# EFFECT OF FREEZING ON THE BIOCHEMICAL AND ENZYMATIC ACTIVITY OF LACTOBACILLUS BULGARICUS

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**Abstract:** The problem of preserving the viability, stability and activity of thermophilic lactic acid bacteria Lactobacillus bulgaricus upon freezing is considered. The effect of different freezing conditions and low-temperature storage on the biochemical and morphological properties and stability of the DNA of L. delbrueckii ssp. bulgaricus has been investigated. Sensory evaluation has been carried out for non-frozen bacterial starter cultures containing L. bulgaricus, and their basic physical and chemical parameters (titratable and active acidity and relative viscosity) have been determined. The influence of low temperature on these parameters has been investigated. The effect of freezing and low-temperature storage on the antagonistic activity of L. delbrueckii ssp. bulgaricus strains has been elucidated. The optimum freezing and storage temperatures for the starters containing L. bulgaricus have been determined.

**Key words:** freezing, low temperature storage, biochemical and morphological properties of L. bulgaricus, antagonistic activity

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## INTRODUCTION

The quality of cultured dairy foods depends directly on their production technology and on proper selection, preservation, and subsequent culturing of the starter microflora. The microorganism conservation methods known today consist in bringing the vegetative cells of the microorganisms into an anabiotic state. Since these cells are incapable of passing to the endogenous dormancy state, immersing them into exogenous dormancy (by drying, lyophilization, freezing, etc.) and bringing them out of this state produce stressful situations that cause death of a considerable part of the microorganism population and lead to phenotypic and genotypic changes. Bacterial cells are known to induce nuclease in response to a cold shock, so the lethal effect of low temperatures is due to DNA destruction [1, 2].

Advantages of freezing technologies and low-temperature storage of bacterial starter cultures over the other conservation methods are that they are simple and convenient, require only a small amount of preparative work, and allow rapid recovery of the stored material from the frozen state. Compared to drying and lyophilization, freezing causes less damage to microorganism cultures and leaves them more viable [1, 3–5]. In addition, freezing rarely induces genetic changes [1, 3, 4, 6].

The purpose of this work was to study the effects of various freezing temperatures and conditions and low-temperature storage conditions on the biochemical properties, enzymatic activity, morphology, and genetic stability of thermophilic lactic acid microorganisms of the *L. bulgaricus* genus.

## EXPERIMENTAL

Bacterial starters were obtained from lyophilized bacterial starter cultures produced by Barnaul'skaya Biofabrika Co. (*L. Bulgaricus*; BBV = Bulgarian bacillus, viscous; BBNV = Bulgarian bacillus, nonviscous) and from the *L. bulgaricus* strains B-3964, B-6516, B-3141, B-6543, and B-6515 from the Russian National Collection of Industrial Microorganisms (RNCIM) at the Institute of Genetics and Selection of Industrial Microorganisms.

The lactic acid bacteria culturing medium was reconstituted nonfat dry milk (RF State Standard GOST R 52090-2003), which had no off-flavors or foreign odors and did not contain inhibitors.

The milk was sterilized in an autoclave (steam sterilizer, DGM-500 model) for 10–15 min at a pressure of 0.1 MPa and a temperature of 121 ± 2°C.

The laboratory fermentation starter was prepared under sterile conditions in an abacterial air environment PCR box (Laminar-S). Lyophilized starter cultures were introduced into sterile milk cooled to 38–39°C, which was then thoroughly stirred. Fermentation was performed in a TSO-1/80 SPU thermostat at 40–41°C until the formation of a clot of desired quality.

The starter cultures were frozen at –45, –25, or –10°C in air and in a liquid coolant (ethanol). Freezing was carried out in special-purpose low-temperature chambers.

The starter culture temperature during freezing was measured with chromel–copel thermocouples, whose signal was received by an MVA-8 analog input module

and an AS-4 interface transformer and was recorded on a personal computer.

The DNA of lactic acid bacteria was isolated using a bacterial genomic DNA isolation kit (Sintol, Moscow) [7]. The 16S rRNA gene was amplified on a Tertsik amplifier (DNK-Tekhnologiya, Moscow) using thermostable Taq polymerase (SibEnzim, Novosibirsk) according to the manufacturer's recommendations. The following species-specific primers were used in amplification: 16SbulF: 5'- CAA CAG AAT CGC ATG ATT CAA GTT TG (26) and 16SbulR: 5'- ACC GGA AAG TCC CCA ACA CCT A (22) [7].

