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# Optimization of a cryoprotective medium to increase the viability of freeze-dried *Streptococcus thermophilus* by response surface methodology

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ABSTRACT

*Streptococcus thermophilus* normally exhibits different survival rates in different bacteria medium during freeze-drying. In this study, response surface methodology (RSM) was applied on the design of experiments for optimizing the cryoprotective medium. Our results showed that the most significant factor influencing the resistance of *S. thermophilus* STX2 to freeze-drying was skim milk, followed by sodium glutamate, and then glycerol. These three factors may have interactive effects and produce synergistic protective effects on the growth of *S. thermophilus* STX2. Regression analysis indicated that the optimal concentrations of these variables were determined as: glycerol 79.60 g/L, sodium glutamate 77.40 g/L, and skim milk 116.40 g/L. *S. thermophilus* STX2 freeze-dried in a medium with the optimal formulation obtained a best cell viability up to 93.58%.



## 1. Introduction

Lactic acid bacteria (LAB) is widely used as a part of starter culture in many fermentation processes and it is also considered as probiotics for human health. The industrial exploitation of LAB as a starter culture depends on its concentration and preservation technologies, which can guarantee the delivery of stable cultures in terms of viability and bacterial functions (Palmfeldt & Hahn-Hägerdal, 2000; Yousseef, Lafarge, Valentin, Lubbers, & Husson, 2016).

Freeze-drying, often used for long term storage of biological samples (Heidebach, Forst, & Kulozik, 2010; Kanmani et al., 2011), is suitable to produce concentrated starter culture. Nevertheless, a certain degree of deterioration may occur during the freeze-drying process. A number of factors, such as species (Fonseca, Béal, & Corrieu, 2001), freeze-drying parameters (Abadias, Benabarre, Teixidó, Usall, & Viñas, 2001), physiological state of the cells (Broadbent & Lin, 1999), freeze-drying medium (Chen, Chen, Li, & Shu, 2015; Santos, Gerbino, Araujo-Andrade, Tymczyszyn, & Gómez-Zavaglia, 2014), and rehydration conditions (Chotiko & Sathivel, 2014) can affect the viability of freeze-dried probiotic culture. One of the effective ways to increase the survival rates of microorganisms during freeze-drying is adding protective agents into the medium.

A variety of protective agents have been used to reduce the damaging effects by freeze-drying, such as skim milk, glycerol, manmtol, sorbitol, trehalose, sucrose, maltose, lactose, fructose, glucose, betaine, and amino acids (Bosnea et al., 2009; Carvalho et al., 2004; Chotiko & Sathivel, 2014; Tymczyszyn, Gerbino, Illanes, & Gómez-Zavaglia, 2011). These substances may minimize the changes in the physical state of membrane lipids or diminish the changes in the structure of sensitive protein of the biological systems after freeze-drying, thus improving the cell viability in freeze-drying (Hubálek, 2003). In addition, Daily and Higgens (1973) found that sodium glutamate usually in combination with other compounds like glycerol or milk, were effective in cryoprotecting different microorganisms, especially algae. Although the one-variable-at-a-time approach (OVAT) has been used frequently to study the effect of many protectants on LAB (Santos et al., 2014; Xing, Xu, & Yan, 2013), it consumes a lot of time and

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ignores the interactions between agents, resulting in confusion and a lack of predictive ability.

Response surface methodology (RSM) is an efficient statistical technique for optimizing multiple variables to predict the best performance with minimum number of experiments (Gouda, Thakur, & Karanth, 2001). This statistical technique has been widely applied in many fields such as optimization of enzyme-catalyzed conditions (Murthy, Rakshit, & Kosugi, 2000), food processing conditions (Gültekin-Özgüven, Berktaş, & Özçelik, 2016; Ratnam, Rao, Rao, Rao, & Ayyanna, 2003), and medium conditions (Wang & Lu, 2004). However, it has not yet been reported as a method to optimize culture medium for enhancing the viability of microorganisms after freeze-drying.

*Streptococcus sativarius* subsp. *thermophilus* is one of the most widespread strains used in the production of yoghurt and other dairy products. The aim of the present work was to locate the optimum levels of three important protective agents (glycerol, sodium glutamate and skim milk) in medium for *S. thermophilus* STX2 in order to maximize the cell viability after freeze-drying, and to determine the interactions between pairs of the selected factors by using RSM.

## 2. Materials and methods

### 2.1. Bacterial isolates

*Streptococcus sativarius* subsp. *thermophilus* STX2 was isolated in our laboratory from Rosell yogurt culture (Rosell Institute, Montreal, Canada). The strain was stored in 12% (w/v) skim milk supplemented with 5% (w/v) $CaCO_3$ at 4 °C and subcultured every two months.

### 2.2. Cultures and protective agents

Stock culture was firstly re-activated by at least three successive transfers in 12% (w/v) sterilized skim milk. The pure culture was inoculated to MRS broth medium and then incubated at 37 °C under stationary condition overnight. This culture was subsequently inoculated into a second MRS broth at the level of 1% (v/v) and incubated at 37 °C for use.

