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Incorporation of disaccharides and dimethyl sulfoxide into alginate beads increases post-thaw viability of immobilized *Saccharomyces boulardii* yeast

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Abstract

Gel-immobilized microorganisms are increasingly used in microbiological industries. Currently, the problem of developing technologies for long-term storage of microorganisms immobilized in gel carriers remains urgent. Low-temperature storage is the most effective method of preserving microorganisms. The viability of immobilized cells is affected by cryoprotective properties of gel matrix as well as cooling regimes. Therefore, the effects of incorporation of cryoprotective agents in alginate gel and cooling regimes on the viability of immobilized *Saccharomyces boulardii* cells after cryopreservation were studied. Incorporation of non-permeable cryoprotectants (sucrose, lactose, and trehalose) and permeable one dimethyl sulfoxide (DMSO) in alginate gel beads promoted an increase in the viability of immobilized cells after freeze-thawing. The highest viability rates of the gel-immobilized cells were observed in the alginate gel beads incorporating combinations of DMSO (5 – 10 % v/v) and one of the disaccharides (10 – 20 % w/v). In all experiments, slow cooling provided significantly higher viability if compared to the rapid immersion of the samples into liquid nitrogen.

Introduction

Microorganisms immobilized in gels are widely used in medicine, veterinary medicine, pharmaceuticals, food industry, animal feed production and biotechnology for environmental restoration. Gels of polysaccharides (alginate, chitosan, carrageenan) are most often applied to immobilize probiotic products (Shori 2017). Bioreactors based on immobilized microorganisms in various industries have the number of technological and economic advantages (Gbassi and Vandamme 2012; Martín *et al.* 2015; Żur *et al.* 2016; Shori 2017). The problem of storing immobilized microorganisms both at the stages of biotechnological production and in the form of commercial products is urgent. Techniques for preserving gel-immobilized microorganisms are under development. Cryopreservation and lyophilization are the most effective ways of preserving microorganisms of various taxonomic groups (She and Petti 2015). It is logical that low temperatures and lyophilization can also be used to store the microorganisms immobilized in gel beads. Previously the results of such studies have been reported (Kearney *et al.* 1990; Tsen *et al.* 2007; Solanki *et al.* 2013; Cagol *et al.* 2018).

Cryopreserved samples are usually stored in liquid nitrogen (-196 °C) or in liquid nitrogen vapors at temperatures below -150 °C. During the cryopreservation, microorganisms are exposed to a number of damaging physicochemical factors at the cooling and warming stages, the severity of those is associated with water crystallization – recrystallization. It is possible to minimize the damaging effect of these factors by adjusting the cooling rates and introducing various cryoprotective agents (CPAs) into the preservation medium (Mazur *et al.* 1970; Gordienko and Pushkar' 1994; Pegg 2007). Investigation of the effect of the composition of preserving media and cooling rates on the viability of *S. boulardii* cells when freezing cell suspensions to -196 °C has shown that the highest viability was provided by cooling at a rate of 1 °C.min^{-1}, at which the initial number of viable cells was observed (Vysekantsev *et al.* 2012). With an increase in the cooling rate up to 5 – 40 °C.min^{-1}, the number of viable cells decreased and was minimal after rapid cooling by immersing the samples into liquid nitrogen. The addition of sucrose as a CPA to the preserving medium contributed to a significant rise in cell viability in the samples frozen at a rate of 5 – 20 °C.min^{-1} (Vysekantsev *et al.* 2012). It was also shown that freezing *S. boulardii* and *Escherichia coli* cells to -196 °C in solutions of polysaccharides sodium alginate, carrageenan and agar also increased the cell viability compared to cell suspensions in physiological saline (Vysekantsev *et al.* 2015).