The antagonistic activity of *L. bulgaricus* was determined by perpendicular-stroke coculturing [8] on the surface of dense nutrient medium no. 2. Experiments were performed on 16- to 16-h-old bacterial cultures grown in liquid nutrient medium no. 1.

Liquid nutrient medium no. 1 had the following composition: skim milk hydrolysate (amine nitrogen, 200–250 mg%), 250.0 mL; concentrated yeast autolysate (amine nitrogen, 200–250 mg%), 100.0 mL; agar, 0.8 g; distilled water, to 1 L; 20% NaOH solution, to pH 6.4 ± 0.1. Dense nutrient medium no. 2 had the following composition: skim milk hydrolysate (amine nitrogen, 200–250 mg%), 250.0 mL; concentrated yeast autolysate (amine nitrogen, 200–250 mg%), 100.0 mL; agar, 20.0 g; distilled water, to 1 L; 20% NaOH solution, to pH 6.4 ± 0.1.

The morphological properties of thermophilic lactic acid bacterial cultures were studied by immersion microscopy using a Rathenow microscope with lens 90. The specimens were stained by Gram's method or with methylene blue.

The following physical and chemical properties of the laboratory fermentation starters were determined: titratable and active acidity (pH), relative viscosity at 25°C (measured with Ostwald's capillary viscometer), number of viable lactic acid microorganisms (measured by the limiting dilution analysis), and pathogenic microflora content (quantified according to the USSR State Standard GOST 9225-84).

## RESULTS AND DISCUSSION

The most important properties characterizing the industrial applicability of a starter culture are its acid production capacity and fermentation activity, the structural and mechanical properties of its clot, the micropattern and organoleptic properties of the resulting clot, and viable microflora content.

The bacterial starter cultures obtained in this study were characterized by a white, delicate, and uniform clot with slight signs of whey separation. The fermented milk clots were readily dividable and acquired a uniform texture upon stirring. All of the starters had a pleasant odor, a fermented milk flavor, and no off-flavors or off-odors. The color of the clot was milk white and was uniform throughout the product bulk. There was no pathogenic microflora in the laboratory starters. The lactic acid microorganism content and some physical and chemical properties of the fresh bacterial starter cultures are listed in Table 1.

**Table 1.** Characteristics of the fresh bacterial starters

| Bacterial culture | Lactic acid microorganism content, CFU/cm$^3$ | pH | Relative viscosity |
|---|---|---|---|
| BBV | $6.0 \cdot 10^9$ | 4.2 | 4.31 |
| BBNV | $3.0 \cdot 10^9$ | 4.2 | 1.75 |
| B-3964 | $4.5 \cdot 10^9$ | 4.2 | 1.72 |
| B-6516 | $5.0 \cdot 10^9$ | 4.0 | 1.80 |

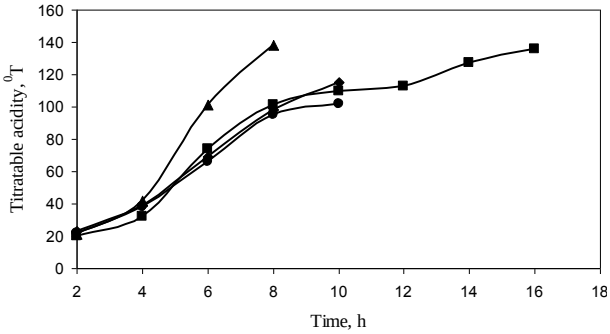


**Fig. 1.** Titratable acidity as a function of fermentation time: ■, BBNV; ▲, BBV; ♦, B-6516; ●, B-3964.

The variation of the titratable acidity of milk during clotting is illustrated in Fig. 1.

All of the bacterial starters had the necessary acidity. The relative viscosity of the bacterial starter obtained using BBV was 2.6 times higher than that of the starters from the nonviscous starter cultures.

The next step of our study was investigation of the effect of low temperature on the viability of the starter microorganisms.

