



# Using Some *Lyoprotectants* for Shelf Life Improvement of a Lyophilized *Intravesical Immune BCG*

Maryam Ziaei Nafchi<sup>1,2</sup>, Seyed Mehdi Hassanzadeh<sup>3\*</sup>, Hooman Kaghazian<sup>3</sup>, Ramin Yaghoobi<sup>4</sup>

<sup>1</sup> Department of Microbiology, Fars Science and Research Branch, Islamic Azad University, Fars, Iran.

<sup>2</sup> Department of Microbiology, Shiraz Branch, Islamic Azad University, Shiraz, Iran.

<sup>3</sup> Research & Production Complex, Pasteur Institute of Iran, Tehran, Iran

<sup>4</sup> Shiraz Transplant Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.

## ABSTRACT

Intravesical immune BCG (Bacillus Calmette-Guérin), strain: 1173P2 as an immune response modifier agent against superficial bladder cancer is being produced in Iran with high potency in form of suspension that requires storage and distribution in frozen state with extra care and cost. Since successfulness of lyophilization process is a technical and somewhat strain dependent; effectiveness of some admissible lyoprotectants (LPs) including sucrose, lactose, trehalose, glucose, dextran-40, and monosodium L-glutamate (MSG) upon biological and physical characteristics of the lyophilized BCG bacilli was investigated with aim to provide an stable lyophilized product through and after lyophilization and during long storage period. BCG bulk was formulated with different amounts of the Lps and submitted to freeze drier with different primary drying stages ranged below the theoretically glass transition temperature (T<sub>g</sub>) of the bulk for the best results. Required characteristics have been significantly improved by a sample contained a combination of lactose, MSG, dextran-40 and tween-80 so that the satisfactory results were achieved for viability, moisture content, appearance, reconstitution time, and shelf life period.

**Key Words:** BCG, Vaccine, Lyoprotectant, Stabilizer, lyophilization, Viability, Shelf life.

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## INTRODUCTION

Tuberculosis (TB) is still between the biggest causes of killer infectious listed by WHO [1, 2]. Bacillus Calmette-Guérin (BCG) is an attenuated strain of *Mycobacterium bovis* and originally was developed and created in the 1920s as the only vaccine against TB although there is still little understand as to how it produces an immunological response. The antitumor effects of BCG bacilli were described as a biological immune response modifier in treatment of early phase (superficial) bladder cancer and recently prostate cancer [3-5]. Potency of intravesical BCG is determined mainly by counting of viable bacilli as colony forming unit (CFU) or by biological activity test such as ATP assay and O<sub>2</sub> uptake measurement [6]. The most satisfactory

preservation method thereby sufficient long term viability would be conserved for BCG products is afforded by lyophilization process which offers a fine alternative to freezing [7]. Lyophilization is a gentle process by which the frozen substance is converted directly to a stable solid state by heat transfer under vacuum in two stages including: mass transfer (frozen free water sublimation which referred to as the primary drying) and isothermal desorption of intra and extra cellular remained frozen water (secondary drying) to reduce moisture content to a value that biological activity will be not supported longer. Partially live mass dropping due to the physical and rarely chemical injury to the infra structure of the bacterial cells is a normal drawback of lyophilization process [8, 9] but it has to be minimized. Since lethal damages to the cells would be arisen in cooling, freezing, sublimation,

