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A Meta Development of a Biotechnological Ferment based on a Consortium of the Genus *Bacillus* for the Optimization of the Fermentation Process of Cassava Tubers

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Abstract: ***No screening and characterization of potential Bacillus starter cultures for fermenting of cassava tubers has been published. No biological consortium ferment has been proposed to date on the fermentation of cassava tubers for the manufacture of cassava-derived products. Bacteria of the genus Bacillus are ubiquitous and present themselves as a value-added solution. This study aims to develop a biotechnological stater using bacteria of the genus Bacillus in the cassava tubers fermentation. 105 isolates have been obtained. The different isolates can secrete essential enzymes for the biotransformation of cassava like amylases (63.80%), cellulases (56.19%) and proteases (41.90%). In addition, isolates were able produce biosurfactants-like molecules (38.09%). The penetrometry test revealed 60% of bacteria of the monofermentative Bacillus genus whose penetrometry indices (PI) were ranged from 5 to 10 between 48 hours and 72 hours. The consortium between homofermentative isolates increased the ability of Bacillus to reduce fermentation time to 24 hours. The consortia (B. safensis + B. subtilis), (B. safensis + B. subtilis), (B. safensis + B. subtilis, (B. safensis + B. tequilensis, (B. tequilensis + Bacillus sp), (B. tequilensis + B. pumilus), (B. safensis + Bacillus sp), (B. safensis + B. pumilus) created a stimulus in cell growth during the fermentation process of cassava tubers. The retting time was reduced from 48 to 24 H. With three consortia having the highest penetrometry indices: (B. Safensis + B. Pumilus), (B. tequilensis + B. Pumilus), and (B. subtilis+ B. Safensis). The development of a starter is a first step in this area and could help reduce the fermentation time of cassava tubers.***

Keywords: Bacillus, Cassava tubers, Consortia, Fermentation

1. Introduction

Traditional biotechnology includes fermentation, a process known and used empirically for centuries for food processing [1]. Bacteria, yeasts and molds are the main actors in this mechanism [2-7]. Food bioconversion occurs spontaneously by these microorganisms that exist naturally in raw materials and contaminants from the equipment used and the environment [8-12].

The lack of control of this process is the major problem that generates a fluctuation in the quality of fermented products. This is the case of cassava fermentation in the Republic of the Congo, which remains artisanal to this day [13, 14]. The rudimentary processing and production of cassava is undoubtedly among the factors that plunge the Congolese population into food insecurity. Several genera of microorganisms initiate retting, including the genus *Bacillus* [8, 10, 12-14]. These are ubiquitous bacteria that are found in different types of fermentation. *Bacillus* actively work with other genera of microorganisms such as lactic acid bacteria, saccharomyces, and candida yeasts, as well as molds, in the degradation of organic compounds in foods through their enzymatic activities to achieve fermentation [15].

This traditional process consists of immersing cassava tubers in water (river, pond, and barrels) for 3 to 6 days to facilitate handling and reduce the content of cyanide compounds [9, 16, 17].

Biotechnologies open up many perspectives in the fields of industry to improve product manufacturing processes and ensure quality control. Starters made up of selected microorganisms are used to initiate fermentation. This technique should improve food security; improve food stability; offer product diversity and provide health benefits [18].

The choice of *Bacillus* as a paradigm of microorganisms used as a starter in cassava retting is such that *Bacillus* are bacteria that tolerate unfavourable growth conditions and therefore have the ability to be cultivated on a large scale [18-24]. *Bacillus* are capable of producing various antimicrobial molecules in fermented foods. These are bacteriocins, biosurfactants of lipopeptide origin such as iturin, fengycin and lichenisin [25-30]. These molecules are involved in the inhibition of spoilage microorganisms (molds, fungi) in fermented foods by extending their shelf life. They can also

block pathogens. Biosurfactants also contribute to improving the organoleptic qualities of the fermented product. *Bacillus* are known to secrete a wide range of enzymes [24, 28-30]. *Bacillus* are equipped with a metabolism capable of contributing to the organoleptic, technological, and nutritional characteristics of fermented foods [31].

The exact role Bacillus as ferment seems to be unclear in fermentation processes because the model of starters with fermentative capacity. In this work, we chose to develop a biotechnological ferment based on bacteria of the genus *Bacillus* to improve endogenous knowledge in the context of cassava retting in the Republic of Congo. Direct inoculation of *Bacillus* as a starter with multiple potentialities in the fermentation of cassava tubers would be a creative solution in the Republic of Congo for large-scale cassava production. This biotechnological ferment would overcome the restrictions related to the production of fermented cassava and would be among the answers to achieve food self-sufficiency. We were interested in the bacteria of the genus *Bacillus* contained in fermented cassava. The choice of fermented cassava as a sample to isolate these bacteria is such that, firstly, the *Bacillus* species are ubiquitous [32]. Finally, cassava would be an ecological niche favourable to the growth of Bacillus species due to the nutrient substrate found there [7-10, 12].

This work aims to isolate and evaluate *Bacillus* species to select those favourable to the development of the ferment that can optimise the fermentation process of cassava tubers.

2. Methods

Isolation and purification

10 g of sample were weighed on aluminium foil and diluted in 90 ml of sterile physiological water. After complete homogenisation, serial decimal dilutions (10^{-2} to 10^{-7}) were prepared from this basic solution. Secondly, we carried out the isolation by spreading 100 µl of each dilution on the surface of the previously poured and solidified in Petri dishes. The incubation of the dishes was carried out at 37 ° C for 24 to 48 hours in an aerobatic incubator. After incubation, each colony of different appearances was isolated separately. The purification of the isolates was carried out rigorously by successive cultures on nutrient agar LB or PCA until obtaining distinct and homogeneous.

The morphological characterization was performed using a light microscope (OPTIKA, Italy). Gram staining was performed using 3% potassium hydroxide (KOH) [33]. Speculation efficiency was assessed to determine the ability of isolates to form endospores [34]. Briefly, bacterial cells cultured in LB at 37°C for 24 were heat inactivated. The number of spores was measured by measuring heat-resistant colonies (70°C for 30 min) in LB-agar plates, while viable cells were measured as total CFU in LB-agar plates. Spore % = (spores/mL)/(viable cells/mL) × 100%. The rapid test for detection of the cytochrome oxidase and catalase enzyme activity was performed for all bacterial strains according to the manufacturer guidelines (LIOFILCHEM, Italy). All purified isolated cultures were stored at −20°C in Lysogeny broth (LB) (10 g tryptone, 5 g yeast extract, and 10 or 5 or 0.5 g NaCl) containing 20% (v/v) glycerol.

Cellulase and Amylase Research

We used cellulose and starch as substrates to perform cellulolytic and amylolytic activities, respectively, with the following protocol: for 100 ml, 0.5% cellulose and 1% starch were added separately to the LB agar medium. After sterilising the medium in an autoclave at 121 ° C for 15 minutes, we poured the Petri dishes after cooling the medium (reduction of heat leaving the medium in a fluid state). After solidification, we performed spots with the colonies of the isolates to be tested using a sterile Pasteur pipette. The dishes are incubated at 37 ° C for 48 to 72 hours. The test was revealed by sprinkling Lugol's in the Petri dishes. Lugol's is an iodized solution; it naturally causes a colouration in contact with the starch (dark blue or purplish colouration depending on the purity of the Lugol's). On the other hand, it gives a mahogany brown colouration in the presence of cellulose. The presence of enzymes (cellulases or amylases) is marked by a clear halo around the spot. This reflects the degradation of the substrate by the enzyme; the test is then positive. The percentage of lysis diameters (DL) of the enzymatic activities (amylo and cellulo) was determined according to the following formula: DL=(DT-DC)/DTx100. With: DT: total lysis diameter (lysis zone + colony diameter); DC: colony diameter; DL: lysis diameter.

