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Enhancement of Viability for *Staphylococcus Aureus* During Freeze-Drying Using the Response Surface Methodology

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Abstract. Response surface methodology (RSM) was used to optimize a protective agent for enhancing the cell viability of *Staphylococcus aureus* during freeze-drying. Using a previous Plackett-Burman design, it was found that sodium glutamate, trehalose, L-cysteine hydrochloride and bovine serum albumin were the most effective freeze-drying protective agents for *S. aureus*. Through the comparative analysis of single protectant, the complex protective agents show better effect on the *S. aureus* viability. The paper was carried out by Box-Behnken Design to determine the optimum levels of these four protective agents to seek for maximum viability of *S. aureus* during freeze-drying. The experimental data allowed the development of an empirical model ($P<0.0001$) describing the inter relationships between the independent and dependent variables. By solving the regression equation, and analyzing the response surface contour and surface plots, the optimal concentrations of the agents were determined as: 15.73% sodium glutamate, 3.43% trehalose, 0.50% L-cysteine hydrochloride, 0.47% bovine serum albumin. *S. aureus* freeze dried in this medium obtained a cell viability of up to 96.78%.

Introduction

The World Health Organization (WHO) has announced, foodborne illness covers a wide range of diseases worldwide, which has been a public health problem for decades, to ensure food products are safe enough to consume, the amount of disease-causing pathogenic microorganism is obviously an important aspect of food safety work. *S. aureus* is one of the most important causes of bacterial food poisoning pathogens, widely distributed in nature, such as air, soil, water and so on, as well as exists in human and animal skin, which is a kind of important pathogenic bacteria that can cause human and animal purulent infection, and it is also one of the common pathogenic bacteria cause human food poisoning. In recent years, food poisoning cases that caused by *S. aureus* share 25% [1]. At present in the Chinese food hygiene standards have *S. aureus* inspection as a regular inspection item.

Nowadays, a lot of *S. aureus* in the form of a lyophilized powder supplied to the market, but directly performing freeze drying, *S. aureus* bacteria went through the mechanical action, the cell membrane permeability changes, will inevitably make some enzyme protein molecule passivation inactivation, thereby causing cell damage or death [2-3], but protective agents can reduce the damage of the freeze-drying process for microbial cells, protect cell biological activity, improve the rate of living bacterium [4].

During attempts to reduce adverse impacts on the freeze-dried bacteria cells, several compounds have been examined as protective agents in freeze-drying, for instance, skim milk, trehalose, sucrose, lactose, amino acids and their salts [5-6], the results indicated that sodium glutamate, trehalose, L-cysteine hydrochloride and bovine serum albumin were the four most significant factors for the protection of freeze-dried *S. aureus*. The aim of our study was to find the optimum levels of the important protective agents (sodium glutamate, trehalose, L-cysteine hydrochloride and bovine serum albumin) considered for the freeze-drying of *S. aureus*, improving the survival rate and the number of viable cells during freeze-drying, and to determine the mutual interactions between pairs of the selected factors simultaneously through response surface methodology (RSM).

Materials and Methods

Bacterial Strains and Cultures

S. aureus standard strain (CICC 21600) was provided by the China Center of Industrial Culture Collection was used in this study. Lyophilized preservation of standard strain were reconstituted with stroke-physiological saline solution, sterile conditions on a nutrient agar crossed the resurrection, the cells were incubated at 36 ±1 ℃ for 18 ~ 24h. Within 7 days if not used, should be reactivated to maintain a high degree of vitality.

Preparation of Freeze-Dried Powder

All the protective agents used in the experiment were dissolved with distilled water and formulated into different levels of concentrations. For the former two agents, they were autoclaved for 15 min at 113 ℃ while L-cysteine hydrochloride and bovine serum albumin were sterilized by filtration through a 0.22 μm filter. Cells in the early stage of the stationary phase were harvested under aseptic conditions by centrifugation at 5,400 g for 10 min at room temperature (Mini Spin, Eppendorf, Germany). Then the cells were washed twice with saline (0.85% NaCl) and centrifuged again. Aliquots (1 mL) of each resuspension were transferred into sterilized vials (7 mL, Schott Glaswerke AG, Germany) and were frozen at -80 ℃ for 2 ~3 h. Cells mixed with distilled water were considered as control. Then the samples were immediately frozen at - 40 ℃, 6.8 Pa for 16 ~ 24 h using a vacuum freeze dryer (2-6D plus, Christ, Germany).