**Corresponding author:** Seyed Mehdi Hassanzadeh

**Address:** Research & Production Complex, Pasteur Institute of Iran, Tehran, Iran .

**E-mail:** ✉ m\_hassanzadeh@yahoo.com

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secondary drying, sealing, storage, and rehydration; accordingly concepts on each one have to be considered. Moisture content and Physical features of lyophilized product are among the most important index for preliminary confirmation of integrity of the process [8, 9]. By a desired formulation of BCG bulk and a properly lyophilization cycle; a thoroughly solidified matrix with the lowest damages to the cells, the highest viability and quick reconstitution time would be assured [10-12]. Integrity and viability of lyophilized BCG could be greatly supported by those stabilizer excipients which act as lyoprotectant (LP) agents in order to control cell dehydration and retaining the entire matrix through freezing, water removal steps and storage [13] including trehalose, lactose, sucrose, glucose, dextrane and mono sodium glutamate (MSG) [8] along with mannitol, bovine serum albumin, and gelatin which are considered as the excellent matrix forming (caking) agents and as the collapse temperature modifier during lyophilization [13, 14]. In this regard, two nonexclusive mechanisms pointed to *vitrification* of water (glassy state or hard viscose) and *water replacement* (substitution) before cells are to be frozen and dehydrated [8, 15, 16]. Although the related mechanism is almost controversial, but the vitrification (solidification) has been totally accepted because most of the common LPs are impermeable [16, 17] especially against hydrophobic cell wall of mycobacterium bacilli. Vitrification leads to immobilizing of extracellular small ice crystals and native cells, and also promotes removing of free water molecules from the cells before and during freezing to eliminate intracellular icing. A none toxic LP [10] with lower water activity, higher water solubility, higher polar hydroxyl groups, higher  $T_g$  or  $T_c$ , and better performance as a caking agent would be superior to other ones [18]. It should be noted that cryoprotectant agents such as glycerol are different from LPs in terms of their ability for crossing the cell membranes (penetrating) and their high evaporation point and hygroscopic properties which can lead to a none desirable highly viscous lyophilized products [15]. Lyophilization parameters would be assigned regarding to composition of BCG bulk

mainly the kind and amount of LPs. In this regard, considering the eutectic temperature ( $T_e$ ) of the crystalline solids such as monitol and MSG (and sucrose), and the transition temperature ( $T_g$ ) of amorphous (glassy) solids (super cooling liquids) including most of carbohydrates are definitely important [13, 19]. If a product exceeds its  $T_e$  or  $T_g$  during sublimation then drying would take place from liquid phase because of melting or collapsing of the substance [20]. Although attempts to develop lyophilized BCG products have been already described [4, 21-23], but lyophilization process of BCG products is not a conventional balance that could be performed in the same way, rather it could be vary from one strain to another one, and depends on the components, equipment, expected characteristics and so on. The current study has focused on development of a lyoprotected BCG (1173P2 strain) bulk formula and lyophilization cycle to achieve a well dried intravesical BCG with robust physical and biological features.

## MATERIALS AND METHODS

*Mycobacterium bovis* BCG secondary seed lot (1173P2 strain) was obtained from Pasteur Institute of Paris. Trehalose dehydrate (99.5%), sucrose (99.5%), lactose monohydrate (98%), glucose (99.5%), monosodium l-glutamic acid (98%) and dextran-40 were all purchased from Sigma Aldrich. Culture media, solutions and guinea pigs provided by Pasteur Institute of Iran.

### Preparation of BCG bulk

The LPs (Table 1) were chosen according to the related articles and combined with the BCG bulk. Preparation, composition and concentration of the BCG bulk was determined according to routine method as described by Farmer et al., [14] for comply the requirements. LP solutions were prepared in distilled water and autoclaved for 15 min at 121°C. The LP solutions were used separately as the diluents during preparation of BCG bulks from harvested semi dried BCG bacilli.

**Table 1. Some characteristics of the Lyoprotectants used in preparation of the samples.**

Lyoprotectants	MW <sup>(a)</sup>	Solubility <sup>(a)</sup> (g/100ml)	aW <sup>(b, c)</sup> (10% in water)	-OH groups <sup>(d)</sup> (No.)	T <sub>g</sub> <sup>(d)</sup> (°C)	T <sub>c</sub> <sup>(d)</sup> (°C)	UFW% <sup>(d)</sup>	G/C <sup>(e)</sup>
Trehalose	342.3	68.9	0.980	8	-30	-30	17	G
Sucrose	342.3	200	0.992 <sup>a</sup>	8	-32	-32	36	G
Lactose	342.3	19	0.970	8	-28	-31	40	G
Glucose	180	91	0.985 <sup>b</sup>	5	-43	-40	29	G
Dextran 40	40,000	>3		-	-	-10	-	G
MSG	169	74		-	-	-	-	C

Table 1 MW: molecular weight; Solubility: g/100 ml water; aw: water activity (10% in pure water); TG: glass transition temperature; Tc: collapse temperature; UFW: unfreezable water; G/C: glassy or crystal formation. Based on data from a: www.sigma alderich.com; b: Robinson and Stokes (1959); c: Taylor and Rowlinson (1955); d: Yong G, 2010; e: Levine H 1988.

