# Biological Characteristics of Trehalose and Its Protective Effect on Food Fermentation Starters during Lyophilization

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**Abstract** This paper reviewed the unique biological function of trehalose and its mechanism of stabilizing biological macromolecules and the research progress in the protective effect of trehalose on lactic acid bacteria fermentation starters during lyophilization in food production. The application of trehalose in food industry was prospected.

**Key words** Trehalose; Biological Characteristics; Lyophilization

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Trehalose is a natural disaccharide with stable properties. It is non-toxic and harmless, and has high resistance to adversity (high temperature, freezing, drying, high permeability, *etc.*) and protective effects on biological cells, and no side effects on the human body. It is commonly used as a protective agent for lactic acid bacteria during lyophilization. In recent years, many scholars have conducted extensive research on the anti-freezing protective effect of trehalose on lactic acid bacteria. This paper aimed to summarize the research progress in this area as below.

## Structure and Physicochemical Properties of Trehalose

### Structure of trehalose

Trehalose, also known as α-D-glucopyranosyl-α-D-glucopyranoside, is a non-reducing disaccharide condensed from two glucose molecules through hemiacetal hydroxyl group, with a molecular formula of $C_{12}H_{22}O_{11}$ and molecular weight of 342.31[1]. Trehalose has three optical isomers, namely αα type, αβ type and ββ type, of which αβ type and ββ type are rarely found in nature, and only a small amount of αβ trehalose found in honey and royal jelly. The αα type is the most common trehalose (also known as mushroom sugar) in nature, which is commonly present in bacteria, fungi, algae, lower plants, and insects[2].

### Physical and chemical properties of trehalose

The anhydrous trehalose has a melting point of 210.5 ℃ and dissolution heat is 53.4 KJ/mol. Trehalose is easy to crystallize and has good crystallinity. Crystallized trehalose has a density of 1.512 g/cm and dissolution heat of 57.8 KJ/mol, and it loses water at 130 ℃. Over 99% of trehalose can still be stored when heated at 100 ℃ for 24 h. Trehalose is easily soluble in water, hot ethanol and glacial acetic acid, but not in ether and acetone. Its solubility in water changes significantly with temperature. At a low temperature, its solubility in water is lower than that of sucrose, and the same as that of maltose.

Trehalose has a sweetness equivalent to 45% of sucrose, making it a high-quality sugar with a soft sweet taste. Its mild sweetness is more long-lasting than granulated sugar, so it can improve the sweetness of foods with high sugar content. When combined with other sweeteners, it can enhance the unique taste of food materials.

Trehalose has the highest glass-transition temperature among disaccharides, up to 115 ℃. Therefore, when trehalose is added to other foods, it can effectively increase the glass-transition temperature of foods, making it easier to form a glassy state. It can play a role in maintaining vitrification and freshness.

Anhydrous crystallized trehalose has a strong water-absorption property. When encountering water-containing substances or under the condition of sufficient water vapor, it can effectively absorb water molecules in water-containing substances or free water molecules, and becomes aqueous crystallized trehalose itself. Most trehalose sold on the market exists in the form of crystals containing two molecules of crystal water. Trehalose has low hygroscopicity, and water-retaining property. Trehalose dihydrate has no hygroscopicity at relative humidity below 92%, while anhydrous trehalose has hygroscopicity at relative humidity above 30%. Such property enables it to have both low hygroscopicity and high moisture-retaining property.

## Biological characteristics and action mechanisms of trehalose

### Unique biological function of trehalose

Trehalose has unique biological characteristics that other disaccharides do not have. Trehalose exists in a variety of forms in organisms, both in the form of free sugars and as a component of trehalose glycolipids. Different forms of existence correspondingly exhibit different biological functions of trehalose, which are mainly manifested in three aspects. ① It can be used as the basic component of trehalose glycolipids in organisms. Trehalose glycolipids

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have several different structures, widely exist in *Corynebacterium*, *Mycobacterium*, *Nocardia* and other bacteria, and are one of the main components constituting the cell wall of above bacteria. ② Trehalose is used to provide energy. The content of trehalose in organisms varies with the growth status, nutritional status, and environmental conditions of organisms. External pressure can cause the synthesis and accumulation of trehalose. For example, drying will lead to rapid accumulation of trehalose, while after absorbing water, trehalose will be rapidly metabolized in organisms. That is to say, trehalose is catalyzed to decompose into two glucose molecules through trehalase. As an energy source and metabolic intermediate, trehalose provides a calorific value equivalent to other compounds, playing the role of carbohydrate storage. ③ Trehalose is a stress metabolite of many organisms, which has the function of protecting biological cells and bioactive substances from damage under adverse environmental conditions such as dehydration, drought, high temperature, freezing, high osmotic pressure and toxic compounds. Trehalose exists as a natural non-specific biological protective substance[1]. Moreover, trehalose molecules can regulate the distribution of dipoles on the head group of phospholipids, thereby affecting the distribution of surface charge on the membrane, and changing the action environment of charge-sensitive protective substances or harmful particles.

