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Research Paper

Fermentation of bitter cassava flour using *Bacillus subtilis as a starter*

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Summary

Cassava flour was purchased from Hebei Kaikuo Food Group Co., Ltd. in the People's Republic of China. This flour was fermented with Bacillus subtilis to improve its nutritional value and facilitate the degradation of high hydrocyanic acid content. The high protein (3.65%), phosphorus (3.39%), calcium (0.33%); pH (5.25), and degradation of hydrocyanic acid content (69.04%).

Keywords: *Cassava flour, bitter, Bacillus subtilis, fermentation, starter*

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I. Introduction

Cassava (Manihot *esculenta Crantz)* has significant importance in human and animal nutrition, gaining its place among staple foods, especially in Africa, Latin America and parts of Asia Campos et *al.*, (2017).

Many of the countries in these regions produce cassava in large quantities to meet the caloric needs of humans and livestock Parmar et *al.*, (2017). It is a rich source of carbohydrates and essential minerals such as iron, calcium, potassium, copper, magnesium, manganese and zinc.

Cassava can be processed into various value-added products to reduce post-harvest losses and improve the availability of cassava-based foods. Methods such as fermentation, drying, frying, lattice, and baking are commonly used, individually or in combination, to achieve this. In addition, fermentation improves fiber digestibility and breaks down anti-nutrients. The quality of cassava roots is crucial, especially with regard to cyanide content, which should remain below 250 ug/g to meet WHO safety standards; Abass et *al.*, (2022).

Cassava is traditionally processed into a wide variety of fermented products with different local names (attiéké, gari, fufu, agbelima, chikwangue placali, attoukpou) (Sahouegnon et al., 2014; Yao et *al.*, (2015).

Fermentation is controlled by several microorganisms, some of which have positive effects such as product preservation, flavor development, cyanide reduction, and changes in functional properties.

In recent years, various probiotic strains of *Bacillus,* including *Bacillus coagulans, Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus velezensis* and *Bacillus clausii*, were tested in vitro and in vivo for possible probiotic activities. Probiotic strains have been shown to *Bacillus* species produce a variety of enzymes, including cellulases, amylases, proteases and lipases. In addition, they can produce metabolites antimicrobials, such as bacteriocins and peptides, which hinder the growth and reproduction of dangerous bacteria (Soares et *al.*, 2023; Zhao, Yu, & Yan, 2023).

Probiotic Bacillus strains can solve flavor problems unique and long fermentation time of fermented products caused by lack of certain functional genes and the insufficient metabolic capacity of the strains of fermenters (Lactobacillus and Bifidobacterium) at the current stage, and also have great potential to improve the safety of fermented products Shan et *al*., (2023).

Fermented foods are obtained after microbial reproduction and metabolism of food raw materials under natural conditions or by adding microbial agents. They have high nutritional value and unique flavor, are safe, and have high long-term preservation potential (Zhang, et al., 2023). During the fermentation process, various microorganisms change the chemical composition of the raw materials, thereby improving the nutritional content of fermented foods and providing customers with health benefits (Ashagrie et al., 2023; Louw, Lele, Ye, Edwards & Wolfe, 2023).

Based on differences in fermentation processes, fermentation can be broadly classified into liquid-state fermentation, semi-solid fermentation, and solid-state fermentation. Examples of typical liquid-state fermented foods include fermented dairy products (Tian, Xiong, Yu, Chen, & Lou, 2023) and fermented fruit juice drinks (Zhang, et al., 2023). Semi-solid fermented foods encompass fermented plant products (Torres, Veron, Contreras, & Isla, 2020) and fermented fruit products (Li, Chen, et al., 2023).

Solid-state fermented foods mainly include fermented grain and legume products (Lingua et al., 2022; Xie et al., 2019), fermented tea products, and fermented meat products (Ojha, Kerry, Duffy, Beresford, & Tiwari, 2015).