### Filling and Lyophilization cycle

Amber flatted bottom type1 glass vials (10R), were washed, sterilized and de-pyrogenized by the washing machine (Bush Strobel, Germany). The vials were filled with 3 ml of BCG bulk suspensions (120mg wet BCG) manually and submitted to the production freeze dryer (Tofflon, China). Three different lyophilization cycles composed of three steps: freezing to reach -40°C during 1 hour for rapid and completely solidifying of samples, starting desiccation after 4 hours under 0.1 mbar at -40°C (below the  $T_g$  of the LPs), -30°C and -20°C (below the ice nucleation or supercooling) for 20 hours in the 1<sup>th</sup>, 2<sup>ed</sup> and 3<sup>rd</sup> cycle respectively, and secondary drying at 0.01 mbar and 25 °C for 2 hours. At the end of the process the vials were closed by rubber stoppers (Samsung, Korea) under vacuum (0.1 mbar or less) and sealed by aluminum caps and stored at 2-8°C [11, 14].

### Quality control tests:

#### 1. moisture content measurement

Moisture content of the samples was measured using standard colorimetric Karl-Fischer titration (Mettler Toledo, DL37 and Switzerland) [24, 25].

#### 2. Viability assay

Colony forming units (CFU) of the samples (10 vials of each formula) were measured before and promptly after lyophilization, and after 3, 6, 12, 18 and 24 months storage at 5°C and after 28days at 37 °C. Each sample was reconstituted and diluted using sterile serum saline and spread on the surface of Lowenstein-Jansen's medium, incubated at 37 ± 0.5°C for 30 days according to BP 2012. CFUs were visually checked and counted [25].

#### 3. O<sub>2</sub> Uptake test

Measurement of oxygen uptake by the classical Warburg respirometric technique was carried out following the methodology [17].

#### 4. Skin sensitivity reaction (SSR) test

According to WHO TRS No.771- annex 12, six healthy guinea pigs (250-400 g) with no history of any antibiotic intake were used. Randomly, 1mL of each samples of reconstituted lyophilized BCG was injected intradermally. The size of each lesion was measured after 4 weeks [23].

### Statistical analysis

The error bars in the figures represented the standard deviations for 10 samples in each experiment. The significance of the data for viability, moisture contents and O<sub>2</sub> uptake were assessed as p<0.1 and evaluated using the analysis of variance. The results for estimation of shelf life were statistically analyzed for regression followed by stability study using Minitab, v17.3 software (2016). Shelf life is equal to time period in which it can

be 95% confident that at least 95% of response is within spec limits (8.5 – 300 × 10<sup>5</sup> cfu/mg).

## RESULTS

### Lyophilization cycle

Drying cycle 1 and 2 failed completely when samples were not solidified properly. By the running of cycle 3, the higher temperature in primary drying has led to higher long term viability, and lower residual moisture content for all samples although, promptly after the process, live germ and physical appearance of the solidified matrixes almost were near together. Here the results of cycle 3 were reported.

### Skin Sensitivity Reaction (SSR) test:

Except for MGS and dextran, the size of lesions was slightly increased (p>0.1) in accordance with the increasing of the lyoprotectants (LPs) as shown in Table 2, and in compare to blank sample 1 (fresh bulk, without LPs) with highest SSR response, blank 2 (without LPs) and blank 3 (only 10% of LPs) with a lower responses (data not shown). This is on account of positive correlation between concentrations of some LPs with viability and potency due to their higher efficacy of LPs whereas increase of MSG or dextran to 10% led to slightly decrease of efficacy and then lower SSR. These results in compare to the fresh bulk appear to show the safety (biocompatibility) of these LPs even at high concentration.