Protease research

For 100 ml of PBS (0.01 X), we added 1 g of agarose. The mixture was heated in the microwave until the agarose was completely dissolved. After reducing the heat, 10 ml of skimmed milk are added under sterile conditions. After homogenisation, the Petri dishes are poured. To inoculate the inoculum, we made the wells using a sterile tip after solidification of the medium. We separately cultured each isolate to test in the tubes containing 5 mL of LB broth at 37 ° C with shaking (200 rpm) for 24 hours (overnight culture). Each culture is taken from Eppendorf tubes and then centrifuged at 6000 rpm for 10 min. The inoculum that is inoculated in the wells for the search for proteases is none other than the supernatant (50 µL) of each culture. Then we incubated the Petri dishes at 37 ° C for 20 to 24 hours. The presence of a clear halo around the wells indicates the hydrolysis of casein by proteases. The lysis diameters of each isolate are measured using a graduated ruler.

Biosurfactant Production Assay

The emulsification activity was carried out using the method used by Uyar and Sağlam [35].

The emulsifying activity of a biosurfactant is its ability to retain an emulsion of hydrocarbons or oils in water. 5 mL of washed cells and 5 mL of acellular supernatant of each isolate were poured into a test tube containing 5 mL (v/v) of gasoline. The mixture was shaken vigorously for 3 min using a vortex mixer (VELP Scientifica, Italy). The tubes were then incubated at room temperature for 24 h. The height of the emulsion layer and the total height of the mixture were then measured. All the experiments were performed in triplicate, and the emulsification index (EI24%) was calculated using the standard formula for EI24% (He/Ht) × 100, with He being the emulsion height, Ht being the total height of mixture, and EI24% being emulsification percentage after 24 h.

Fermentation Test

The fermentation capacities of cassava tubers were evaluated of the isolates during the retting. The strains used each have the capacity to produce at least 2 different biomolecules. To begin with, the tubers are peeled and washed with tap water. We sized, chopped and weighed them respectively using a graduated ruler, a knife, and a scale so that we obtain a piece of size approximately 2 cm^3 with a weight of 12.30 g. We then added under aseptic conditions three (3) pieces of freshly cut tubers to jars containing 100 mL of autoclaved distilled water (121°C for 15 minutes). A volume of 3 ml of the overnight culture of each strain was inoculated into the different jars (Figure 1). A 5-day fermentation was launched to identify isolates that could have fermentation potential and to build a softening scale. Photo 4 illustrates the process of this test). We had launched in parallel a natural control retting (without inoculum) with sterile distilled water (EDT) and tap water (ERT).

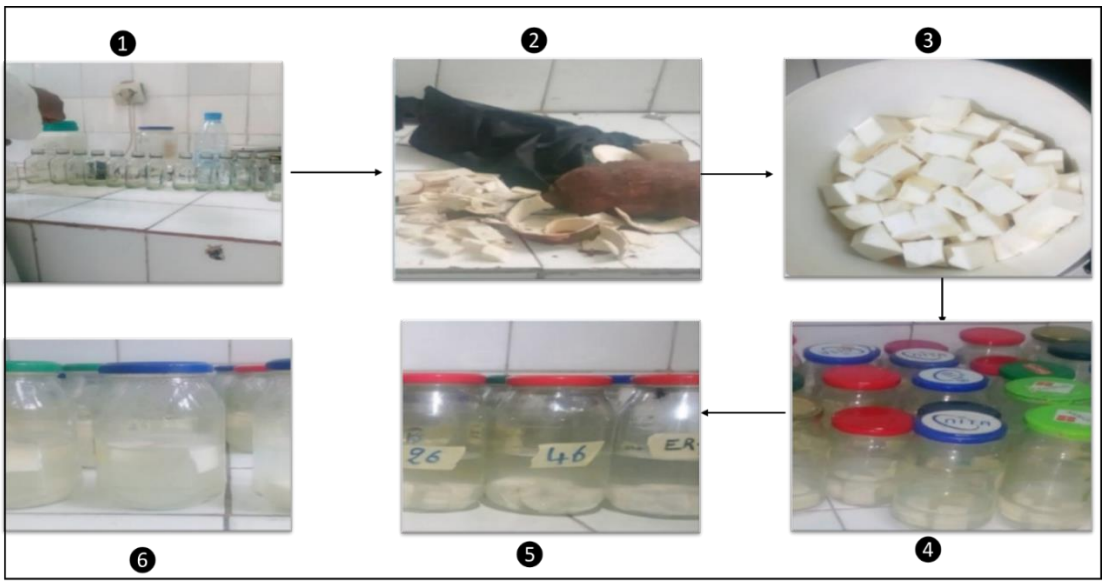

Figure 1: Process of fermentation of cassava tubers. ①Preparation of the inoculum; ② Preparation of the cassava pieces; ③Cassava pieces of 2 cm^3 each and weighing 12.5 g; ④Three cassava pieces immersed in 100 ml of sterile distilled water containing 3 mL of inoculum, ⑤Fermentation of cassava pieces; ⑥Fermentation on the fifth day.

Fermentation Monitoring

The optical density (O.D.) was taken before and after inoculation. After 16 hours of incubation at room temperature, the O.D. was read three times a day at an interval frequency of 3 hours, during fermentation. To do this, we used a spectrophotometer (752 UV / VIS). The pH was measured using a pH meter. This parameter provides information on the acidity or alkalinity of the retting water during fermentation. It was read three times a day at an interval frequency of 3 hours, during fermentation.

Penetrometry indices (PI). The softening of cassava tubers was evaluated taking into account their resistance to the penetrometer. We used a mechanical penetrometer (graduated platinum loop) to probe the pieces of fermented cassava tubers under sterile conditions. Values were established according to the texture of the tubers: a score of ten (10) was associated with tubers whose penetrometer was completely inserted and broke the tubers. An index of seven (7) to eight (8) was associated with tubers with penetration of the instrument creating cracks. An index of five (5) was associated with tubers that the penetrometer was inserted but did not break. Then zero (0) for tubers whose maximum resistance was not admissible The Figure 2 illustrates the texture of the pieces of fermented cassava tubers with the associated indices. Isolates were selected basing in PI.

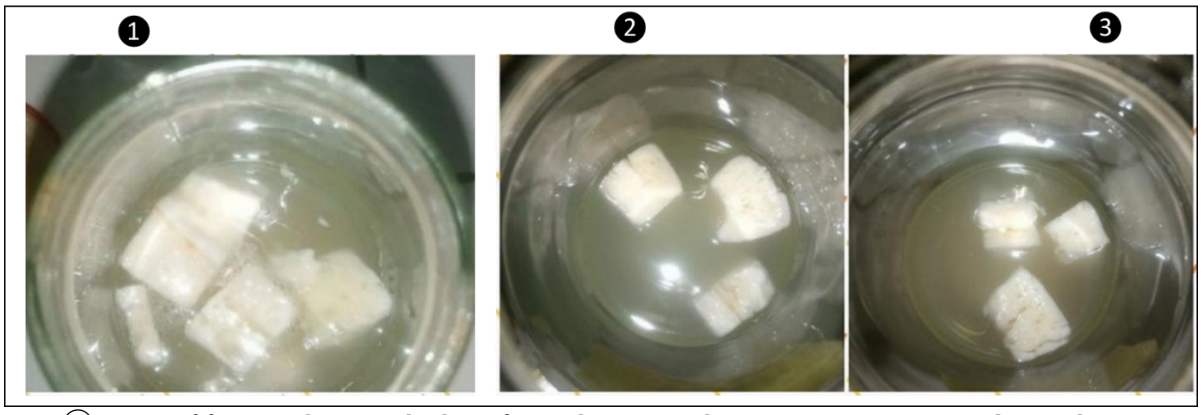

Figure 2: ① Pieces of fermented cassava broken after probing using the penetrometer corresponding to the penetrometry index PI = 10. ② Pieces of fermented cassava cracked after probing using the penetrometer corresponding to the

penetrometry index PI= 7-8. ③pieces of fermented cassava not broken after probing using the penetrometer corresponding to the penetrometry index IP = 5.

