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Cocoa Residues as Support Material for *Acetobacter pasteurianus* Starter Culture Freeze-Drying

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Abstract Microbial starters are of great economic importance to the food industry and many other sectors. These organisms ensure that food is processed to produce reproducible products of consistent quality. Maintaining their stability and viability during processing and storage is therefore a priority. Of all the methods proposed for their preservation, freeze-drying remains the most suitable for bacteria. In this study, cocoa pod flour and/or pulp was used as a cryoprotectant, in comparison with mannitol, the cryoprotectant that best protects acetic bacteria. *Acetobacter pasteurianus* strains were grown in YEPG broth and centrifuged. The pellets were then collected and mixed with 20% mannitol, cocoa pulp juice, cocoa pericarp flour, and a combination of cocoa pulp juice and cocoa pericarp flour. The samples were freeze-dried, and the survival rate after freeze-drying and during storage as well as acid production were assessed. The results show that the combination of cocoa pericarp and cocoa pulp juice preserved the strains better during lyophilization and storage (79.24 ± 0.32%) than the control (76.05 ± 0.37%) and the other trials. In addition, the acid production of the strain was better preserved by the combination of cocoa pericarp flour and cocoa pulp juice than in any of the other trials. Cocoa pericarp flour combined with cocoa pulp juice could be used as an alternative cryoprotectant for freeze-drying of *Acetobacter pasteurianus*. This could enable the valorisation of cocoa residues, such as pod and pulp.

Keywords: *Acetobacter pasteurianus, cocoa pulp, cocoa pericarp, cryoprotectants*

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1. Introduction

Acetic acid bacteria (AAB) are obligate aerobic bacteria, and their dependence on oxygen strongly influences their strategies for adapting to the environment [1,2,3,4,5]. This adaptive capacity allows AAB, in particular *Acetobacter pasteurianus*, to participate in the transformation processes of several foods, such as wine, beverages, vinegar, and cocoa bean fermentation, by enhancing their aroma. In addition, these microorganisms play a role in protecting certain foods from pathogenic microorganisms, mainly moulds [6,7,8,9].

In cocoa fermentation, several studies show that *Acetobacter pasteurianus* dominates, and this strain is commonly used in starter culture cocktails to improve the quality of cocoa beans [10,11] and in the bioproduction of various industrial foods, especially vinegar [12]. *Acetobacter pasteurianus* strains were selected as starters for cocoa fermentation because of their ability to produce high levels of acetic acid. This metabolite (acetic acid) plays a crucial role in the cocoa fermentation process and in the development of cocoa bean flavour [11].

However, *Acetobacter pasteurianus* starters are usually preserved in broth form. In this form, starters rapidly lose viability and are often subject to contamination [13,14]. For this reason, several industrial techniques have been proposed for the preservation of *Acetobacter pasteurianus*. Among these, freeze-drying appears to be the best technique, as it is less time-consuming, less costly, simpler, and easier to apply [15].

During lyophilization of *Acetobacter pasteurianus*, mannitol is commonly used as a cryoprotectant [16,17]. Recently, we have shown that soy flour can be used as an alternative to mannitol for the freeze-drying of *Acetobacter pasteurianus* [18]. However soy flour is used for human and animal food. As a result, the use of soy flour for the industrial production of *acetobacter pasteurianus* starter would not be viable, hence the need to find substrates of less economic importance such as cocoa residues.

The aim of this work is to identify alternative cryoprotectant to mannitol, derived from cocoa residues (cocoa pod and bean pulp), for freeze-drying of

Acetobacter pasteurianus.

2. Materials and Methods

2.1. Materials

The *Acetobacter pasteurianus* strain used in this study was isolated from cocoa bean fermentation in Côte d'Ivoire [10]. The strains were preserved in YEPG broth (1% yeast extract, 4% ethanol, 1% peptone, 1% glucose) supplemented with 20% glycerol at -80°C. The cocoa used in this study was from the Agnéby-Tiassa (Côte d'Ivoire).

2.2. Methods

2.2.1. Microbial Culture of *Acetobacter pasteurianus* for Freeze-drying

Acetobacter pasteurianus was first reactivated in YEPG broth, at 37°C for 24 hours then streaked onto a YEPG agar plate and incubated at 37°C for 48 hours. A pure colony was used to inoculate 120 mL of YEPG broth. Cultures were incubated at 30°C for 5 days with agitation.

