

Involvement of *Bacillus* Species in the Understanding of the Softening Process of Safou Pulp (*Dacryodes Edulis* H.J.Lam)

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Abstract: For the last twenty years, techniques including preservation of traditional fermented foods have been considerably grown. Safou is a fruit from *Dacryodes edulis* widely consumed in Africa. The Safou preservation poses a myriad of problems. In the Republic of Congo, a fermentation technique has been developed in order to preserve fruits. This work aims to study the bacteria of the genus *Bacillus* involved in the softening of Safou. Using classical microbiological techniques, bacteria have been estimated with number about $2.5 \pm 0.7 \cdot 10^2$ FCFU / g from the softened safous and at $4 \pm 0.8 \cdot 10^6$ CFU / g from the non-softened safous. By using PCR multiplex of *fibE* gene, *Bacillus subtilis* and *Bacillus safensis* have been mostly identified and strains have shown to degrade pectin, amylose and proteins with the percentages up to 55%, 55% and 63 %, respectively. 45% of isolates including *Bacillus subtilis* and *Bacillus safensis* can secrete pectinases, amylase and proteases. The assessment of Penetrometry index showed that *B. subtilis* and *B. safensis* are able to contribute in the softening of african pears.

Keywords: Safou, Fermentation, preservation, PCR multiplex, *Bacillus*

1. Introduction

Dacryodes edulis occurs naturally in the countries bordering the Gulf of Guinea. It is cropping from Sierra Leone east to Angola along the Atlantic and further inland to Uganda. It has also been reported from northern of Zimbabwe. Safou or African pears are typical fruits can be produced by *Dacryodes edulis* crop. The culture of *Dacryodes edulis* has spread an attracting growing interest and is currently in strong progression in the continent of Africa. In Africa population this fruit is very well appreciate [1, 2]. Safous have been found not only in Africa countries, but also it is exported in Europe including France, Nederland, Germany and Belgium.

More and more the demand of safou on the Congolese market has become strong. But the local production is weak and African pear is still a seasonal fruit in the Republic of Congo. One of the major and worrying problems is the preservation of the safou. The post-harvest rot diseases of fruits of the African pear are one the economic concern in Africa [3, 4]. In order to preserve fruits the Congolese people have developed a traditional strategy consisting in fermenting the safous and drying them. Most of the scientific research carried out on *D. edulis* has focused on the nutritional value of African pear pulp and oil extraction processes [5-7]. By drafting this paper no studies have been focused to understand the fermentation process of safous. In addition endogenous knowledge states that the safou once softened can be eaten or dried for future preservation. The microorganisms involved in the softening of African pear have not yet been studied. This study aims to specifically study the understanding in the role of *Bacillus* species in the softening of the traditional process of Safou preservation.

2. Methods

Isolation of Microorganisms

The safous freshly sold on stalls at the Total market in Brazzaville (Arrondissement 2, Bacongo) were purchased and transported in a cooler and sent to the laboratory as quickly as possible. The samples were sorted on the basis of texture. Those with a nice texture were stored at room temperature for 4 to 5 days to observe the softening process. At the end of 5 days of softening, the pulp of safou was sample for microbiology assessment. Dilutions were done, and microorganism suspension was streaked with Mossel supplemented with polymyxin B. Enumeration of colonies was done in triplicate on Plate Count Agar (PCA). The Petri dishes were incubated at 37°C for 24 h to 48 h. After the first isolation on Petri dishes, each colony of different appearance was separately isolated. Purification of the isolates was rigorously done by successive and alternating subcultures. Purity was estimated by using a microscope for morphological characterization. Gram status was determined by using 3% KOH. Sporulation, hydrogen peroxide (H₂O₂), and oxidases tests were used for biochemical characterization.

