-maltodextrin (Prot + MD) significant (P<0.05) improved the viability of the strain after freeze drying with the survival rate of 98.13 and 97.58%, respectively. Among all protective agents tested, Prot + Tre was found to maintain high degrees of viable cell number with the lowest specific rate of cell death *(k)* of 7.45 x 10^{-4} and 1.79 x 10^{-2} day^{-1} after storage at 4°C for 168 days and 30°C for 84 days, respectively. Additionally, the accelerated storage tests using accelerated temperatures of 40, 50, 60 and 70°C were used to develop a model system in order to estimate the viability of freeze-dried probiotic *L. plantarum* TISTR 2075 in different protective agents for long-term storage. It was concluded that accelerated storage testing is a useful technique with certain predictability in this study.

Introduction

Food products containing probiotic microorganisms are gaining interest and available in market worldwide (Vasudha and Mishra, 2013; Ashraf and Smith, 2015). The global market for probiotic food is expected to reach US$52.34 billion by 2020 (James, 2014). Over the last few years, the development of non-dairy probiotic products is a challenge for the food industry due to the ongoing trend of vegetarianism and a high prevalence of lactose intolerance in many people around the world (Nualkaekul *et al*., 2012). Probiotics are available for consumers in an increasing variety of non-dairy applications such as fruit and vegetable beverages, dessert products, cereal products, meat products and health supplements for direct consumption (Yeo and Liong, 2010; Behboudi-Jobbehdar *et al*., 2013).

The viability of probiotics should be maintained during processing, storage and delivery to target site in gastrointestinal tract (Ying *et al*., 2010). The minimum concentration of viable probiotic bacteria at least 10^6-10^7 CFU/mL was typically proposed at the time of consumption to provide health benefit (Kosin and Rakshit, 2006). In order to preserve probiotic bacteria for long-term viability and functionality, dehydration process which involved the transition of microorganisms from a liquid to a solid medium is commonly used for production of dried powder of probiotics (Iaconelli *et al*., 2015). Freeze drying is considered as a suitable method for stabilizing microorganisms that are greatly sensitive to high temperature (Goderska, 2012; Fonseca *et al*., 2015). However, freezing and subsequent sublimation of frozen water could be attributed to cellular injuries including damage to cell membrane and DNA (Tripathi and Giri, 2014). Additionally, the changes in the physical state of membrane lipids during storage may result in severe loss of bacterial viability during storage (Fonseca *et al*., 2015). A number of cryoprotective agents such as proteins, sugars and carbohydrates have been used to minimize the bacterial inactivation after freeze drying and subsequent storage (Carvalho *et al*., 2004). According to Jofré *et al*. (2015), survival rate of *L. rhamnosus* CTC1679, *L. casei/paracasei* CTC1677 and *L. casei/*

