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Cost Effectiveness of Cryoprotective Agents and Modified De-man Rogosa Sharpe Medium on Growth of *Lactobacillus acidophilus*

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Abstract: The effect of cryoprotective agents (namely, sodium chloride, sucrose, dextran, sorbitol, monosodium glutamate, glycerol, skim milk and skim milk with malt extract) and modified De-Man Rogosa Sharpe (MRS) medium, on the viability and stability of *L. acidophilus* ATCC 4962, was investigated. The modified MRS medium was not only economical, but it gave a relatively higher yield of *L. acidophilus* ATCC 4962 than the commercial MRS. Monosodium glutamate, skim milk and skim milk with malt extract provided significantly higher viable counts, with optimum concentration at 0.3%. Nevertheless, at concentration above 0.5%, there was a reduction in cell viability, which could be attributed to cell shrinkage associated with osmotic pressure changes inside the cells. It was also found that *L. acidophilus* ATCC 4962 was stable at 28°C for eight weeks. Skim milk demonstrated a significant growth of probiotics. Skim milk was the preferred cryoprotective agent, as it is of low cost, easily available and demonstrated a significant growth of probiotics. In conclusion, modified MRS medium with skim milk is suggested for the remarkable growth and yield of *L. acidophilus*.

Key words: Cryoprotective agents, freeze-drying, *Lactobacillus acidophilus*, viability, stability

INTRODUCTION

Lactobacillus species have attracted much attention as probiotics due to their natural habitat in the human gastrointestinal system and the proposed roles they play as part of normal beneficial human microflora. These are specifically important for dairy and nutraceutical industries. Abadias *et al*. (2001) reported that fermented food containing lactic acid bacteria contributes about 20% of the total economic value of fermented food products worldwide. During freeze-drying, the viability of microbial cells is affected on several factors including the drying media (Carvalho *et al*., 2004), the growth phase (Otero *et al*., 2007; Edward *et al*., 2011), the initial concentration of microorganisms (Costa *et al*., 2000; Morgan *et al*., 2006), the presence of cryoprotective agents and the rehydration conditions (Abadias *et al*., 2001). The major factors of injury from drying of bacterial cells are probably osmotic shock with membrane damage and the removal of bound water which affects the properties of many hydrophilic macromolecules in cells (Selmer-Olsen *et al*., 1999). Thus, retaining critical levels of bound water, perhaps by adding suitable cryoprotective additives plays an important role in the conservation of the cell viability. Therefore, a good cryoprotective agent should provide cryoprotection and efficient drying to the cells during the freeze drying process. It also provides a good matrix to allow stability and ease of rehydration (Costa *et al*., 2000; Zhao and Zhang, 2005). Cryoprotectants such as glycerol, sorbitol and mannitol may be added during the process of freeze-drying of Lactobacilli, which helps in preventing inactivation of the cells during drying and stabilizes the microorganisms during storage (Ross *et al*., 2005).

Probiotic Lactic Acid Bacteria (PLAB) are heterotrophic and fastidious with complex nutritional requirements. De-Man, Rogosa and Sharpe (MRS) medium (De Man *et al*., 1960) is commonly used to grow PLAB (Sathyanarayanan *et al*., 2011). The types of growth media used play an important role in the growth activity. A number of growth media such as MRS broth, M-17, Elliker's broth, skim milk and whey permeates, have been reported to be used for cultivation of PLAB (Mayra-Makien and Bigret, 1993). Some factors should be considered in the choice of growth medium are costs, ability to produce large number of cells and harvesting method. Simple synthetic media, with these factors taken into account, have been studied (Stanley, 1977). Because of the cost-effective preparation and easy harvest, the tryptone yeast extract lactose medium and tryptone meat extract glucose medium have been used to cultivate and concentrate the cultures of lactic acid bacteria

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(Peebles *et al*., 1969). In one of the comparative studies, three growth media, namely MRS medium, the optimum-point medium and the center-point medium have been assessed and it was found that the MRS medium produced the largest number of viable cells. However, the MRS medium is an expensive, uxuriant medium with complicated ingredients. PLAB are very fastidious microorganisms (Oh *et al*., 1995), known to have limited biosynthetic ability and requiring several amino acids and vitamins for growth (Hujanen *et al*., 2001). These growth factors are usually supplied by a complex nitrogen source like yeast extract. Thus, there is a requirement of an inexpensive and easy to prepare media to grow lactic acid bacteria with maximum yield.