Bacillus *subtilis* is a Gram-positive aerobic soil bacterium widely used for the production of heterologous proteins (Earl AM et *al., 2008)*. It secretes numerous enzymes to degrade a variety of substrates, allowing it to survive in a constantly changing environment.

Since the anti-nutritional factors present in cassava flour, thus limiting its consumption in humans as well as in animals, fermentation is very important to improve the quality of the food after fermentation.

This study focuses on the fermentation of cassava flour by *Bacillus subtilis* to degrade hydrocyanic acid and also improve food quality by increasing protein content.

II. Materials and methods

2.1. Test material

Strain Source: The strain used in this study was isolated by the research team from cassava flour residue at an early stage.

CF: Purchased from Hebei Kaikuo Food Group Co., Ltd.

Liquid medium: Dissolve 2.0 g of tryptone, 1.0 g of yeast extract and 2.0 g of sodium chloride in 200 ml of distilled water.

Sterilize at 121℃ for 15 min.

Aerobic fermentation bags: purchased from Beijing Baiwangtongda Technology Co., Ltd.

2.2. Test methods

2.2.1. Identification of strains

First, strains isolated from cassava flour waste were cultured in solid medium for 24 h, and strains were isolated from cassava flour waste and cultured in LB solid medium for 24 h.

The strain was initially identified by observing its morphology according to the “Strain Identification Manual”. Then, the strain was transferred into liquid medium and incubated at 37 °C.

Incubate at 200 rpm for 24 h. After culture was completed, the strains were Gram stained and their cellular characteristics were observed.

2.2.2. Determination of the deformation growth curve

Referring to the method of Guo Baozhu et *al*., (2020), we added the activated bacterial solution into a 96-well plate at a volume of 200 μL per well and cultured it at 37 °C and 200 r/min for 24 h. During the culture process, the optical density (OD value) of the bacterial solution at a wavelength of 600 nm was measured every hour to plot the growth curve of the strain.

2.2.3. Preparation of fermentation broth

The frozen bacteria were activated and cultured for 24 h. Take 5 ml of the activated bacterial solution and inoculate it into 500 ml of liquid culture medium. Then place these inoculated 500 ml of liquid culture medium into a constant temperature shaker at 37 °C and 200 rpm for fermentation for 24 h to prepare the fermentative bacterial solution for reserve use.

2.2.4. Determination of the capacity of strains to degrade cyanogenic glycosides in cassava flour

In this study, the solid-state fermentation process was used to carry out cassava flour fermentation. Referring to the research of Guo Baozhu et *al*., (2020), the fermentation parameters were slightly adjusted. The specific fermentation parameters are as follows: fermentation time of 5 days, temperature of 37 °C, matter-water ratio of 1 : 0.4 and bacterial inoculation amount of 5%.

Stages of the fermentation operation:

First, we have 400g cassava flour and mix it in a matter-water ratio of 1 : 0.4, add 3% molasses, and then inoculate 5% of the pre-cultured bacterial solution (the number of viable bacteria is 1.8×10^{8} CFU/ml). After thorough mixing, the mixed samples were placed in aerobic fermentation bags (23 × 30 cm), sealed, and placed in a constant temperature incubator at 37 °C for fermentation for 5 days. The method includes three replicates. After fermentation, the samples were removed from the fermentation bags, dried naturally in the open air, and then ground. Part of the samples was kept for the determination of cyanogenic glycoside content to evaluate the degradation effect of cyanogenic glycosides (CG) after fermentation.

2.2.5. Indicators and detection methods

(CG) content in unfermented and fermented cassava flour (CF) samples as well as crude protein (CP), calcium (Ca), total phosphorus (P), water potential (pH).

Detection method: In this test, the CG content was determined by the colorimetric method of GB/T13084-2006. Conventional nutrients were determined by the methods specified in the national standards of the People's Republic of China GB/T 6435-2006, GB/T 6438-2007, GB/T 6432-1994, GB /T 6433-2006, GB/T 6436-2002 and GB/T6437-2002, respectively, for DM, Ash, CP, EE, Ca and P. The FDN and FDA were determined according to the method described in previous literature (VAN SOEST et *al*., 1991).