Fermentation with strains in Consortium

We mixed in pairs the 48-hour isolates with a penetrometry index IP = 10. At a rate of 3 mL of the inoculum, 1.5 mL of each culture were taken. The experience of *Bacillus* in coculture has already been demonstrated and appreciated in optimising the duration of the fermentation duration. The fermentation activity with isolates in association was also very effective during our experiments compared to pure isolates. The same operation was repeated 3 times to confirm the effectiveness of the consortia. The retting juice were stored in 2 ml eppendorf tubes according to the fermentation duration (72 H,, 48 H and 24 H) and kept in the refrigerator for the realisation of the dilutions. To control the ferùmentation in terms CFU, 1 ml of each type of retting juice were diluted in 9 mL of physiological sterile water and vigorously stirred for about 30 seconds. We diluted up to 10^{-2}. The spreading of each dilution was carried out on Mossel medium and Sabouraud chloramphenicol agar (SCA) medium. The incubation lasted 24 to 48 hours at 37 ° C. Microscopic observations were made after incubation.

Extraction of genetic material and gel electrophoresis

To do this, 3 ml of each culture were taken and placed separately in sterile Eppendorf tubes. Tubes were centrifuged at 10,000 rpm for 1 min. After destroying the supernatant, we added 200 µL of the EP lysis solution to the pellet. This solution consisted of 200 mM sodium chloride + 300 mM sodium acetate and 20 µL of lysozyme at 10 mg / ml. We then incubated the tubes at 37 ° C for 10 min in an incubator (INCU-Line VMR). After incubation, we added 400 µL of another lysis solution (lysis binding buffer: Roche High-pure RNA isolation kit Roche). The tubes were shaken vigorously using a vortex and centrifuged for 20 min. The supernatant is collected and transferred to other sterile Eppendorf tubes and we added a volume of 600 µL of cold ethanol (95%). After adding the ethanol, we again incubated the tubes, this time at a temperature of -20 ° C for 20 min. After this operation, the tubes were centrifuged at 10,000 rpm for 20 min. The supernatant obtained is destroyed before adding 1 ml of cold ethanol (95%) and incubating the mixture again at -20 ° C for 10 min. After incubation, centrifugation was carried out for 10 min. The resulting pellet constitutes the DNA extract. The tubes are dried on a bench for 20 min and a volume of 40 µL of the elution buffer was added to dilute the DNA.

The electrophoresis gel with 1% agarose has been performed. UV lamp (UVP TM-20E has been performed in terms of DNA visualisation using.

Genomic DNA Extraction, PCR and Sequencing

Genomic DNA extraction and purification were performed using adapted NucleoSpin Microbial DNA kit (Macherey-NAGEL). Briefly, the targeted isolate is grown in 5 mL of LB broth for 24 h at 37°C with stirring. DNA purity was assessed by electrophoresis on 1% agarose gel and by the ratio of optical densities 260/280 nm. The amplification of fibE gene has been performed according to the previous describe method [36]. The housekeeping 16S rRNA gene has been amplified by PCR (Thermal Cycler, Bio-Rad) by using universal primers fD1 (5′-AGACTTTGATCCTGGCTCAG-3′ and rP2 (5′-ACGGCTACCTTGTTACGACTT-3′). 5 µL of each amplification product was mixed with 2 µL of loading buffer (BIOKÉ). Mixtures were subjected to electrophoresis on 1% agarose gel (w/v). The 10 kb 2-Log (BIOKÉ) was used as a molecular weight marker. The PCR products were purified using the solution of Gel Extraction kit (Omega Bio-tek), and the purified products were subjected to sequencing by the Sanger technique (3130 × l Genetic Analyser (Applied Biosystems)). The sequences obtained were aligned with the software BioNumerics 7.5 (Applied Maths, Belgium) and corrected manually to resolve discrepancies between the sense and antisense strands. Sequences were compared with homologous sequences contained in the sequence databanks through NCBI (National Center for Biotechnology Information (http://www.ncbi.gov/Blast.cgi) using the BLASTn program based on the identification criterion published by Drancourt [20]. All sequences have been stored in NCBI GenBank data.

3. Results

Isolation, purification, and characterisation

105 isolates had been obtained in this work after isolation from fermented cassava. All have variable characteristics. Some colonies are small, others medium or large. They have several shapes with different textures, white, beige, yellowish, etc. All the phenotype variations of these cultures are multifactorial. These variations are related to the conditions under which these bacteria develop. Microscopic observation reveals rod-shaped bacteria with positive mobility, grouped in pairs, in chains, or isolated. They were all sporulating, Gramme positive, and catalase positive.

Cellulolytic, amylolytic and proteolytic activities

The ability of bacteria isolated from retted and fermented cassava were tested to secrete several exocellular enzymes including amylase, cellulase and protease and to coordinate the action with the fermentation process of cassava tubers. This allows us to find out for enzymatic activities. Isolates have been tested for their ability to degrade cellulose, starch and milk casein. As explained in the methods, the profiles of different enzymatic tests carried out are illustrated in Figure 4. Lysis plaques demonstrate enzyme activity which reflects a degradation of the substrate according to a given dimension

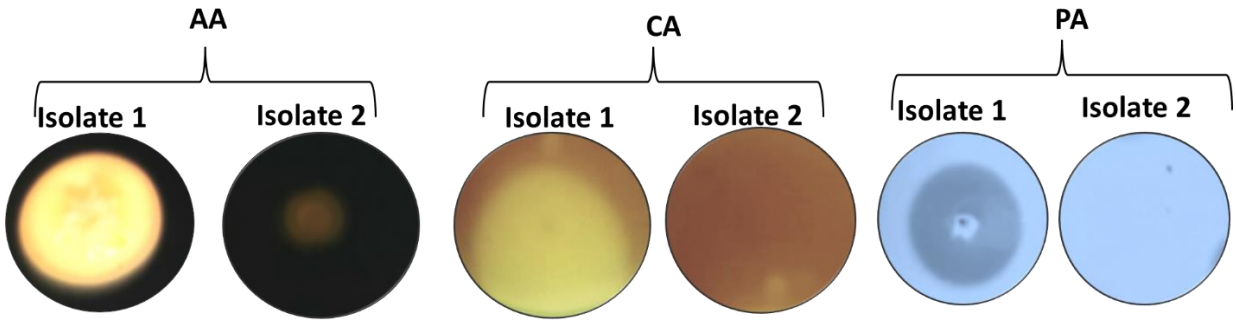

Figure 3: Amylolytic, cellulolytic and Proteolytic activities. AA: Amylolytic activity, CA: Cellulolytic activity and PA: proteolytic activity

The 105 isolates of our work were used for the production of amylases, cellulases and protease. The lysis diameters were calculated according to the methods mentioned afore. The positive results are included in the interval that ranges from 21.78% to 80%. Among the 105 isolates tested, 63.8% (67/105) were positive for the production of amylases (Figure 4). S1, S21, S49, S65, S73, S77 and S99 were the higher.

