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Cocoa By-products As An Alternative to Expensive Cryoprotectants for Freeze-drying of *Lactobacillus plantarum*

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Abstract *Lactobacillus plantarum* is a lactic acid bacterium involved in a number of fermentations, including cocoa. This strain is subject to genetic modification if poorly preserved, hence the importance of lyophilisation. However, synthetic cryoprotectants are used to protect the strain during freeze-drying. However, these cryoprotectants are expensive and increase the cost of freeze-drying. The aim of this work is to develop a cheaper freeze-drying medium for *Lactobacillus plantarum* from cocoa by-products. To achieve this, the *Lactobacillus plantarum* strain was cultured, centrifuged and the resulting pellet was recovered. Cocoa pulp and cocoa pod powder were prepared and autoclaved at 121°C for 15 minutes each, then added to the pellet alone or in combination. A sucrose control was made samples were frozen and lyophilised. Survival rate and acid production were assessed after freeze-drying. The highest viability rate after freeze-drying was recorded with cocoa bean liquor (82.88 ± 0.37) compared to 76.83 ± 0.21 for the control. The acidity of the cocoa bean liquor after freeze-drying was identical to that of the control. Cocoa bean liquor could be used as an alternative to cryoprotectants, which are too expensive for freeze-drying *Lactobacillus plantarum*.

Keywords: *Lactobacillus plantarum, Cocoa bean liquor, Cocoa pods, Freeze-drying, Cryoprotectants*

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1. Introduction

Lactobacillus plantarum is a very important microorganism that is widely used as a probiotic due to its beneficial properties for human health and food processing [1,2,3,4,5]. This microorganism has positive effects on the reduction of digestive and chronic diseases, the immune system, cholesterol levels, food fermentation and provides biological control against harmful microorganisms [6,7,8]. As a result, *Lactobacillus plantarum* is generally recognised as a safe probiotic with GRAS status [1,6].

To protect this strain of global interest and make it more stable and available, several technologies such as microencapsulation, spray drying, spray drying and vacuum drying have been proposed [9,10,11]. However, of all these technologies, only vacuum drying or lyophilisation is the most widely used, as this technology both removes moisture from the cells without increasing the temperature and better preserves cell viability [12]. The safest way to maintain the stability and use of this strain is to render it in powder form [13].

However, according to the work of Chen *et al.*, [14], Han *et al.*, [15], Velly *et al.*, [16], unavoidable damage during lyophilisation can affect microorganisms and have negative effects. This damage can lead to disorganisation of the inner and outer membranes, denaturation of DNA and proteins, or even a reduction in the lytic activity of the microorganism. According to Fonseca *et al.*, [17], O'Brien *et al.*, [18], Stephan *et al.*, [19], this damage is mainly related to the freezing step that takes place just before lyophilisation. Therefore, to prevent and reduce the undesirable effects associated with lyophilisation, protective substances or cryoprotectants are added before freezing the microorganism [20]. The cryoprotectants belonged to either the carbohydrate, amino acid or protein groups, with a high frequency of use of cryoprotectants belonging to the carbohydrate group. In the case of lyophilisation *Lactobacillus plantarum*, the most frequently used cryoprotectants were skimmed milk, sucrose and trehalose Li *et al.*, [13], Gul *et al.*, [21].

Carbohydrates are among the most widely used substances, because they block the formation of ice crystals, facilitate cell adaptation between the space and

surfaces where the molecules are located, and form glassy structures with low molecular interactions [22,23]. However, these protective substances are not produced in all countries, and purchasing them for use can increase the cost of lyophilisation. Therefore, over the years, a variety of materials have been proposed as protective substances to replace the commonly used ones that are too expensive. The work of Hongpattarakere *et al.*, [24] with aqueous extracts and crude fibres of maize, bean and soy, followed by that of Chotiko *et al.*, [25] with purple rice bran fibres and finally that of Savedboworn *et al.*, [26] with rice protein and fructooligosaccharide has shown that it is possible to use substances other than those commonly used to ensure good protection and preservation of the *Lactobacillus plantarum* strain. Côte d'Ivoire has also been the world's leading producer of cocoa beans for several decades, producing over 2.4 million tons of cocoa in the last harvest [27]. However, after shelling for fermentation, the shell or pod, which represents around 70% of the fruit, is generally discarded, and the pulp that runs off is not used, causing some environmental pollution. However, the pulp is said to be rich in glucose, fructose and sucrose, while the shell is rich in starch, fibre and cellulose. These molecules are among those used as cryoprotectants for bacteria and yeast. Cocoa shell and pulp could therefore be used as cryoprotectants for freeze-drying *Lactobacillus plantarum*. Such a study has never been carried out in the literature, hence the interest in this work. The general aim of this work is to develop a cheaper freeze-drying medium for *Lactobacillus plantarum* from cocoa by-products.