The aim of the present work was to modify the MRS (De-Man Rogosa Sharpe) medium with respect to its economic and biological effect and to examine the effect of various cryoprotective agents on the survival of freeze-dried *L. acidophilus* ATCC 4962 cultivated in the modified MRS medium. In addition, the stability of freeze-dried *L. acidophilus* ATCC 4962 powder at different temperatures was studied by determining the total viable count for a period of 8 weeks.

MATERIALS AND METHODS

Bacterial culture and growth conditions: *Lactobacillus acidophilus* ATCC 4962 was purchased from the American Type Culture Collection (Rockville, MD). The cultures was stabbed in De-Man Rogosa Sharpe Agar (MRSA) medium (Difco, USA) and stored at 4°C. The strain was activated by two subcultures in 100 mL MRS broth prior to experimental use.

Preparation of modified MRS medium: The modified MRS broth medium was prepared using 20.0 g dextrose (R and M Chemicals, UK), 8.0 g Bacteriological peptone (Oxoid, UK), 8.0 g meat extract (Fluka, Germany), 4.0 g yeast extract (Fluka, Germany), 5.0 g sodium acetate (AJAX Chemicals, Sydney, Australia), 2.0 g disodium phosphate (Sigma, UK), 2.0 g ammonium citrate (Oxoid, UK), 1.0 g Tween 80 (Sigma, UK), 0.1 g magnesium sulfate (Sigma, UK) and 0.05 g manganese sulfate (Fluka, Germany) in 1 L of distilled water. The mixture was boiled until the ingredients were completely dissolved. The pH of the mixture was adjusted with 1 M HCl to 6.0±0.1. The mixture was autoclaved at 121°C for 15 min and cooled to 45°C prior to use.

In addition, the modified MRS agar medium was prepared similar to that described for the modified MRS broth medium but with the addition of 15.0 g agar (Becton, Dickinson and Company, USA) per liter of distilled water.

Preparation of cryoprotective solutions: Various compounds, namely, sodium chloride, sucrose, dextran, sorbitol and monosodium glutamate (Sigma, USA), glycerol (R and M Chemicals, UK), skim milk (Sunlac, New Zealand) and skim milk with malt extract (Becton, Dickinson and company, USA) were examined as cryoprotective agents. The cryoprotective solutions of 0.1% w/v were prepared by dissolving or dispersing the cryoprotectants in sterile distilled water. Sodium chloride, glycerol, skim milk as well as skim milk with malt extract (1:1 w/w) solutions were autoclaved at 115°C for 10 min, while sucrose, dextran, sorbitol and monosodium glutamate solutions (heat labile) were sterilized by membrane filtration using 0.20 μm cellulose nitrate membrane (Whatman, UK) (Zayed and Roos, 2004). In addition, monosodium glutamate, skim milk and skim milk with malt extract at concentrations of 0.1 - 0.7% w/v were also studied.

Cultivation of strains of *L. acidophilus* and freeze dried with cryoprotective solutions: *L. acidophilus* ATCC 4962 was cultivated in modified MRS medium for 36 h at 37°C. After incubation, 20 mL ($\approx 2\times10^9$ CFU mL^{-1}) of the medium was centrifuged at 3500 rpm for 10 min at 4°C (Beckman J-6M/E, USA). Free-cell concentration was estimated by the pour plate method on modified MRS agar, after aerobic incubation at 37°C for 36 h. After centrifugation, the pellet (cell biomass) was collected and washed with Ringer solution (Merck, Germany) twice. Each time, Ringer solution was discarded after centrifugation. The cell pellet was mixed with 0.02 mL distilled water or various cryoprotective solutions before freeze drying (Labconco Lyph Lock 6 Freezer Dryer, Shell Freeze System, USA).