Determination of Proteolytic activity

Some *Bacillus* strains were assessed for the ability to secrete proteases as described and modified by Kaya-Ongoto et al, 2019 [8]. Briefly, 1 g of agarose was weighed and mixed with 100 mL of PBS. The mixture was heated in a microwave for 3 min until agarose was completely dissolved and then cooled in a water bath at 40 ° C. Then 10 mL of skim milk was added to the mixture. After homogenization, the mixture was poured into the Petri dishes. Once solidified, wells were carefully and aseptically generated into the gels. A volume of 50 µL of the overnight culture supernatant is deposited in the wells made on the medium

composed of 1% agarose gel, 0.01 M PBS, pH 7.4 and supplemented with skimmed milk. The Petri dishes are incubated at 37 ° C for 24 hours. The presence of proteolytic activity is detected by a clear halo around colonies indicating hydrolysis of casein. The halo diameter was measured.

Amylolytic and pectinolytic activities

To assess the amylolytic, pectinolytic activity of isolates, an overnight culture in LB medium was carried out on Petri dishes. Then a young bacterial colony was deposited on the surface on solid LB medium separately supplemented with 1% of starch 0.5 % of pectin. The Petri dishes were incubated 24h to 48h. The revelation is made with Lugol. A test is positive if there is a clear halo around the colony corresponding to a lysis range. The percentage of enzymatic activity was determined according to the following formula: $\% = \frac{DT-DC}{DT}$ with DT: Total lysis diameter (Lysis area + Colony diameter); DC: Colony diameter; %: Percentage of lysis.

Molecular Identification of Microorganisms

The recent molecular identification developed in our laboratory using the amplification of *fibE* gene encoding for the fibrinolytic enzyme has been used to identify strains like *B. amyloliquefaciens*, *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. altitudinis*, *B. mojavensis*, *B. safensis*, and *B. atrophaeus* [8]. Briefly, extraction and purification of isolate genomic DNA were performed according to the NucleoSpin Microbial DNA (Macherey-NAGEL) kit. Briefly, isolates were grown in 5 mL LB broth for 24 h at 37°C with stirring. The DNA purity was assessed by electrophoresis on agarose gel and by the ratio of optical densities 260/280 nm. 5 µL of each amplification product was mixed with 2 µL of loading buffer (BIOKE). Mixtures were subjected to electrophoresis on 1% agarose gel (w/v). The 10 kb 2-Log (BIOKE) was used as a molecular weight marker.

The influence of identified bacteria in the softening

The penetrometer resistance is an effective and reliable method for evaluating safou puple softening. A mechanic penetrometer has been used to introduce in softened African pears. Five overnight culture corresponding to Nic3, Nic32, Nic37, Nic39 and Nic45 were done. The pure culture have been assessed to reach 0.8 to 1 of D.O. Safou have been first

washed with aseptic water and sterilized with 70 % and 90% of ethanol. The aseptic safous were immersed for 5 to 10 min in each of the cultures and in the culture put together. The samples were stored at room temperature to follow the softening process. The penetrometry indices were calculated during the fermentation process. The values were established according to the texture of the softening safou. A score of ten (10) was associated with the safou whose the penetrometer completely has been penetrated. A score of five (5) was associated with the safou whose the penetrometer has not been completely penetrated. Zero (0) was associated with the safou puple whose peak strength and maximum stress were not allowable.

3. Results

Microbiological assessment and Enzymatic Activities in the softening of Safou

A PCA medium have been used to enumerate bacteria. $2, 5 \pm 0.7$. 102 CFU/g from softened safou and 4 ± 0.8 . 106 CFU/g from non-softened African pear have been obtained. A total of 45 isolates have been randomly chosen for future experiments. Macroscopic and microscopic analysis, Gram-positive bacteria with 3% KOH, spore-forming bacteria test, and the positive catalase reaction had been allowed the classification of 24, 4% (11/45) working bacterium as candidates from the genus *Bacillus*. All eleven (11) bacteria were spore-forming colonies excepting for 75, 6% (34/45). To easily discriminate *Bacillus* spp. at the genera level, pure cultured isolates were assessed for their ability to produce proteases, pectinases and cellulase. A screening of the amylolytic, proteolytic and pectinolytic activities was carried out among 11 isolates with *Bacillus* oriented phenotype. The presence of enzymatic activities was detected by using starch, casein and pectin as following substrates (Figure 1). Among 11 isolates 55% (6/11) were able to show a very interesting the clear halo in terms of amylolytic and pectinolytic activity (Figure 1left and center) including Nic 3, 32, 37, 39, 42 and 43 strains. Neither activity was observed in 45% (5/11). This is concerning Nic1, 5, 33, 40 and 45. Regarding proteolytic activity 63% of isolates had positive activity including Nic1, 32, 33, 37, 39, 42 and 43 (Figure 1 right).