*Corresponding author.
Email: *wanticha@yahoo.com*
Tel: +66 37217300

paracasei CTC1678 was ≥ 94% after freeze drying with glucose, lactose and skim milk. Furthermore, soy protein isolate mixed with maltodextrin provided protective capability of >80% survival on the viability of freeze-dried *Bifidobacterium longum* 1941 (Dianawati *et al*., 2013). Prebiotics are non-digestible carbohydrates that resist hydrolysis and absorption in the upper parts of the gastrointestinal tract and affect the host by selectively stimulating the growth and/or activity of colonic microflora (Roberfroid, 1998). Prebiotics were also applied as protective agents during freeze drying process. Inulin is a natural polysaccharide composed of a chain of fructose units with a terminal glucose unit. The application of the inulin in food industry is related to its capability of substituting sugar and fat with a low count of calories (Toneli *et al*., 2010). The presence of inulin as a stabilizing agent in freeze drying process was reported. Nualkaekul *et al.* (2014) suggested a reduction in viable cell number of 0.92 log CFU/g when inulin was used as protectant of freeze-dried *L. plantarum* NCIMB 8826 in pomegranate powder after storage at room temperature for 12 months. Also, trehalose as stabilizer is widely used to stabilize protein during drying (Hinrichs *et al*., 2001). It has been demonstrated that trehalose is an effective cryoprotectant during freeze drying of *L. rhamnosus* GG and *L. plantarum* IFA No. 278 (Pehkonen *et al*., 2008; Strasser *et al*., 2009). This is probably due to the remarkably high glass transition temperature (*T*g) of trehalose and the strong ion-dipole interactions and hydrogen bonding between trehalose and the biomolecule (Meng *et al.*, 2008). Additionally, maltodextrin as bulking agent and stabilizing agent in drying process was reported. The survival rate of 79.9% with the viable cell number of approximately 10 log CFU/g was achieved after freeze drying of *L. plantarum* G2/25 (Yao *et al*., 2009). Besides, an accelerated storage testing is a useful method for the prediction of storage stability and for the estimation of shelf-life (Tsen *et al.*, 2007). Several studies have proposed a model to extrapolate the shelf life of probiotics in powdered form during storage. A successful prediction of storage stability of freeze-dried *L. acidophilus* BCRC 10695, *L. acidophilus* CCRC 10695 and *L. brevis* ATCC 8287 (Desmond *et al*., 1998; King *et al*., 1998; Tsen *et al*., 2007) using the accelerated storage testing method based on Arrhenius theory has been proposed. Moreover, no information regarding accelerated storage testing of freeze-dried *L. plantarum* has been reported. In order to find the efficient protective media which have a great capability to stabilize probiotic cells during freeze drying and storage, protein and protein supplemented with prebiotic maltodextrin, fibersol-2, trehalose and inulin were used as protective media for *L. plantarum* TISTR 2075 in freeze drying. The protective ability of these materials to enhance the stability of the strain during subsequent storage was also determined. An accelerated storage testing based on the Arrhenius equation was applied so as to develop a model system to predict storage stability of freeze-dried probiotic for long-term storage.

Materials and Methods

Microorganisms

The probiotic *Lactobacillus plantarum* TISTR 2075 isolated from fermented vegetables was obtained from Microbiological Resource Center, Thailand Institute of Scientific and Technological Research (TISTR), Thailand. The strain was preserved in de Man-Rogosa-Sharpe (MRS) broth (Difco, Detroit, MI, USA) with 20% (v/v) glycerol content at -20°C. For routine analysis, the strain was subcultured twice in MRS broth and was incubated at 37°C for 24 h under microaerobic-static conditions to maintain freshness and then used as inoculum.

Preparation of cereal extracts fermentation

Fermented Plai Ngahm Prachin Buri rice extract was prepared according to the procedures described by Savedboworn and Wanchaitanawong (2015). Plai Ngahm Prachin Buri rice was washed and soaked in distilled water. The soaked rice was mixed with distilled water (rice:water = 1:10 w/v). After decanting the soaking water, the soaked rice was mixed with distilled water and then comminuted in a blender for 3 min. The resultant slurry was filtered through double-layered cheesecloth 2 times to yield cereal extracts. Rice extract was dispensed into containers and sterilized by heating at 121°C for 15 min. Sterilized rice extract was inoculated with overnight culture of 1% (v/v) *L. plantarum* TISTR 2075. The fermentations were performed under no pH control in Duran screwcapped glass bottles at 37°C for 24 h. Viable cell counts were determined by the standard plate count method with MRS medium supplemented with 0.5% $CaCO_3$ at 37°C for 24 h. pH was measured with a pH meter.

Freeze-drying of probiotic L. plantarum *TISTR 2075*

Prior to freeze drying, the 24-h incubated culture of probiotic *L. plantarum* TISTR 2075 grown in Plai Ngahm Prachin Buri rice extract was mixed with 15% (w/v) protein (Prot; All plant protein, Nutrilite, Amway, USA) and 5% (w/v) of each protective agent used as follows: trehalose (Tre; Hayashibara, Japan),

fibersol-2 (Fib; Matsutani, Japan), maltodextrin DE 10 (MD; Du Zhi Xue, China) and inulin (Inul; Nutrition Sc Co., Ltd, Thailand) for 30 min by a magnetic stirrer. The suspensions were transferred into lyophilized flask under aseptic conditions and frozen at -18°C for 17 h. A freeze-drier was operated at 0.110 mbar and -50°C for 18 h. Freeze-dried samples were analyzed immediately for the viability.