Determination of Cell Viability

To determine the viable counts of the *S. aureus* as the formation of freeze-dried powder, freeze-dried samples were balanced for 30 min at room temperature, then with a serial dilution on sterile saline solution (0.85% NaCl), Each dilution degrees from 1 mL sample well liquid in 0.3 mL, 0.3 mL and 0.4 mL quantity of added three Baird - Parker plate, then coated with a sterile L-rod whole tablet. After cultivated at 36 ±1 ℃ for 45 ~ 48 h, viable cells were enumerated before (initial count) and after freeze drying. A plate with a typical colony of *S. aureus* was selected, and the same dilution degrees of three tablet number all colonies combined between 20 ~ 200 CFU (colony forming units)

tablet, and the number of colonies was counted. Viability was calculated by using the following equation:

$$\text{Viability (\%)} = \frac{\text{Viable cells after freeze} - \text{drying (CFU/ml)}}{\text{Viable cells before freezing (CFU/ml)}} \times 100\%. \quad (1)$$

Design of the Response Surface Optimization

RSM was conducted to design this experiment with the aid of Design-Expert software. Cell viability was set as the response value. Based on the four determined key factors, a mathematic model was developed through the Box-Behnken design (BBD) to find out the optimal point in the pre-optimized area. The levels of these four variables were given in Table 1. The design was meant to find the optimal protective agents composition by fitting a polynomial model through RSM. In this experiment, each significant factor was represented at three levels: low (-1), medium (0) and high (+1). It was made up of totally 29 experiments with 24 B-B design and 5 central points design. A second degree polynomial equation was employed as follows:

Table 1. The factors levels of Box-Behnken experimental design

Factors	Coded levels		
	-1	0	1
Trehalose(x_1)	2%	4%	6%
Sodium glutamate(x_2)	12%	15%	18%
L-cysteine hydrochloride(x_3)	0.5%	1%	1.5%
Bovine serum albumin(x_4)	0.25%	0.5%	0.75%

$$Y=\beta_0+\sum \beta_i x_i+\sum \beta_{ii} x_i^2+\sum \beta_{ij} x_i x_j . \quad (2)$$

where Y is the predicted responses, β_0 is the intercept term, x_i and x_j are independent variables, β_i is the linear term, β_{ii} is the squared term, and β_{ij} is the interaction term.

Statistical Analysis of the Data

Design Expert software (Version 8.0.6, Stat-Ease. Inc., Minneapolis, MN, USA) was used for regression and graphical analysis of the experimental data obtained. The optimum levels of the selected variables were obtained by solving the regression equation and also by analyzing the response surface contour and surface plots.

Results

Optimization Design Results and Analysis of Box–Behnken

The objective of the present study was to find the optimum combination of trehalose, sodium glutamate, L-cysteine hydrochloride and bovine serum albumin level to maximize the cell viability of *S. aureus* during freeze-drying. The design matrix of BBD and results of Y (responses) were listed in Tables 1 and 2. The experimental and predicted responses for cell viability were illustrated in Table 2. Survival rate of vacuum freeze-dried *S. aureus* cells was represented by Y (%), therefore, the major influence of protective agents was measured by Y. Using multiple regression analysis on the experimental data, the following quadratic polynomial equation was found to express the cell viability of freeze-dried *S. aureus*:

$$Y=96.65-3.55x_1+5.49x_2-1.82x_3-1.22x_4-1.02x_1x_2-2.08x_1x_3-0.36x_1x_4-0.92x_2x_3+0.87x_2x_4 -0.17x_3x_4-2.97x_1^2-13.51x_2^2+1.23x_3^2-3.01x_4^2 \cdot \quad (2)$$

Table 2. Experimental Design and Results of Box–Behnken

Run	x_1	x_2	x_3	x_4	Viability (%)	
					Actual value	Predicted value
1	-1	-1	0	0	78.03±1.50	77.21
2	1	-1	0	0	72.82±2.13	72.16
3	-1	1	0	0	89.87±2.10	90.24
4	1	1	0	0	80.56±2.03	81.09
5	0	0	-1	-1	97.62±2.30	97.72
6	0	0	1	-1	95.24±1.72	94.44
7	0	0	-1	1	95.13±0.86	95.64
8	0	0	1	1	92.05±2.90	91.66
9	-1	0	0	-1	95.52±2.75	95.08
10	1	0	0	-1	89.28±1.54	88.69
11	-1	0	0	1	94.04±1.03	93.36
12	1	0	0	1	86.37±1.96	85.54
13	0	-1	-1	0	80.32±2.15	79.78
14	0	1	-1	0	93.42±2.14	92.60
15	0	-1	1	0	78.43±1.92	77.98
16	0	1	1	0	87.85±2.06	87.12
17	-1	0	-1	0	97.82±0.54	98.20
18	1	0	-1	0	94.88±2.28	95.25
19	-1	0	1	0	97.54±1.30	98.73
20	1	0	1	0	86.27±2.40	87.45
21	0	-1	0	-1	75.41±0.81	76.73
22	0	1	0	-1	85.56±2.68	85.97
23	0	-1	0	1	71.40±3.80	72.55
24	0	1	0	1	85.04±1.20	85.28
25	0	0	0	0	96.34±1.72	96.65
26	0	0	0	0	96.87±2.54	96.65
27	0	0	0	0	95.76±2.19	96.65
28	0	0	0	0	97.25±1.14	96.65
29	0	0	0	0	97.04±1.13	96.65

