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RESEARCH ARTICLE

MAIZE FLOUR, AN ALTERNATIVE CRYOPROTECTANT FOR FREEZE-DRYING BACILLUS THURINGIENSIS FOR COCOA FERMENTATION IN CÔTE D'IVOIRE

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Abstract

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Bacillus thuringiensis is an important micro-organism in organic farming and food fermentation due to its metabolites, which have fungicidal and insecticidal properties. To ensure stability and avoid genetic modification, the strains are freeze-dried. However, the cryoprotectants used are very expensive. The aim of this study was to design a more economical freeze-drying environment for Bacillus thuringiensis using local flours. Bacillus thuringiensis was grown and then centrifuged to recover the resulting cell pellets. Local flours were prepared individually and added to each pellet before freezing and lyophilisation. Survival after lyophilisation and survival over time at 30°C were evaluated. Maize and sorghum flours gave survival rates after lyophilization of 93.05 ± 0.69 and 92.07 ± 0.35 respectively, close to those of sucrose (96.31 ± 0.42) and mannitol (94.91 ± 1.51). All flours maintained more than 50% of strain survival for 1.5 months. Sorghum and maize flours could be used as an alternative to cryoprotectants, which are too costly, to preserve Bacillus thuringiensis strains.

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Introduction:-

Bacillus thuringiensisare microorganisms commonly found throughout various environnements **(Ongena and Jacques, 2008)**. These Gram-positive, spore-forming bacteria are notable for their ability to producecrystalline inclusion bodies that contain insecticidal proteins known as Cry and Cyt proteins **(Pohare et al., 2021)**.These proteins repel and inhibit insects of the orders Lepidoptera, Coleoptera, Diptera and Hymenoptera, as well as nematodes. In addition, a study by **Kim et al. (2004)** showed that Bacillus thuringiensis is involved in the control of microbial plant diseases thanks to its fungicidal, bactericidal and insecticidal properties. These different properties of Bacillus thuringiensis are related to its ability to produce non-ribosomal peptides, commonly known as lipopeptides **(Sanahuja et al., 2011 ; Bel et al., 2017 ; Daquila et al., 2019)**. Thanks to these properties, Bacillus thuringiensis metabolites have been used for several years as biological control agents to replace chemical molecules in some Asian countries **(Lacey et al., 2015 ; Arthurs and Dara, 2019 ; da Silva et al., 2022)**. In Côte d'Ivoire, the work of **Yao et al. (2017)** on cocoa allowed the isolation, identification and characterization of Bacillus thuringiensis. These bacteria were screened and the one with exceptional properties in terms of flavour, acid and protein production was selected as a starter to improve the quality of Ivorian cocoa. However, this strain is stored in liquid broth or on petri dishes, which limits its shelf life and increases the risk of contamination and genetic modification of its genome **(Ávila et al., 2015 ; Guo et al., 2020 ; Zhang et al., 2020 ; Chantorn et al., 2022)**.

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However, the formation of ice crystals in the freezing step of lyophilization causes damage to the bacterial membrane, which can lead to cell lysis **(Rathore et al., 2013)**. This requires the addition of cryoprotectants prior to lyophilization to ensure bacterial protection **(Chen et al., 2023)**. The cryoprotectant that best protects and preserves Bacillus is sucrose. As the sucrose used in freeze-drying is too expensive, its use could lead to an increase in the cost of freeze-dried products. However, Ivorian agricultural resources contain many substances, such as local cereal flours, which are rich in simple and complex sugars that are commonly used as preservatives for bacteria and yeast. The aim of this research was to develop a more economical freeze-drying medium for Bacillus thuringiensisusing locally produced flours.

Materials and Methods:-

Materials:-

The Bacillus thuringiensis strain used in this study was isolated from cocoa bean fermentation in Côte d'Ivoire **(Yao et al., 2017)**. This strain was preserved in nutrient broth (0.2% yeast extract, 0.5% sodium chloride, 1% casein peptone, 0.1% beef extract) containing 20% glycerol at -80°C. The five flours (maize flour, sorghum flour, rice flour, millet flour, soy flour) used in this study were purchased in Abidjan, Côte d'Ivoire.

Methods:-

Microbial culture of Bacillus thuringiensis strain for freeze-drying

The Bacillus thuringiensisinoculum was first reactivated in nutrient broth for 24 hours and then cultured on a nutrient agar plate. The agar containing the strain was then incubated at 37°C for 24 hours. A pure colony was used to prepare a concentrated suspension using 120 mL of nutrient broth. The cultures were placed in an oven at 30°C for 3 days.