The freezing resistance of microorganisms depends on several factors, including the microorganisms' genus and species, the stage of their development, temperature, freezing rate, freezing medium, and storage time. The effect of low temperature on microorganisms is characterized in terms of intracellular and extracellular changes. The heaviest damage is caused by intracellular ice formation, which disrupts plasma membranes and cell walls. In addition, ice formation increases the concentration of intracellular and extracellular solutions, and this leads to protein denaturation and to the disruption of permeability barriers [2].

The fresh starters were poured into 10-mL test-tubes under sterile conditions and were then frozen at –45, –25, or –10°C in air or in the liquid coolant. After freezing, the tubes with fermentation starters were stored in heat-insulated containers at the temperature equal to the freezing temperature. The frozen starters were stored for 6 months.

The frozen starters were examined to determine their microbiological, biochemical, and physicochemical characteristics. Before being examined, the starters frozen at –10°C were thawed in a refrigeration chamber at 4–8°C. The starters frozen at –25 or –45°C were thawed in a water bath at 20°C.

The data characterizing the dependence of lactic acid microorganism content of the laboratory starters on the

freezing technique and storage time are presented in Table 2.

**Table 2.** Number of *L. bulgaricus* cells in the starters after freezing

| Storage time, days | Number of microorganisms, CFU/cm$^3$ · 10$^{-9}$ | | | | | |
|---|---|---|---|---|---|---|
| | $t$ = –10°C | | $t$ = –25° | | $t$ = –45°C | |
| | 1* | 2** | 1 | 2 | 1 | 2 |
| BBV | | | | | | |
| 1 | 2.19 | 3.00 | 4.50 | 4.60 | 5.21 | 6.00 |
| 14 | 1.10 | 2.10 | 3.30 | 3.80 | 4.00 | 4.50 |
| 30 | 0.50 | 1.30 | 2.50 | 3.00 | 3.50 | 3.60 |
| 60 | 0.08 | 0.45 | 1.50 | 2.00 | 2.20 | 2.50 |
| 90 | 0.03 | 0.12 | 0.60 | 1.10 | 1.50 | 1.70 |
| 180 | 0.009 | 0.009 | 0.30 | 0.50 | 0.5 | 1.10 |
| BBNV | | | | | | |
| 1 | 1.10 | 1.20 | 1.90 | 2.00 | 2.50 | 2.70 |
| 14 | 0.30 | 0.50 | 1.40 | 1.50 | 2.00 | 2.20 |
| 30 | 0.10 | 0.25 | 1.10 | 1.20 | 1.60 | 2.00 |
| 60 | 0.01 | 0.07 | 0.60 | 0.70 | 1.1 | 1.40 |
| 90 | 0.007 | 0.007 | 0.30 | 0.45 | 0.80 | 1.10 |
| 180 | 0.003 | 0.005 | 0.12 | 0.20 | 0.30 | 0.50 |

* Freezing in air;** freezing in the liquid coolant.

The freezing temperature and rate and the storage temperature can significantly affect the survival of the microorganisms [9–11]. Throughout the storage period, the highest survival rate of the microorganisms was observed in the starters frozen and stored in the liquid coolant at –45°C, i.e., at the minimum storage temperature used in this study. This finding is in agreement with data of other authors [9–11]. After 6-month-long storage, the average survival rate of the microorganisms was 17% of their initial number; that is, the microorganism content decreased by less than one order of magnitude. The survival rate of the microorganisms in the starters frozen in air at –45°C was somewhat lower: in 6 months: their number was approximately 10 times smaller than their initial number. The number of microorganisms in the starters frozen at –25°C was, on the average, 5–10% of their initial number, depending on the freezing regime. The lowest survival rate was observed for the microorganisms in the starters frozen at –10°C.

The rather high survival rate of the microorganisms frozen in the liquid coolant can be explained as follows. The efficiency of heat transfer in the freezing of the starters in the liquid coolant is much higher than in their freezing in air. As a consequence, the freezing time for the starters frozen in the liquid coolant is much shorter than for the starters frozen in air. Accordingly, the water crystallization rate for the starters in the liquid coolant is one order of magnitude higher. These freezing conditions minimize the destructing factors associated with water crystallization in the cells and intercellular space, which cause death of a large number of microorganisms in the starter.