2.3. Statistical analysis

The tests were carried out in triplicate and the numerical values obtained are expressed by the assigned arithmetic mean, standard deviation and corresponding coefficient of variation.

Using R software version 4.3.1., the data that followed a normal distribution after the normality test and equality of variances by the Leven test allowed us to perform the one-way analysis of variance (ANOVA $_{1}$) in order to compare the means of the substrates. And the data that did not meet the conditions, the Kruskal-Wallis test was used.

III. RESULTS

3.1. Strain identification results

Observation of colony morphology revealed that after culturing the strain on solid medium for 24 h, the colonies were off-white, translucent, circular, slightly convex in the middle, with a moist surface and sharp edges. Using the Gram staining method, microscopic observation revealed that the strain was purple in the Gram stain and was a Gram-positive spore-producing bacterium (as shown in Figure 1 and 2). The obtained sequence was compared with the NCBI GenBank database for homology analysis. The 16S rRNA sequence of the strain was consistent with that in the NCBI GenBank database. The similarity of the 16S rRNA sequence of *Bacillus subtilis is* 100%, which indicates that the strain is *Bacillus* subtilis and is named *Bacillus subtilis* FRI (as shown in Figure 3).

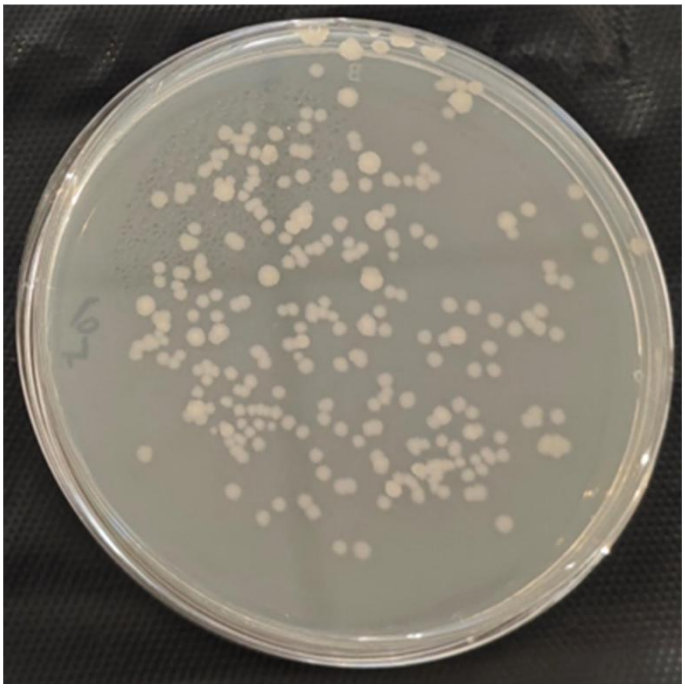

Figure 1 : Morphology of the deformation

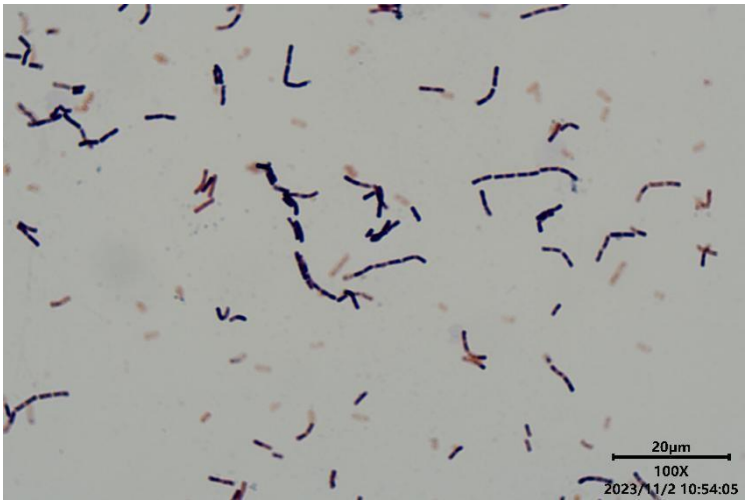

Figure 2: Gram stain results of bacterial strains

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus subtilis strain DSM 10 16S ribosomal RNA, partial sequence	Bacillus subtilis	2569	2569	100%	0.0	100.00%	1517	NR_027552.1
Bacillus subtilis strain IAM 12118 16S ribosomal RNA, complete sequence	Bacillus subtilis	2564	2564	100%	0.0	99.93%	1550	NR_112116.2
Bacillus inaquosorum strain BGSC 3A28 16S ribosomal RNA, partial sequence	Bacillus inaquosorum	2564	2564	100%	0.0	99.93%	1538	NR_104873.1