During the immobilization by ionotropic gelation, the beads (microspheres) acquire a rigid shell due to the cross-linking of sodium alginate molecules with divalent cations (Ca^{2+}) (Woodward 1988). The manifestation of physicochemical factors during the cryopreservation of cells in alginate beads and in polysaccharide solutions will differ. This can affect the survival of probiotic microorganisms immobilized in the beads and, therefore, the therapeutic effect of their use. The probiotic strain of the yeast *S. boulardii* is widely exploited in medical practice and the food industry to treat and prevent the dysbiosis of various origins (McFarland 2010; Moré and Swidsinski 2015). The immobilization of *S. boulardii* in gel beads and the development of methods for long-term storage of such products may hold promise for the pharmaceutical and food industries. The research was aimed to develop the composition of gel carriers and experimentally substantiate cooling regimes that will ensure a high viability rate of immobilized *S. boulardii* cells during the cryopreservation.

Experimental

Research object

The *S. boulardii* CNCM I-745 were isolated from the commercial product Enterol® (Biocodex, France). They were grown in wort agar plates with sugar content 8 ° according to Balling scale (Afanas'yeva 1976) at 30 °C for 48 h. The cells were harvested and suspended in physiological saline (0.9 % w/v). The yeast concentration in the suspension was 1×10^8 cells.mL^{-1}.

Immobilization

To immobilize yeast cells, three series of alginate carriers incorporating CPAs were prepared. In the first series, 1 % (w/v) sodium alginate gel incorporated one of the disaccharides: sucrose, trehalose or lactose. The concentration of disaccharides in the gel made 10 % and 20 % (w/v). In the second series, the 1 % sodium alginate gel contained dimethyl sulfoxide (DMSO) at concentrations of 5 %, 10 %, and 15 % (v/v). In the third series, a 1 % sodium alginate gel contained various combinations of each of the disaccharides with DMSO: 10 % or 20 % disaccharide + 5 %, 10 %, or 15 % DMSO (Table 1). All solutions were tyndalized (Poliak *et al.* 2008). Gel beads with the cells immobilized in them were obtained by ionotropic gelation (Tsen *et al.* 2007). The bead diameter was 2000 ± 100 μm. The stabilization of the beads in the 0.2 M $CaCl_2$ solution lasted 20 min. Before freezing, the samples were kept at 0 °C in melting ice.

Freezing

The beads (by 100 pieces) were placed into 2.0 mL cryovials (Nunc, USA). The samples were frozen in two ways: 1) rapid freezing down to -196 °C by

immersion into liquid nitrogen; 2) slow cooling at a rate of 1 °C.min^{-1} up to -40 °C in the programmable freezer (Cryoson, Germany) followed by immersion into liquid nitrogen. Frozen samples were thawed after 10 days in a water bath at 37 °C for 10 min.

Viability assessment

The beads were dissolved in 4 % EDTA (w/v) in a water bath at 30 °C. The viability of yeast cells was assessed by the plate Koch's method by colony formation on wort agar (Lusta and Fikhte 1990). The number of macrocolonies formed on the agar surface was recorded. Each macrocolony was formed after the reproduction of one viable cell, i.e. the colony-forming unit (CFU). This easy method makes it possible to assess the integral characteristics of the viability of microbial cells. Cell viability in beads was assessed before and after cryopreservation. In this case, the viability of the yeast before freezing served as a control. The result of assessing viability after freezing was presented as % of the control (Eq. 1):

$$Viability\ [\%] = \frac{Number\ of\ viable\ cells\ after\ freezing}{Number\ of\ viable\ cells\ before\ freezing} \times 100\% \quad (1)$$

Statistical analysis

For statistical processing of experimental data, the averages $\bar{X}$ and standard deviations $S\bar{x}$ were determined. The results were presented as $\bar{X} \pm S\bar{x}$. To determine the statistical significance of differences between the comparison groups, Student's t-test was used. The significance level was 0.05. The data obtained were statistically processed using the MS Excel software. Each series of experiments was performed six times (n = 6).