**Table 2: Skin reaction test report (mm), 42 days after 1mg/ml injection of the lowest and highest amounts of each lyoprotectants (LPs).**

Sample with	Legion diameter (mm)
Lyoprotectants (LPs)	
Fresh Bulk (1.5% MSG)	6.8±0.41
Lyophilized Blank-1(no LPs)	1.8±0.08
Lyophilized Blank-2(only LPs)	0
Lactose 2.5 %	4.6±0.31
Lactose 10 %	4.2±0.30
Glucose 2.5 %	4.1±0.30
Glucose 10 %	4.0±0.32
Trehalose 2.5 %	4.9±0.34
Trehalose 10 %	4.2±0.30
Sucrose 2.5 %	4.5±0.31
Sucrose 10 %	4.8±0.32
MSG 2.5 %	4.5±0.31
MSG 10 %	5.3±0.40
Dextran-40 2.5%	3.9±0.25
Dextran-40 10%	4.8±0.33

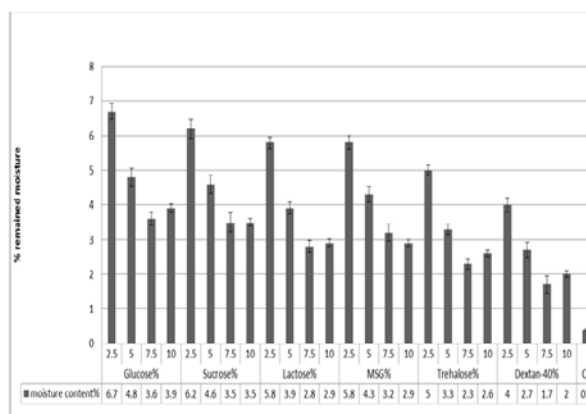
MSG: Mono Sodium Glutamate. Accepted criteria 4.2-7.5, 2.0-6.5, and 0-6.5 for 1.0, 0.1 and 0.01 mg/ml injection respectively. Standard deviation was calculated between 3 repeats for each sample.

### Appearance and Physical properties:

Forming a well solid state matrix comparable to the volume, shape and color of their relative frozen mass, with minimum moisture content and readily rehydratable with cold diluent in a short reconstitution time; would be the primary requirements for the lyophilized BCG product. All formulations at equivalent amounts of the LPs were solidified with their own physical characteristics in lyophilization cycle 3. By visual study Fig. 1, samples contained MSG was solidified in form of a loosely and no coherent cake so that reformed to small formless beads by shaking the vials. For all samples of lactose, trehalose and dextran-40; and for sucrose and glucose at 5% to 10% concentrations, drying was proceeded successful and without any sign of adverse reactions such as big shrinkage, back melting, puffing, or collapsing, while a puffed matrix achieved by 2.5% of glucose and sucrose. A semi rubbery cake was created by all concentration of dextran-40. A minor shrinkage was seen in all samples especially in lower concentration but by MSG and dextran-40 it was greater than did by others. As indicated in Fig. 2, moisture content shows a minor increase for all samples as the LPs were increased from 7.5% to 10% so that for samples prepared using 7.5% of dextran and 2.5% glucose it stayed at lower and higher respectively. The mean reconstitution (with 3ml cold water) time less than 1 minute for the samples ( $p \leq 0.01$ ) suggested the good dispersing ability of the formula at the low moisture content. The Combined sample contained 1.5% of dextran-40, MSG 1.5%, and lactose 7.5%, showed the better physical appearance and the lowest moisture content (0.6%) promptly after lyophilization that revealed the effectiveness of combination of LPs. By addition of tween-80 (0.04%) to combined formula a better porosity, lower aggregation of bacteria, minimum remained moisture (0.4%) and better reabsorption of water was achieved.

