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Production of Lyophilized Culture of *Lactobacillus acidophilus* with Preserving Cell Viability

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Optimal lyophilization process was developed for manufacturing the dried product of *Lactobacillus acidophilus* with high cell viability. Three major factors, freezing rate, specific surface area of samples, and stabilizer type and their synergy were shown to play a crucial role in the development of an effective lyophilization process. Finally we found an optimal combination among three process parameters mentioned above: an exceptionally high cell survival percentage of 90% was achieved using the 8.28 cm^{-1} specific surface area of samples, slow freezing rate, and a stabilizer composition of 4% skim milk +1% glycerol +0.1% calcium chloride.

Key words: *Lactobacillus acidophilus*, lyophilization, survival percentage

INTRODUCTION

Lactobacillus has a long history of its utilization for the manufacture of many value-added products. A number of reports have encouraged the incorporation of some strains of lactobacillus in food due to their property of stabilizing human gastro-intestinal microflora. *Lactobacillus acidophilus* has been reported to be an extremely effective agent for prevention and treatment of gastro-intestinal disorders [1,2]. The application potential of lactobacillus has also been identified in the field of oncology. Dietary lactobacilli have been reported to decrease colon cancer risk factors such as soluble faecal bile acids and faecal bacterial enzymes [3]. Recently lactobacilli have been found to be effective in inhibiting the growth of tumor cells *in-vitro*, which suggests their possible role in cancer therapy [3-5]. Moreover, lactic acid, the major product of lactobacillus fermentation, has been reported to be useful in the production of biodegradable polymer, eco-friendly solvents and non-hazardous cleaning agents [6].

Due to wide ranging applications of lactobacilli and lactic acid a large number of industrial fermentation processes have been developed [7-9] with the objective of maximizing biomass and/or product yield. In our previous study we had reported a high biomass concentration (50 g/L) in a continuous culture of *Lactobacillus acidophilus* through cell recycle [10].

The application potential of lactobacilli necessitates the development of an effective process of biomass preservation. Various methods such as fludization, spray drying and freeze drying (lyophilization), have been reported for the preservation of lactobacilli [11-13]. However, many of the processes developed from aforementioned methods had the disadvantage of low viability of the preserved cells. In order to reduce the incidence of cell death during the cell preservation process several additives such as adonitol, carbohydrates, dimethyl sulphoxide, glycerol, serum, peptone, glutamate and milk products were suggested [13,14].

In the present study, we have identified the major factors responsible for affecting the viability of *Lactobacillus acidophilus* during lyophilization. By optimization of freezing rate, specific surface area of samples, and stabilizer composition we could achieve a high cell survival percentage during the freeze-drying process.

MATERIALS AND METHODS

Microorganism and Culture Medium

Lactobacillus acidophilus ATCC 4356 (human origin) used in this study, was grown on MRS medium. The details of medium compositions were well described in our previous report [10].

Cultivation and Cell Recovery

L. acidophilus was cultivated in 5-L Bioflo III laboratory fermenter under total cell recycle. The culture conditions and bioreactor operation employed were the same as previously reported [10]. The culture broth was centrifuged at 8,000 g for 20 min. at 4℃. The pellet was washed twice with 0.1% peptone water. The washed cell mass was subsequently used for cell preservation experiments.

Lyophilization of *L. acidophilus* Cultures

The cell mass obtained by the recovery process was lyophilized stepwise through freezing and subsequent drying. After being mixed with 10% skim milk (unless otherwise mentioned), the samples at room temperature were frozen to -60℃ through four different freezing

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rates, as described below:

1) Method A: frozen to -60℃ in four steps i.e. 4℃ → 0℃ → -20℃ → -60℃;
2) Method B: frozen to -60℃ in three steps i.e. 4℃ → -20℃ → -60℃;
3) Method C: frozen to -60℃ in two steps i.e. -20℃ → -60℃; and
4) Method D: frozen to -60℃ in one step process.