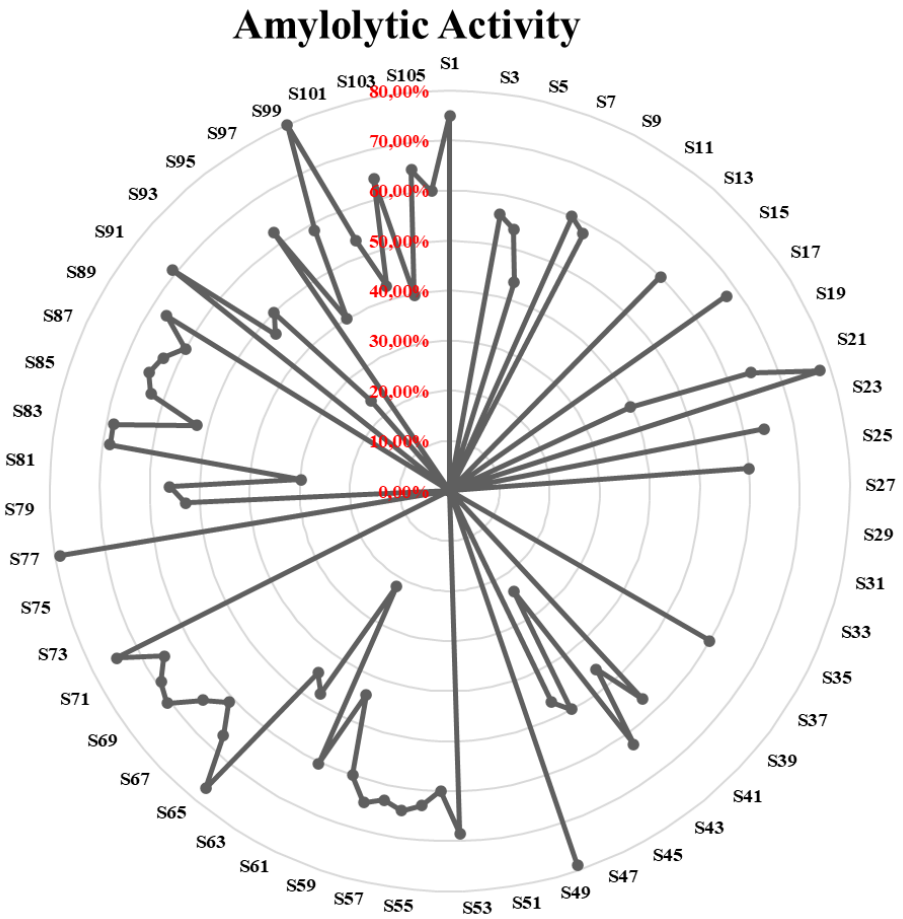

Figure 3: Amylolytic activity of each isolate. The figure shows the behaviour of the isolates (S1, S2,... S105) in relation to their lysis diameter. Each circle constituting this figure represents in percentage the amylolytic ability of each isolate according to the spot performed.

Regarding cellulolytic activity, the activity percentages ranged from 5.8% to 80%. Among the 105 isolates evaluated, 56.19%. (59/105) were positive for the production of cellulase. 10.4 % (11/105) S3, S5, S25, S41, S45, S59, S65, S77, S89, S99 and S101 were higher (Figure 4).

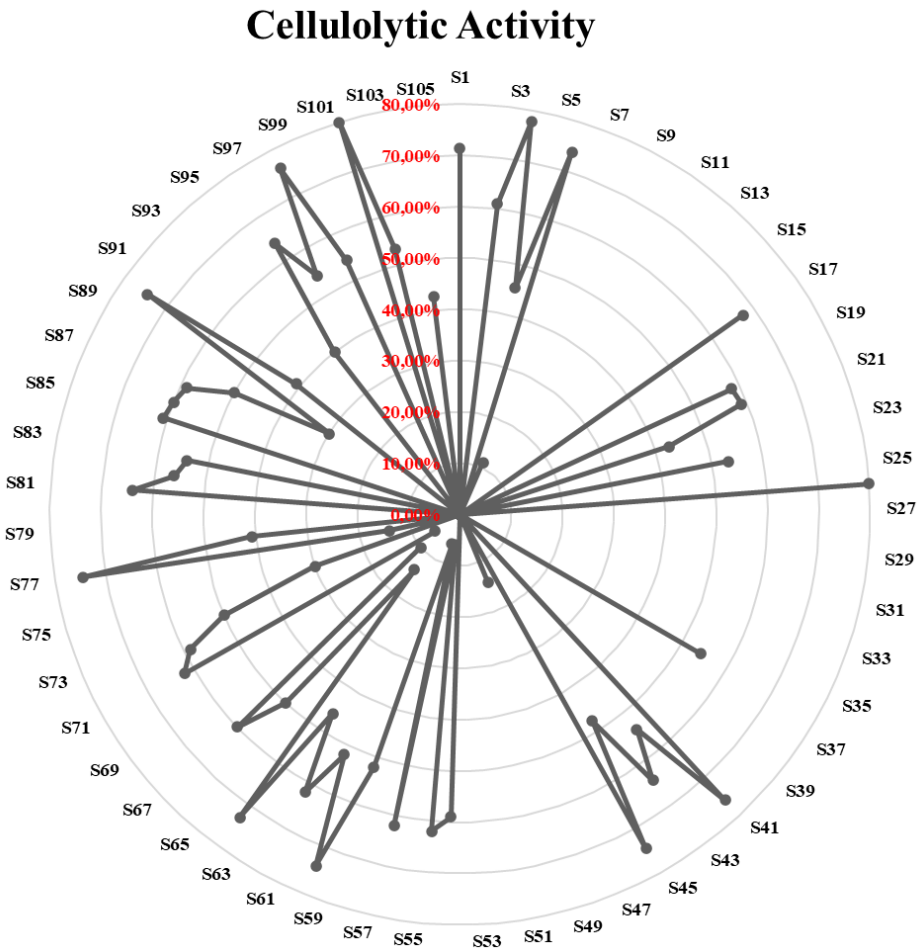

Figure 4: Results of the cellulolytic activity of each isolate. The figure above shows the behaviour of the strains (S1, S2, ... S105) according to their enzymatic digestion. Each circle in the figure expresses the percentage dimension of the diameter of the cellulase lysis range of certain isolates.

Regarding protease production, the lysis areas (halos) were evaluated in centimetres (cm) after 24 hours of incubation at 37 ° C. 41.90% (44/105) of isolates were positive for protease production.