The high survival rate of the lactic acid microorganisms after freezing does not ensure that they completely retain their properties and viability. The functional activity of the starter bacteria was judged from acid formation intensity data.

The acid formation activity was determined from the time required to reach pH 4.5 in culturing the microorganisms in reconstituted dry skim milk. Table 3 lists acid production activity data for the lactic acid bacterial cultures frozen in the liquid coolant at –45°C.

**Table 3.** Acid production activity data for *L. bulgaricus* bacteria

| Bacterial starter | Fermentation time for 100 mL milk + 1 mL starter, h | |
|---|---|---|
| | before freezing | after freezing |
| BBV | 7–8 | 7–8 |
| BBNV | 15–16 | 16–17 |
| B-3964 | 10–11 | 10–12 |
| B-6516 | 10–11 | 10–12 |

These data demonstrate that, after 6-month-long storage, the starters retained their high biochemical activity; the fermentation time increased, on the average, by 1 h. The growth dynamics of the thawed *L. bulgaricus* cultures that were frozen in the liquid coolant at –45°C and were stored for 6 months is illustrated in Fug. 2.

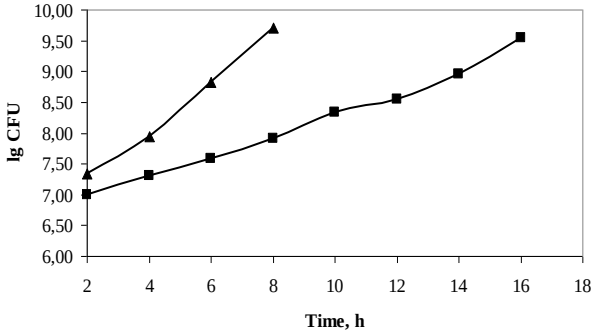


**Fig. 2.** Growth dynamics of *L. bulgaricus* cells in milk fermentation with the (■) BBNV and (▲) BBV starter cultures.

An important physicochemical property of a starter culture is its viscosity. Fig. 3 shows how the relative viscosity of BBV starter cultures as a result of freezing and storage under different conditions.

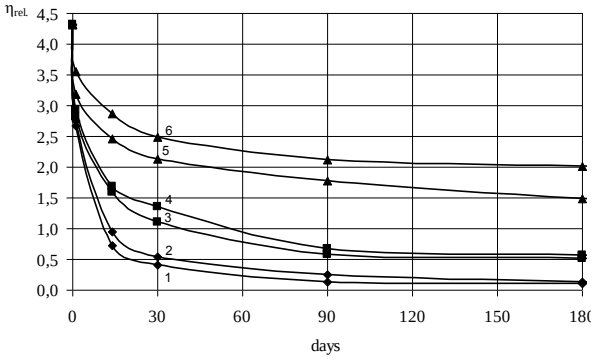


**Fig. 3.** Effect of freezing under different conditions on the relative viscosity of the BBV starter culture. Freezing in air: (1) –10°C, (3) –25°C, and (5) –45°C. Freezing in the liquid coolant: (2) –10°C, (4) –25°C, and (6) –45°C.

These data suggest that the strongest viscosity-reducing effect is exerted by freezing as such: the relative viscosity decreases by a factor of 1.2–2.4, depend-

ing on the kind of starter culture and freezing technique. The largest, 2.4-fold decrease in relative viscosity was observed for BBNV frozen in air at –10 C; the smallest decrease in relative viscosity, for BBV frozen in the liquid coolant at –45°C. The decrease in relative viscosity of the bacterial starter cultures at –10°C is nearly independent of whether they were frozen in air or in the liquid coolant. In 14 days, the relative viscosity of the bacterial cultures frozen in air decreased, on the average, by a factor of 4.6 and that of the cultures frozen in the liquid coolant decreased by a factor of 3.7.

The marked decrease in the relative viscosity of the bacterial starter cultures frozen in air or in the liquid coolant at –10°C is due to the fact that slow freezing yields large ice crystals outside the cells, thus changing the initial ratio of the volumes of the intercellular and intracellular spaces through water redistribution and water-to-ice transition.