2.2.2. Preparation of Support (cryoprotectant) for Freeze-drying

The harvested cocoa was opened with a knife, and the pulp-coated beans were removed, weighed, and one (1) kilogram of pulp-coated cocoa beans was combined with two (2) liters of distilled water. The mixture was vigorously homogenised for five minutes, and then left to stand for five (5) minutes to allow the cocoa beans to separate from the juice. The pulp juice was immediately placed in an Erlenmeyer flask and autoclaved at 121°C for 15 minutes. After removing the beans from the pods, the empty cocoa pericarps were cut into small pieces and dried in an oven at 40°C for 48 hours. The cocoa pericarp was then ground into a powder (pericarp flour) using a blender (Moulinex, France). Pericarp flour was prepared at a concentration of 4% in distilled water. Mannitol (20%) was also prepared at a concentration of 4% in distilled water, and both solutions were autoclaved at 121°C for 15 minutes before use.

2.2.3. Determination of Sugars Contained in Cocoa Pericarp Flour and Cocoa Pulp Juice

Five (5) grams of cocoa pulp juice and cocoa pericarp were placed in a 200 mL volumetric flask respectively.

Next, they were supplemented with 100 mL of distilled water heated to 60°C. The mixture was stirred until completely cooled and then filtered using Whatman paper. The resulting filtrate constituted the water-soluble extract of the fermented and dried beans.

The method used for the determination of total sugars is that of [19]. For thus purpose, 100 µL of cocoa pericarp flour and cocoa pulp juice respectively, are added to test tubes, followed by the addition of 1 mL of concentrated sulfuric acid and 200 µL of phenol. The mixture is allowed to cool for 5 minutes. After cooling, 2.7 mL of distilled water is added, and the optical density is measured with a spectrophotometer at 490 nm. A standard curve is plotted under the same conditions using a 1 mg/mL glucose solution to determine the sugar content of the samples.

In addition, the method described by [20] was used to determine the reducing sugars. A volume of 100 µL of cocoa pericarp flour, and cocoa pulp juice is added to test tubes respectively, followed by the addition of 200 µL of 3,5-dinitrosalicylic acid (DNS). The mixture is then placed in a boiling water bath for 5 minutes. After cooling, 2 mL of distilled water is added, and the absorbance is measured with a spectrophotometer at 540 nm. The concentration of reducing sugars is determined using a standard curve prepared from the same conditions with a 1 mg/mL glucose solution.

2.2.4. Lyophilization of *Acetobacter pasteurianus* Strain

The cell suspension of *Acetobacter pasteurianus* obtained in section 2.2.1 was centrifuged at 12,000 × g for 5 minutes at 4°C in a centrifuge (Laboao, China). The pellets were then washed with sterile saline (0.9% NaCl). After washing, the required amount of pulp juice, cocoa pericarp flour, and mannitol were added to the cell pellet in a 100 mL Erlenmeyer flask, according to the experimental design (Table 1). The mixtures were homogenized and frozen at -60°C for two (2) hours before being freeze-dried at -60°C ± 3°C and 1 Pa for 48 hours in a freeze-dryer (Laboao, China).

Table 1. Different compositions of support (cryoprotectant) used for freeze-drying

Essay	Pulp juice (mL)	Cocoa pericarp flour (mL)	Mannitol (mL)	Saline solution (mL)
1	0	0	4	21
2	2.5	0	0	22.5
3	0	2.5	0	22.5
4	2.5	22.5	0	0

2.2.5. Determination of the Survival Rate of *Acetobacter pasteurianus* Strain After Freeze-drying

The successive decimal dilution method proposed by [21] was used to determine the survival of the *Acetobacter pasteurianus* strain on YEPG agar media from the microbial culture prepared in section 2.2.1 before lyophilization. After freeze-drying, 0.1 g of lyophilizate from each sample was suspended in 4 mL of YEPG broth. The suspensions were incubated for 2 hours at 30°C. A series of dilutions of each incubated sample was then prepared, and a 100 µL volume of each cell suspension was inoculated uniformly onto YEPG agar to determine the microbial load after freeze-drying. Agar plates were then incubated at 30°C for 24 hours. Cell viability was determined by standard enumeration on nutrient agar, with an average of three plates used for each test and dilution. Plates with bacterial counts between 30 and 300 CFU were used to calculate the microbial load. The survival rate of *Acetobacter pasteurianus* after the freeze-drying process was expressed according to the method of [22]. The survival factor (SF) for each test was expressed as a percentage using the following equation:

$$\mathbf{SF} = \frac{1-(logCFUbefore - logCFUafter)}{logCFUbefore} X100$$

Where:

CFU_{before} = $CFU.mL^{-1}$ X total volume culture (ml) before the freeze – drying process