Moisture content measurement: The moisture content was measured using moisture analyzer (Mettler Toledo Delta range, PM 480, Switzerland). Five gram of the freeze dried powder was measured for 10 min at 105°C.

Determination of total viable count: The freeze-dried powder of *L. acidophilus* ATCC 4962 of 1.0 g was rehydrated separately by mixing with the original volume (20 mL) of modified MRS broth medium and then incubated at 25°C for 10 min (Font de Valdez *et al*., 1985; Sinha *et al*., 1982). After serial dilution, *L. acidophilus* ATCC 4962 was incubated aerobically at 37±1°C for 72 h in modified MRS agar medium and the viable count was determined by pour plate method (Hekmat and McMahon, 1992).

Determination of percentage of loss in viable count: The percentage of loss in the viable counts of *L. acidophilus* ATCC 4962 after freeze-drying with various cryoprotective agents was calculated using the following equation:

$$\text{Loss of the cells (\%)} = \frac{N_0 - N}{N_0} \times 100\%$$

Where:
N_0 = No. of viable cells before freeze-drying
N = No. of viable cells after freeze-drying

Stability evaluation of freeze-dried strains of *L. acidophilus*: The freeze-dried *L. acidophilus* ATCC 4962 with 0.3%w/v of monosodium glutamate, skim milk and skim milk with malt extract were stored at three different temperature of 5°C (refrigerated) 28°C (ambient room temperature) and 40°C (oven). Every 7th day, the powder of both strains was analyzed separately for 8 weeks. The stability of the powder was assessed by obtaining the total viable count.

Statistical analysis: All data are presented as Mean±SD. Comparison of means of modified MRS and commercial MRS were analyzed by Student's t-test. One-way analysis of variance was used for statistical analysis (version 15.0, SPSS Inc. Software, USA), with a significance level at $p<0.05$. When there was a statistically significant difference, post-hoc Tukey-HSD test was performed. The experiments were repeated three times.

RESULTS AND DISCUSSION

Freeze-drying has been studied as a dehydration process for bacteria to produce a dry solid formulation. This study showed the impact of modified MRS medium and cryoprotective agents on the viability of *L. acidophilus* after freeze-drying ATCC 4962. In addition to the stability of freeze-dried *L. acidophilus* ATCC 4962 powder at different temperatures, the influence of the initial cell concentration for both strains was also estimated by determining the total viable count for a period of eight weeks.

Modified MRS medium: The modified MRS medium used in the present study was not only more economical because of the use of less amount of expensive nitrogen source (meat extract, yeast extract and peptone) which reduce cost of fermentation medium but also gave a higher yield of *L. acidophilus* ATCC 4962 (90.6×10^8 CFU mL^{-1} after 36 h incubation) as compared to the commercial MRS medium (87.4×10^8 CFU mL^{-1} after 36 h incubation of *L. acidophilus* ATCC 4962) as shown in the Fig. 1 ($p<0.05$). Cost of raw materials is one of the major factors in economics of production of media for lactic acid fermentation (Altaf *et al*., 2006). A critical stepin the development of economical fermentation process is selection and optimization of carbon and nitrogen sources (Naveena *et al*., 2005). Yeast extract is used in most of the fermentation studies as supplement, but high price hinders its use in large quantities (Yoo *et al*., 1997). In an economic analysis for *Lactobacilli* growth, the largest contributor was found to be yeast extract accounting for about 38% of total production medium cost (Tejayadi and Cheryan, 1995). Yeast extract and peptone have profound influence on the cell concentration (Monteagudo *et al*., 1993).

Several studies demonstrated the significance of amino acids and vitamins for cell growth and emphasized about pyramidines and B vitamins present in yeast extract which are essential for the viability and growth of the cells (Amrane and Prigent, 1994).

All the above reports state that peptone and yeast extract are significant nitrogen sources for probiotics production and if decrease nitrogen sources are used either there is decrease in growth production or increase in incubation period. As per the finding of the present study, though we reduced the concentration of peptone, yeast extract and meat extract to 20% than the commercial MRS in modified MRS medium yet the growth was more significant than the commercial MRS medium.