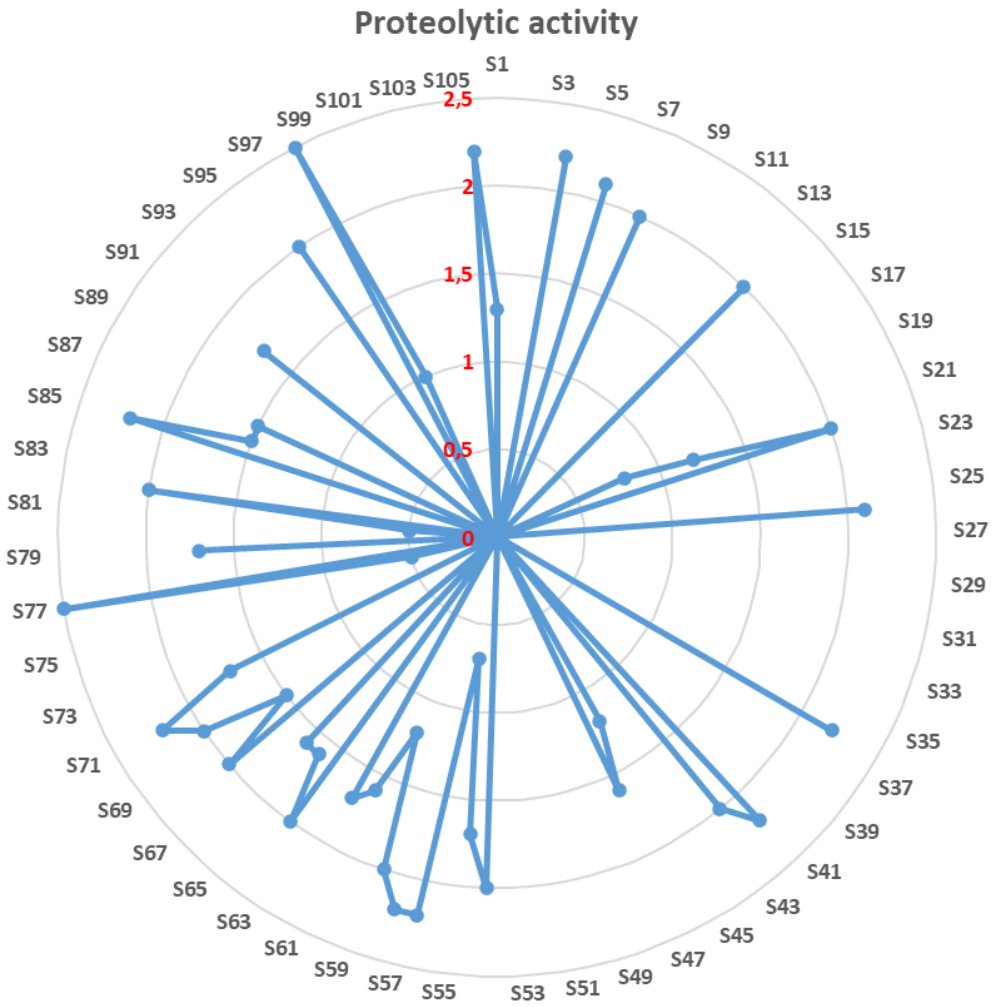

Figure 5: Proteolytic activity of each isolate

Biosurfactant Production

Isolates were tested for their ability to produce biomolecules like biosurfactants. The emulsion index was determined after 24 hours of incubation of the strains (IE24) at room temperature for the tubes showing a positive result. After measuring the heights (height of the emulsion layer, total height of the solution), the emulsion index (IE24) of each isolate was determined according to the formula stated in the methods. The results obtained, and Figure 6 presents all the results**.**

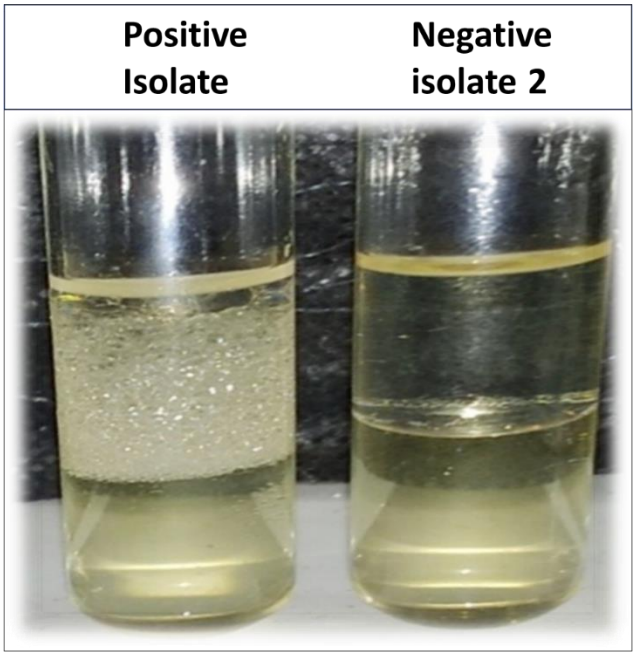

Figure 6: Biosurfactant production

The production of biosurfactants was carried out by highlighting the isolates and the essence according to the protocol stated in the methods. In total, 38.09%. (40/105) isolates among the tested were able to produce biosurfactants.

The positive results range from 20% and 100%. Many isolates had percentages above 70%.

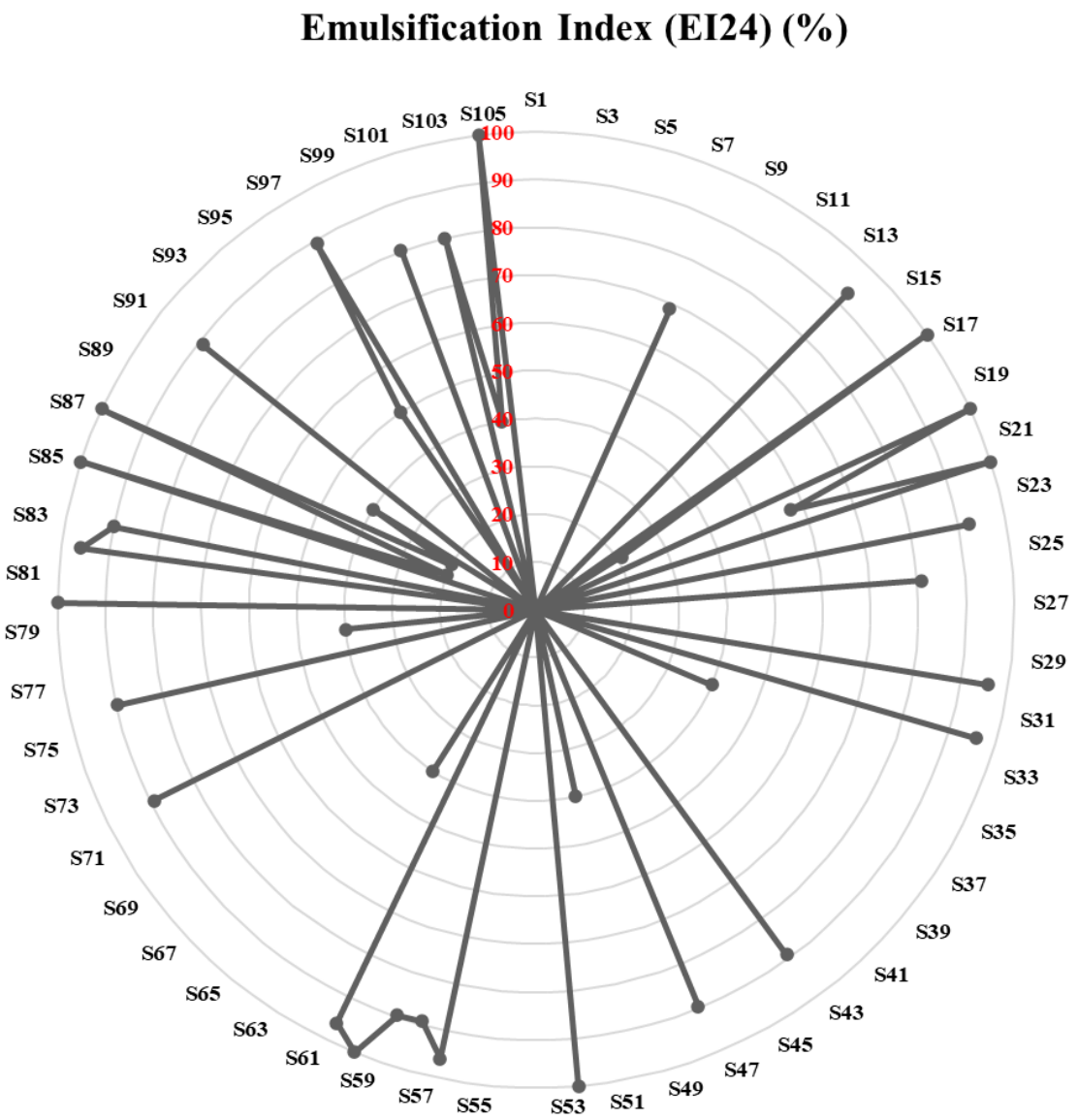

Figure 7: Biosurfactant production presents the results of biosurfactant production of all isolates (S1, S2…S105).