Lyophilization of Bacillus thuringiensis starters

The concentratedBacillus culture obtained after three days was centrifuged at 12,000 x g for 5 min at 4°C using a refrigerated centrifuge (Laboao, China). The pellets obtained were then rinsed with a saline solution (0.9% NaCl) and collected in a sterile 100 mL Erlenmeyer flask. The different flours obtained were prepared individually to a final concentration of 4%. Two grams of each flour were dissolved in 50 mL of distilled water. The mixture was heated to a final temperature of 70-80°C for 20-30 minutes with continuous stirring. The mixture was further cooled to approximately 30-40°C **(Coulibaly, 2016)**. Two additional chemical cryoprotectants, mannitol and sucrose, were used as controls. The initial studies consistedof 20% sucrose, 20% mannitol and a combination of 20% sucrose and 20% mannitol,followed by various experiments with the flours. Table I shows the composition of the different lyophilisation tests. Following the incorporation of cryoprotectants, the samples were frozen at -60°C for 1 hour and then freeze-dried at 1 Pa and -55°C for 48 hours using a freeze dryer (Biobase, China).

Table 1:- Different formulations of cryoprotectants used in freeze-drying.

Essay	Flour (mL)	Sucrose (mL)	Mannitol (mL)	Physiological saline (mL)
1	0	0	5	20
2	0	5	5	15
3	2.5 millet flour	0	0	22.5
4	2.5 maize flour	0	0	22.5
5	2.5 rice flour	0	0	22.5
6	2.5 soy flour	0	0	22.5
7	2.5 sorghum flour	0	0	22.5
8	0	5	0	20

Determining the survival rate of Bacillus thuringiensis starters after lyophilization

The successive decimal dilution method developed by **Cui et al. (2022)** was used to estimate the survival of Bacillus thuringiensis strains on nutrient agar media from the microbial culture performed in 2.2.1 to determine the microbial load before freeze-drying. After lyophilisation, 0.1 g of lyophilisate from each Bacillus thuringiensis assay was reconstituted in 4 mL of nutrient broth. Suspensions were incubated at 30°C for 2 hours. Subsequent dilutions of each incubated sample were prepared, and a 100 µL volume of each cell suspension was uniformly inoculated onto the nutrient agar to assess the microbial load after freeze-drying. Agar plates were then incubated at 30°C for 17 hours. Cell viability was assessed by standard enumeration on nutrient agar. The mean of the three plates was used

for each assay and dilution. Plates with bacterial counts between 30 and 300 CFU were used to assess the microbial load. The viability ofBacillus thuringiensis after lyophilisation was quantified using the methodology of **Mendoza et al. (2013)**. Cell viability for each test was expressed as a percentage survival factor (SF), calculated using the following equation:

$$SF = \frac{1-(\log CFU before - \log CFU after)}{\log CFU before} X100$$

CFU_{before}= CFU.mL^{-1}X total culturevolume (ml) before lyophilisation
CFU_{after}= CFU.g^{-1}X total weight of dry bacterial sample (g)

Evaluation of the ability of Bacillus thuringiensis strains to produce pectinolytic enzymes after lyophilisation
The production of pectinolytic enzymes in solid media was carried out according to the protocol described by **Soares et al. (1999)** and adapted by **Ouattara et al. (2008)**. The pectinolytic activity was evaluated on an agar medium composed of 0.28% $(NH_4)2SO_4$; 0.6% K_2HPO_4; 0.2% KH_2PO_4; 0.02% yeast extract; 0.01% $MgSO_4$; 1.7% agar and 1% polygalacturonic acid at pH 7. The microbial load of the culture before freeze-drying and of the suspensions after freeze-drying was determined and set at 10^5 cells/mL. Aseptic wells 0.5 cm in diameter and 3 mm deep, were then drilled into the agar. A sample was added to each well, taking into account the loading. Assays were performed in triplicate for each sample and incubated for 48 hours at 30°C. After incubation, the Petri dish containing the culture medium was flooded with a solution of iodine and potassium iodide (5 g potassium iodide +1 g iodine +330 mL distilled water) to exposebright areas around the wells, indicating pectinolytic activity. The diameters of the halos were quantifiedto determine the percentage of enzyme produced before and after lyophilisation, and the percentage of enzyme retained after freeze-drying. The enzyme content (TE) after lyophilisation was calculated using the formula below.