Moisture content study: Water content is an important parameter for the stability of dried cultures (Clementi and Rossi, 1984). It is known that microorganisms survive better at low-water activity and generally the viability decreases when the water activity is increased (Kurtmann *et al*., 2009) The results of moisture content values are given in Table 1.

Table 1: Moisture content of freeze-dried *L. acidophilus* with different cryoprotective agents

Cryoprotective agents	Moisture content (%)
Sodium chloride	1.93±0.01
Sucrose	1.98±0.02
Glycerol	1.81±0.04
Dextran	1.96±0.09
Skim milk	1.86±0.06
Skim milk with malt extract	1.82±0.12
Sorbitol	1.98±0.07
Monosodium glutamate	1.89±0.06

Mean±SD, N = 3, S+M: Skim milk with malt extract, MSG: Monosodium glutamate

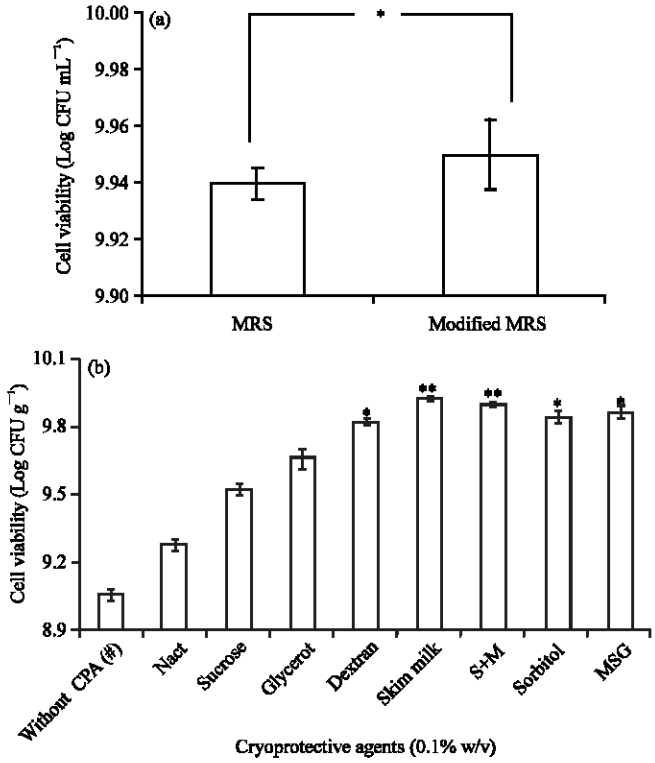

Fig. 1(a-b): (a) Viability comparison between MRS medium and modified MRS medium and (b) Effect of cryoprotective agents on the viability of *L. acidophilus* cultivated in modified MRS medium, Mean±SD, N = 3. Without (#): No cryoprotective agents; S+M: Skim milk with malt extract, MSG : Monosodium glutamate, **Significant when compared to the control ($p<0.01$), *Significant when compared to the control ($p<0.5$)

The moisture content values for freeze-dried *L. acidophilus* ATCC 4962 containing different cryoprotective agents was between 1.81 and 1.98%. It was reported that the moisture content in a range of 1-4% was acceptable for freeze-dried *L. acidophilus* (Ekdawi-Sever *et al*., 2003). Moisture content up to 4% was acceptable for freeze-dried *L. acidophilus* (Gardiner *et al*., 2000).

Recovery and viability of freeze-dried *L. acidophilus*: Rehydration is an important process in the recovery of freeze-dried microorganisms. An organism which survives the various processing steps, such as freezing, drying and storage, may lose its viability during rehydration. Poor recovery of cells may be attributed to inadequate rehydration procedure. If rehydration was performed under inappropriate condition, the cells that were subjected to injury might not be able to repair its damage (Costa *et al*., 2000). The study of Wang and colleagues indicated that higher rehydration temperature of 30-40°C enabled better recovery of *L. acidophilus* (Wang *et al*., 2004). Speck and Myers (1946) reported that increasing the rehydration temperature from 20-25°C to 37-50°C increased and decreased the recovery of spray-dried and freeze-dried cells of lactobacillus bulgaricus, respectively. on the other hand, similar studies by other researchers (Sinha *et al*., 1982) reported that rehydration at the refrigeration temperature may cause leakage of intracellular substances from the cells, thereby resulting in low viability. Font de Valdez *et al*. (1985) found that 20°C was the optimum rehydration temperature for freeze-dried lactic acid bacteria. nevertheless, in the present study, it was found that 25°C was the optimum rehydration temperature for both tested freeze-dried strains of *L. acidophilus*.