Results and Discussion

Effect of cooling regimes on the viability of yeast cells immobilized in gel beads without CPAs

It was found that after rapid cooling, 14.69 % of yeast cells remained viable in gel beads without CPAs. After slow cooling, 56.48 % of viable cells remained. The findings indicate that the cooling regimes affect the viability of *S. boulardii* yeast cells immobilized in alginate gel beads during the cryopreservation. Higher viability of the immobilized cells was provided by slow cooling (1 °C.min^{-1}). These results were similar to the data obtained in studies on suspensions of free cells *S. cerevisiae* (Mazur 1961) and *S. boulardii* (Vysekantsev *et al.* 2012). Similar results were obtained with rapid and slow freezing of *S. boulardii* cells embedded in various gels, in particular sodium alginate without beads formation (Vysekantsev *et al.* 2015). This dependence of the viability of yeast cells on the cooling rate was consistent with the main provisions of the two-factor and multifactorial theories of cell cryoinjury (Mazur *et al.* 1970; Pegg 2007). Under slow cooling a more thermodynamically stable structure is known to be formed in samples to be frozen. This eliminates mechanical damage to cells during the freeze-warming.

The alginate hydrogel itself exhibits cryoprotective properties due to its three-dimensional structure inhibiting the growth of ice crystals (Brockbank and Ga 1988; Zhang *et al.* 2018), as well as due to hydrogen bonding with intracellular water molecules (Brockbank and Ga 1988) and the formation of a highly viscous intercellular environment, which acts as a mechanical barrier and protects cells against extracellular ice crystals (Ponomareva *et al.* 2018). Since the hydrogels contain mainly bound water (Rehm 2009), during freeze-thawing in alginate gels, inhibition of crystallization-recrystallization occurs in comparison with aqueous solutions. Additionally, the cooling rate also affects the dynamics and severity of these processes.

Viability of S. boulardii cells immobilized in gel beads incorporating DMSO after rapid and slow cooling

Since the viability rates of cells immobilized in beads of 1 % (w/v) alginate gel after freezing were not high enough, in subsequent experiments the influence of cryoprotective substances with different mechanisms of protective action (permeable and impermeable) (Fuller 2004) on the viability of immobilized cells was studied. DMSO