Bacillus cabrialesii strain TE3 16S ribosomal RNA, complete sequence	Bacillus cabrialesii	2564	2564	100%	0.0	99.93%	1550	NR_180419.1
Bacillus subtilis strain JCM 1465 16S ribosomal RNA, partial sequence	Bacillus subtilis	2560	2560	99%	0.0	100.00%	1472	NR_113265.1
Bacillus subtilis strain NBRC 13719 16S ribosomal RNA, partial sequence	Bacillus subtilis	2560	2560	99%	0.0	100.00%	1475	NR_112629.1
Bacillus subtilis subsp. subtilis strain 168 16S ribosomal RNA, complete sequence	Bacillus subtilis subsp. subtilis	2558	2558	100%	0.0	99.86%	1550	NR_102783.2

Figure 3: Results of the comparison of 16S strain rRNA sequences

3.2. Observed chemical parameters

Figures 4,5,6,7 and 8 illustrate the degradation to cyanogenic glycosides (GC), water potential (pH) and crude protein (CP), calcium (Ca), total phosphorus (P) contents in unfermented and fermented cassava flour (CF) samples.

3. 2.1. Degradation into cyanogenic glycosides (CG)

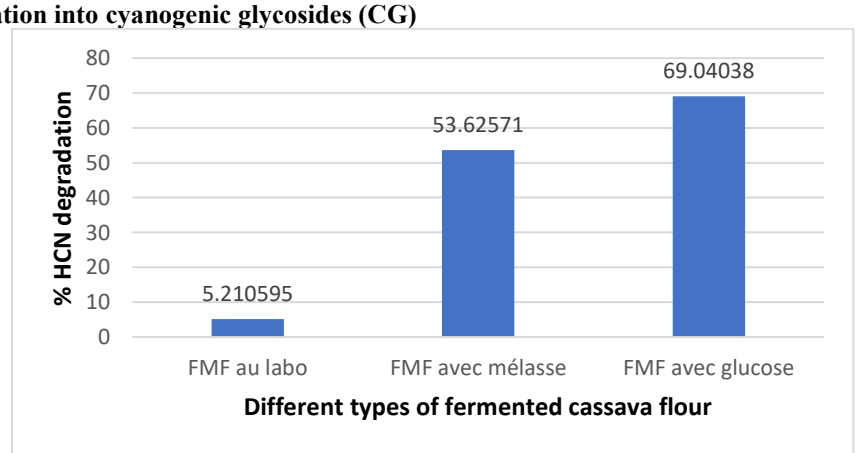

Figure 4 : Degradation into hydrocyanic acid in different types of fermented cassava flour.

Figure 4 shows a degradation of the hydrocyanic acid content in fermented cassava flours. The cassava flour fermented in the laboratory shows a degradation of the hydrocyanic acid content of 5.21%, by adding molasses in

the fermentation process, the degradation of hydrocyanic acid in the cassava flour increased by 53.62%; while by fermenting the cassava flour with glucose, we obtained a degradation of the hydrocyanic acid content of 69.04%. The one-way ANOVA test (df=3, p-value= 1.177E-10) at the 5% level reveals a very significant variation in pH in these various fermented and unfermented flours. This strong difference in degradation is evident from the fact that after fermentation in the laboratory, drying was carried out in the oven while drying after fermentation with molasses and glucose was carried out in the open air.