In extracellular ice formation, the growth of ice crystals in the intercellular space reduces the cell size. This leads to cell compression and to the formation of folds in the cell wall, causing mechanical damage to the protoplasm. The dehydration of the cell can lead to intimate contact between protoplasm layers located opposite to one another. As water enters the cell during thawing, the joined walls separate. This is often accompanied by protoplasm separation from the walls, with damage happening to the protoplasm structure [2, 9, 10, 12]. The disruption of the structure of the object being frozen causes a dramatic decrease in its viscosity.

Long-term storage of the frozen starters at –10°C leads to the growth of larger crystals through a decrease in the size of smaller crystals (recrystallization). In turn, this causes a still severer structural disruption in the frozen object. The viscous starter cultures BBV displayed a larger decrease in relative viscosity than the nonviscous ones. Upon 6-month-long storage, the relative viscosity of BBV frozen in air decreased by a factor of 45, while that of the nonviscous cultures decreased by a factor of 25 and 16, respectively.

The smallest decrease in viscosity was observed for the starter cultures frozen at –45°C. Upon 6-month-long storage, the relative viscosity of the starter cultures frozen in air decreased by a factor 3.8 and that of the cultures frozen in the liquid coolant decreased by a factor of 2. This insignificant decrease in the liquid viscosity is due to the high freezing rate. Rapid cooling (to –25°C or –45°C) prevents considerable water and solute redistribution by diffusion and favors the formation of small, uniformly distributed ice crystals, thus causing the least possible damage to the structure of the object.

The genetic stability of the lactic acid microorganisms in the bacterial starter cultures frozen at different temperatures and under different conditions was evaluated in terms of preservation of morphological and biochemical properties and antagonistic activity, as well as in terms of the retention of the molecular weight of, and the number of fragments in, the DNA of the bacteria—information gained using genus-specific primers (16S for–16S rev) [7].

Cell morphology depends on many factors, including the culturing conditions, the age of the culture, and the composition of the medium. In the culturing of *L. bulgaricus* in the MRS Agar medium, the bacteria appear as bacilli, either separate or aggregated in chains. Figure 4 shows the micrographs of the lactic acid starter cultures recorded before their freezing and after their freezing and 6-month-long storage.

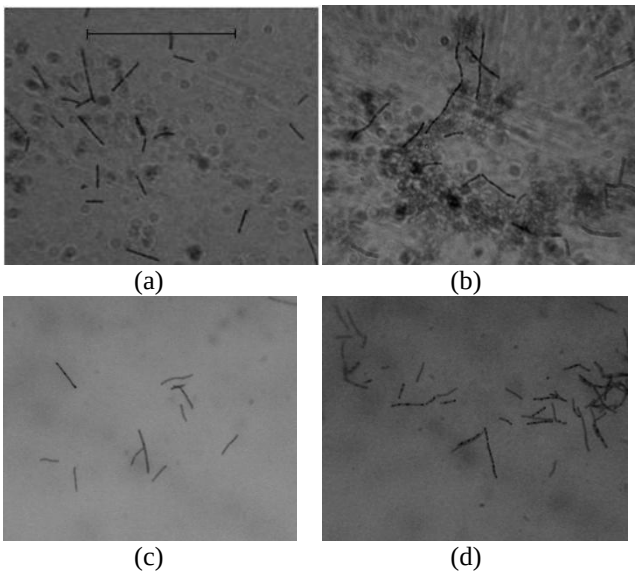


**Fig. 4.** Morphology of *L. bulgaricus* cells: (a) nonfrozen BBV, (b) nonfrozen BBNV, and (c, d) BBNV frozen and stored for 6 months at (c) –10 and (d) –45°C.

Considerable morphological changes were observed for all starter cultures frozen in air in all temperatures regimes and for the cultures frozen at –10°C in the liquid coolant. The microorganisms after freezing had curved cell walls, and most of then were separate. The BBNV starter cultures had lost their volutin granules, which were retained only in rare cases (Fig. 4c).

After freezing in the liquid coolant at –25 or –45°C, microorganisms with affected cell walls occurred sparsely, mostly in viscous starter cultures, and were separate or chained, and some of the bacilli had an elongated shape. Cells without volutin granules were observed in the BBNV cultures (Fig. 4f). Therefore, the freezing and low-temperature storage conditions were nondestructive.