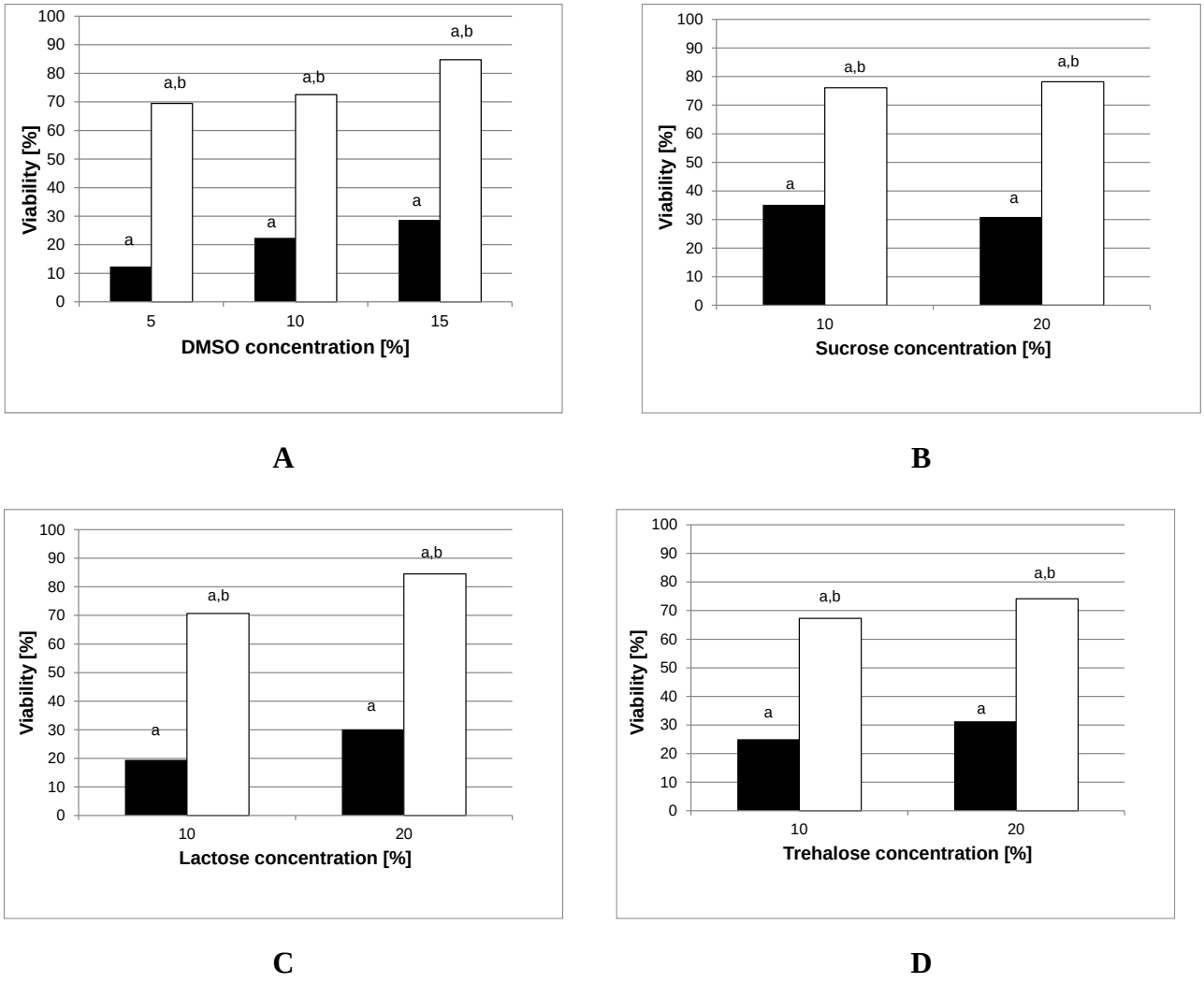

Fig. 1. Viability of *S. boulardii* cells immobilized in alginate gel beads incorporating DMSO (**A**); sucrose (**B**); lactose (**C**) and trehalose (**D**). ■ – rapid cooling; □ – slow cooling.
Note: a – significant differences as compared to the control (number of viable cells prior to freezing), $p<0.05$; b – significant differences between viability after rapid and slow cooling.

was used as a permeable CPA. This CPA is most widely used in cryopreservation of eukaryotic cells (Awan *et al.* 2020). It was found that the addition of 10 % or 15 % DMSO to the composition of the alginate gel led to increasing of the number of viable immobilized yeast cells after rapid and slow freezing compared to the gel with no additives. Cell viability after slow cooling of the beads significantly exceeded the viability of rapidly cooled cells. Thus, after rapid cooling of the gel beads containing 10 % and 15 % DMSO, the cell viability was 22.43 % and 28.67 %, and after slow cooling that made 72.54 % and 84.75 %, respectively (Fig. 1A).

In alginate gel beads incorporating 5 % DMSO, a significant increase in cell viability (69.48 %) was observed after slow cooling. After rapid cooling of the beads incorporating 5 % DMSO, cell viability did not increase.

DMSO has the ability to bind water that reduces the free water content in extracellular and intracellular spaces. The presence of DMSO inside a cell reduces the probability of intracellular crystallization. Due to this, a mechanical injury to cells was reduced owing to structural rearrangements of ice during freeze-thawing, and cell viability was increased.

Viability of S. boulardii cells immobilized in gel beads incorporating disaccharides after rapid and slow cooling

The effect of incorporation of impermeable CPAs, i.e. disaccharides to the alginate gel beads on the post-cryopreservation viability of immobilized yeast cells was studied. After rapid cooling to -196 °C in gel beads with 10 or 20 % sucrose, the viability was 35.1 and 30.9 % of cells, respectively (Fig. 1B). After slow cooling, the beads incorporating 10 % sucrose retained 76.0 % of viable cells, and in the ones with incorporation of 20 % sucrose viability was kept at 78.2 % of the cells. In beads of alginate gel incorporating 10 or 20 % lactose, the viability of 22.43 and 28.67 % of cells, respectively, was observed after rapid cooling (Fig. 1C). After slow cooling, 70.7 and 84.5 % of viable cells remained, respectively.

The viability of yeast cells in gel beads with the addition of 10 and 20 % trehalose after rapid cooling was 25.0 and 31.3 % (Fig.1D), and after slow cooling this was 67.3 and 74.1 %.