Figure 1a demonstrates that the modified MRS medium has more significant effect on cell viability than the commercial one. The viable count in modified MRS medium was found to be 90.6×10^8 CFU mL^{-1} for *L. acidophilus* ATCC 4962 whereas, it was significantly ($p>0.05$) lower in commercial MRS medium (87.4×10^8 and 83.2×10^8 CFU mL^{-1} for *L. acidophilus* ATCC

Table 2: Loss in viable counts (%) of *L. acidophilus* after freeze-drying

Cryoprotective agents	Loss in viable count (%)
No cryoprotective agent	88.24±0.23
Sodium chloride	78.85±1.71
Sucrose	43.66±4.04
Glycerol	35.42±5.66
Dextran	26.41±3.22
Skim milk	12.77±4.09
Skim milk with malt extract	14.26±3.58
Sorbitol	23.15±4.59
Monosodium glutamate	27.89±2.57

Means with different uppercase letters are significantly different ($p<0.05$), Mean±SD, N = 3

4962). Figure 1b shows the viable count of freeze-dried *L. acidophilus* ATCC 4962 with different cryoprotective agents. It is clear from the results that cryoprotective agents improved the viability of the cells when compared with that in absence of cryoprotective agent ($p<0.05$). Monosodium glutamate, skim milk and skim milk with malt extract provided significantly higher viable counts than other cryoprotective agents ($p<0.05$). There was no statistically significant difference between monosodium glutamate, skim milk and skim milk with malt extract with respect to the viable count ($p>0.05$). However, skim milk was preferred over monosodium glutamate and skim milk with malt extract as the former is of low cost, safe and has beneficial properties. A higher survival rate of the freeze-dried cells with skim milk as cryoprotective agent was also reported by other researchers (Ross *et al*., 2005)

The percentage of loss results in the viable counts of *L. acidophilus* ATCC 4962 after freeze-drying with various cryoprotective agents are given in Table 2. The viable count of freeze-dried *L. acidophilus* ATCC 4962 without cryoprotective agents was approximately 11.3×10^8 CFU g^{-1} (9.05 log CFU g^{-1}). This indicates that about 88.24% of *L. acidophilus* ATCC 4962 bacterial cells lost their viability after freeze-drying. This is in line with the results of the earlier work (Cordero and Voltolina, 1997; Blanquet *et al*., 2005) who found that entrapment of probiotic cells without protective solution did not offer good protection in freeze-drying while improved viability was achieved by adding cryoprotective agents. The loss in the viability without any cryoprotective agent is a consequence of cell injury at several target sites, namely the cell wall, the cell membrane and the DNA (Teixeira *et al*., 1995), as well as a result of membrane lipid oxidation (Castro *et al*., 1997; Teixeira *et al*., 1996). The primary cell damage in freeze-drying was probably attributed to ice crystal formation, high osmolarity due to high concentrations of internal solutes with membrane damage, macromolecule denaturation and the removal of water, which affected the properties of many hydrophilic macromolecules in cells (Thammavongs *et al*., 1996). On the other hand, cry protective agents could promote cell survival during freezing or drying process (Broadbent and Lin, 1999). The cryoprotective agents play two main functions in preserving the viability of freeze-dried cells. The first is to provide a dry residue with definite physical structure acting as a support material and as a receptor in rehydration and the second is to protect the living cells against damage during freezing or drying (Berny and Hennebert, 1991).