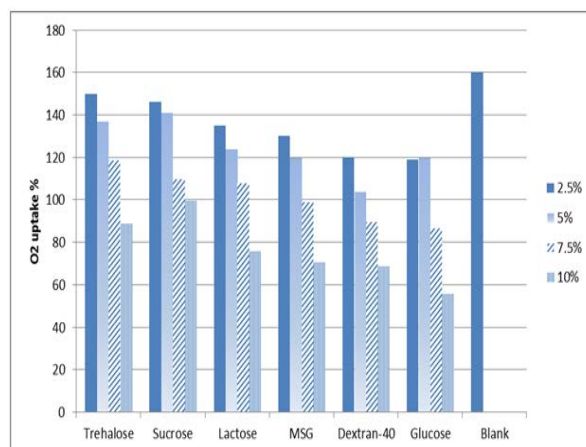


**Fig. 1: Appearance of lyophilized matrix of intravesical BCG samples a)final sample with combination of lactose, MSG and tween 80, b) dextran-40 10%, c)sucrose 10% and MSG 10%.**



**Fig. 2. Residual moisture content of lyophilized samples containing different amount of the lyoprotectants in compare to combined sample (CS) contained 7.5% lactose, 1.5% MSG, 1.5% dextran-40 and 0.04% of tween80**

As shown in Fig. 3, value of  $O_2$  up-take (Warburg test) of lyoprotected BCG bulks in compare to no lyoprotected bulk (160 $\mu$ l) accounts for the temporary marked decrease of biological activity. Lower  $O_2$  uptake was observed in the order of trehalose<sucrose<lactose<MSG<dextran-40. The combined sample showed lower  $O_2$  uptake.

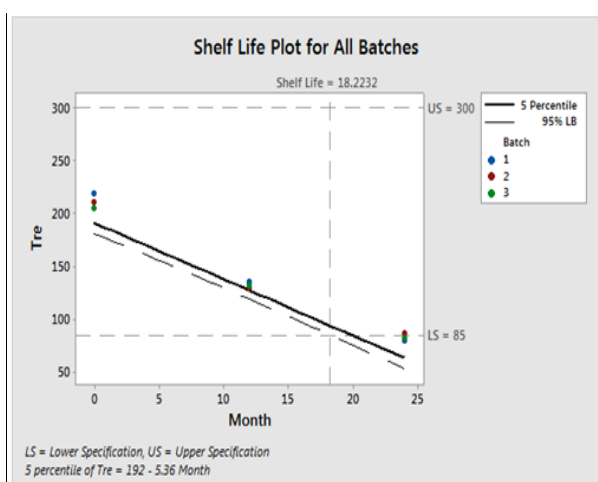


**Fig. 1.  $O_2$  uptake rate in lyophilized samples contained different amount of lyoprotectants (LPs)**

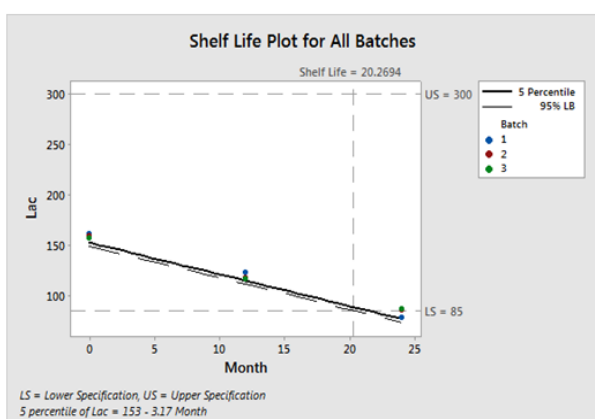
### Viability:

Real colony forming units (cfu) was counted by diluting of the samples in saline solution containing 2% Tween-80 before spreading on solid culture media. The viability values of lyophilized samples by 28% to 97% dropping right after preparation was significantly ( $P < 0.1$ ) lower than the related bulk ( $30 \times 10^6$  cfu/mg). In compare to the blank sample (in absence of LPs) that has been lost 99% of viability, results point out that generally all of the LPs in different concentration retained higher viability at the end of process although viability decreased gradually with various slope during long storage (Fig 4-8) Among the six