We studied the antagonistic activity of *L. delbrueckii ssp. bulgaricus* strains contained in the starter cultures that had been frozen at –45°C in the liquid coolant and had been stored for 6 months. All of the strains retained a high antagonistic activity: the test bacteria growth suppression zone changed, on the average, by only 1–2mm (Table 4).

**Table 4.** Antagonistic activity of the *L. bulgaricus* cultures before and after freezing

| RNCIM number | Growth suppression zone, mm (ε ± 1.0) | | | | |
|---|---|---|---|---|---|
| | *E. coli* | *Sh. Flexneri* | *S. aureus* | *Proteus vulgaris* | *Proteus mirabilis* |
| B-3964 | 22/20 | -/- | 26/24 | 23/20 | 19/16 |
| B-6516 | 13/11 | 12/10 | 9/7 | 19/18 | 17/15 |

In the study of the biochemical properties of the frozen bacterial starter cultures, it was observed that the saccharolytic activity of the lactic bacteria in the frozen

cultures was unchanged (Table 5).

**Table 5.** Biochemical properties of *L. delbrueckii ssp. bulgaricus* strains

| Strain number | Cellobiose | Galactose | Lactose | Maltose | Mannitol | Mannose | Melibiose | Raffinose | Salicin | Sucrose | Trehalose | Arabinose | Sorbitol | Xylose | Aesculin | Melezitose |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| B-3964 | – | – | + | – | – | – | – | – | – | – | – | – | – | – | – | – |
| B-6516 | – | – | + | + | – | + | – | – | – | – | – | – | – | – | – | – |

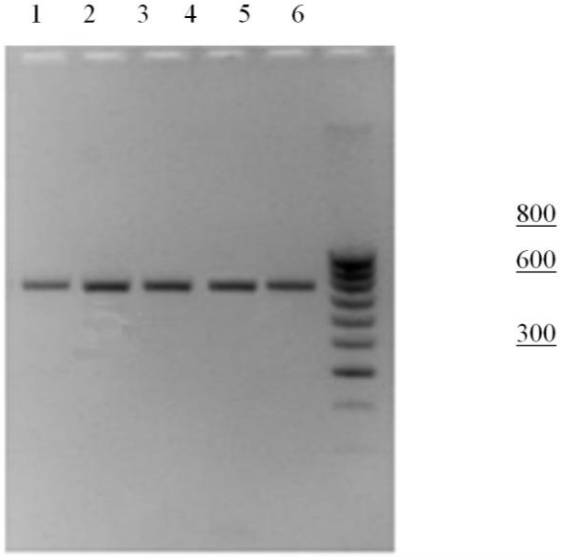


**Fig. 5.** Electrophoregram of the products of amplification of a DNA fragment of the 16S rRNA gene of *L. bulgaricus* bacteria: (1) B-6543, (2) B-6515, (3) B-6516, (4) B-3964, (5) B-3141, and (6) Medigene molecular weight marker.

We investigated the effect of freezing and lowtemperature storage on the stability of the DNA of five commercially valuable *L. delbruesckii ssp. bulgaricus* strains (B-3964, B-6516, B-3141, B-6543, and B-6515). Single-strain *L. delbruesckii ssp. bulgaricus* cultures were poured into 10-mL glass test-tubes under sterile conditions and were frozen at –45°C in the liquid coolant. The frozen starter cultures were stored in heat-insulated containers at –45°C for 6 months.

The DNA of the five *L. delbruesckii ssp.bulgaricus* strains was isolated via a procedure suggested by Bespomestnykh et al. [7]. Figure 5 shows the electrophoregram of the products of amplification of a DNA fragment of the 16S rRNA gene with the species-specific primers 16SbulF and 16SbulR for the five strains of the lactic acid bacteria frozen and stored for 6 months.

The electrophoresis data indicate that the molecular weight and the number of DNA fragments of the DNA of the bacteria are not affected by freezing and 6-month-long storage and, therefore, the lactic acid bacteria must have retained their morphological and biochemical properties.

These data suggest that temperatures of –25 to –45°C are optimal for freezing and storage of the starter cultures of the thermophilic lactic acid bacteria *L. bulgaricus*. After thawing, the starters that were stored under these conditions afford fermented milk products with a sufficiently dense clot, good organoleptic properties, and a high concentration of viable microorganisms.

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