Sodium chloride was used as a cryoprotective agent to promote the survival of lactic acid bacteria throughout storage in the dry state (Carvalho *et al*., 2003a; Linders *et al*., 1997). Glycerol, sorbitol and other sugars were selected as cryoprotective agents due to their functions in the prevention of damage utilizing their anti-oxidant properties (Wisselink *et al*., 2002). Monosodium glutamate was considered as a superior agent to increase the survival of the majority of lactic acid bacteria during freeze-drying (Font de Valdez *et al*., 1985; Carvalho *et al*., 2003b) Our results showed that these media can be used to increase the survival of the LAB strains studied. Other researchers suggested that skim milk powder should be selected as drying medium for LAB (Carvalho *et al*., 2004; Carvalho *et al*., 2003b) Therefore, in the present study, skim milk, which was used as drying medium, showed a faster repair of the cell injury and the ability to dry easily, skim milk solids are expected to prevent cellular injury by stabilizing cell membrane constituents (Castro *et al*., 1995). In addition, milk proteins may form a protective coating on the cell wall of probiotics and calcium in milk helps in stabilization of probiotics after freezing or freeze-drying (King and Su, 1994). Skim milk contains many solutes such as phosphates and citrate that would prevent cellular injury by stabilizing cell membrane constituents and pH (Modler and Villa-Garcia, 1993) In addition, milk has proteins that act as a cryoprotective coating on the cell wall, thus increases the survival of bacteria after freezing or freeze-drying (King and Su, 1994). Malt extract was added to skim milk to enhance the effectiveness of skim milk because malt extract contains vitamins and proteins. However, higher cell loss after freeze drying has been noted by other researchers who reported 30-50% loss in viability after freeze drying of pure cultures in the presence of various cryoprotectants (Zamora *et al*., 2006). On the other hand, about 65-70% loss of viability after freeze drying by using three different cryoprotective

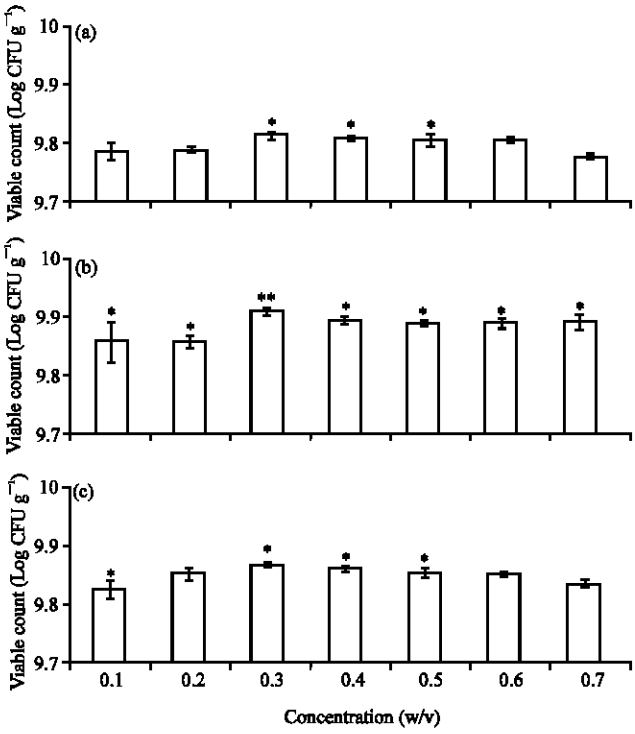

Fig. 2(a-c): Effect of various concentrations of cryoprotective agents (a) Monosodium glutamate (b) Skim milk and (c) S+M: Skim milk with malt extract on *L. acidophilus* viability in modified MRS broth medium, Mean±SD, N = 3, **Significant when compared to the control ($p<0.01$), *Significant when compared to the control ($p<0.5$)

agents, namely 10% skim milk, calcium alginate or 0.85% physiological saline (Jagannath *et al*., 2010).