Fermentation test

The selection of strains for the fermentation test was made according to their ability to produce biomolecules (amylases, cellulases, proteases, and biosurfactants). Indeed, we preferably selected strains that have the capacity to produce at least 2 different biomolecules. Thus, among the 105 strains tested, 63 have multiactivity. This is included S1, S4, S5, S6, S8, S14, S17, S20, S21, S22, S24, S26, S36, S41, S42, S43, S44, S45, S46, S47, S54, S55, S56, S57, S58, S59, S60, S61, S62, S63, S64, S65, S66, S67, S68, S69, S70, S71, S72, S76, S77, S78, S79, S80, S81, S82, S83, S85, S86, S87, S88, S89, and S91, S96, S97, S98, S99, S100, S101, S102, S103, S104, and S105. Direct fermentation with the isolates was started for 5 days. Since softening was obviously the primary parameter of this experiment, the penetrometry index (PI) was measured using a mechanical penetrometer on the fifth day of fermentation to identify strains with fermentative capacity. The pH values were determined. By simple observation, pieces that had reached maximum softening were noted by the formation of a sort of paste at the bottom of the jar; some pieces showed swelling and were suspended, others had kept their initial volume. At the end of the survey, all pieces were almost softened with a pH of around 5. The following scores are given for the distinction related to the softening of the tubers before moving on to the controlled fermentation itself. A score of ten (10) is associated with tubers whose penetrometer was completely introduced and broke the tubers. An index of seven (7) to eight (8) is associated with tubers that create cracks during penetration of the instrument. An index of five (5) was associated with tubers that the penetrometer was introduced but did not break the pieces. Then zero (0) is given for tubers whose maximum resistance is not admissible. Figure 7 shows the penetrometry indices of all strains (63 strains) on the fifth day of fermentation.

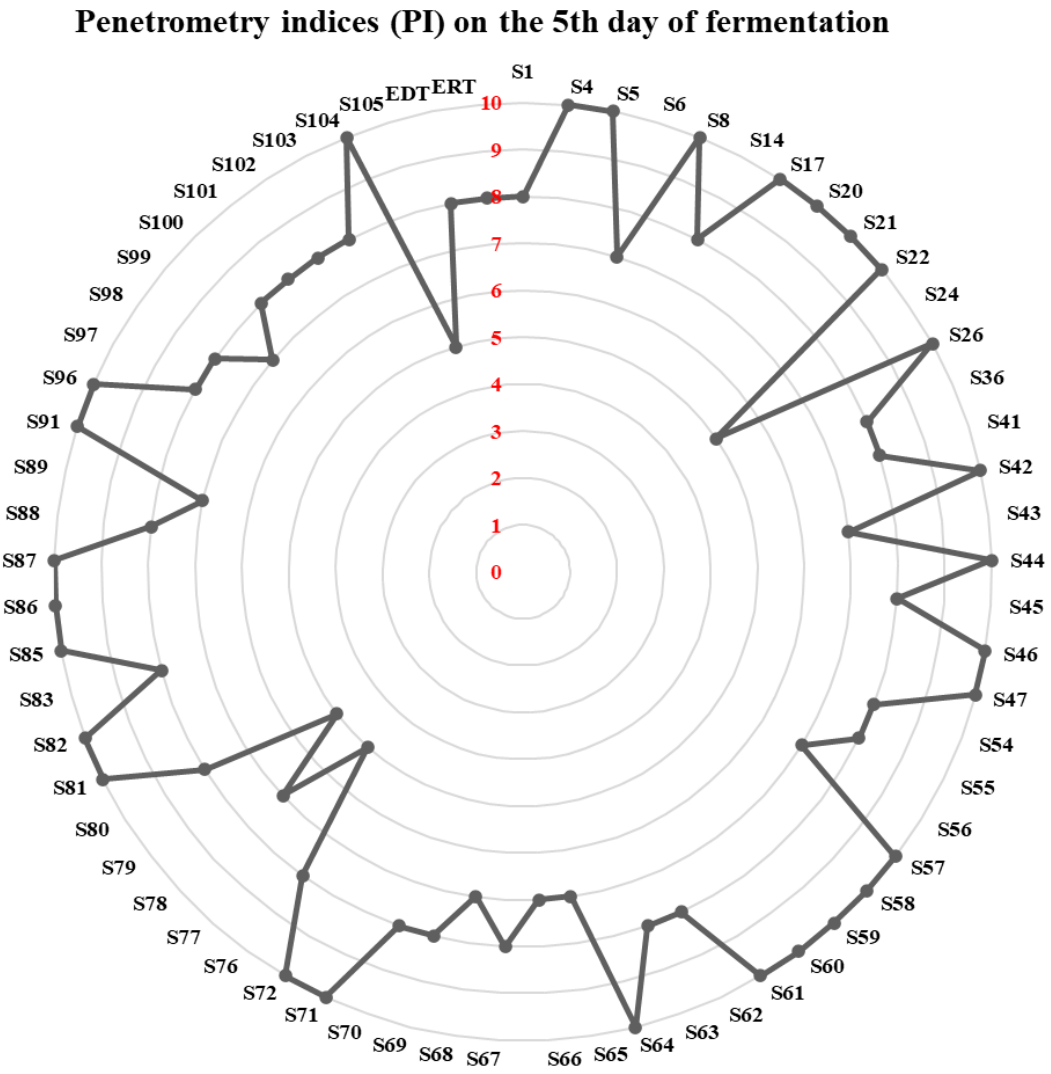

Figure 8: Penetrometry indices on the fifth day of fermentation: Each circle represents the penetrometry index of the cassava pieces fermented with the 63 isolates. On the fifth day of fermentation, all cassava pieces were softened with IP indices ranging from 5 to 10 including cassava pieces launched into natural fermentation (control) with sterile distilled water (ERT) and tap water (EDT).

Strains that can constitute the biotechnological fermentation of cassava

Given that the duration of natural retting took 4 days in our experiment, we selected strains (ferments) capable of softening cassava tubers in less than 4 days. Physicochemical parameters such as pH, O.D., pH, and PI were measured after 24h, 48h, 72h and 96h.

After 24h, the PI was 0 % for all strains. The pH descendait entre 5.6 et 6.

However at 48-hour fermentation showed 30% (19/63) of pure ferments including S1(5), S4 (8), S5 (10), S21 (8), S22 (10), S26 (10), S42 (10), S44 (5), S46 (10), S57 (10), S58 (5), S71 (8), S72 (5), S81 (10), S82 (5), S85 (8), S91 (8), S96 (10), and S104 (8) have fermentative ability in 48 hours with a pH ranging from 5 to 6.