**Bioprotective mechanisms of trehalose**

Trehalose can stabilize biological macromolecules and protect cell membranes and proteins from harsh environments such as high temperature, freezing, drying and high osmotic pressure.

At present, the mechanism of trehalose to stabilize biological macromolecules is not very clear, and the "water substitution" hypothesis and the "glassy state" hypothesis are the two main hypotheses that currently exist. The "water substitution" hypothesis suggests that biological macromolecules are surrounded by a layer of water film, which plays a role in maintaining their structure. When the water film is lost, trehalose can form hydrogen bonds with the dehydrated part of the biological molecule, allowing it to maintain its original structure without losing its activity. The "glassy state" hypothesis suggests that trehalose can tightly envelop adjacent biomolecules under dry conditions, forming a "glassy-state compound" with an amorphous structure. Glass has a very high viscosity and low mobility, leading to an increase in the stability of the preserved material, thereby effectively protecting biomolecules during drying.

Liu[3] revealed the molecular mechanism of trehalose's water substitution effect, volume effect and vitrification effect, and proposed the mechanisms of nucleation-inhibiting effect, charge regulation effect and hydropexis effect from the perspective of the solution-side microstructure, the dipole distribution of phospholipid head group and the stability of hydration layer. Several effects did not conflict with each other and exist simultaneously, but the degree to which they could exert varied in different situations. The stabilization mechanism of "nucleation-inhibiting effect" suggested that trehalose alleviated the dehydration trend on the phospholipid membrane side by reducing the crystallization trend on the solution side, thereby avoiding dehydration damage to the biological membrane and stabilizing the biological membrane. Trehalose/water/POPC membrane systems with different compositions could be established to simulate the dehydration environment of biological membranes by controlling the degree of hydration. It was found that trehalose could replace water to bind with phospholipid molecules, thus increasing the average area of a single phospholipid molecule, reducing the thickness of the double-layer membrane, the order of acyl chains and the mobility of each group, and inhibiting the transition of phospholipid membrane to the gel state. The hypotheses of "water substitution effect", "volume effect" and "glassy state" were confirmed, and their specific action modes were explained at the atomic level.

Liu[3] proposed the protection mechanism of "charge regulation effect". The research results showed that under heating conditions, the stabilizing effect of trehalose was not only reflected in the improvement of static structure, but also in the reduction of dynamic fluctuation amplitude before and after heating. Finally, MD simulation of trehalose/water/POPC double-layer membrane system under mechanical stress was carried out in the study. It was found that the application of transverse compressive stress was similar to the dehydration effect, while the transverse tensile stress was similar to the enhancement of hydration degree. Under tensile stress, the main stabilizing mechanism of trehalose on the membrane was the water substitution effect. However, under compressive stress, due to insufficient hydrogen-bond binding sites exposed at the head group of phospholipids, the water substitution effect was limited. At this time, the main mechanism of trehalose was the "hydropexis effect". In specific, when the trehalose content was high, more than 20% of the water molecules bound by the phospholipid head group form hydrogen bonds with nearby trehalose, so this part of water molecules was equivalent to being captured by the cage-shaped space formed by phospholipids and trehalose molecules, and would not diffuse into the solution easily, and the hydration layer of the phospholipid head group became more stable.

## Study on the Protective Effect of Trehalose on Lactic Acid Bacteria During Lyophilization

**Protective effect of trehalose on of *Lactobacillus* during lyophilization**

The vacuum freeze-drying technology of *Lactobacillus plantarum*, mainly directing at the shortcomings of *L. plantarum* including short storage time and inconvenient transportation, is to extend its storage time and maintain high activity of *L. plantarum*.