$$TE(\%) = \frac{1-(diameterbefore - diameterafter)}{diameterbefore} X100$$

diameter before : halo diameter in Cm of the strain in the culture broth
diameter after : halo diameter in Cm of strain after freeze-drying

Survival assessment of freeze-dried Bacillus thuringiensisafter storage at ambient temperature (30°C±2°C)
The bacterial count approach used in section**2.2.3** was used to assess cell viability during storage. The bacterial count and viability were determined using the method of **Chen et al. (2023)**.

Bacterial count (CFU/mL) = mean number of colonies from three replicates of the identical dilution multiplied by the dilution factor.
Survival rate (%) = (viable cell count after lyophilisation / viable cell count before lyophilisation) x 100%.

Determination of the percentage of enzyme produced by freeze-dried Bacillus thuringiensisafter storage at ambient temperature (30°C±2°C)
The enzyme production method described in section **2.2.4** was used to evaluate enzyme production over time.
The percentage of enzyme (TD) produced over time was expressed using the following formula:

$$TD(\%) = \frac{diameter\ \ lyophilization}{diameter\ \ after\ \ lyophilization} X100$$

freeze-dried diameter = diameter obtained from the powder suspension on the given day
diameter after lyophilization = diameter obtained immediately after lyophilisation

Survival assessment of lyophilised Bacillus thuringiensis cultures after different freezing times
A pure colony of Bacillus thuringiensis (Bt)grown on nutrient agar was used to prepare a concentrated suspension with 120 mL nutrient broth. Thecultures were incubated for 72 hours at 30°C in an oven. The concentratedBacillus culture obtained after 3 days was centrifuged at 12,000 x g for 5 min at 4°C using a refrigerated centrifuge (Laboao, China). The pellets were then rinsed with a saline solution (0.9% NaCl) and collected in a sterile 100 mL Erlenmeyer flask. Maize flour was formulated to a final concentration of 4%. The strain was lyophilised by adding 2.5 mL of prepared 4% maize meal, followed by the addition of 22.5 mL of physiological saline. **Table II** shows the different freezing times prior to lyophilisation.

Table 2:- Bacillus thuringiensis freeze-drying device as a function of temperature and freezing time.

Essay	Time of freezing (hours)	Time of freezing (-20°C)	Time of freezing (-60°C)
Bt+maize 0.4%	1	0	X

	2	X	X
	3	X	X

Bt : Bacillus thuringiensis
After the addition of maize meal, the samples were frozen as described in the table above and lyophilised for 48 h at 1 Pa and -55°C in a lyophiliser (Laboao, China). Strain viability was determined as described in section **2.2.3**.

Statistical analysis
All experiments were performed in triplicate and data are presented as mean ± standard deviation. Data were entered and calculated using Excel 2019. Survival and performance of lyophilised cultures were analysed using descriptive statistics. Means were compared using one-way ANOVA. Tukey's error rate multiple comparison test was used in XLSTAT software to separate means. Differences were considered significant at $p < 0.05$.

Results:-

Survival rate of strains after lyophilization
Table 3 shows the survival rateof the strains after lyophilisation with the different flours. Survival rates were above 81% in all trials. Furthermore, maize flours provided very good protection for the strains during lyophilisation, with survival rates of 93.05 ± 0.69 this rate are closer to those obtained with sucrose (96.31 ± 0.42), mannitol (94.91 ± 1.51) and sucrose + mannitol (95.21 ± 0.22).

Table 3:- Survival rate of Bacillus thuringiensis strains in various local flours.

Freeze-dried samples	**Survival rate of strains after lyophilization (%)**
strain+Mannitol 4%	94.91±1.51ab
strain +Sucrose 4% +mannitol 4%	95.21±0.22ab
strain +Mil 0.4%	81.68±1.96c
strain+maize 0.4%	93.05±0.69ab
strain +rice 0.4%	83.72±1.22c
strain +soy 0.4%	81.91±1.63c
strain +Sorghum 0.4%	92.07±0.35b
strain +sucrose 4 %	96.31±0.42a

Values are presented as mean ± SD

Amount of enzyme produced by the strains after freeze-drying
Table 4shows the ability of Bacillus thuringiensis strains to retain their enzyme production after freeze-drying in the different flours. The amounts of enzyme produced by the Bacillus strain in the flours were statistically identical to those produced by the strain in the reference cryoprotectants (mannitol and sucrose).
Table4:- Ability of Bacillus thuringiensis strains to maintain enzyme production after freeze-drying in different flours.