In the freezing procedures given above, the cell samples were allowed to stand at 4, 0 and -20℃ for 2 hr and at -60℃ for 12~18 hr before being subjected to lyophilization. The frozen cell samples contained in containers with constant radius (1 cm) were lyophilized at -40℃ under 0.2~0.3 mbar vacuum in a lyophilizer (CHRIST, alpha 1-4, Osterode am Harz, Germany) for 2 hr (unless otherwise mentioned).

The cell samples were lyophilized with different specific surface areas, which is defined as $2(V+\pi r^3)/(rV)$ where V and r represent sample volume and radius of sample container, respectively. Three stabilizing additives (stabilizers), skim milk, glycerol and calcium chloride (Sigma) were used in this study, and in order to find an optimal composition of the stabilizers, concentrations of skim milk and glycerol were varied with constant concentration of calcium chloride (Table 1).

Estimation of Cell Survival Percentage

The freeze-dried samples were diluted with 0.1% peptone water and incubated on MRS agar plates at 37℃. The number of colonies obtained on the same MRS agar plates before freezing and lyophilization, considered as 100%, was subsequently used to calculate the cell survival percentage in the freeze dried samples.

RESULTS AND DISCUSSION

Effect of Freezing Method on Cell Viability

The effect of sample freezing method was investigated in plastic centrifuge bottles with radius 1 cm, using four different methods, A, B, C, and D (as described in Materials and Methods). The Method A, in which samples were stepwise frozen to -60℃ depicts the slowest freezing rate, whereas the Method D, being a one step freezing method, is the fastest among the four methods. The specific surface area of cell samples was fixed at 3.26 cm^{-1} in all these experiments.

The cell survival percentage was lowest in the lyophilization with the Method A (Fig. 1). The difference of salt concentration between interior and exterior of the bacterial cell could result in the diffusion of water across the cell membrane down the concentration gradient. The above phenomenon seemed to be responsible for osmotic shock and cell death at the freezing rate of Method A. The cell survival progressively increased as the freezing rate increased from A to C. With rapid freezing in Methods B and C the resultant decreased mobility of water seemed to have decreased the deleterious effect of osmotic shock. The decrease in cell survival, though small, took place as the freezing rate increased to maximum (D). Rapid freezing, as in D, might cause formation of intracellular ice crystals, which disrupt the integrity of the cell membrane, leading to cell death.

Table 1. Variation in composition of mixed stabilizers

Experiment number	Concentration of components in the mixed stabilizer		
	Skim milk (%)	Glycerol(%)	Calcium(%)
1	0	5	0.1
2	1	4	0.1
3	2	3	0.1
4	3	2	0.1
5	4	1	0.1
6	5	0	0.1

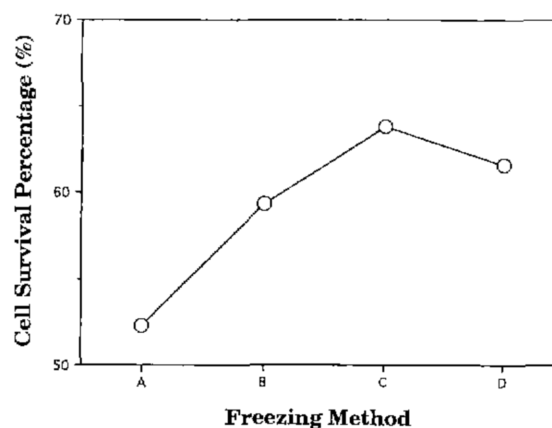

Fig. 1. Effect of freezing rate on survival percentage of *L. acidophilus* in freeze drying.