Effect of concentration of cryoprotective agents on *L. acidophilus* viability: The effect of concentration of three cryoprotective agents, monosodium glutamate, skim milk and skim milk with malt extract on the viability of freeze-dried *L. acidophilus* ATCC 4962 was investigated to select the optimum concentration. The results of viable counts are shown in Fig. 2. There was a statistically significant difference ($p<0.05$) in the viable count of freeze-dried *L. acidophilus* among the different concentrations of skim milk, skim milk with malt extract, as well as monosodium glutamate. Based on the obtained results, 0.3%-0.5% was the optimum concentration for all the three cryoprotective agents. Monosodium glutamate, skim milk and skim milk with malt extract might be able to coat the cells, reducing the exposure of the cells to the environment and thus effectively protecting the cells from damage. At higher concentration, there was a reduction in the cell viability, which could be attributed to the shrinkage of the cells as a result of changes in the osmotic pressure inside the cells. In an earlier study, it was found that the optimum concentration for freeze drying of *Lactobacillus* strains are, 6% w/v skim milk, 6% w/v skim milk with 6% w/v lactose and 6% w/v skim milk with 6% w/v sucrose (Otero *et al*., 2007).

Storage stability of freeze-dried *L. acidophilus*: One of the most important aspects of the freeze-dried probiotic productions is the stability (Brennan *et al*., 1983). The functional food industry requires an improvement of probiotic strain stability during storage, especially when they are stored at room temperature (Savini *et al*., 2010). It is known that storage condition critically affected the viability and stability of freeze-dried cells (Castro *et al*., 1995; Gardiner *et al*., 2000). The stability profiles of monosodium glutamate, skim milk and skim milk with malt extract protected freeze-dried *L. acidophilus*, are depicted in Fig. 3. The results showed that *L. acidophilus* ATCC 4962 was stable at 5°C and 28°C until eight weeks as there was a negligible decrease in the viable counts. Both strains showed approximately 60-68% reduction in viable count at the end of 8 weeks, when stored at 40°C.

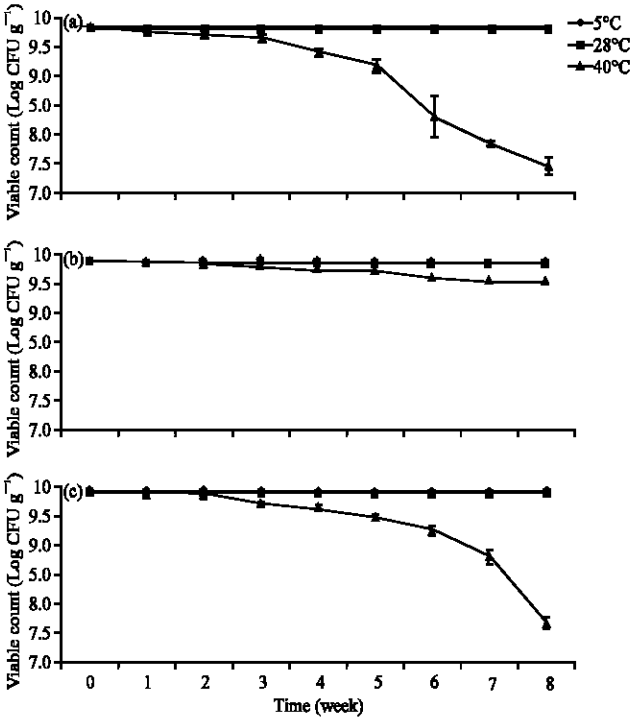

Fig. 3(a-c): Effect of the storage temperature on the stability of freeze-dried *L. acidophilus* in edible broth medium, Mean±SD, N = 3, (a) Skim milk, (b) S+M: Skim milk with malt extract and (c) MSG: Monosodium glutamate

CONCLUSION

In conclusion, the modified MRS medium used in the present study is more economical and gives a high yield of the probiotic bacterial strains of *L. acidophilus* ATCC 4962, alternative replacing commercial MRS medium, without compromising the quality of the medium with respect to the viability and stability of the bacteria. The presence of cryoprotective agents improved significantly the cell viability of tested strain of *L. acidophilus* ATCC 4962. Among the cryoprotective agents studied, monosodium glutamate, skim milk and skim milk with malt extract provided significantly high viable counts, with optimum concentration of 0.3%. *L. acidophilus* strain was stable at 28°C until 8 weeks. Skim milk powder is the preferable cryoprotective agent, as it is of low cost, easily available, functions well and has nutritional value as well.

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