Freeze-dried samples	**Relative enzyme production (%)**
strain+Mannitol 4%	98,35±3,77a
strain +Sucrose 4%+mannitol 4%	99,19±1,41a
strain +Mil 0.4%	100,00±0,00a
strain+maize 0.4%	100,00±0,00a
strain +rice 0.4%	100,00±0,00a
strain +soy 0.4%	100,00±0,00a
strain +Sorghum 0.4%	99,17±1,44a
strain +sucrose 4%	98,05±3,55a

Values are presented as mean ± SD

Effect of freezing time before freeze-drying on Bacillusthuringiensis survival rate
The survival rates of strains after freezing at different temperatures and times are shown in **Figure 1**. The results show very high survival rates (93.0.50 ± 0.69%) for the strain frozen at -60°C for 1 hour. Also, strain survival decreased with freezing time increase. For example, strains lyophilized after freezing for 2 hours at -20°C and -60°C

gave survival rates of 77.58 ± 0.44% and 76.02 ± 0.43% respectively. As for the strain lyophilized after 3 hours of freezing at -20°C and -60°C, the results showed survival rates of 64.65 ± 0.37% and 65.38 ± 0.37% lower than those obtained at 1 and 2 hours.

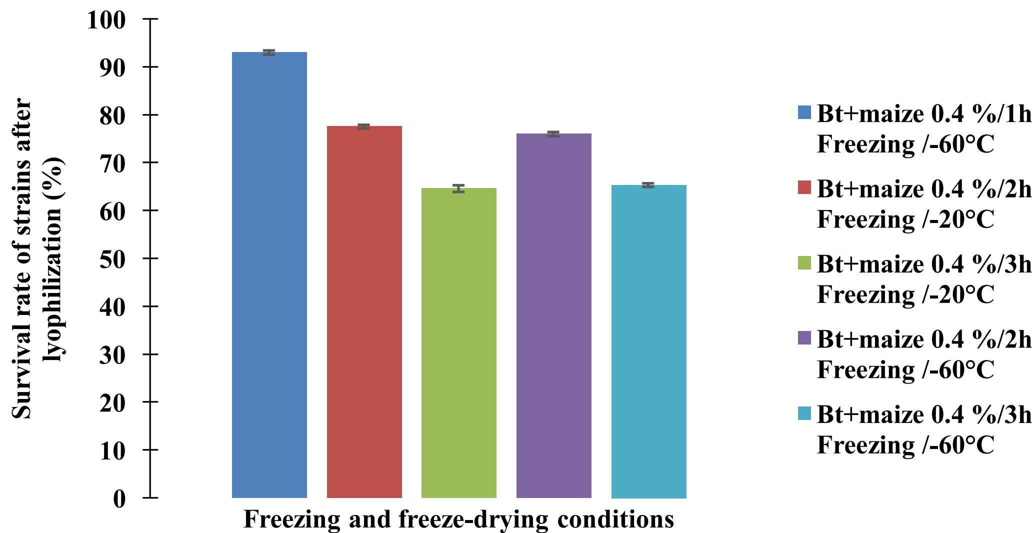

Figure 1:- Effect of temperature and freezing time on the survival of Bacillus thuringiensis strains after freeze-drying.

Storage of freeze-dried starter at room temperature (30 °C ± 2°C)

Figure 2 shows the relative growth of Bacillus thuringiensis strains in the different lyophilized media during storage at 30°C ±2°C for 3 months. All strains preserved with flour had a survival rate of more than 50% after one and a half months of storage at 30°C. The same trend was observed for strains preserved with sucrose and mannitol. The exception was sorghum flour, which showed a strain survival rate of 48.04 ± 0.03.