2. Materials and Methods

2.1. Materials

The lactic acid bacteria strain (*Lactobacillus plantarum*) used in this study was isolated from cocoa bean fermentation in Côte d'Ivoire [28]. This strain was stored at -80°C in MRS (Man Rogosa Sharpe) broth supplemented with 20% glycerol. The empty cocoa shells or pods and cocoa liquor used in this work came from the Agneby-Tiassa region of Côte d'Ivoire.

2.2. Methods

2.2.1. Preparation of Cryoprotectants Based on Empty Pods, Cocoa Liquor Extract and Sucrose

After extraction of the cocoa beans, the cocoa shells were cut into small pieces with a knife. These shells were dried in the oven at 45°C for 3 days. After drying, the shells were ground using a blender (Moulinex, France) to obtain cocoa flour. This flour was placed in a lidded glass jar and stored at 27°C±3°C for later use. Cocoa flour was prepared at 4 %, by mixing four (4) grams of the previously obtained flour with 100 ml of distilled water. The mixture was stirred and then placed on a hot plate with constant stirring. When the solution began to boil, stirring was continued for five (5) minutes and the solution was transferred to a glass jar and allowed to cool to 30°C before being used as a cryoprotectant

The bean liquor was prepared by placing 500 grams of fresh beans and one (1) litre of distilled water in a two (2) litre Erlenmeyer flask. The mixture was vigorously homogenised for ten (10) minutes, then transferred to a clean basin. Wearing gloves, the beans were kneaded for five (5) minutes. The mixture was filtered and the resulting liquid was autoclaved at 121°C for 15 minutes. The solution obtained constituted the cocoa liquor extract.

Sucrose was prepared at 25%, in a 500 ml Erlenmeyer flask. That is, 25 grams of sucrose were weighed and placed in an Erlenmeyer flask, then 500 ml of distilled water were added to the sucrose powder. The whole mixture was vortexed until all the sucrose was dissolved, before being transferred to Pyrex jars for sterilisation in an autoclave at 121°C for 15 minutes.

2.2.2. Microbial Culture of *Lactobacillus plantarum* Starter for lyophilisation

The *Lactobacillus plantarum* strain was first reactivated in MRS broth for 24 hours and then replicated on an MRS agar plate. The agar containing the strain was then incubated at 37°C for 48 hours. A pure colony was used to prepare a dense suspension with 120 ml of MRS broth. Cultures were incubated for 48 hours at 37°C in an oven.

2.2.3. Lyophilisation of *Lactobacillus plantarum* Starter

The method proposed by [29] was used in this study. The dense culture of *Lactobacillus plantarum* obtained after 48 hours of cultivation was centrifuged at 12 000 x g for 5 min at 4°C in a refrigerated centrifuge (Laboao, China). The bases obtained were then washed with a saline solution (0.9% NaCl) and collected in a sterile 100 ml Erlenmeyer flask. Cocoa pod meal prepared at 4% was added to 10% of the bases While cocoa liquor extract was added to the bases. Sucrose, used as a control, was added to the base at 20 %. The mixtures were homogenised and then placed in a freezer at -60°C for two (2) hours before being freeze-dried for 48 hours at -50°C ± 3°C and 1 Pa using a freeze-dryer (Laboao, China). Table 1 shows the various tests carried out in this study.