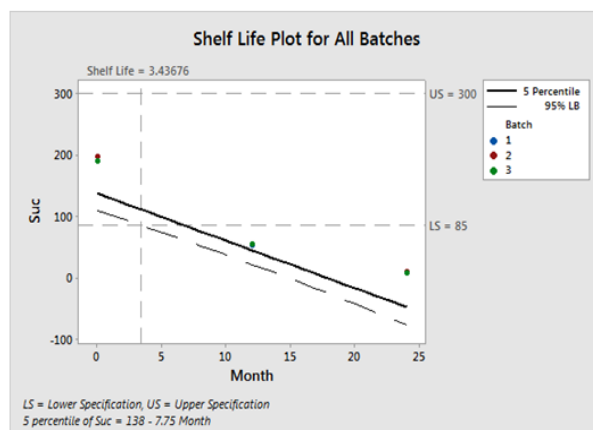
LPs, 10% of trehalose, sucrose, and lactose showed higher viability respectively promptly after the process ( $p < 0.1$ ). The longest shelf life was achieved by lactose, trehalose and MSG respectively while faster declining occurred by dextran-40 following by glucose and sucrose especially when they were more concentrated. Higher heat stability (28 days incubation at  $37^{\circ}\text{C}$ ) equal to 12% and 8% was resulted for 10% trehalose and lactose respectively ( $P < 0.1$ ). All LPs at higher concentration showed higher viability but on the contrary, MSG and dextran were better at  $\leq 5\%$ .



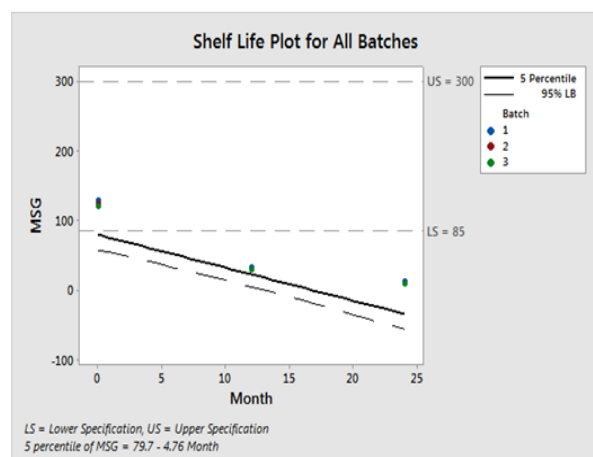
**Fig. 2.** Shelf life estimation for lyophilized samples contained 10% of trehalose (10%) equal to 18 months with 95% confidence within spec limits (lower cfu/mg=  $85 \times 10^5$ , upper cfu/mg= 300)



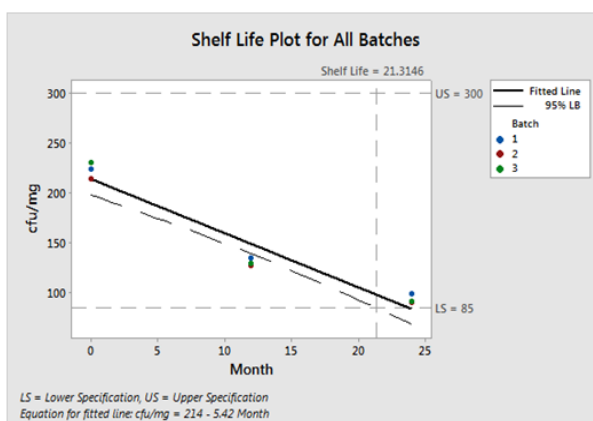
**Fig. 3.** Shelf life estimation for lyophilized samples contained 10% of lactose (10%) equal to 20 months with 95% confidence within spec limits (lower cfu/mg=  $85 \times 10^5$ , upper cfu/mg= 300)



**Fig. 4.** Shelf life estimation for lyophilized samples contained 10% of sucrose (10%) equal to 3.4 months with 95% confidence within spec limits (lower cfu/mg=  $85 \times 10^5$ , upper cfu/mg= 300)



**Fig. 5.** Shelf life was not estimated for lyophilized samples contained MSG (2.5%) with 95% confidence within spec limits.



**Fig. 6. e.** Shelf life estimation for lyophilized combined sample (CS) contained MSG (1.5%), Dextran-40 (1.5%), Lactose (7.5%), and Tween-80 (0.04%) equal to 21.3 months with 95% confidence within spec limits (lower cfu/mg=  $85 \times 10^5$ , upper cfu/mg= 300)

Combined sample showed improvement for viability, shelf life and heat stability equal to 72%, 21months and 14% respectively. In continuation, addition of 0.04% of Tween-80 led to higher viability (75%), shelf life (21.3 months) and lower residual moisture (0.4%) but heat stability decreased to 8%. Tween-80 as a nonionic surfactant helped to recovery of bacilli during rehydration.