Qiu *et al.*[4] applied Plackett Burman, the path of steepest ascent method and Box Benhnken to jointly design, screen and optimize the best formula of lyophilizing protectant for *L. plantarum* M1-UVs29. The formula of the best protectant was: skimmed milk 13.0%, trehalose 7.6%, and sorbitol 2.7%, and the survival rate of bacterial powder was 86.28%. The bacterial powder added

with the protectant was better than that without the protectant in artificial gastric juice, artificial intestinal juice, storage capacity, and heat treatment tolerance. In order to improve the biological activity of the direct-vat cholesterol-reducing *L. plantarum* I4 fermentation starter, Zhu *et al.* [5] investigated the protective effects of five protective agents on strain I4 during vacuum freeze drying, and selected four protective agents with good effects for response surface optimization. The results showed that when trehalose 4.04%, sodium glutamate 2.9%, sucrose 10.38% and skimmed milk powder 14.84% were used as the composite protective agent, the survival rate of I4 was higher during lyophilization, reaching 83.32%. Ye *et al.* [6] studied the formula of lyophilizing protectant for *L. plantarum*, and determined the optimal protectant formula as follows: skimmed milk powder 8%, trehalose 10%, maltodextrin 8% and water 66%, through a four-factor three-level orthogonal test.

Wu *et al.* [7] found that the protein content in the surface material of bacteria was about three times that of polysaccharides. With trehalose or stachyose as a protective agent, the lyophilizing survival rate of the bacteria was improved after stripping the surface material. Tan *et al.* [8] systematically analyzed the protective effects of different types of protective agents on *L. reuteri*, in order to improve its lyophilizing survival rate and industrial preparation efficiency. The results showed that oligosaccharides had the best protective effect on *L. reuteri* during lyophilization, and the mixtures of substances with different molecular weights in different proportions could not improve the protective effect. Glutathione, betaine, amino acids, nucleotides, inorganic salts, vitamins and other substances did not significantly improve the protective effect of oligosaccharides on the strain during lyophilization, and the role of surface substances of *L. casei* in the freeze-drying process was analyzed. Qin *et al.* [9] used response surface methodology to optimize the formula of lyophilizing protectant for *L. reuteri*, in order to improve the survival rate of *L. reuteri* in the freeze-drying process. The optimized protectant formula was fructooligosaccharide 2.66%, skimmed milk powder 10.11%, and trehalose 5.90%. The freeze-drying survival rate reached 89.43%, which was 13.76% higher than that before optimization.

Gong[10] loaded trehalose into *L. bulgaricus* cells through electroporation technology to strengthen the protection of cell membrane. The results showed that when the intracellular trehalose content was greater than 3.47 $\mu g/10^7$ CFU, the protective effect began; and when the intracellular trehalose content reached 10.09 $\mu g/10^7$ CFU, the survival rate of lactic acid bacteria could reach 99.59% after spray drying. Electroporation will damage the cell membrane. Since intracellular trehalose can protect the cell membrane of lactic acid bacteria during electroporation and spray drying, moderate electroporation treatment will not reduce the overall survival rate of lactic acid bacteria after spray drying. The survival rate of *Lactobacillus* loaded with trehalose using two pulses at 2.5 kV/cm was 61.30% after electroporation and spray drying. After trehalose loading and enzyme treatment of acidic and basic proteins were combined, the survival rate of lactic acid bacteria in spray drying was increased to 93.5%.

**Protective effect of trehalose on *Lactococcus* during lyophilization**

*Pediococcus acidilactici*, which can produce pediocin, has probiotic functions such as antibacterial and cholesterol-lowering functions, and is mainly used in food fermentation, preservation, and other fields. Probiotics refer to live bacteria that can have beneficial effects on hosts' physical health by ingesting appropriate amounts. In recent years, due to the gradual discovery of their functions in regulating the intestines, enhancing immunity, lowering cholesterol, and fighting cancer, their research and application have received increasing attention.

In order to improve the survival rate of *P. acidilactici* during lyophilization, Zhou *et al.* [11] used the Plackett Burman experimental design and the path of steepest ascent method combined with the response surface method to study the effects of different lyoprotectants on the survival rate during lyophilization. The results showed that the concentrations of trehalose, mannitol and manganese sulfate had a significant impact on the survival rate of bacteria. The interaction between trehalose and mannitol was significant, while the interaction was not significant between trehalose and manganese sulfate and between mannitol and manganese sulfate. The obtained formula of the optimal protective agent was trehalose concentration 73.64 g/L, mannitol concentration 43.76 g/L, and manganese sulfateconcentration 1.91 g/L, with which the survival rate was 98.10% ± 1.03% during lyophilization.