3.2.2. The water potential (pH) of fermented and unfermented cassava flour

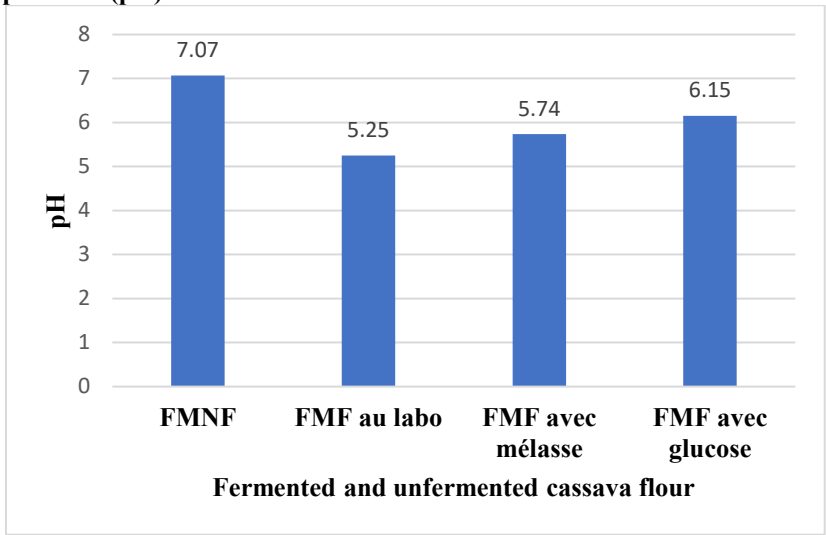

Figure 5 : pH of fermented and unfermented cassava flour.

Figure 5 reveals that unfermented cassava flour (NFCF) has a neutral pH of 7.07 while fermented cassava flour (FMF) with glucose has an acidic pH of 6.15 followed by fermented cassava flour (FMF) with molasses which also has an acidic pH of 5.74; and lastly comes the pH of fermented cassava flour in the laboratory which is also acidic of 5.25.

The one-way ANOVA test (df=3, p-value= 1.152E-08) at the 5% level reveals a very significant variation in pH in these various fermented and unfermented flours.

This difference is explained by the fact that the cassava flour fermented in the laboratory was of low quantity, which facilitates good homogeneity of the cassava flour with the microorganisms compared to the cassava flour fermented by molasses and glucose which had a large quantity.

3.2.3. Crude protein (CP) content

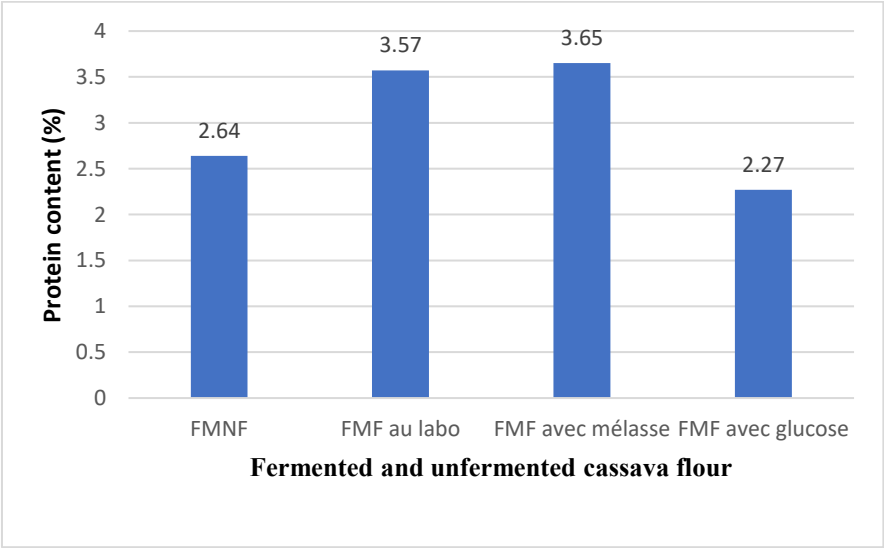

Figure 6 : Protein content of fermented and unfermented cassava flour.