Table 1. different tests and compositions of cryoprotectants used for freeze-drying

Tests	Fresh cocoa bean liquor (ml)	Shell of cocoa (ml)	Sucrose (ml)	Saline solution (ml)
1	0	0	5	20
2	25	0	0	0
3	0	5	0	20
4	20	0	5	0

2.2.4. Determination of the Survival Rate of *Lactobacillus plantarum* Starters after Lyophilisation

The successive decimal dilution method proposed by Cui *et al.*, [30] was used to determine the survival rate of *Lactobacillus plantarum* strains on MRS agar media from the microbial culture performed in **2.2.2** to determine the microbial load before freeze-drying. After lyophilisation, a mass of 0.1 g of lyophilisate from each *Lactobacillus plantarum* assay was suspended in 4 ml of MRS broth. The suspensions were incubated for 2 hours at 30°C. A series of dilutions were then prepared from each incubated

sample and a 100 µL volume of each cell suspension was inoculated uniformly onto MRS agar to determine the microbial load after freeze-drying. The agar plates were then incubated at 37°C for 48 hours. Cell viability was determined by standard agar enumeration. The average of the three plates was used for each assay and dilution. Plates with bacterial counts between 30 and 300 CFU were used to determine microbial load. The survival rate of *Lactobacillus plantarum* after the lyophilisation process was expressed according to the method of Mendoza *et al.*, [31]. The cell viability obtained for each (assay) was expressed as a percentage survival factor (SF), calculated using the following equation (1):

$$SF = \frac{1-(\log CFU_1 - \log CFU_2)}{\log CFU_1} \times 100 \quad (1)$$

CFU_1 : CFU.ml^{-1} X Total culture volume before freeze-drying

CFU_2 : CFU.g^{-1} X Total dry mass of *Lactobacillus plantarum* lyophilisate (g)

2.2.5. Determination of Strain's Ability to Maintain Acid Production

After lyophilisation, 0.1 gram of each condition for the strain was added to four (4) ml of MRS broth and then incubated for two (2) hours. At the end for this time, the microbial load was determined, set at 10^5 Cells/ml and used to inoculed 15 ml of MRS broth. The same treatment was carried out with the liquid culture from question **2.2.2** before lyophilisation. Samples were incubated for 72 hours, after which pH and acidity were assessed. The titratable acidity was determined in 5 ml of each culture with a 0.1 N sodium hydroxide (NaOH) solution. The acidity of the different cultures was determined using the following formula (2):

$$P_a = \frac{N_b \times V_b \times 1000 \times M}{V_a} \quad (2)$$

Va: Test volume (ml)
Nb: NaOH normality (ml)
Vb: Volume of NaOH (ml)
M: Molar mass of lactic acid

Finally, the amount of acid produced was determined from the following form (3):

$$Acidity(\%) = \frac{A_1}{A_2} \times 100 \quad (3)$$

A_1: Acidity produced by the strain after freeze-drying
A_2: Acidity produced by the strain in the liquid medium before freeze-drying

2.2.6. Determination of Viability of Freeze-dried *Lactobacillus plantarum* During Storage At Room Temperature

The lyophilisates were stored for one (1) month at room temperature (30°C ± 2°C) in the dark, and the strain lyophilised with fresh cocoa bean liquor and the strain lyophilised with the combination of pod (10%) + cocoa liquor extract was used for this work. Each week, 0.1 of each lyophilisate was resuspended in four (4) ml of EPT and incubated at 37°C for two (2) hours. A series of dilutions of each previously incubated sample was then prepared and a 100 µL volume of each cell suspension was inoculated uniformly onto MRS agar. The agar plates were then incubated at 37°C for 48 hours. Cell viability was determined by standard agar counting. The average of the three plates was used for each assay and dilution. Plates with bacterial counts between 30 and 300 CFU were used to determine the microbial load. Viability over time was assessed using the formula proposed by [32].

$$SR(\%) = \frac{\log CFU_2}{\log CFU_1} \times 100 \quad (4)$$

UFC_1: logarithm of the load determined each week
UFC_2: logarithm of the load determined immediately after freeze-drying
SR: Survival Rate

2.2.7. Determination of the Amount of Lactic Acid Produced by Freeze-Dried *Lactobacillus Plantarum* During Storage At Room Temperature