The experimental data (viability of bacterium after freeze-drying) in Table 2 were fitted to a full quadratic second-order polynomial equation by applying multiple regression analysis using Design Expert software. The coded variables were used in the regression analysis because they resulted in a better fit than the actual values [7]. The fitted function was tested for adequacy and fitness using analysis of variance (ANOVA). Table 3 shows the results of the model analysis for *S. aureus*. The regression model for preservation of *S. aureus* was highly significant (P <0.0001). Insignificant lack of fit (P = 0.1240) implied that the obtained model was adequate to represent the experimental data.

Table 3. The ANOVA of Regression Equation Y of S. aureus

Source	SS	DF	MS	*F*	Pr>*F*	Significance
Model	1891.94	14	135.14	137.00	<0.0001	**
x_1	151.51	1	151.51	153.60	<0.0001	**
x_2	361.79	1	361.79	366.77	<0.0001	**
x_3	39.64	1	39.64	40.18	<0.0001	**

Source	SS	DF	MS	*F*	Pr>*F*	Significance
x_4	17.76	1	17.76	18.01	0.0008	**
$x_1 x_2$	4.20	1	4.20	4.26	0.0581	
$x_1 x_3$	17.35	1	17.35	17.59	0.0009	**
$x_1 x_4$	0.51	1	0.51	0.52	0.4834	
$x_2 x_3$	3.39	1	3.39	3.43	0.0851	
$x_2 x_4$	3.05	1	3.05	3.09	0.1008	
$x_3 x_4$	0.12	1	0.12	0.12	0.7298	
x_1^2	57.24	1	57.24	58.03	<0.0001	**
x_2^2	1183.36	1	1183.36	1199.63	<0.0001	**
x_3^2	9.74	1	9.74	9.88	0.0072	**
x_4^2	58.89	1	58.89	59.70	<0.0001	**
Residual	13.81	14	0.99			
Lack of fit	12.36	10	1.24	3.41	0.1240	
Pure error	1.45	4	0.36			
Cor total	1905.75	28				

Table 3, cont. The ANOVA of Regression Equation Y of S. aureus.

Note: SS, Sum of squares; DF, degree of freedom; MS, mean square, Pr>F, P values, ***p*<0.01, very significant; R^2 = 0.9928**,** R_{adj}^2 = 0.9855

The goodness of fit of the polynomial model was checked via the determination coefficient and the correlation coefficient. In this case, the value of R^2 (0.9928) meant that only 1% of the variability in the responses was not explained by the model. A high value of the correlation coefficient indicated a good agreement between the experimental and predicted values of cell viability. Besides, the value of the adjusted determination coefficient (R_{adj}^2=0.9855), which was close to the R^2 value, also confirmed that the model was highly significant.

The significance of each coefficient of the model was determined by Student's t-test and P-value (Table 3). The larger the magnitude of the t-value and smaller the P-value, the more significant the corresponding coefficient [8]. Among the interaction effects, the effects of trehalose × L-cysteine hydrochloride was highly significant (P_{x1x3}=0.0009), whereas those of trehalose × sodium glutamate, trehalose × bovine serum albumin, sodium glutamate × L-cysteine hydrochloride, sodium glutamate × bovine serum albumin and L-cysteine hydrochloride × bovine serum albumin were insignificant (P_{x1x2}=0.0581, P_{x1x4}=0.4834,P_{x2x3}=0.0851,P_{x2x4}=0.1008, P_{x3x4}=0.7298). This meant that the effect of one agent concentration on survival rate was dependent on the level of another one.

The isoresponse contour and surface plots of RSM as a function of two factors at a time, holding all other factors at fixed level (zero, for instance), were helpful for understanding both the main and the interaction effects of these two factors [2]. The response values for the variables could be predicted from these plots. Figures 1-3 represent the was response contour and surface plots for the cell viability of *S. aureus* during freeze-drying.