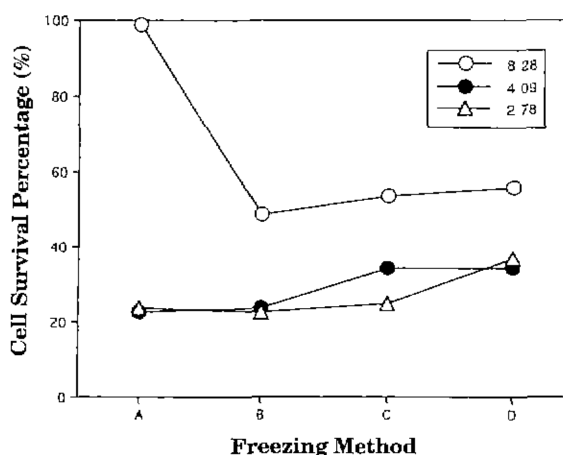

Fig. 2. Effect of specific surface area of culture samples (cm^{-1}) on survival percentage of *L. acidophilus* in freeze-drying at four different freezing methods (A, B, C, and D).

Importance of Specific Surface Area of Culture Samples

The *L. acidophilus* cultures were lyophilized in glass containers of radius 1 cm. Different volumes of the samples were taken to obtain three different specific surface areas i.e. 8.28, 4.09, and 2.78 cm^{-1}. As a whole, the sample having highest specific surface area (8.28 cm^{-1}) showed higher percentage of cell survival (Fig. 2). Evidently from Figs. 1 and 2, both the specific surface area of culture samples and freezing rate are of crucial importance in maintaining the cell viability during the lyophilization. Lyophilization involves phase transition (freezing of water and sublimation of ice) for which proper heat transfer is required, and at the same time water vapors should be removed from the samples. The lower specific surface area of the samples might cause the higher resistance to heat and mass transfer,

and the resistance, so created, could generate local gradients in the sample temperature, water mobility, and water vapor diffusion. It has been reported that in many cases the difference between the glass transition temperature (the temperature at which the diffusion of water molecules ceases) and the cake collapse temperature (the temperature at which molecular mobility begins) was in the range of mere 2~3℃ [15]. Therefore, the local changes in water mobility and vapor diffusion might occur with a small shift in temperature and could affect the performance of the freeze-drying process.

Effect of Stabilizer on Cell Survival

During the freezing and subsequent drying, a number of unfavorable changes in cell viability take place, which may ultimately lead to cell death. In order to prevent them, or at least to mitigate these unfavorable changes, certain stabilizers can be added to samples before freezing. De Valdez *et al.* [16] examined the protective effects of glycerol, adonitol, and four other related polyhydric alcohols on 12 strains of lactic acid bacteria subjected to freeze-drying and showed that the presence of adonitol most markedly protected the viabilities of the strains tested, due presumably to the esteric structure of its hydroxyl groups. Glycerol also provided effective protection for *L. leichmannii* ATCC 4797 (90% survival) [17], and the addition of 0.04 M cysteine was reported to result in a high viability (78%) of *L. bulgaricus* ATCC 11842 [17]. Tsvetkov and Brankova [18] showed that the mixture of nonfat milk and peptone at concentrations 8 and 5%, respectively, led to 100% viability of *L. plantarum* strain L4.

The effect of stabilizer components was studied at two levels i.e. the effect of individual component and the synergy of the stabilizer mixture. The specific surface area of samples was fixed at 8.28 cm^{-1} in all the experiments pertaining to effect of stabilizer(s) on cell survival.

Effect of individual stabilizer component: Three stabilizing components, skim milk, glycerol and calcium chloride were individually tested for their effect on cell survival at each of the four freezing rates. With the same specific surface area, as shown in Fig. 2 and 3(a), the effect of freezing rate on the percentage of cell survival was nearly unchanged with 5 and 10% skim milk. The low cell survival at B, C and D suggests that skim milk might not be as effective in checking the cell death due to osmolysis or intracellular ice crystal formation. The skim milk ingredients, especially lactose, seem to be most effective at the concentration of 20% in improving the percentage of cell survival in all freezing rates.