The results obtained showed that the addition of sucrose, lactose, and trehalose disaccharides to the alginate gel contributed to significant increasing in the viability of immobilized *S. boulardii* cells after freezing to -196 °C. In all the samples, the number of viable yeast cells after slow cooling significantly exceeded the number of viable cells in rapidly cooled samples.

Impermeable CPAs, i.e. disaccharides used in the experiments due to hydration reduce the amount of free extracellular water, and as osmotic agents they diminish the content of intracellular water (Fuller 2004). This leads to a rise in the viscosity of solutions and to a decrease in cryodamage to cells during the formation of ice crystals. This assumption is supported by the studies of other authors, who demonstrated that the addition of trehalose and β-cyclodextrin to alginate beads reduced the content of free water and decreased its activity (Calvo and Santagapita 2016). During freezing, the content of freezable water as well as its activity decreased with increasing sucrose concentration in the sodium alginate solution (Pongsawatmanit *et al.* 1999).

Viability of S. boulardii cells immobilized in gel beads incorporating the combinations of one of disaccharides and DMSO after rapid and slow cooling

The effect of incorporating combinations of a permeable CPA DMSO and one of the disaccharides in alginate gel on viability of immobilized *S. boulardii* yeast cells after rapid and slow cooling was studied. After rapid cooling in gel beads incorporating combinations of 10 % sucrose and 5 %, 10 % or 15 % DMSO, remained 27.22 %; 38.31 %; 26.52 % of viable cells (Table 1), and in gel beads incorporating the combinations of 20 % sucrose and 5 %, 10 % or 15 % DMSO the viability made 48.25 %; 38.7 %; 26.01 %, accordingly. After slow cooling in gel beads with the addition of combinations of 10, 20 % sucrose and 5, 10 % DMSO, the number of viable cells did not differ from the control (before freezing). In samples of gel beads with the addition of combinations of 10 %, 20 % sucrose and 15 % DMSO, cell viability was 84.22 % and 48.84 %.

The viability of yeast cells immobilized in gel beads incorporating 10 % lactose combined with 5 %, 10 %, 15 % DMSO after rapid cooling was 21.07 %; 30.33 %; 23.18 %, and in gel beads incorporating the combinations of 20 % lactose and 5 %, 10 %, 15 % DMSO the viability was 35.93 %; 27.59 %; 14.47 % (Table 1). After slow cooling in the gel beads with the addition of 10 % lactose and 5 %, 10 %, 15 % DMSO, remained 80.07 %; 79.67 %; 63.04 % viable cells. In gel beads incorporating 20 % lactose and 5 %, 10 %, 15 % DMSO after slow cooling the viability was 87.01 %; 81.28 %; 64.00 %.

The number of viable yeast cells immobilized in gel beads incorporating the combinations of 10 % trehalose and 5 %, 10 %, 15 % DMSO after rapid cooling was 29.49 %; 47.54 %; 30.41 %, and in gel beads with the addition of 20 % trehalose and 5, 10, 15 % DMSO – 25.52; 44.2; 36.71 % (Table 1). After slow cooling, the viability of cells immobilized in gel beads incorporated 10 % trehalose combined with 5 %, 10 %, 15 % DMSO was 80.1 %; 87.43 %; 64.19 %. In the gel beads containing 20 % trehalose and 5 %, 10 %, 15 % DMSO the viability made 74.9 %; 89.36 %; 83.25 %. Slow cooling in samples of beads with the

addition of 5 % or 10 % DMSO in combination with one of the disaccharides increases the viability rate of the cells in comparison with the samples containing one CPA. In this case, the combined protective effect of permeable and impermeable cryoprotective substance was observed

Table 1. Viability of *S. boulardii* yeast immobilized in alginate gel incorporating the combinations of DMSO and disaccharides after freezing to -196 °C.