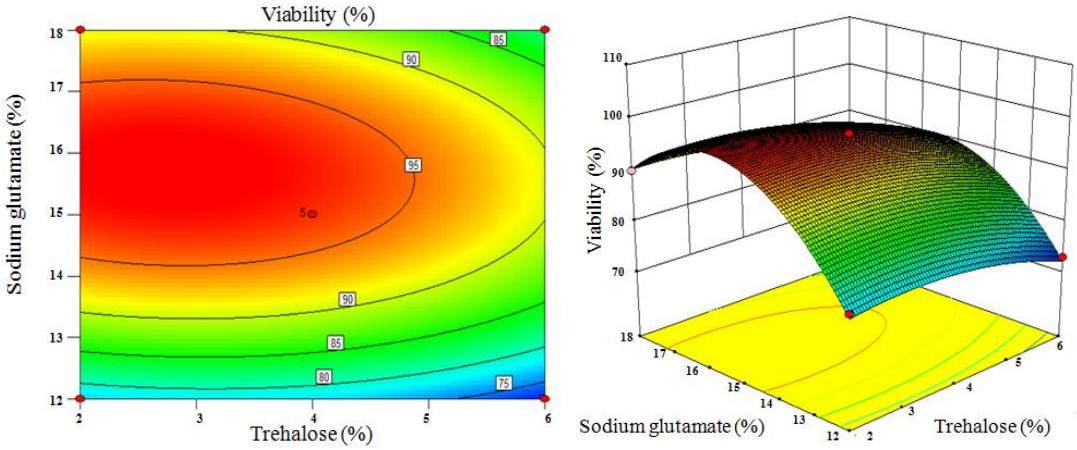

Figure 1. Response surface andcontour plot of trehalose versus sodium glutamate on the cell viability

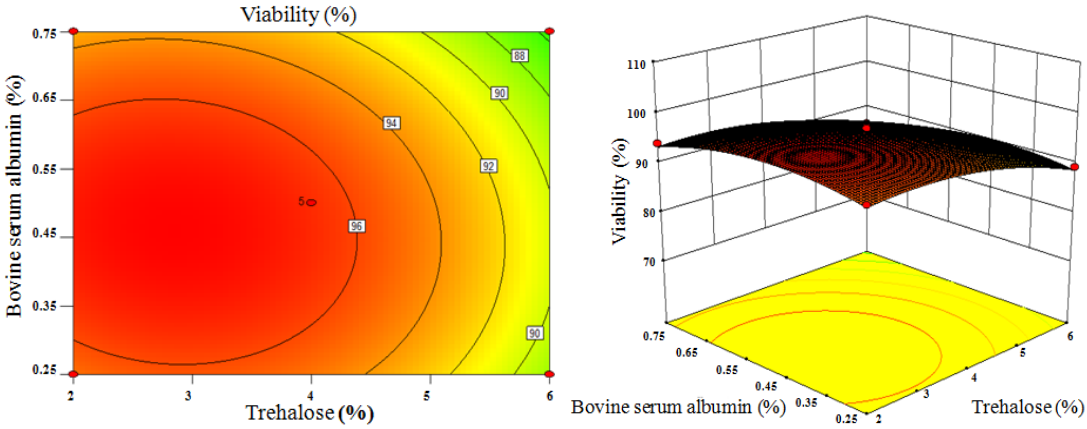

Figure 2. Response surface andcontour plot of trehalose versus bovine serum albumin on the cell viability

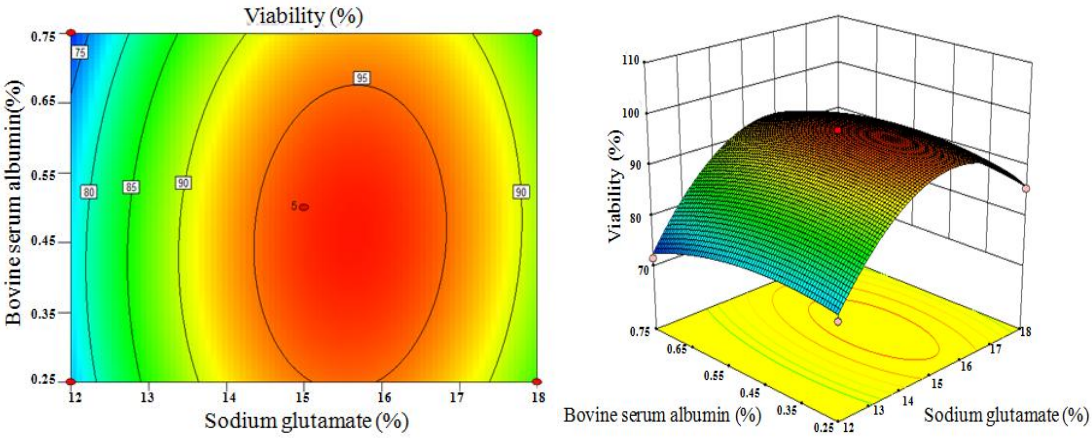

Figure 3. Response surface andcontour plot ofsodium glutamate versus bovine serum albumin on the cell viability