The determination of lactic acid produced by the strain in the different lyophilisates was carried out using the suspension prepared each week as described in **2.2.6**. The load of each suspension was determined by counting the strains under freeze-drying conditions using a light microscope (×40). For this purpose, 25 µL of each lyophilisate was placed on the Thoma cell slide, to which 25 µL of methylene blue was added, and covered with a coverslip. Live cells present on the white background of the slide were counted. The formula proposed by Fossi *et al.*, [33] was used to determine the loading of each suspension. The different loadings were set at 10^5 Cells/ml and used to seed 15 ml of each MRS broth for each condition and incubated at 37°C for 48 hours. At this time, the cultures were titrated with NaOH solution in the presence of phenolphthalein as a colour indicator. Finally, the amount of acid produced over time was determined from the following form (5):

$$Acidity(\%) = \frac{A_1}{A_2} \times 100 \quad (5)$$

A_1: Acidity produced by the strain each week (ml)
A_2: Acidity produced by the strain after freeze-drying (ml)

2.2.8. Statistical Analysis

All experiments were repeated three times and the raw data generated were expressed as mean ± standard deviation. Data were entered and calculated using Excel 2019. Descriptive statistics were used to analyse survival rates and performance of lyophilised cultures. One-way analysis of variance (ANOVA) was used to compare means. Means were separated by Tukey's error rate multiple comparison test using XLSTAT software, and differences in means were considered statistically significant at $p < 0.05$.

3. Results

3.1. *Lactobacillus plantarum* Viability after

Lyophilisation with Cryoprotectants

The different survival rates recorded after freeze-drying of the *Lactobacillus plantarum* strain with fresh cocoa bean liquor and/or empty cocoa pod solution are shown in Table 2. The survival rates recorded after lyophilisation are all higher than 67.26 ± 0.76%. In addition, the strain lyophilised with fresh cocoa bean liquor gave a survival rate of 82.88 ± 0.45%, higher than the sucrose control (76.83 ± 0.63%). The strain lyophilised with the combination of fresh cocoa bean liquor and empty cocoa pod solution showed a survival rate of 71.89 ± 0.68%, significantly closer to that of the sucrose control. The strain freeze-dried with empty cocoa pod solution gave the lowest viability (67.26 ± 0.76%) of all the other tests.

Table 2. Viability rate of *Lactobacillus plantarum* in the presence of various cryoprotectants after freeze-drying

Essay	Survival Rate (%)
Lp + fresh cocoa bean liquor	82,88 ± 0,45a
Lp + shell	67,26 ± 0,76b
Lp +fresh cocoa bean liquor + shell	71,89 ± 0,68c
Lp + Sucrose	76,83 ± 0,63d

Lp : Lactobacillus plantarum

3.2. Ability of *Lactobacillus plantarum* to Produce Acid After Freeze-drying

Table 3 show, the pH and acidity produced by the strain after freeze-drying in the presence of different cryoprotectants. The pH values in the acid-producing liquid media ranged from 5.17 ± 0.00 to 5.43 ± 0.05. Statistical analysis showed no significant difference between the pH values obtained in the tests with different cryoprotectants. However, with regard to acidity, the amount of acid retained varied widely, with values ranging from 73.45 ± 2.67% to 97.03 ± 2.56%. The freeze-dried strain with fresh cocoa bean liquor retained up to 90.60 ± 1.27%, much closer to the control test with sucrose (97.03 ± 2.56%) and the test combining fresh cocoa bean liquor and empty cocoa pod solution (86.21 ± 4.22%). As for the strain freeze-dried with empty cocoa pod solution alone, it retained much less acid production than the other trials (73.45 ± 2.67%).

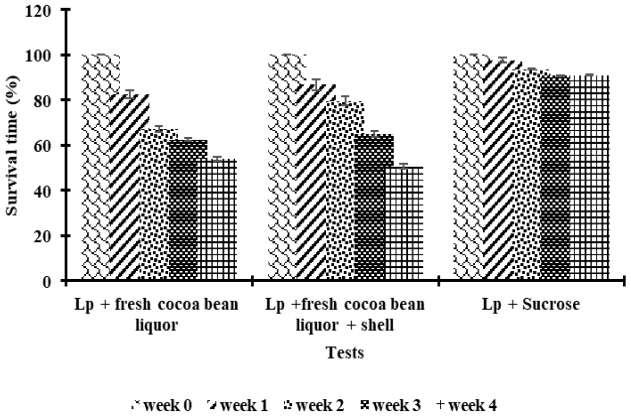
Table 3. Hydrogen potential and amount of acid produced after freeze-drying *Lactobacillus plantarum* in the presence of different cryoprotectants