Storage of freeze-dried probiotic L. plantarum *TISTR 2075*

The freeze-dried powders were kept in sealed aluminum foil bags (7.5 x 12 cm) and stored at 4 and 30°C. The viability was determined every month at 4°C and every 2 weeks at 30°C. The specific rate of cell death (k, day^{-1}) of freeze-dried *L. plantarum* TISTR 2075 was calculated as a first-order reaction from $k = \ln (N_0/N)/t$, where N refers to the bacterial cell count at a particular storage period (CFU/g), N_0 represents the bacterial cell count at the beginning of the storage (CFU/g) and t is the storage time (day) (Tsen *et al*., 2007).

Accelerated storage test

Accelerated storage test was determined according to the procedures described by Tsen et al. (2007) with minor modifications. The freeze-dried samples were incubated at 40, 50, 60 and 70°C. The residual viable cell number was evaluated on each sample collected at constant time intervals to calculate the specific rate of cell death (k). Samples were taken every 24 h for 6 days at 40°C, every 12 h for 3 days at 50°C, every 6 h for 1.5 days at 60°C and every 1 h for 6 h at 70°C.

Scanning electron microscopy (SEM)

The freeze-dried powders were attached to a brass stub with double-sided adhesive tape and sputter coated with a layer of gold. Digital images were recorded with a scanning electron microscope (JSM 6400, JEOL, Tokyo, Japan) and captured at the required magnification.

Enumeration of viable cell number

Freeze-dried powder (1 g) was resuspended in 9 mL of sterile 0.85% NaCl solution for 30 min at room temperature. The appropriate serial dilutions were prepared before pour plating on MRS agar (added with 0.5% $CaCO_3$) and incubated at 37°C for 24 h. The percentage of cell survival was defined as follows : survival rate (%) = (N/N_0) x 100, where N represents the number of viable cell count after freeze drying (CFU/g) and N_0 denotes the viable cell count before freeze drying (CFU/g).

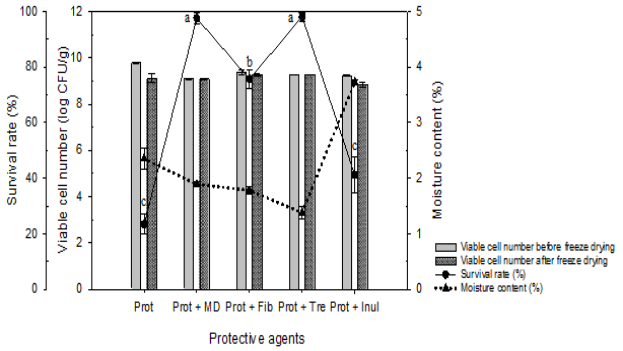

Figure 1. Viable cell number, survival rate and moisture content of probiotic *L. plantarum* TISTR 2075 after freeze drying with various protective agents.
Values with different lowercase letters (a-c) are significant differences by Duncan's multiple range test ($P < 0.05$).
Prot: Protein; MD: Maltodextrin; Fib: Fibersol-2;Tre:Trehalose; Inul: Inulin

Water activity and moisture content

Water activity was measured after freeze drying using an Aqualab water activity instrument (Aqualab, Model Series 3TE, USA). The residual water content of the freeze-dried powders was evaluated in a drying oven at 105°C until a constant weight was attained.

Statistical Analysis

Each result was expressed as the mean ± S.D of two determinations. The data were assessed using analysis of variance (ANOVA) with a level of significance at $P < 0.05$. Significant divergences among mean values were determined with Duncan's multiple range tests. All statistical analyses were performed using SPSS Software, version 12 (SPSS, White Plains, NY, USA).