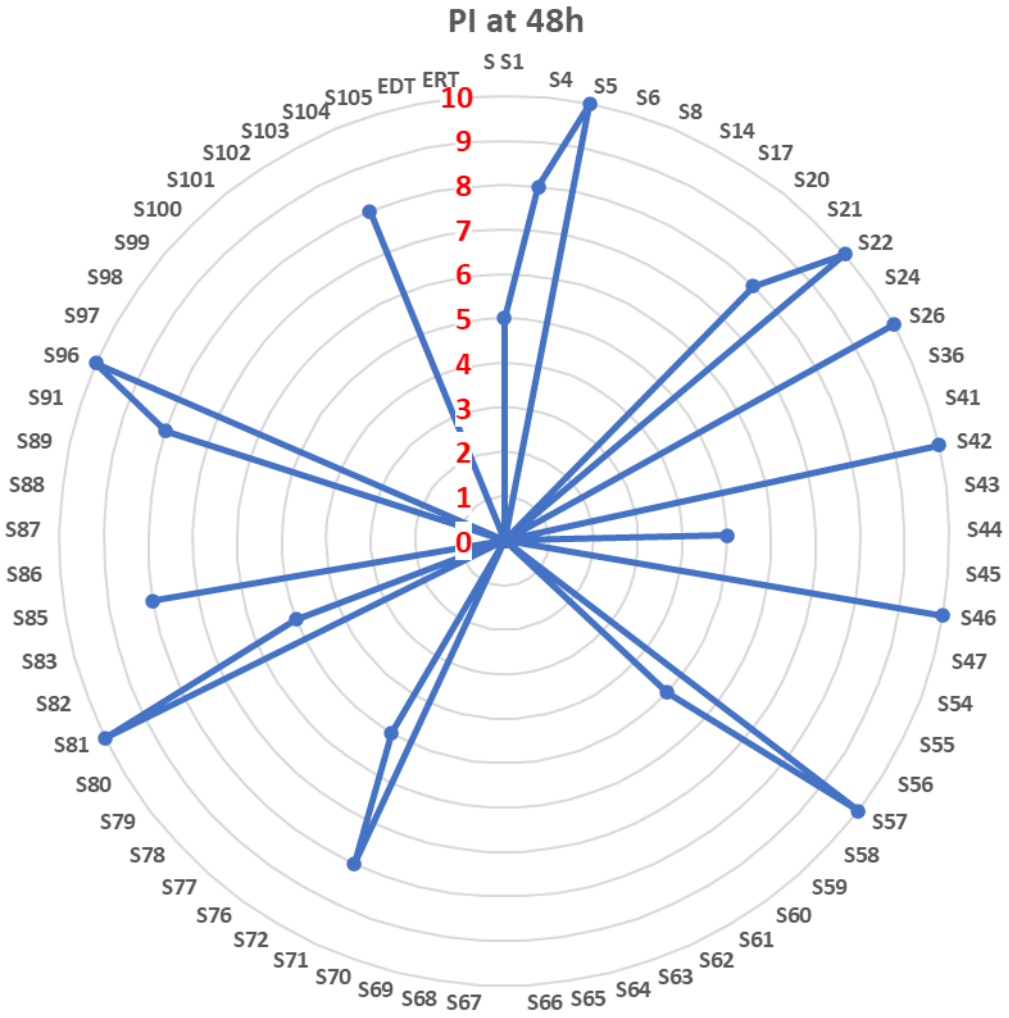

Figure 9: Isolates with the ability to ferment for 48 hours

At 72-hour fermentation showed 39.6% (25/63) of pure ferments. Alongside the isolates mentioned at 48 hours, Five other isolates were able to ferment cassava tubers. This included S8 (10), S20 (10), S60 (8), S64 (8), and S86 (8) **(Figure 9).**

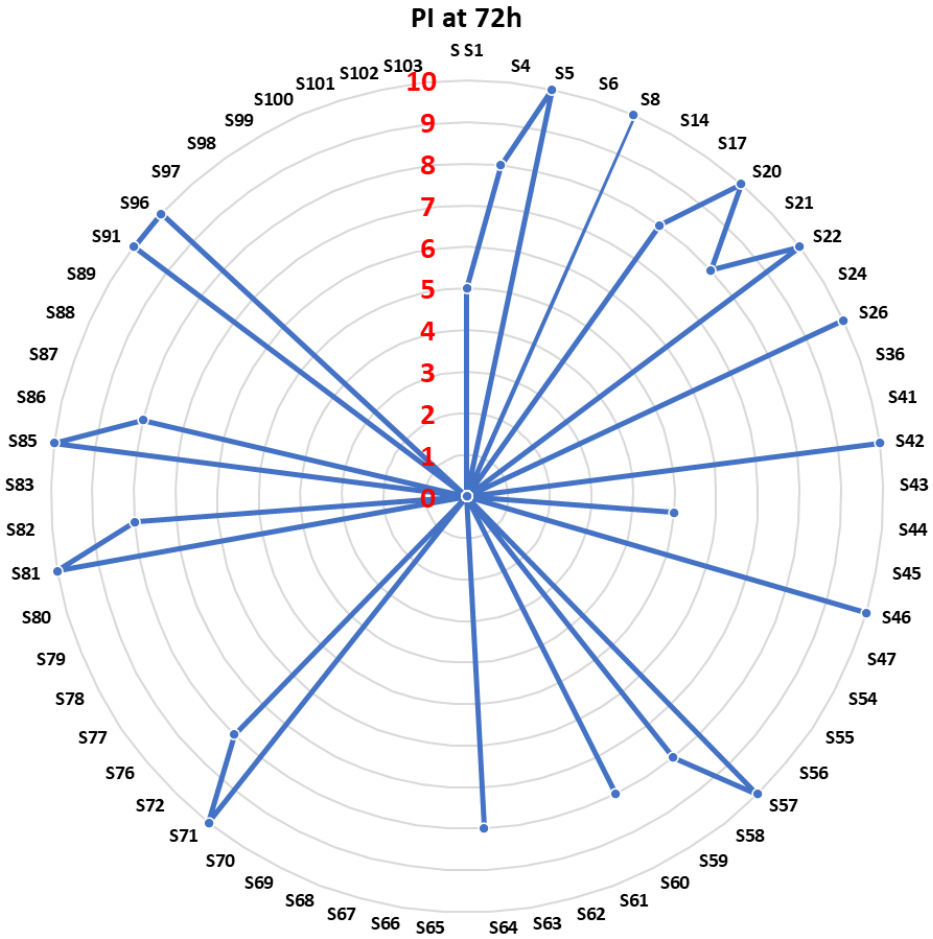

Figure 9: Isolates with the ability to ferment for 48 hours.

Fermentation with Consortium strains

The 48-hour isolates that softened the cassava tuber pieces very well (with IP = 10) were mixed in pairs. These are S5 (10), S22 (10), S26 (10), S42 (10) S46 (10), S57 (10), S81 (10), and S96 (10). In total, 8 strains were chosen to carry out the coculture fermentation, which allowed us to obtain 28 pairs of isolates. At a rate of 3 ml of the inoculum, 1.5 mL of each culture was taken. Coculture was effective and reduced the fermentation time of cassava tubers from 48 H to 24 H. Among the 28 strain pairs used to start fermentation, we obtained 8 pairs with a fermentation capacity of 24 H with the penetrometry index varying from 8 to 10 and pH 5 and 6. The other pairs showed fermentation power beyond 24 H and do not obey the objectives set in this work. Table IX shows the fermentation test with isolates in the consortium.