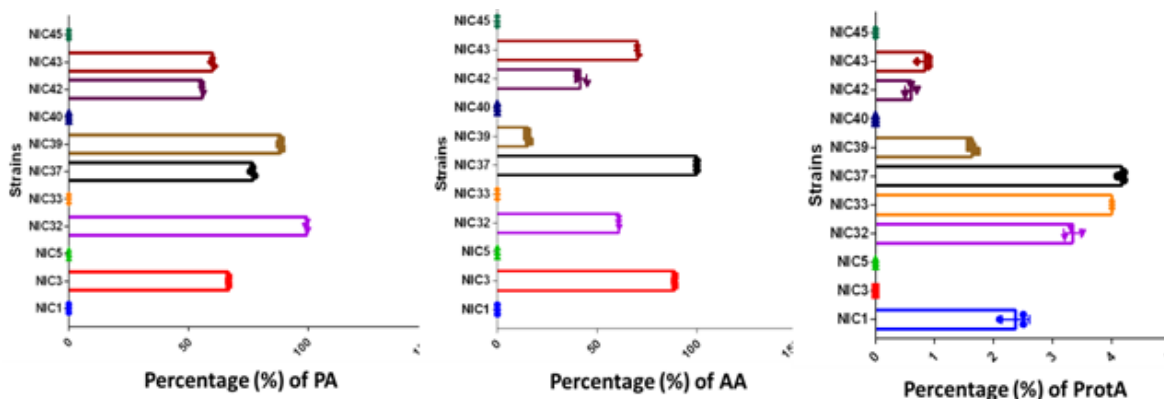


Figure 1: Enzymatic Activities of isolates in the softening of Safou. Left (Pectinolytic activity (PA)), Center (amylolytic activity (AA) and Right (Proteolytic activity (PA)). Nic3, Nic32, Nic33, Nic37, Nic40, Nic42 and Nic45: Bacteria isolated with *Bacillus* oriented phenotype.

Molecular identification of isolates

A strategy has been implemented in our laboratory as part of a previous study. We therefore sought to identify by using this strategy. The 11 *Bacillus*-oriented bacteria which colonize Safou have been identified. After purification of the genomic DNA, amplifications of the *fibE* gene encoding the fibrinolytic enzymes (FibE) were carried out. By using 6 pairs of primers including Ba.IdMa-F / Ba.IdMa-R for *B. amyloliquefaciens*, Bl.Id.Ma-F / Bl.Id.Ma-R for *B. licheniformis*, Bs.Id.Ma-F / Bs.Id.Ma-R for *B. subtilis*, Bp.Id.Ma-F / Bp.Id.Ma-R for *B. pumilus*, Bm.Id.Ma-F / Bm.Id.Ma-R for *B. mojavensis*, Bsa.Id.Ma-F / Bsa.Id. Ma-R for *B. safensis*. Isolates Nic3, 32 and 37 have been associated to *Bacillus subtilis* meanwhile isolates Nic39 and 49 avec been associate to *Bacillus safensis* (Figure 2). Nothing has been amplified Nic 3, 32, 40, 42, 43 and 45.

The influence *Bacillus* in the softening

By mixing safou with the *Bacillus* cultures including Nic3, Nic32, Nic37, Nic39 and Nic45, the safous have been stocked at room temperature for softening. The penetrometry index has been calculated. As results we show that the strains were able to soften the safous faster than expected. *Bacillus* Nic32 and Nic45 strains exhibited an interesting phenotype in softening the safou after two days. Finally we assessed for consortium including both Nic32 and Nic45 strains. Safous were able to quickly soften after one daycap are to the safous that were not mixed with the culturable bacteria (Figure 3).