Results and Discussion

Viability of L. plantarum *TISTR 2075 after freeze drying with different protective agents*

After freeze drying, the strain survival rate of 23.45% was achieved when Prot was used as protective agent. The addition of protective agents were found to significantly ($P < 0.05$) improve the survival rate of the strain with different degrees of protection. Among all protectants tested, Tre, MD and Fib enhanced the viability of *L. plantarum* TISTR 2075 with the survival rates of 98.13, 97.58 and 75.71%, respectively. However, no significant difference in survival rate was observed when Inul was added comparing with Prot alone (Figure 1). Freeze-drying might cause cell membrane damage, protein and DNA denaturation resulting in the loss of cellular viability and activity (Meng *et al*., 2008; Tripathi and Giri, 2014; Fonseca *et al*., 2015). Protective agents play an important role in the

Table 1. Experimental *k* values of freeze-dried *L. plantarum* TISTR 2075 during storage at 4°C for 168 days and 30°C for 84 days and predicted *k* values of freeze-dried *L. plantarum* TISTR 2075 during storage at 4 and 30°C

Protective agents	Experimental *k* values (day^{-1})		Predicted *k* values (day^{-1})	
	$k_{4°C}$ (R^2)	$k_{30°C}$ (R^2)	$k_{4°C}$	$k_{30°C}$
Prot (Control)	2.71×10^{-3} (0.887)	3.13×10^{-2} (0.964)	7.95×10^{-4}	4.43×10^{-2}
Prot + MD	1.63×10^{-3} (0.835)	2.14×10^{-2} (0.933)	4.19×10^{-4}	2.80×10^{-2}
Prot + Fib	1.17×10^{-3} (0.710)	2.15×10^{-2} (0.840)	2.80×10^{-4}	2.93×10^{-2}
Prot + Tre	7.45×10^{-4} (0.856)	1.79×10^{-2} (0.860)	2.17×10^{-4}	2.36×10^{-2}
Prot + Inul	3.48×10^{-3} (0.897)	5.77×10^{-2} (0.869)	1.04×10^{-3}	1.18×10^{-1}

Prot: Protein; MD: Maltodextrin; Fib: Fibersol-2; Tre: Trehalose; Inul: Inulin

conservation of viability. The protective capability of trehalose could be due to the stabilization of cell membranes by replacing the water between lipid headgroups and the prevention of unfolding and aggregation of protein by hydrogen bonding with polar group of protein (Crowe *et al*., 2001). The greater flexibility in the glycosidic bond between the two D-glucose molecules, as compared to other disaccharides, may allow trehalose to conform to the irregular polar groups of macromolecule (França *et al*., 2007).

Also, maltodextrin exhibited high protective capability after freeze drying process. Many researchers suggested that maltodextrin has the ability to retain water, stabilize enzyme, prevent cellular injuries, provide good oxidative stability and overcome the stickiness (Bhandari *et al*., 1993). Incorporation of maltodextrin could be beneficial due to their relative high *T*g values (Semyonov *et al.*, 2010) and amorphous form are able to prevent protein unfolding during drying (DePaz *et al.,* 2002). Furthermore, protein as carrier was found to have a great protective effect on the survival of probiotic in this study. Protein is capable of preventing cellular injury by forming a protective coating on the cell wall (Gharsallaoui *et al*., 2007). Protein macromolecules may not pass through the structure of the peptidoglycan layer that covers the plasma membrane of lactic acid bacteria. It is only capable of acting as inactive bulking agents, forming a protective coating around the cells and lowering the probability of a large number of cells coming closer and fusing with each other (Ghandi *et al*., 2012).

Moisture contents of the strain after freeze drying with various protective agents were in the range of 1.38-3.83%. Zayed and Roos (2004) revealed that a certain amount of water must remain in dehydrated state for a satisfactory survival rate. The residual moisture in freeze-dried materials is directly related to the type of freeze-drying medium. Moreover, the morphology of freeze-dried *L. plantarum* TISTR 2075 with different protective agents was illustrated in SEM micrographs. It was observed that *L. plantarum* TISTR 2075 were entrapped and covered by protective matrices (Figure 2). All freeze-dried powders exhibited a similar particle shape with a porous-sheet-like structure. This result is in concordance with Xu *et al.* (2016) that freeze drying process created a porous structure. Additionally, Poddar *et al*. (2014) suggested that freeze-dried material has connected porosity giving internal surface area for water absorption.