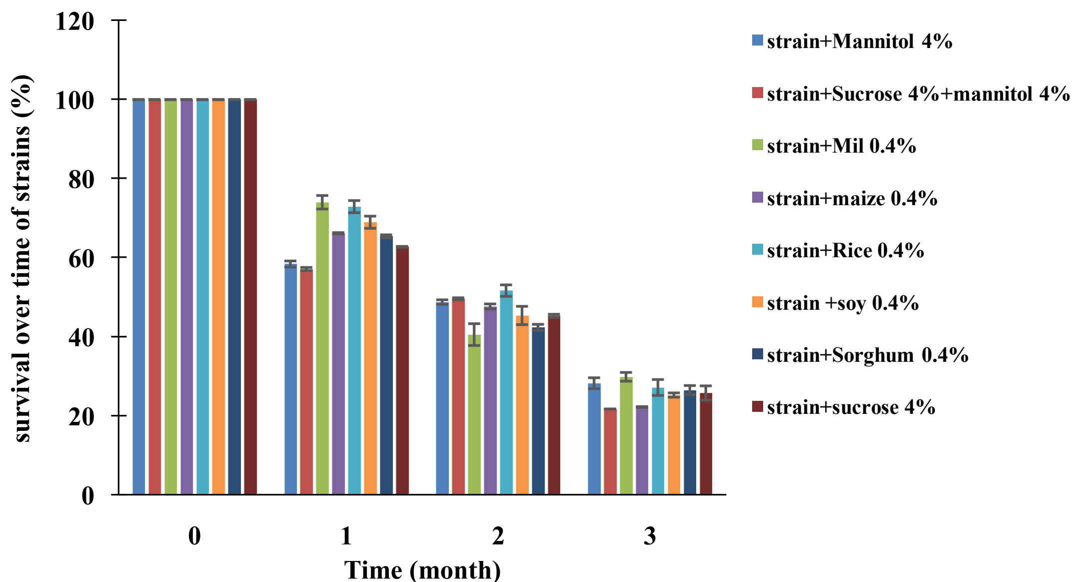

Figure 2:- Comparison of Bacillus thuringiensis survival rates with different cryoprotectants and local flours over 3 months at 30°C ± 2°C.

Enzyme production from freeze-dried starter at ambient temperature 30°C ± 2°C

Figure 3 shows the evolution of the amount of pectinolytic enzyme produced by the Bacillus thuringiensis strain with cryoprotectants (mannitol and sucrose) and millet, maize, rice-soya and sorghum flours. The flours maintained the strains ability to produce pectinolytic enzymes above 50% for nine (9) weeks at room temperature in millet

(72.27 ± 0.13), maize (51.60 ± 0.09) and rice (55.44 ± 0.12) flours and in the mannitol (53.36 ± 0.23) cryoprotectant control. In contrast, soy flour (43.88 ± 0.19), sorghum flour (26.94 ± 0.28) and the sucrose control (46.17 ± 0.20) showed relatively low enzyme production levels after nine weeks storage.

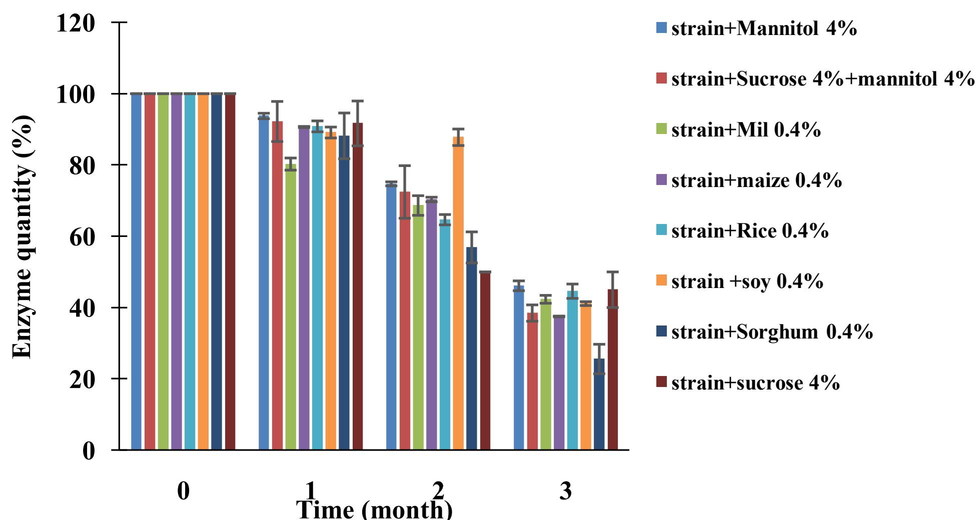

Figure 3:- Relative pectinolytic enzyme production by Bacillus thuringiensis in the presence of different cryoprotectants and local flours stored at 30°C ± 2°C for 3 months.