CFU_{after} = $CFU.g^{-1}$ X total weight of the dry bacterial sample (g)

2.2.6. Determination of Acid Production Capacity of *Acetobacter pasteurianus* After Freeze-drying

After freeze-drying, 0.1 g of lyophilizate from each sample was suspended in 4 mL of YEPG broth. The suspensions were incubated at 30°C for 2 hours, and a cell suspension standardized to 10^5 cells/mL was used to inoculate 15 mL of YEPG broth. Cultures were incubated at 30°C for 72 hours with agitation. After incubation, the titratable acidity was determined in 5 mL of culture supernatant using 0.1 N sodium hydroxide (NaOH) solution. The same method was used to measure the acid production of *Acetobacter pasteurianus* using a pure colony.

The acid production of the *Acetobacter pasteurianus* strain before and after freeze-drying was determined using the following formula:

$$P_a = \frac{N_b \times V_b \times 1000 \times M}{V_a}$$

Where:

Va: Volume of the sample (mL)

N_b: Normality of NaOH (mL)

V_b: Volume of NaOH (mL)

M: Molar mass of acetic acid

$$\textbf{RAP}\ (\%) == \frac{Acid\ production\ with\ bacterial\ powder}{Acid\ production\ with\ pure\ colony} \times 1000$$

Where:

RAP: Relative Acid Production

2.2.7. Storage of Freeze-dried *Acetobacter pasteurianus* Starter at Ambient Temperature

The best freeze-drying condition was chosen, and the starter powders produced under these conditions (cocoa pericarp flour + pulp juice) were stored in a laboratory at room temperature. Each week, the survival rate of *Acetobacter pasteurianus* was determined as described in section **2.2.5**.

3. 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 analyze survival rates and performance of lyophilized 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 $p < 0.05$.

4. Results

4.1. Biochemical Characterization of Cocoa Pericarp Flour and Pulp Juice

The total sugar concentration of cocoa pericarp flour and cocoa pulp juice ranges from 0.45 ± 0.083mg/g to 0.64 ± 0.07 mg/g. While their reducing sugar content is between 0.12 ± 0.00 mg/g and 0.20 ± 0.01 mg/g (Table 2).

Table 2. Sugar content of cocoa residues

Supports (cryoprotectants)	Total sugar (mg/g of MF)	Reducing sugar mg/g of MF)
Cocoa pericarp flour (0.4 %)	0.45 ± 0.083	0.12 ± 0.00
Cocoa pulp juice	0.64 ± 0.07	0.20 ± 0.01

MF: Fresh material

4.2. Survival of *Acetobacter pasteurianus* Strains After Freeze-drying with Different Cryoprotectants

Freeze-drying of *Acetobacter pasteurianus* strain with cocoa pericarp flour (0.4%) combined with cocoa pulp juice resulted in the best survival rate (79.243 ± 1.241%). Then, the freeze-drying conditions using mannitol and cocoa pericarp flour gave similar survival rates (73.27 ± 2.92% and 75.97 ± 3.59%, respectively), while freeze-drying *Acetobacter pasteurianus* with cocoa pulp juice resulted in the lowest survival rate (58.267 ± 1.68%) (Table 3).

Table 3. Survival rate of *Acetobacter pasteurianus* after freeze-drying using differents supports or cryoprotectants

Supports/ cryoprotectants	Survival rate (%)
Mannitol	73,38 ± 4,97b
Cocoa pericarp flour (0.4 %)	73,84 ± 1,62b
Cocoa pulp juice	58,27 ± 1,68c
Cocoa pericarp flour and pulp juice	79,24 ± 1,24a

4.3. Relative Acid Production by *Acetobacter pasteurianus* Strains After Freeze-drying

Contrary to the survival rate, the freeze-drying conditions with cocoa pericarp flour as support gave a relative acid production (74.66 ± 0.12%) higher than the other conditions. The condition using cocoa pericarp flour + cocoa pulp juice as support gave an acid production of 69.141 ± 0.07%. The other conditions, including cocoa pulp juice and mannitol, showed relative acid production levels between 48.95 ± 1.80% and 58.83 ± 0.16% (Table 4).