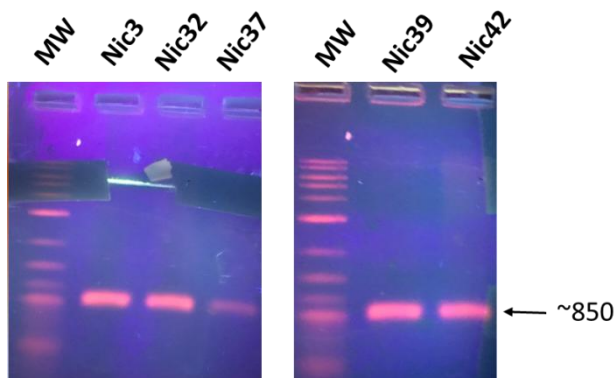


Figure 2: Molecular identification of isolates. MW: molecular weight. Nic3, 32, 37 are matching to *Bacillus subtilis*. Nic 39 and 45 are matching to *Bacillus safensis*

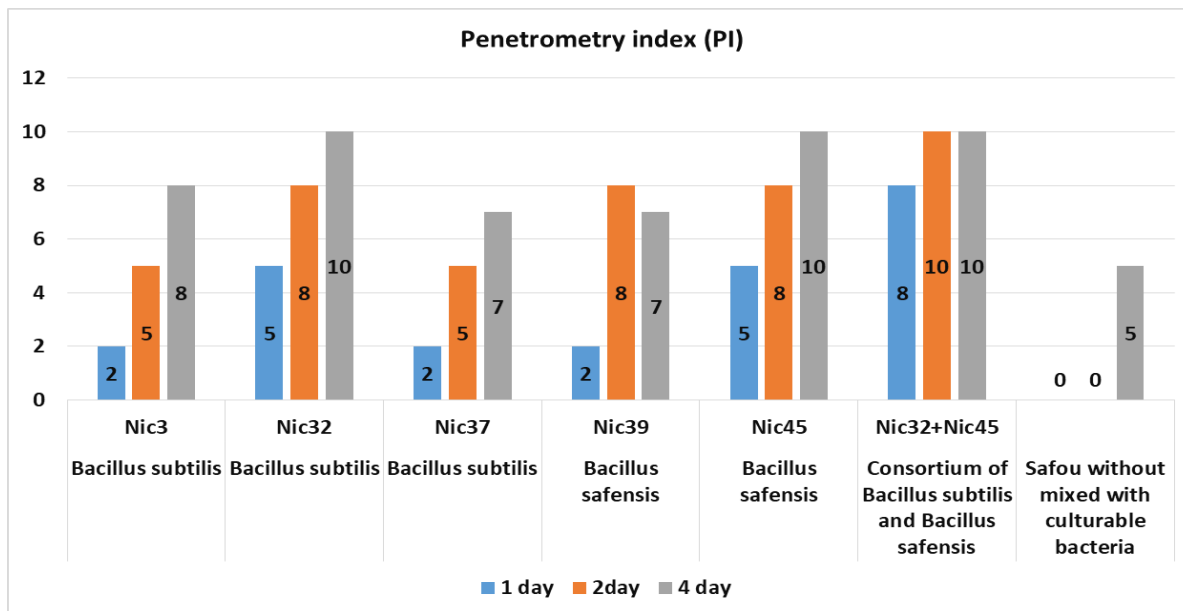


Figure 3: The Penetrometry Index (PI) of strains have been assessed during four (4) day of fermentation. Nic3, 32, 37 are matching to *Bacillus subtilis* and Nic 39 and 45 are matching to *Bacillus safensis*

4. Discussion

In this work we have worked on ripe fruits of safou collected in local markets in *Brazzaville*. One of major trouble shooting of safou comes to the short duration of natural softening. The post-harvest losses by natural softening of safou pulp have been studied [6]. This work aimed to

isolate and to molecularly identify the microorganisms like *Bacillus* and to understand the enzymatic activities in the softening of safou.