Discussion:-

The main objective of this study was to develop a low-cost freeze-drying medium for Bacillus thuringiensisusing local flours. Freeze-drying can cause unavoidable damage to cells, leading to adverse effects on their internal structures, mechanical injury, lytic consequences and denaturation of DNA and proteins **(Velly et al., 2014 ; Chen et al., 2015 ; Han et al., 2018)**. To reduce these effects, cryoprotectants are added to microorganisms prior to freezing and lyophilisation **(Chen et al., 2019 ; Gul et al., 2020)**. However, these commonly used cryoprotectants are expensive and their use results in higher lyophilisation costs. As a result, research has been conducted worldwide for several years to find alternatives to these substances **(Savedboworn et al., 2019)**. Work by **Hongpattarakere et al. (2013) ; Chotiko and Sathivel (2014)** has shown that it is possible to use aqueous extracts and crude fibres from maize, beans, rice and soya as cryoprotectants for Lactobacillus plantarum. In this work, survival rates of over 81% could be attributed to the ability of Bacillus strains to sporulate, which gives them greater resistance to freeze-drying **(Shu et al., 2018)**. Also, according to the work of**(Costa et al., 2000)**, the rehydration medium of freeze-dried strains can influence the repair of cells damaged by the freeze-drying process. For other authors, cryoprotectants play the most important role because, according to **Morgan and Vesey (2009) ; Cayra et al. (2017) ; Merivaara et al. (2021)**, when mannitol is used as a protector, it promotes the formation of crystalline structures for the protection of functional cell proteins, increases mechanical resistance and favours the replacement of water contained in the lipid membrane.

In contrast,glucose acts as a protective agent by facilitating water exchange through hydrogen bonding in bacterial membrane bilayers and by forming amorphous glass matrices that reduce molecular mobility within and around the cell**(Morgan et al., 2006 ; Morgan and Vesey, 2009)**. Therefore, the near control survival rates of mannitol and sucrose observed with sorghum and maize meal may indicate that these two matrices contain similar components to the controls, such as sugars or their derivatives. These substances could therefore be proposed to the food and pharmaceutical industries as an alternative to reduce the cost of lyophilisates. Regarding the preservation of enzymatic activity, statistical analysis showed no difference between the results. These results are consistent with those reported by **Park et al. (2021)**.According to these authors, if the cryoprotectant used prior to lyophilization is in phase with the microorganism, no important gene of the microorganism will be denatured at the end of lyophilization. Similarly, the results of temperature and freezing time optimisation show survival rates above 65% for all assays, with values approaching 95% when samples are frozen at -20°C or -60°C for between one (1) and two (2) hours prior to freeze-drying. These results differ from those reported by**Farfan Pajuelo et al. (2023) ; Ma et al. (2024)** who recommend a longer freezing time of 12 hours at -80°C and -60°C respectively prior to lyophilisation.

These relatively different freezing time results could be related to a number of factors, including the microorganism, cryoprotectant, apparatus and method of use.

These stable crystals would affect the cell membrane by inducing instability in membrane bilayers, thereby destabilising proteins due to the lackof amorphous crystal formation, which is essential during storage **(Kumar et al., 2017 ; Merivaara et al., 2021)**. While studies by **Han et al. (2018) ; Merivaara et al. (2021)** using glucose as a cryoprotectant showed a constant level of microbial viability, this could be attributed to the ability of monosaccharides to form amorphous glassy structures. These amorphous structures preservethe components produced by the cells before and during storage,thuspreventing irreversible electrochemical changes in the cell membrane **(Palmfeldt et al., 2003 ; Wolkers and Oldenhof, 2015)**.

Conclusion:-

Bacillus thuringiensis are microorganisms of great interest as biocontrol agents against insects, moulds and microbial parasites. These strains were freeze-dried using local flours to reduce the cost of preservation. All flours ensured good preservation of the strain and its enzymatic activity, with a better result for maize flour. In fact, maize flour proved to be the best at protectingagainst cold and storing at room temperature. In addition, the optimisation results showed that it is necessary to reduce the freezing time before freeze-drying in order to achieve higher survival rates. As a result, maize flour could be recommended for freeze-drying strains for biotechnological applications. For further research, it would be useful to identify all the optimal conditions for the use of maize flour.

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