Essay	pH	Acidity (%)
Lp + fresh cocoa bean liquor	5,43 ± 0,05 a	90,60 ± 1,26ab
Lp + shell	5,22 ± 0,00b	73,45 ± 2,67c
Lp +fresh cocoa bean liquor + shell	5,17 ± 0,00bc	86,21 ± 4,42b
Lp + Sucrose	5,28 ± 0,00c	97,03 ± 2,57a

Lp : Lactobacillus plantarum

3.3. Viability Rates of Freeze-dried *Lactobacillus plantarum* Strains Stored at Room Temperature

The viability of *Lactobacillus plantarum* during storage at room temperature (30°C ± 2°C) is shown in figure 1. All protective substances ensured *Lactobacillus plantarum* storage with a strain survival rate of over 50% for one (1) month. However, a more moderate decrease in survival rate in the control but more intense in the trials was recorded. In addition, the combination of fresh cocoa bean liquor + empty cocoa pod solution provided better protection than fresh cocoa bean liquor during the first two weeks, with values of 79.61 ± 1.59% and 67.04 ± 1.21% respectively. As for the control, the survival rates obtained during storage did not drop slightly, reaching 91.11 ± 0.37% after one (1) month.



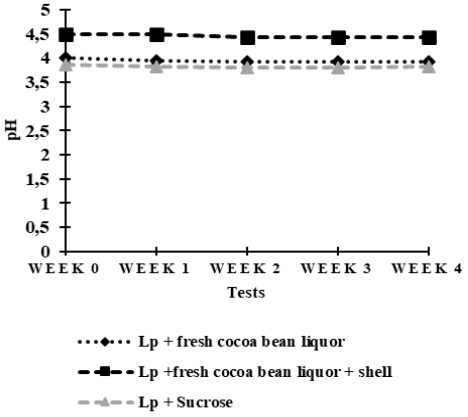
Lp: Lactobacillus plantarum

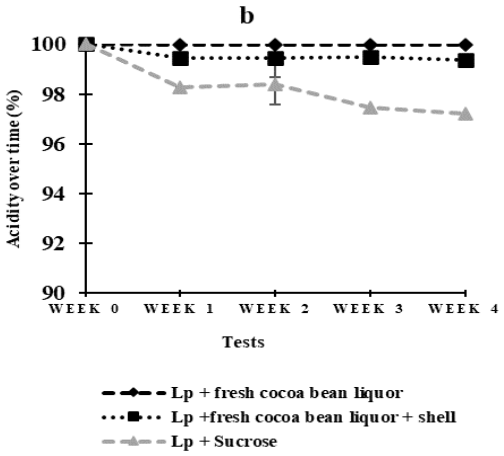
Figure 1. Viability of *Lactobacillus plantarum* in the presence of various protective substances during storage at room temperature

3.4. Ability of *Lactobacillus Plantarum* to Produce Acid During Storage of Lyophilised Strain At Room Temperature

The pH and acidity recorded in the freeze-dried strain with different cryoprotectants are shown in Figures 2a and 2b respectively. In general, the pH in lyophilisates varies very little during storage at room temperature. With regard to acidity, the amount of acid produced by the strains lyophilised with fresh cocoa bean liquor and the combination of fresh cocoa bean liquor + empty cocoa pod solution decreased slightly from 100.00 ± 0.00% to 99.95 ± 0.00% and 100.00 ± 0.00% to 99.37 ± 0.00% respectively. However, a slightly greater decrease was observed in the sucrose control, with values falling from 100.00 ± 0.00% to 97.21 ± 1.51%.

a





Lp: Lactobacillus plantarum

Figure 2. Variation in acidity produced by *Lactobacillus plantarum* strains lyophilized with different protective substances during storage at room temperature