Effect of protective agents on the viability of freeze-dried L. plantarum *TISTR 2075 during storage*

The stability of freeze-dried *L. plantarum* TISTR 2075 in various protective agents was evaluated during storage temperature of 4°C for 168 days and 30°C for 84 days. It was obvious that storage temperature was a crucial parameter affecting the survival of freeze-dried cells. The viability of the strain was quite stable during storage at 4°C. A high storage temperature led to a great decrease in the number of viable probiotic cell for all protective agents. The viability loss of

Table 2. Moisture contents of freeze-dried *L. plantarum* TISTR 2075 with various protective agents during storage at 4°C for 168 days and 30°C for 84 days

Protective agents	Moisture content (% ± S.D.)	
	Storage temperatures	
	4°C	30°C
Prot (Control)	4.74 ± 0.10	4.55 ± 0.23
Prot + MD	2.31 ± 0.12	1.82 ± 0.09
Prot + Fib	2.60 ± 0.19	1.94 ± 0.13
Prot + Tre	2.13 ± 0.12	2.00 ± 0.14
Prot + Inul	4.64 ± 0.02	4.44 ± 0.33

Prot: Protein; MD: Maltodextrin; Fib: Fibersol-2; Tre: Trehalose; Inul: Inulin

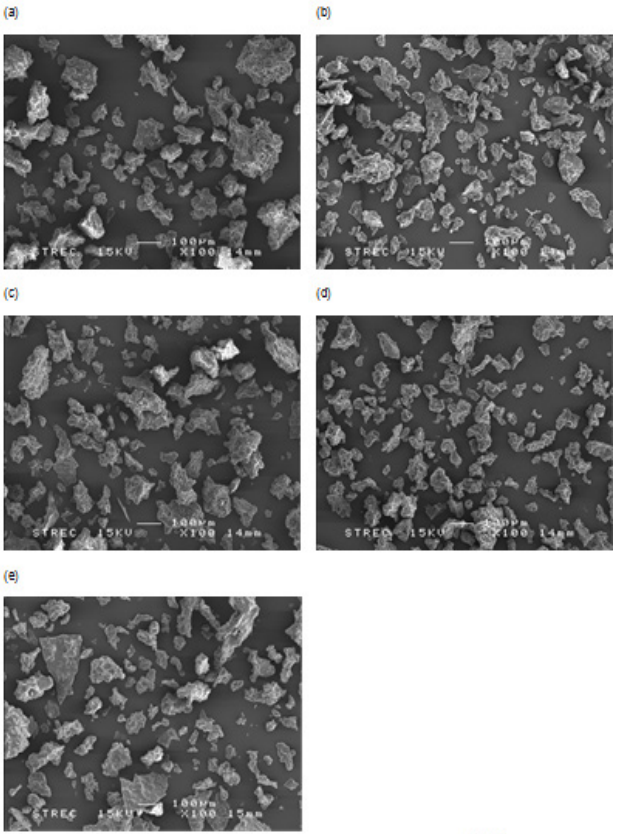

Figure 2. Scanning electron micrographs of freeze-dried *L. plantarum* TISTR 2075 powders with different protective agents; Prot (a), Prot + MD (b), Prot + Fib (c), Prot + Tre (d) and Prot + Inul (e)
Prot: Protein; MD: Maltodextrin; Fib: Fibersol-2; Tre: Trehalose; Inul: Inulin

0.05-0.24 and 0.70-2.86 log CFU/g was detected during storage at 4 and 30°C, respectively. The rising temperature was not only increasing the metabolic activity in the cells, but also modified the molecular mobility of water as the environmental temperature approached *T*g (the *T*-*T*g gradient). As the results, the matrix will move closer to the rubbery state and water molecular mobility will increase (Behboudi-Jobbehdar *et al.*, 2013).