## DISCUSSION

This study has attempted to provide a more stabilized lyophilized intravesical BCG (1173P2 strain). In several researches stabilization of lyoprotectants (LPs) contained biological formula such as BCG vaccine during and after lyophilization has been discussed with details [11, 13, 26, 27]. Strasser et al., [18] and Lugosi et al., [28] reported that influence of some LPs on viability, is bacterial species specific and therefore their related efficacy needs to be determined on case-to-case. Investigations have been revealed that some substances including carbohydrates and amino acid derivatives that act as LP to enhance stability of lyophilized biological products via preservation of the native cells structure first during freezing and then during drying stages [4, 7, 9, 29-31]. Safety of a LP is between the first criteria in order to be used in drug formula [10]. The skin reaction test results of the blank samples confirmed the safety of the six LPs at their specific concentrations. Since lower water activity of BCG bulks could be considered as a sign of effectiveness of LP and successfulness of lyoprotection activity; accordingly, lower level of O<sub>2</sub>-uptake by sugars could be revealed better protectivity effect of them in compare to MSG and dextran. As a result, substances with lower water activity and higher osmotic, apparently make lower intercellular free water so lower inner ice crystals. On the other hand, samples containing dextran also had lower residual moisture content that means water molecules were entrapped in the solidified matrix rather than substituted via H-bonds so that higher desiccation had happened. Accordingly, better caking performance was achieved by dextran following by the other ones. Between 62 preservatives that have been studied by Miller et al., [23] on stability of lyophilized BCG vaccine the most effectiveness achieved by sucrose, lactose, glucose, galactose, trehalose, MSG and sodium aspartate respectively. We achieved higher long stability in the order of lactose>trehalose>MSG>sucrose>glucose>dextran, yet the higher viability promptly after lyophilization achieved by trehalose, sucrose, lactose, MSG, glucose, and dextran respectively. On the other hand, samples contained glucose and dextran lost more than 99% of viability

rapidly at the end of 6<sup>th</sup> month storage while the highest remained viability achieved by lactose and trehalose. Such different results might be due to used different lyophilization process or even kind of the strain.

Lactose alone has been included in the licensed TICCE BCG vaccine with 2 years expire time [32]. Kovalcik et al., [33] demonstrated that hygroscopic amorphous lactose by which water molecules is held and water available in the microenvironment of product is decreased is a weak caking agent. On the contrary Dalia Mohamed [4] reported BCG vaccine contained lactose (15%) was superior over trehalose or trehalose-gelatin mixture. He estimated shelf life of 330 days with trehalose, and more than two years with lactose compared with 100 days for liquid BCG. Effectiveness of lactose better than trehalose during lyophilization of lactobacillus spp. has been reported by Nag et al., [34]. Our results for higher viability achieved by lactose are in correlate with the lasts and also with the report of Liao et al., [35] and Tonnis et al., [36]. They showed small humectant sugars such as lactose and trehalose with sufficient flexibility to replace the hydrogen bonds and also having higher *T<sub>g</sub>* to remained in glassy state during storage; usually have more water binding capacity and have better protectivity than crystalline sugar such as sucrose [31] although this effectiveness does not scale with the *T<sub>g</sub>* for dextran-40 polymer [16, 37].

Although trehalose and sucrose was shown as the non-reducing disaccharides which are superior to lactose and glucose that induce Maillard reaction [15] but in low moisture content products Maillard reaction would be suppressed. Studies of Bissoyi et al., [38] and Lerbret et al., [37] said that, trehalose is superior to sucrose due to its 2.5 times greater hydration radius, binding with large numbers of water molecules in glassy state, its higher ability to displace water molecules, and finally, presence on both sides of the cell membrane by which adequate protection of intracellular components and cell membrane could be attained.

Despite better characteristics of trehalose; since, it is not being used (as of early 1998) in any Food and Drug administration (FDA) approved parenteral products; [15] therefore it would not be our first choice apparently. On the other hand our results with lactose are closely similar to those achieved with trehalose.