In order to improve the survival rate of *Streptococcus thermophilus* powder obtained by vacuum freeze drying, Liu *et al.* [12] optimized the formula of composite protective agent for lyophilization of *S. thermophilus* by response surface analysis. According to the results of single-factor experiments, lactose, tryptone and trehalose had better protective effects on *S. thermophilus* during lyophilization among various freeze-drying protective agents. With the cell survival rate of *S. thermophilus* as the index, Box Behnken design and response surface analysis were conducted to optimize the composite formula of *S. thermophilus* protective agent. The optimization results showed that the optimal composite protective agent formula was lactose 5.58 g/100 ml, tryptone 6.27 g/100 ml, and trehalose 7.73 g/100 ml, with which the theoretical survival rate of freeze-dried bacterial powder was 77.33% after rehydration. After verification, the survival rate of freeze-dried cells after rehydration of freeze-dried bacterial powder was 77.85%, which was close to the theoretical prediction value. The prepared bacterial powder was stored at 4 ℃ for 8 weeks, and maintained a viable bacterial count of over 1 010 cfu/g.

Jiao *et al.* [13] studied the protective effects of different lyoprotectants on *S. thermophilus* M5-5 cells during vacuum freeze drying. Based on single-factor and orthogonal experiments, the survival rate of the freeze-dried cells was used as an evaluation index to investigate the protective effects of nine lyoprotectants on M5-5 cells during the freeze-drying process, in order to obtain the

optimal protectant formula. The results showed that when trehalose, glycerin and skimmed milk were used as single lyoprotectants, the cell survival rates were 43.85%, 47.51% and 40.86%, respectively, which were significantly higher than those of other six protectants ($P < 0.05$); and the optimal formula of lyoprotectants was determined by orthogonal experiments as trehalose 60 g/L, sodium glutamate 20 g/L, glycerin 20 g/L, and skimmed milk 150 g/L. Meanwhile, the freeze-drying survival rate of *S. thermophilus* M5-5 could reach 88.41%. The results of scanning electron microscope showed that the bacterial cells in the group added with the protectant formula were complete and smooth, indicating that the protectant formula could effectively reduce damage to the bacterial cells during the freeze-drying process.

Gu *et al.* [14] used the Plackett Burman design to screen the protective agent of lactic acid bacteria for fermented composite walnut powder from seven factors: sodium glutamate, skimmed milk powder, trehalose, maltodextrin, sucrose, arabic gum, and glycerol. They then determined the center point of the response surface by the path of steepest ascent method, and finally optimized the protective agent by the Box Behnken test. The results showed that trehalose, skimmed milk powder and maltodextrin had a prominent impact on the survival rate of lactic acid bacteria after Plackett Burman design screening; and after optimization by response surface methodology, the optimal protectant formula was trehalose 2.23%, skimmed milk powder 2.50%, and maltodextrin 2.77%. And a protectant powder was obtained after vacuum drying, and the total number of viable bacteria was $9.71 \times 10^8$ CFU/ml, so the protectant could maximize viable lactic acid bacteria.

## Application Prospects of Trehalose

Trehalose has excellent non-specific protective effects on organisms or biological macromolecules, protecting organisms from environmental pressures such as high temperature, low temperature, dryness, and peroxide. Trehalose has the functions of preventing starch retrogradation and protein denaturation, and stabilizing tissue and cell structure in food processing. Adding trehalose to various foods containing protein can effectively protect the natural structure of protein molecules, thereby keeping food flavor and texture unchanged. Trehalose can attach to the cell wall of some microorganisms as a structural part of the organisms, and is also a high-quality protective agent for biomembrane proteins, unicellular organisms, animals and plant tissues and organs, and pharmaceutical preparations. As a non-permeable protective agent, trehalose has been widely used in low-temperature preservation of tissues such as cells, embryos, skin, trachea, and lungs[15]. In the process of plants responding to abiotic stress, trehalose plays an important role in maintaining osmotic pressure in plants, keeping membrane structure, participating in signal transduction process, *etc.*, and has important significance for crop cultivation and breeding improvement[16]. It is precisely because trehalose has these special physicochemical properties and functions that it is widely used in fields such as food, medicine, and agriculture. It is safe and has no toxic and side effects on people. It can play a good role whether used alone or in combination with other cryoprotectants. Although the protective mechanism of trehalose has not yet been fully elucidated, it is believed that with the continuous deepening of research, trehalose will definitely be widely applied in a broader field.

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