From the results, the protective capability of protectants could be considered in terms of a specific rate of cell death (*k* value). The *k* values were various depending on storage conditions and types of protectants. As shown in Table 1, Prot + Tre as protective agent was found to be relatively effective with the lowest k values at both storage temperatures. The k values of 7.45 x 10^{-4} day^{-1} with the final viable cell count of 9.22 log CFU/g and 1.79 x 10^{-2} day^{-1} with the final viable cell number of 8.79 log CFU/g were achieved at storage temperature of 4 and 30°C, respectively. Unfortunately, Prot + Inul (k_{4oC} = 3.48 x 10^{-3} day^{-1} and k_{30oC} = 5.77 x 10^{-2} day^{-1}) were found to be less effective at 4 and 30°C, respectively, however the viable cell numbers were still > 8 log CFU/g higher than recommended effective dosage of probiotic products. As shown in Table 2, moisture contents of freeze-dried cells were 2.13-4.74% and 1.82-4.55% after storage at 4°C for 168 days and 30°C for 84 days, respectively. Consistent with the report of Zayed and Roos (2004) that the optimum moisture content for the storage of *L. salivarius* subsp. *Salivarius* (UCC 500) ranged from about 2.8 to 5.6%. The inactivation of freeze-dried lactic acid bacteria during storage almost resulted from chemical reaction such as oxidation and protein denaturation (Passot *et al*., 2012). Among all possible degradation events, lipid oxidation of membrane fatty acid was mainly deemed responsible for cell death during storage. Lipid oxidation is also accompanied by the formation of free radicals which mainly damage DNA and cell membrane during long-term storage (Albadran *et al*., 2015).

Prediction of the viability of freeze-dried L. plantarum *TISTR 2075 by accelerated storage test*

The accelerated storage testing was used to develop a model system in order to predict the long-term preservation of freeze-dried probiotic *L. plantarum* TISTR 2075. The specific rate of cell death *(k)* of freeze-dried microorganism in various

protective agents kept under accelerated temperatures at 40, 50, 60 and 70°C could be determined from Equation 1.

$$\ln N = \ln N_0 - kt \qquad [1]$$

where N_0 is the initial viable cell number (CFU/g), N is the viable cell number at any time (CFU/g), k is the specific rate of cell death (day^{-1}) and t is the storage time (day).

The correlation between temperature and k value could be described by the Arrhenius equation as shown in Equation 2.

$$k = Ae^{(-E_a/RT)} \qquad [2]$$

where k is the specific rate of cell death (day^{-1}), Ea is the energy of activation ($J.mol^{-1}$), R is the gas constant (8.32 $J.mol^{-1}.K^{-1}$), and T is the absolute temperature (K). When taking the natural logarithm of both sides of Equation 2, the Equation 3 is achieved.

$$\ln k = \ln A - \frac{E_a}{R} \cdot \frac{1}{T} \qquad [3]$$

The Arrhenius g om the determined k values in terms of natural logarithms versus the reciprocals of their absolute temperatures (Figure 3). Consequently, k_{4oC} and k_{30oC} were estimated. The predicted k values of the strain freeze-dried with various protective agents during long-term preservation at 4 and 30°C were shown in Table 1.