MSG (1 to 1.5%) as a crystallizer and anti-oxidative agent are being widely used to keep potency of some vaccines including BCG vaccine unchanged during storage or exposure to heat, light, and humidity [7, 22, 28, 37]. In our experimental samples, MSG retained viability of BCG bacilli moderately, while it failed to prepare a stable matrix. Obayashi et al., [21, 39] suggested that thermostability of the lyophilized BCG vaccine prepared

with MSG (1.5%) was consistently higher than that did with 1% of sucrose.

According to the present study, dextran-40 in appropriate concentration mainly acted as a bulking agent and it made the cake structure harder. Foster et al., [40] reported that dextran polymers alone could not preserve biomolecules. This least Potency could be explained in standpoint of the role of the molecular size,  $T_g$  and hydroxyl groups so that trehalose with lower  $T_g$  but the most number of hydroxyl groups acts better as a LP, whilst dextran as a large polymer with the highest  $T_g$  but the least number of hydroxyl groups is a better bulking agent, and D-glucose, on the contrary, has a low  $T_g$  and a small number of hydroxyl groups is feeble for both [40]. It is well known that as the molecular weight of a carbohydrate is increased, the glassy state is formed more readily [15] so this could also explain the better performance of dextran and disaccharides rather than monosaccharides as showed by Jeong et al., [41].

According to reports, amino acids derivatives, small sugars or polymer glasses alone could not support completely the stability of the products by inhibiting collapsing or shrinkage due to higher residual moisture [35]. On the other hand removal of cell structural water could cause severe damages to viable cells [7]. For these reasons, combination of two or more LPs sometimes leads to better results. In a study due to collapse problems associated with glucose; product has been stabilized better with combination of glucose and lactose [11, 20]. Farmer et al., [14] prepared lyophilized BCG contained 8.3% dextran solution with 0.025% triton WR1339 containing 0% to 20% glucose. He reported that with dextran (higher  $T_g$ ) alone virtually all of the water was removed while viability highly reduced, and by addition of 7.5% glucose (lower  $T_g$ ) maximum retention of viability is achieved. Jahanbakhsh et al., [22] improved thermal stability and viability of lyophilized BCG vaccine by adding a combination of monosodium glutamate (1%), glucose (0.5%), lactose (2%) and dextran (1%). Apparently in our combined sample, MSG acts mainly as an antioxidant and  $T_g$  booster, dextran-40 as a caking agent and lactose as a vitrifying agent. Tween-80 as a common biocompatible nonionic surfactant is provided dispersed bacilli during preparation of BCG bulk and inhibited aggregation upon rehydration of lyophilized products [42, 43]. Furthermore tween-80 may promote the penetration of LPs; removing of free water molecules from the cells and dehydration process via reducing the aggregation of bacteria [15]. As well as, lyophilization of small clumps and individual BCG cells is easier generally because cooling and dehydration could be done more quickly [37].

We found lyophilization cycle 3 with primary temperature above the  $T_g$  but below the super cooling and ice

nucleation temperature was more effective on solidification, decreasing residual moisture and longer viability of the samples and resultant combined formula consists of lactose, sodium glutamate, dextran-40 and tween-80 for two years after.

Apart from the influence of LPs and freeze drying process on survival of BCG bacilli, It is crystal clear that success to reach desired survival rate of lyophilized BCG bacilli is dramatically impacted not only by LPs but by BCG strain, quality of BCG culture, physical and chemical characteristics of BCG suspension, type of vials and rubber stoppers, setting up a proper freeze drying process and so on [28, 44].

## CONCLUSION

The excipients individually possess a different ability for vitrifying,  $T_g$ , water replacement and collapse temperature modifying but the choice for having a more stabilized lyophilized BCG product should be those which provide better vitrified state with much lower intra cellular icing during freezing that appear to be a prerequisite during freeze-drying. Despite this, there were no significant difference ( $p < 0.5$ ) between the sugars for a stable solid when they were combined with dextran-40 although LPs alone had different behavior in terms of solidification and protective ability.

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