Table 4. Relative acid production by *Acetobacter pasteurianus* after freeze-drying using different supports or cryoprotectants

Supports or cryoprotectants	Relative acid production (%)
Mannitol	48,95 ± 1,80d
Cocoa pericarp flour (0.4 %)	74,66 ± 0,12a
Cocoa pulp juice	58,83 ± 0,16c
Cocoa pericarp flour and pulp juice	69,14 ± 0,07b

4.4. Evolution of the Survival Rate of *Acetobacter pasteurianus* During Storage at Ambient Temperature

When *Acetobacter pasteurianus* lyophilisates are stored at room temperature, the survival rate decreases from week to week. Moreover, this decrease is much faster for lyophilisates obtained with mannitol as cryoprotectant. Up to week 3, the freeze-dried products obtained with cocoa pericarp flour + cocoa pulp juice showed a survival rate of 86.25 ± 0.48%, while the survival rate of the freeze-dried products obtained with mannitol during the same period was 55.27 ± 1.15% (Figure 1).

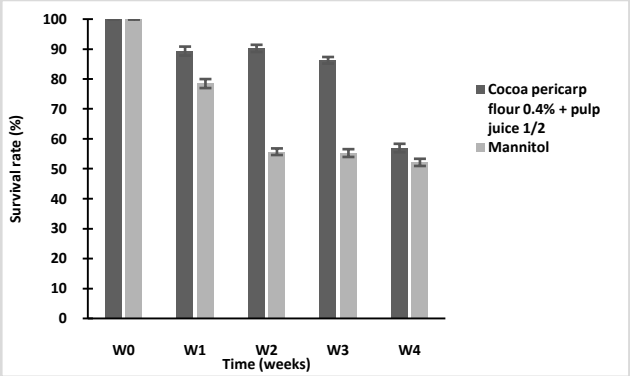

Figure 1. Survival of Acetobacter pasteurianus strain during storage at ambient temperature

5. Discussion

Microbial starters can be preserved for an extended period of time by dehydrating them using the freeze-drying method [23]. Cryoprotectants are employed during the freeze-drying process to shield microorganisms from the cold, which has a detrimental effect on microbial cell survival [24,25].

These cryoprotectants belong of several groups of molecules, including alcohols, proteins, and carbohydrates, are generally used to protect microorganisms during freeze-drying [26,27]. Among these molecules, mannitol is commonly used to protect *Acetobacter pasteurianus* cells during freeze-drying [28,29,30].

The main objective of this study was to find a better carrier or cryoprotectant for the preservation of *Acetobacter pasteurianus* during freeze-drying, using cocoa residues. Freeze-drying of *Acetobacter pasteurianus* with a combination of cocoa pericarp flour and cocoa pulp juice showed higher cell viability (79.24 ± 1.24%) than with mannitol (73.38 ± 4.97%). The high viability observed with these cocoa residues could be related to the high presence of sugars in these matrices. Several studies have shown that cocoa pulp is rich in glucose, fructose, and sucrose, while cocoa pods are rich in starch and cellulose [31,32,33]. It is these sugars, either alone or in combination, that are used as cryoprotectants to protect and preserve many microorganisms, including yeasts, acetic and lactic acid bacteria, and *Bacillus* species [34,35].

The acidification capacity of the strains after freeze-drying was determined by titration of acidic compounds. The results show that *Acetobacter pasteurianus* loses between 30% and 52% of its acid production capacity during freeze-drying, depending on the supports or cryoprotectants used. The loss is more pronounced when mannitol is used as a support. These results suggest that freeze-drying conditions negatively affect the cellular metabolism of *Acetobacter pasteurianus* in terms of organic acid production. In addition, cocoa residues seem to be more effective in maintaining organic acid production during freeze-drying.

Furthermore, the formulation of cocoa pericarp flour and cocoa pulp juice allows good cell viability of *Acetobacter pasteurianus* to be maintained for up to one month, in contrast to mannitol. This suggests that the combination of carbohydrates in cocoa pericarp flour and cocoa pulp juice improves the viability and shelf life of microorganisms during freeze-drying. While mannitol remains the most effective cryoprotectant for *Acetobacter pasteurianus* during freeze-drying [28], the formulation using cocoa pericarp flour + cocoa pulp juice as a carrier or cryoprotectants is recommended as a cheaper and more accessible alternative to mannitol. This formulation offers a high level of viability after freeze-drying and during storage at room temperature, making it an important factor for biotechnological applications in industries worldwide, especially in developing countries.

6. Conclusion

The cocoa pericarp flour and cocoa pulp juice provides better protection for the *Acetobacter pasteurianus* strain during freeze-drying. This mixture preserves the viability of the strain for at least one month at room temperature without affecting its acidifying power. In short, this cryoprotectant production from local residues is a better alternative to conventional cryoprotectants (mannitol) used to protect *Acetobacter pasteurianus*.

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