4. Discussion

Freeze or vacuum drying is a technique used to preserve the structure of bacterial cells [13]. Although effective, this technique causes ice crystals to form in the cell during drying, which affect the integrity, fluidity and permeability of the bacterial membrane and causes mechanical damage that can lead to bacterial cell death [34,35]. For this reason, protective substances or cryoprotectants are added to bacterial suspensions prior to lyophilisation [23,36,37]. These cryoprotectants are mainly used to prevent the formation of ice crystal and contact with oxygen molecules, to provide mechanical protection, increase the glass transition temperature and accelerate the lyophilisation rate. In this study, the different survival rates obtained with the lyophilisates could be related to the thawing temperature after lyophilisation of the bacteria or to a poor adaptation of the cells to the cryoprotectant [38]. In fact, if the thawing temperature is not adapted, bacterial survival is affected by changes in electrolyte concentrations or by water recrystallization. Furthermore, the high cell viability observed with fresh cocoa bean liquor as a cryoprotectant may be related to its richess in monosaccharides and disaccharides. According to Bucheli *et al.*, [39], Goto *et al.*, [40], Lopez *et al.*, [41], cocoa pulp is a sugar-rich medium containing glucose, fructose and sucrose. These sugars, especially sucrose, are commonly used to protect microorganisms prior to lyophilisation. According to Chiu *et al.*, [22], Carvalho *et al.*, [42], monosaccharides and disaccharides are able to fit between the spaces and surfaces where their molecules are arranged, penetrate the cell wall, form glassy substrates with weak molecular interactions, and induce plasmolysis prior to freezing to provide mechanical protection. Furthermore, unlike the other tests, the presence of several cryoprotectants in the pulp could justify this high rate. Other work carried out by Hongpattarakere *et al.*, [24] with a mixture of aqueous extracts of maize, mung bean and soybean crude fibers, by Chotiko *et al.*, [25] with purple rice bran fibers and by Peng *et al.*, [20] with pomelo peel celluloses showed that it was possible to use other substrates to protect *Lactobabillus plantarum* during freeze-drying and achieve high survival rates. Similarly, the lower survival of lyophilisates protected with cocoa shells alone could be explained by their high starch and cellulose content. Starch and cellulose are substrates commonly used as supports for freeze-drying microorganisms. In other words, substances that protect cryoprotectants and microbial cells. Due to their large size, these substances cannot penetrate cell membranes to replace water. They provide external protection. With this in mind, work by Chen *et al.*, [32] showed that the use of 10% skimmed milk on *Lactobacillus plantarum* and *Lactobacillus fermentum* had a positive protective effect on these two strains. Similarly, Qu *et al.*, [43] showed that 15% skimmed milk improved the freeze-drying survival rate of *Lactobacillus gasseri* from less than 20% to 56.5%. The amount of acid retained by the strain after freeze-drying in the presence of cryoprotective differences varied from experiment to experiment. This variability in the amount of acid could be related to the protection of the strains by the different cryoprotectants. Indeed, if the strain is well protected by the cryoprotectant, no difference in acid production should be observed. The variable productions recorded would suggest that freeze-drying has an effect on the DNA of the cells, specifically on the gene responsible for organic acid production, which could explain this result Chen *et al.*, [14], Han *et al.*, [15], Velly *et al.*, [16].

When the tests at room temperature (30°C ± 2°C), the viability of the microorganisms in the cocoa lyophilisate was much lower than in the control. This decrease in viability was more pronounced in the pulp than in the sucrose and may be related to the fact that the pulp contains other molecules in addition to sugars. These molecules lyophilized with the strains were able to degrade progressively during storage, which may have affected the survival of the strains lyophilized with cocoa pulp. However, the pulp maintained the survival rate above 50% for one (1) month, while organic acid production remained virtually unchanged. Similar results were reported by Chotiko *et al.*, [25] in their study of purple rice bran fibre for the protection of *Lactobacillus plantarum*.

5. Conclusion

The effect of fresh cocoa bean liquor and/or flour as cryoprotectants on *Lactobacillus plantarum* strains was evaluated by strain survival, ability of strains to maintain production after freeze-drying, cell viability and preservation of acid production over time. The results showed that cocoa pulp could ensure very good conservation of *Lactobacillus plantarum* strains during lyophilisation and storage at room temperature. Cocoa pulp could therefore be proposed as an alternative for freeze-drying lactic acid bacterial strains, thereby reducing the cost of purchasing cryoprotectants. The best conditions for using pulp as a cryoprotectant need to be determined.

Conflicts of Interest

The authors have declared that they have no conflicts of interest.

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