From the results, predicted k value of freeze-dried *L. plantarum* TISTR 2075 in different protective agents was verified by the experimental k value. The ratio of predicted and experimental k values were approximately 0.24-0.30 and 1.31-2.05 at 4 and 30°C, respectively. Roos (1995) suggested that the phase transition is important causes for the observed deviations from Arrhenius equations. A change in the physical state of freeze-dried powders during storage may change activation energy. Additionally, nonenzymatic browning reaction is also responsible for cell death during storage depended on the physical state (Passot *et al*., 2012). Rate of browning was low below a critical temperature, above which the rate of the reaction increased substantially (Roos, 2001). Nonenzymatic browning is not always prevented in the glassy state. The reaction rates were lower at temperature below *T*g comparing with temperature above *T*g (Kawai *et al.*, 2005). Several studies have been successfully predicted the viability of microorganism during storage. According to Tsen *et al.* (2007), there was no significant difference between predicted and actual results of freeze-dried *L. acidophilus* CCRC 10695 during storage. Consistent with Hamsupo *et al.* (2005) that there was no significant difference in viability between prediction and experimental survival rates of spray-dried *L. reuteri* KUB-AC5 at 4 and 30°C for 4 months. This indicated that the accelerated storage testing is the potential extrapolation tool for estimation of the bacterial shelf-life with certain degree of correctness and predictability (Lapsiri *et al*., 2012).

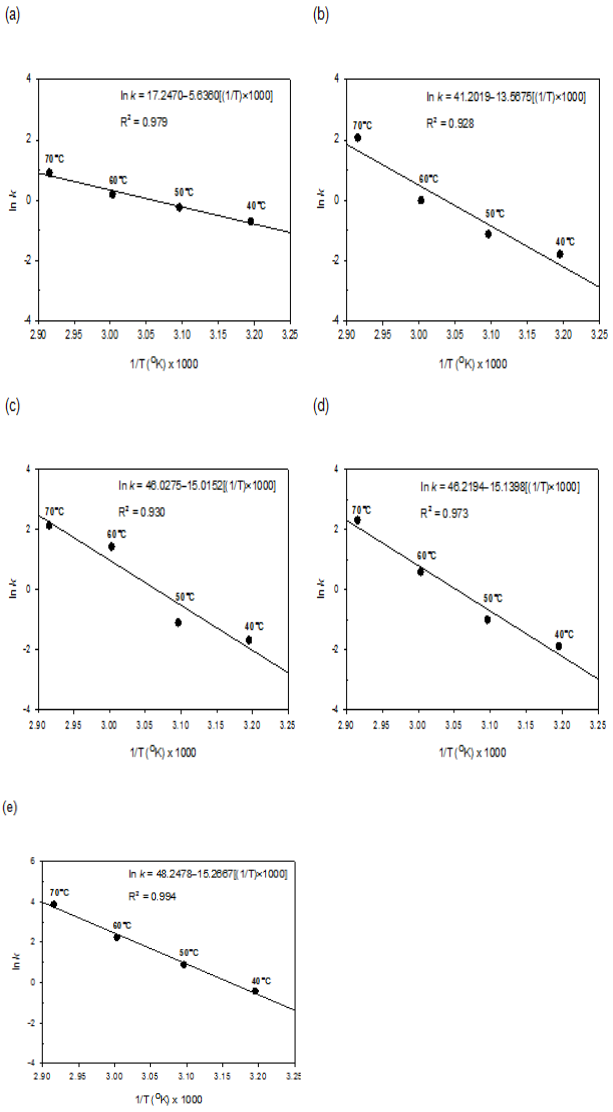

Figure 3. Arrhenius plots of the specific rate of cell death *(k)* of freeze-dried *L. plantarum* TISTR 2075 at various temperatures in different protective agents; Prot (a), Prot + MD (b), Prot + Fib (c), Prot + Tre (d) and Prot + Inul (e) Prot: Protein; MD: Maltodextrin; Fib: Fibersol-2; Tre: Trehalose; Inul: Inulin

Conclusion

In this study, probiotic *L. plantarum* TISTR 2075 has the capability to survive after freeze drying process depending on the type of protective agents.

Prot + Tre, Prot + MD and Prot + Fib were found to be the most effective on probiotic in retaining the viability after freeze drying, especially Prot + Tre which exhibited significant impact on probiotic survival during storage at 4 and 30°C. The kinetic analysis of accelerated storage test data induced the equation indicating the prediction model of probiotic survival during storage. These model could be extrapolated the strain survival stored at 4 and 30°C with certain predictability. The correction factor would require rectifying the models. These predictive equations will be useful for probiotic manufacturers to design and expect the probiotic shelf-life.

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