The selected protective agents were glycerol, sodium glutamate and skim milk. The protective medium was prepared by suspending the above additives in distilled water according to the experimental design in Table 1 and Table 2.

### 2.3. Preparation procedure

Cells were harvested under aseptic conditions at the beginning of the stationary phase at 10,000 rpm at 4 °C for 10 min in the Eppendorf 5804 R centrifuge (Eppendorf Company, Hamburg, Germany). The growth medium was decanted and the harvested cells were washed twice in aseptic distilled water, and centrifuged again. Each pellet was re-suspended in the experimental protective medium to make cell suspension containing approximately $1.0 \times 10^{10}$ CFU/mL. Aliquot (1 mL) of each resuspension was transferred into two sterilized vials (7 mL) and were stored at −20 °C overnight. Then, the samples were freeze-dried at −55 °C (system pressure: 20 Pa) for 24 h in the Alpha 1–2 LDplus freeze-dryer (Martin Christ Company, Osterode, Germany).

### 2.4. Determination of cell viability

Viable counts of cells were determined before and after freeze-drying as colony forming units (CFU). Each sample before being frozen was serially diluted 10-fold in aseptic physiological saline (0.85% NaCl-water) and suitable dilutions were placed on modified TJA agar media, consisting of yeast extract (5 g), beef extract (10 g), lactose (20 g), sucrose (2 g), sodium acetate (5 g), $K_2HPO_4$ (2 g), Tween 80 (1 g), tomato juice (50 mL), and agar 15 g (pH, 6.8 ± 0.2) in 1 L of DI water. The dried samples were re-suspended in 1 mL skim milk, incubated at room temperature for 15 min and plated as described above. Plates were incubated at 37 °C for 48 h before the colonies were counted.

The viability of cell suspension for each protective medium was calculated using the following equation:

$$\text{Viability}(\%) = \frac{\text{Viable cells after freeze} - \text{drying (CFU/mL)}}{\text{Viable cells before freezing (CFU/mL)}} \times 100$$

### 2.5. Experimental design and statistical analysis

Our previous Plackett-Burman design indicated that glycerol, sodium glutamate and skim milk were the most effective freeze-drying protectants for *S. thermophilus* STX2 (Huang, Lu, & Yuan, 2005). Thus, a full factorial central composite design (CCD), with five settings for each of these three factors, was employed to optimize a protective medium for maximization of the viability of freeze-dried *S. thermophilus* STX2, and to explore the interactions between these variables. A total of 20 runs, performed in duplicate, were required for this procedure. The experimental design in the coded ($X_i$) and in actual ($x_i$) level of variables was shown in Table 1. For statistical calculation, the test factors were coded according to the following equation:

**Table 1**
Level and code of variables chosen for central composite design (CCD).

| Variable | Symbol | Coded level | | | | |
|---|---|---|---|---|---|---|
| | | −1.682 | −1 | 0 | +1 | +1.682 |
| Glycerol (g/L) | $x_1$ | 15.90 | 50.00 | 100.00 | 150.00 | 184.10 |
| Sodium glutamate (g/L) | $x_2$ | 15.90 | 50.00 | 100.00 | 150.00 | 184.10 |
| Skim milk (g/L) | $x_3$ | 15.90 | 50.00 | 100.00 | 150.00 | 184.10 |

**Table 2**
CCD experimental design matrix with experimental and predicted values of cell viability of *S. thermophilus* STX2.

| No. | $x_1$ | $x_2$ | $x_3$ | Viability (%) | |
|---|---|---|---|---|---|
| | | | | Experimental | Predicted |
| 1 | −1 | −1 | −1 | 49.55 | 49.36 |
| 2 | 1 | −1 | −1 | 35.91 | 36.58 |
| 3 | −1 | 1 | −1 | 34.09 | 37.31 |
| 4 | 1 | 1 | −1 | 52.73 | 50.09 |
| 5 | −1 | −1 | 1 | 80.00 | 84.27 |
| 6 | 1 | −1 | 1 | 56.36 | 54.78 |
| 7 | −1 | 1 | 1 | 49.09 | 50.06 |
| 8 | 1 | 1 | 1 | 44.32 | 46.14 |
| 9 | −1.682 | 0 | 0 | 72.27 | 68.14 |
| 10 | 1.682 | 0 | 0 | 52.27 | 54.09 |
| 11 | 0 | −1.682 | 0 | 70.00 | 68.91 |
| 12 | 0 | 1.682 | 0 | 52.73 | 51.51 |
| 13 | 0 | 0 | −1.682 | 25.45 | 25.61 |
| 14 | 0 | 0 | 1.682 | 54.09 | 51.63 |
| 15 | 0 | 0 | 0 | 89.09 | 90.29 |
| 16 | 0 | 0 | 0 | 86.82 | 90.29 |
| 17 | 0 | 0 | 0 | 90.91 | 90.29 |
| 18 | 0 | 0 | 0 | 88.18 | 90.29 |
| 19 | 0 | 0 | 0 | 93.64 | 90.29 |
| 20 | 0 | 0 | 0 | 92.73 | 90.29 |