DMSO [%]	Disaccharides		Viability [% from control]			
			Rapid cooling		Slow cooling	
	Disaccharide type	Concentration [%]	$\bar{X} \pm S\bar{x}$	Confidence level	$\bar{X} \pm S\bar{x}$	Confidence level
5	Sucrose	10	27.22 ± 2.64	a, b, e	92.41 ± 3.04	b, e
		20	48.25 ± 2.85	a, b, e	95.42 ± 2.82	b, e
	Lactose	10	21.07 ± 2.16	a, b, e	80.07 ± 3.10	a, b, e
		20	35.93 ± 2.84	a, b, e	87.01 ± 3.18	a, b, e
	Trehalose	10	29.498 ± 2.10	a, b, e	80.1 ± 3.21	a, b, e
		20	25.52 ± 2.58	a, b, e	74.9 ± 2.76	a, b, e
10	Sucrose	10	38.31 ± 2.93	a, b, c, e	93.29 ± 2.55	b, e
		20	38.7 ± 2.74	a, b, c, e	93.71 ± 2.90	b, e
	Lactose	10	30.33 ± 22.41	a, b, c, e	79.67 ± 3.35	a, b, e
		20	27.59 ± 2.70	a, b, c, e	81.28 ± 3.10	a, b, e
	Trehalose	10	47.54 ± 2.16	a, b, c, e	87.43 ± 2.82	a, b, e
		20	44.2 ± 3.05	a, b, c, e	89.36 ± 2.75	a, b, e
15	Sucrose	10	26.52 ± 2.77	a, b, d, e	84.22 ± 3.80	a, b, d, e
		20	26.01 ± 2.69	a, b, d, e	48.84 ± 2.77	a, b, d, e
	Lactose	10	23.18 ± 2.14	a, b, d, e	63.04 ± 3.10	a, b, d
		20	14.47 ± 3.17	a, b, d, e	64.00 ± 3.42	a, b, d
	Trehalose	10	30.41 ± 3.25	a, b, d, e	64.19 ± 2.75	a, b, d, e
		20	36.71 ± 2.90	a, b, d, e	83.25 ± 3.15	a, b, d, e
1 % alginate gel without additives			14.69 ± 0.35	a, b	56.48 ± 2.30	a, b

$\bar{X}$ – average, $S\bar{x}$ – standard deviation, a – confidence level <0.05 between $\bar{X}$ values before and after freezing down to -196 °C, b – confidence level <0.05 between $\bar{X}$ values after slow and rapid freezing, c – confidence level <0.05 between $\bar{X}$ values at DMSO concentration of 5 % and 10 % and corresponding disaccharide concentrations (10 % and 20 %), d – confidence level <0.05 between $\bar{X}$ values at DMSO concentrations of 10 % and 15 % and corresponding disaccharide concentrations (10 % and 20 %), e – confidence level <0.05 between $\bar{X}$ values in 1 % alginate gel samples without additives and in the samples with cryoprotectants.

(Pakhomova *et al.* 2013).

Obtained results indicate that the viability of cells immobilized in gel beads containing the combination of disaccharide with DMSO does not exceed the viability rate of yeast in beads during rapid cooling in comparison with beads containing only one cryoprotective agent. When immersed into liquid nitrogen, the protective mechanisms of both impermeable and permeable cryoprotective substances do not have time to be fully implemented.

Conclusions

Slow cooling (1 °C.min^{-1}) provides a higher viability of *S. boulardii* cells immobilized in alginate gel beads both without additives and with cryoprotective substances. Incorporation of

impermeable CPAs (sucrose, lactose, trehalose) and a permeable one (DMSO) as well as the combinations of these disaccharides with DMSO in the alginate gel increases the viability of *S. boulardii* cells immobilized in the gel after freezing to -196 °C. The viability of *S. boulardii* cells immobilized in alginate gels incorporating the combinations of 5 – 10 % DMSO and one of the disaccharides at a concentration of 10 – 20 %, after slow freezing, exceeds the cell viability rates in gel samples with the addition of one CPA (DMSO or disaccharide). The maximum viability of the immobilized cells after slow cooling was observed in gel beads incorporating 5 – 10 % DMSO combined with 10 – 20 % sucrose or 10 % DMSO combined with 20 % trehalose. Taking into account recommendations of the Food and Agriculture Organization of the United Nations and the World Gastroenterological Organization, regarding the composition of various commercial probiotic and symbiotic products, and our results, the incorporation of disaccharides into the gel carriers for immobilization can be proposed. This is relevant for technologies for long-term storage of immobilized probiotic microorganisms. For freezing low cooling rates are preferable to be used.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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