With glycerol only in the absence of skim milk in the culture samples, the cell survival percentage was drastically decreased to less than 20% at all freezing rates (Fig. 3(b)). Since glycerol has already been reported as a substance, which could prevent the formation of large ice crystals during the freezing process [19], the aforementioned report corroborates that the greater stabilizing effect of skim milk was mainly exerted in the drying process. As expected well, the stabilizing effect of glycerol was evidently higher at higher freezing rate (D).

Calcium chloride was found to be effective at low freezing rates (Fig. 3(c)). The ionic effect of calcium chloride seems to mask the deleterious phenomenon of

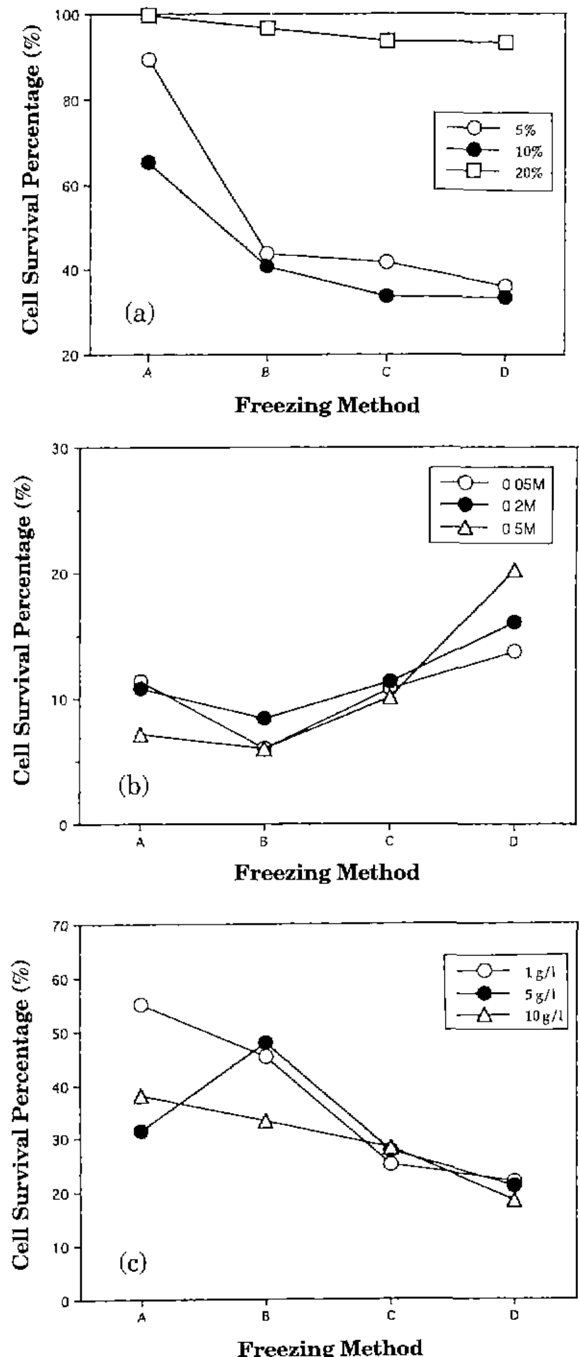

Fig. 3. Effects of (a) skim milk, (b) glycerol, and (c) calcium concentration in culture samples on survival percentage of *L. acidophilus* in freeze-drying at four different freezing methods (A, B, C, and D).

osmotic shock thereby resulting in higher cell survival at low freezing rate of the samples. Moreover, all the three concentrations of calcium chloride tested (1, 5 and 10 g/L) were more or less equally conducive for the manifestation of its stabilizing effect at high freezing rates.