It is evident from Figure 6 that the protein content of unfermented cassava flour is 2.64% while that fermented by glucose is lower with 2.27%. An increase in protein content was observed in cassava flour fermented by molasses with 3.65%; followed by cassava flour fermented in the laboratory with a content of 3.57%.
The Kruskal Wallis test (df = 13.5, p-value = 0.003671) at the 5% level reveals a highly significant variation in crude protein content in fermented and unfermented cassava flours.
This difference lies in the fact that microorganisms are capable of producing a variety of enzymes, including cellulases, amylases, proteases and lipases which have excellent protein secretion ability.

3.2.4. Calcium (Ca) content

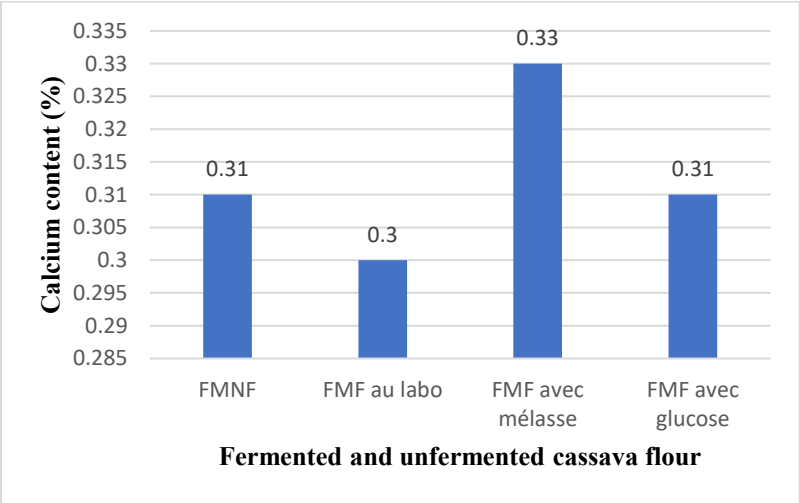

Figure 7: Calcium content of fermented and unfermented cassava flour.

It is observed in Figure 7 that the calcium content in unfermented cassava flour is 0.31% compared to cassava flour fermented with glucose which has the same content, i.e. 0.31%. On the other hand, a slight difference was observed in cassava flour fermented with molasses where the calcium content is 0.33%; and finally, cassava flour fermented in the laboratory contains 0.30% calcium content.
One-way ANOVA test (df = 3, p-value = 0.05172) at 5% level reveals non-significant variation in calcium content in fermented and unfermented cassava flours.

3.2.5. Phosphorus (P) content

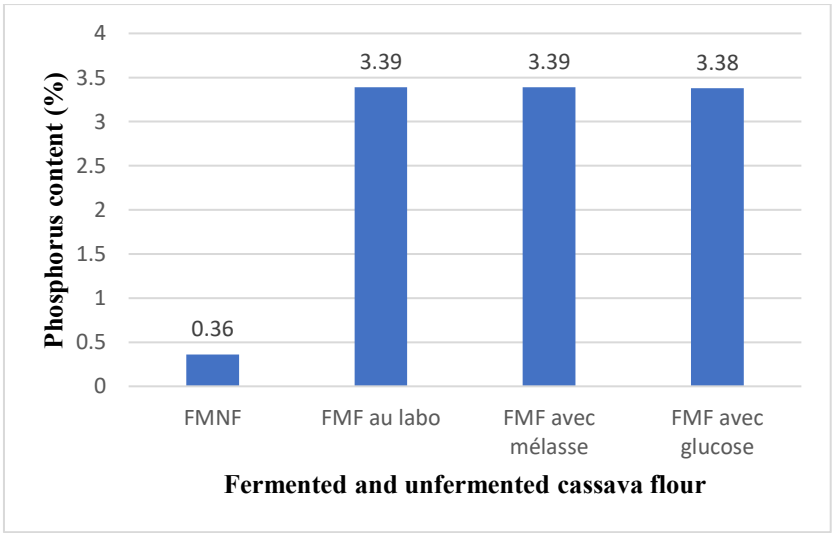

Figure 7 : Calcium content of fermented and unfermented cassava flour.