It can be seen that the two-dimensional contour plots of them seemed to be a circle; this indicates that the mutual interaction of terms $x_1 \times x_4$ was not significant for both of the two responses (Figure 2). Moreover, the oval in the contour plots of $x_1 \times x_2$ and $x_2 \times x_4$ implied that the interaction effect on the corresponding variables between $x_1 \times x_2$ and $x_2 \times x_4$ was significant (Figures 1 and 3), indicating that sodium glutamate may be themost important protective agent influencing the resistanceof *S. aureus* to freeze-drying. In the freeze-drying process, sodium glutamate could interact with water

so that the dry powder retains minute amounts of water to meet the minimum requirements for microbial cells to sustain life [9]. In addition, sodium glutamate also has antioxidant effects, inhibition of triglyceride oxidation and free radical formation, to prevent irreversible damage to the cell membrane [10]. As to trehalose, it is a non-reducing disaccharide with its characteristic of anti-freezing, high heat-resistance and stability. The addition of trehalose can replace the water molecules and cell membrane and hydrophilic groups on the protein to form hydrated bonds, protein structure and function to ensure stability [11], trehalose can also increase the bacterial suspension glass transition temperature, So that bacterial suspension to form a glassy state, sensitive substances embedded in the glass state, thereby protecting cells [12]. L-cysteine hydrochloride was the only amino acid with a reactive thiol group (-SH) and has a reducing property. In addition, the amino group of amino acids could react with microbial cell protein carbonyl, so that the structure of protein stability. Serum albumin was a classic, excellent protein stabilizer, it could make the protein activity against losses, and also effectively prevent the surface of the protein adsorption, freeze-drying process of the majority of proteins have a protective effect [13].

From equations derived by differentiating Eq. 2, the optimum values for the independent variables investigated were 3.43% trehalose, 15.73% sodium glutamate, 0.50% L-cysteine hydrochloride and 0.47% bovine serum albumin, with the corresponding Y=100.75%. To confirm the results, *S. aureus* was freeze-dried in this optimum protective medium, and a viability of (96.78±2.49)% (N=3) was obtained. The good correlation between these two results verified the goodness of fit of the model.

Discussion

Vacuum freeze-drying, which is based upon sublimation, has been used to manufacture microbiology powders for decades. Typically, cells were first frozen and then dried by sublimation under high vacuum [14]. It has been demonstrated that cellular inactivation occurs mostly at the freezing step. Therefore, in order to protect the cells from damage during freeze-drying, many studies have focused on adding protective agents to minimize damage.

On one hand, carbohydrates were reported to exhibit enhanced desiccation tolerance by forming hydrogen bonds to proteins during drying, which help to maintain the tertiary protein structure in the absence of water [15]. On the other hand, proteins could also decrease the injury of cells. Furthermore, loss of viability of dried cultures was a consequence of cell damage at membrane lipid oxidation [6]. The composite freeze-dried protective agent provided by the invention was combined with carbohydrates, protein protective agents and antioxidants so as to achieve synergistic effect and synergistic effect, and effectively improve the survival rate of *S. aureus* lyophilized powder.

Conclusions

In the present study, RSM was employed to determine the effect of protective agents (sodium glutamate, trehalose, L-cysteine hydrochloride and bovine serum albumin) on the viability of *S. aureus* during freeze-drying. The freeze-drying condition was optimized by determination of the best combination of protective agents to enhance the viability of the cells. By solving the regression equation, the optimum protective

medium for *S. aureus* was determined as following: 3.43% of trehalose, 15.73% of sodium glutamate, 0.50% of L-cysteine hydrochloride and 0.47% of bovine serum albumin. This protective media resulted in a viability of 96.78% for freeze-dried *S. aureus*. The research results indicated that RSM not only helped us locate the optimum concentrations of the protective agents in order to enhance the maximum viability of *S. aureus* during freeze-drying, but also proved to be well-suited to evaluating the main and interaction effects of the protective agents on the cell viability.

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