Synergistic effect of stabilizers: After obtaining the information about pronounced stabilizing effect of skim milk (at low freezing rates) and glycerol (at high freezing rates) it was imperative to test their synergy for further improving the cell survival. In six different experiments, keeping the concentration of calcium chloride constant (1 g/L), the concentration of skim

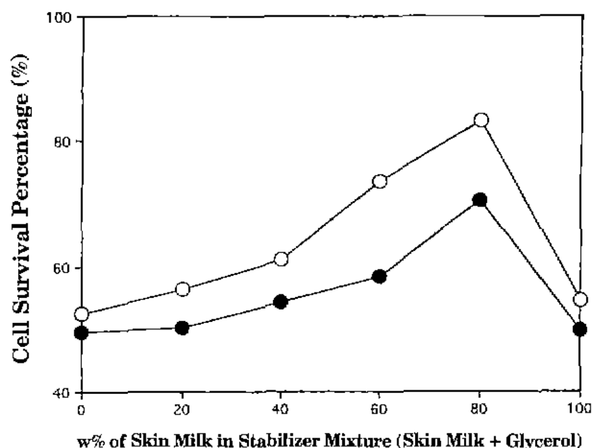

Fig. 4. Effect of compositions of stabilizing additives in culture samples (shown in Table 1) on survival percentage of *L. acidophilus* in freeze-drying at slow (—○—) and rapid (—●—) freezing rate.

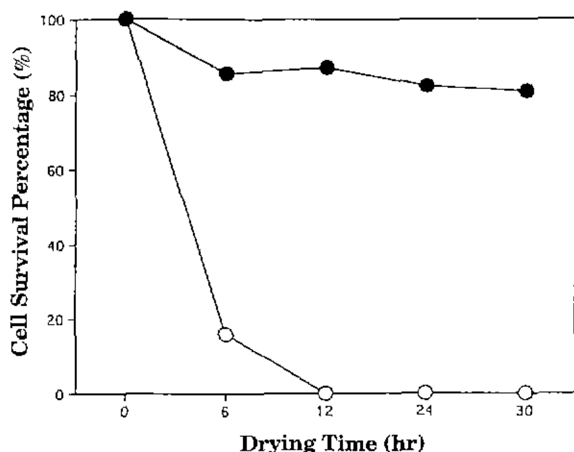

Fig. 5. Time-course variation of survival percentage of *L. acidophilus* during lyophilization process with stabilizing additives at two different compositions, skim milk 4% + glycerol 1% (—●—) and glycerol 5% (—○—) only.

milk and glycerol were varied between 0~5% (Table 1). The cell survival percentage was investigated at lowest (A) and highest (D) freezing rates in each of the six experiments (Fig. 4). As the concentration of skim milk increased, the improvement in cell survival percentage was obtained in the samples at both the two freezing rates, and more significant improvement was achieved with the freezing rate A due to the higher stabilizing effect of skim milk in lower freezing rates (Fig. 4), as explained earlier. The sharp decrease in cell survival in experiment no. 6 (skim milk 5%, glycerol 0%) could be explained on the basis of cell death due to intracellular ice formation. In other words, the absence of glycerol made the phenomenon of intracellular ice formation virtually unabated.

The most effective stabilizer composition (4% skim milk, 1% glycerol, 0.1% calcium chloride) was used to study its efficacy on long duration of freeze-drying process at slow freezing rate (A). Its effectiveness was further confirmed by less than 10% decrease in cell survival percentage during prolonged freeze-drying period of 30 hr (Fig. 5). The above result of 90% cell survival underlined the superiority of 4% skim milk +1% glycerol over the condition where only glycerol was used as a stabilizer. The former combination was effective in protecting the cells from being inactivated in the stressful environment developed during primary and secondary phases of freeze-drying, and hence could be extremely effective in the development of freeze drying processes for bacterial cell preservation.

This study makes it possible to achieve high cell survival percentage in freeze-dried lactobacilli by: (a) finding an optimal combination between the specific surface area of samples and freezing rate and (b) employing effective stabilizers and their optimal composition capable of protecting cells both from osmolysis and intracellular ice formation.

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