Figure 7 states that unfermented cassava flour is devoid of phosphorus, i.e. 0.36%, compared to that which undergoes fermentation by microorganisms. Cassava flour fermented in the laboratory has a phosphorus content

of 3.39%, that fermented with molasses 3.39% and also cassava flour fermented with glucose contains 3.38% phosphorus.

One-way ANOVA test (df = 3, p-value = 0.3169) at 5% level reveals non-significant variation in phosphorus content in fermented and unfermented cassava flours.

IV. Discussions

Previous studies have shown that microbial fermentation can reduce the content of antinutritional factors, such as cyanogenic glucoside (CG), in cassava flour, and that the nutritional value of fermented cassava flour (FMF) is better than that of unfermented cassava flour (UFF). However, despite its potential nutritional value, FFSM has not been adequately studied for use in growing pig feed.

It is well known that cassava roots have low protein content. In our study, an increase in protein content was observed in molasses-fermented cassava flour with 3.65% while that of unfermented cassava flour is 2.64%. While contrary results were recorded by Emmanuel et *al*., (2012) who found in Banye cassava roots white variety an average protein value of 3.48±0.47%. The pH of different fermented and unfermented cassava flours are between 5.27 and 7.07. The different pH values of different flour formulations show that they are acidic. These pH values obtained are similar to those reported by EMPERATRÍZ et *al*., (2008) which were in the range of 4.6 to 6.1 in green plantain flours and by AYO et *al*., (2010) which were 4.18 to 5.77 in plantain flours at different stages of ripening.

As shown by the pH values, the acidity of fermented cassava flour varies considerably from one fermentation to another. The variation in pH of fermented and unfermented cassava flour is found to be statistically significant.

In this study, fermentation processes have an impact on cyanide degradation of cassava flour. Indeed, statistical analysis revealed the absence of significant difference in these various fermented and unfermented flours. This strong difference in degradation is evident from the fact that after fermentation in the laboratory, drying was carried out in an oven while drying after fermentation with molasses and glucose was carried out in the open air. According to Purseglove (1968), cassava roots with a cyanide content of up to 100 mg / kg could be classified as toxic varieties. Therefore, it is necessary to treat them before consumption. Unfermented cassava flour is devoid of phosphorus, i.e. 0.36%, compared to that which undergoes fermentation by microorganisms. Cassava flour fermented in the laboratory has a phosphorus content of 3.39%, that fermented with molasses 3.39% and also cassava flour fermented with glucose includes 3.38% phosphorus with a non-significant difference. Our results differ from those of Zoumenou et al., (1999) who reported in cassava roots of the Bonoua cultivar, a sweet Ivorian species, a phosphorus value of 140± 0.30 mg / 100 g.

In this study, the calcium content in unfermented cassava flour was 0.31% compared to glucose-fermented cassava flour which had the same content of 0.31%. However, a slight difference was observed in cassava flour fermented with molasses where the calcium content was 0.33%; and finally, laboratory-fermented cassava flour had 0.30% calcium content. This was not the case for Chavez et *al*., (2000) who found differences in 20 cassava genotypes from the International Center for Tropical Agriculture (CIAT) in Colombia, with calcium content values ranging from 37.9 to 94.5 mg / 100 g.

V. Conclusion

Based on the fermentation results obtained in this work, *Bacillus subtilis* shows good potential for improving crude protein, calcium, and phosphorus content. This demonstrates the possibility of bioenrichment of cassava flour by facilitating its degradation of cyanogenic glucosides and increasing its acidity.

Further work should focus on coupling microorganisms to optimize even greater enrichment of protein, calcium and phosphorus content.

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