The isolation from ten (10) softened safou fruits showed a low enumeration rate which was around $2, 5 \pm 0.7 \cdot 10^2$ Colony Forming Unit per gram of material. This low rate

would be due to the low specific activity of the water. Isolation of non-softened African pear has been compared with the softened safou. As a result the numeration was about $4 \pm 0.8 \cdot 10^6$ CFU/g. The softening combined with the loss of water could be hallmarks that stop the growth of other microorganisms. Microorganisms like *Shigella*, *Salmonella*, *Lactic acid bacteria* were not isolated at the end of softening.

The new strategy of the molecular identification of the genus *Bacillus* belonging to group I have been published by our research group. This study targeted *Bacillus subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. pumilus*, *B. safensis*, *B. altitudinis*, *B. mojavensis*, *B. circulans*. The primer pairs chosen in that work targeted the amplification of *fibE* encoding a fibrinolytic enzyme. Using the six (6) pairs of primers, we directly identified three (3) isolates corresponding to *B. subtilis* and two (2) isolates corresponding to *B. safensis*. Our inexpensive and rapid method has the advantage of avoiding gene sequencing steps [8].

Bacteria such as *Bacillus thuringiensis* have also been identified by a direct approach using primers which hybridize specifically on the cry gene of course without going through sequencing [9]. Another study on yeasts had shown that, thanks to the direct PCR approach, it was also possible to directly identify yeasts such as *Saccharomyces cerevisiae*, *S. arboricola*, *S. bayanus*, *S. cariocanus*, *S. kudriavzevii*, *S. mikatae*, *S. paradoxus* and *S. pastorianus*. All this by targeting specific genes belonging to these species [10].

Fruit softening is a major issue of multiple cellular processes, including extensive remodeling of cell wall structure [11]. The ripening of the fruits is a complex event and some molecular events occurring during softening of strawberry fruit have been documented [12]. It is obvious to admit that ripening of fruits like safou is also identified by the secretion of a range of pectin degrading enzymes in the cell wall, and changes in the structure of pectin could result from their synergistic combination with the physiology of the cell [11] and enzymes secreted by *Bacillus*. The flesh of the fruit is softened by enzymes that break down cell walls. The natural action of these enzymes is dependent on the temperature [13] and would be accelerated by the enzymes produced by bacteria of the genus *Bacillus*.

The genus *Bacillus* is known for its ability to produce extracellular enzymes such as amylases, pectinases, proteases [14-17]. In this study we demonstrated the correlation between the secretion of enzymes and safou softening. In the context of this work some identified isolates have shown degradation percentages of pectin, amylose, proteolytic up to 55%, 55% and 63%, respectively. 45% of isolates including Nic32, 37, 39, 42 and 43 can secrete in the same time enzyme like molecules including pectinases, amylase and proteases. All the enzymatic background could contribute to the biodegradation of the wall cell of the safou. This also could allow the release of other cytosol enzymes being inside the plant cells of the safou. The enzymatic activities carried out have shown that bacteria of the genus *Bacillus* are able to secrete lytic enzymes produced during the

softening that could contribute to the degradation of cell wall structures. It has been documented that softening is a hallmark of fermentation in most modern or traditional fermented food [18-21].

5. Conclusion

There is a bright future for African pear generally appreciated for its health benefits. In view of the afore information, there is an involvement of microorganisms in the softening of the pulp of safou. The softening process is orchestrated by the coordinated secretion of enzymes such as proteases, pectinases and proteases by the genus *Bacillus* belonging to the phylogenetic group I. This work seems to be very important for the valorization of the African pear. Several enzymatic activities can be studied, such as lipolytic and cellulolytic activity, which may also help explain the softening of safous. We plan to continue this work by studying the protein profile and molecular identification of all cultivable flora.

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