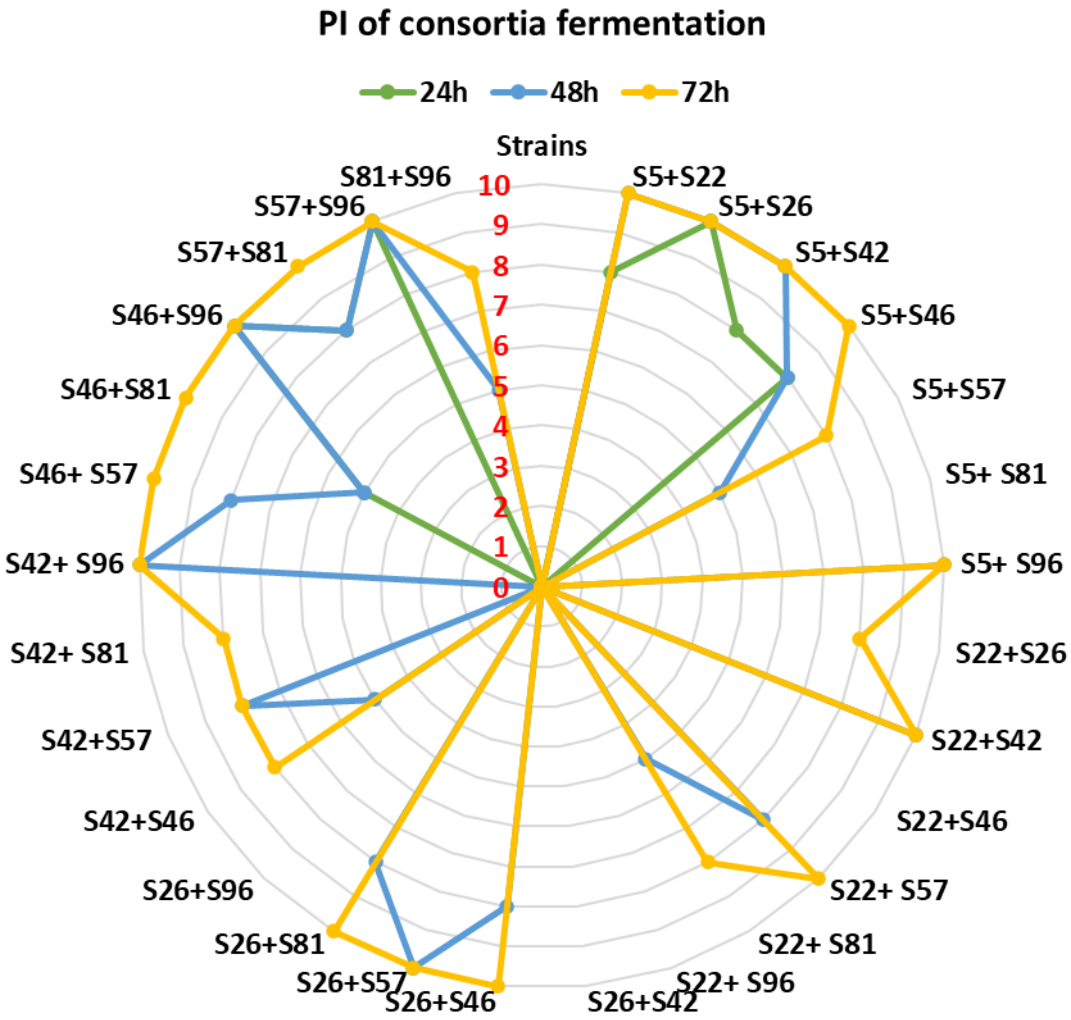

Figure 10: Penetrometry indices for consortia fermentation.

The various fermentation tests have allow us to develop 3 categories of biotechnological ferments, namely 72 H, 48 H and 24 H ferments. Each particularity of the starting strain can depend on the desired goal or determine the desired final product. The scores (5, 8 and 10) corresponding to the texture of the final products will serve as indications for carrying out controlled retting to obtain the cassava-derived product of choice. The strains that constitute these different ferments are: 6 strains of 72 H: S8 (10), S17 (8) S20 (10), S60 (8), S64 (8), and S86 (8). 19 strains of 48 H: S1 (5), S4 (8), S5 (10), S21 (8), S22 (10), S26 (10), S42 (10), S44 (5), S46 (10), S57 (10), S58 (5), S71 (8), S72 (5), S81 (10), S82 (5), S85 (8), S91 (8), S96 (10) and S104 (8). 8 consortia allow to ferment cassava tubers at 24 hours. S5+S22 (8), S5+S26 (10), S5+S42 (8), S5+S46 (8), S46+S81 (5), S46+S96 (10), S57+S81 (8) and S57+S96 (10). The number of consortiums has been added at 48h and 72h (Figure 10). Three other fermentation tests were carried out with the different ferments selected, and the results remained identical to the first tests. The different strains that make up the biotechnological ferments were stored in Eppendorf tubes containing a nutrient broth plus 20% glycerol and preserved in triplicate in the freezer at -20 ° C.

Fermentations were monitoring by plating retting juice on Mossel and Sabouraud chloramphenicol agar (SCA) media, the incubation lasted 24 - 48 H at 37 °C. As a result, bacteria and yeast were able to grow, the CFU were up to 300 (CFU>300).

The examination of the retting juice was carried out. After incubation, colony growth is efficient in the petri dishes containing the Mossel and SCA media for the 72-hour retting juice. Microscopic observations confirm the presence of Bacillus and yeasts for each type of medium. Regarding the 48-hour retting juice, colonies appear in all the petri dishes containing Mossel medium and a total absence of colonies in some petri dishes containing the SCA medium. However, a total absence of colonies is observed in all dishes containing the SCA medium for 24-hour retting juice. The colonies are present only in the Petri dishes containing the Mossel medium.

Molecular Identification

Molecular identification was done to confirm the genus of the bacteria used (Bacillus) and reveal their species. Isolates with good fermentation profiles in less time (48 H) were subjected to genomic DNA extraction verified by 1% agarose gel electrophoresis. A total of eight strains has good fermentation profiles in 48 H. These are S5 (10), S22 (10), S26 (10), S42 (10) S46 (10), S57 (10), S81 (10), and S96 (10).

Amplification using a one-step multiplex PCR with the primers showed that four specific primer pairs allowed the amplification of the fibrinolytic enzyme gene. This allowed for the identification of B. Subtilis (S22), *B. Safensis* (S5 and S57), and *B. Pumilus* (S96). All the results of the

amplification of the fibrinolytic enzyme gene of the isolates are in table I.

Sequencing allowed the identification of *B. subtilis* (Gen bank: MT994787.1) corresponding to strain S26, Bacillus subtilis (Gen bank: MT994789.1) corresponding to strain S42 and *B. tequilensis* (Gen bank: MT994788.1) including strain S46. Strain S81 could not be identified. It is associated with *Bacillus* sp.

Table I: Extraction, fibE and rRNA 16S PCR and sequencing.

Isolates	DNA Extraction	FibE PCR	rRNA 16S PCR	Sequencing	Strain
S5	+	+			*B. safensis*
S22	+	+			*B. subtilis*
S26	+	-	+	+	*B. subtilis*
S42	+	-	+	+	*B. subtilis*
S46	+	-	+	+	*B. tequilensis*
S57	+	+			*B. safensis*
S81	+	-	+	-	-
S96	+	+			*B. pumilus*

4. Discussion

The isolates were evaluated for their ability to secrete amylases, cellulases, and proteases in extracellular medium. 67/105 of isolates producing amylases with amylolytic power ranging from 21.78% to 80%; 59/105 of isolates producing cellulases with a cellulolytic power ranging from 5.88% to 80%; 44/105 of isolates producing proteases with lysis diameters between 0.8 cm and 2.5 cm have been obtained. Bacteria of the genus *Bacillus* producing cellulases, amylases, proteases and biosurfactants have been already documented. Amylolytic, proteolytic, and pectinolytic activities were demonstrated on nutrient agar supplemented with 1% starch for the search for amylases and 5% skimmed milk for the search for proteases.

[25, 28-30, 37, 38]. The novelty in this work is that no study has focused on the *Bacillus* genus bacteria isolated from cassava fermentation which is the subject of a study on the development of a biotechnological ferment. The dissimilarity of enzymatic potentialities in *Bacillus* would be linked to the specificity of the growth conditions of each strain (temperature, optimal pH, and substrate concentration).

The production of enzymes by the bacteria isolated in this work could contribute to increasing the bioavailability of nutrients in fermented cassava and bring great value, including the acceleration of physicochemical transformations, as well as the improvement of digestibility by giving them particular organoleptic properties of uniform quality [39, 40].

Our strains were also tested for their ability to produce biosurfactants. We highlighted the strains with the hydrocarbon. A total of 40/105 strains were able to produce biosurfactants with 24 hour emulsification indices (EI24) ranging from 20 to 100%. Our experience has shown that *Bacillus* strains in general are producers of biosurfactants [28, 29, 41]. The secretion of these biosurfactants is not evident in all strains, with their secretion capacity being variable from one another. This could be explained by the optimal growth conditions of certain strains, including their biodegradation rate. In addition, certain genera of bacteria are only able to hydrolyse a limited number of hydrocarbons. These biomolecules help to grow strains and in improving the organoleptic qualities of fermented product [22, 30]. They also interfere in the inhibition of spoilage microorganisms (moulds, fungi) and confer health properties to fermented foods, which would extend their shelf life [42, 43]

Only strains with the capacity to produce at least 2 different biomolecules (amylase, cellulase, protease, and biosurfactant) were selected for the fermentation test. After in vitro the 63 isolates each in particular by direct inoculation in fermentation, 25 possess the fermentation activity in the interval of 48 H to 72 H with penetrometry indices ranging from 5 to 10 and a pH of approximately 6 with: 19 isolates capable of fermenting cassava tubers in 48 H and 6 that carry out the fermentation in 72 H. Our results show that pure isolates of bacteria of the genus *Bacillus* have fermentation potential. In fact, the duration of fermentation was reduced by 24 or 48 H. These results demonstrate that the fermentation capacity is not the same at the level of the isolates. This study is the first in this field; it was not carried out in the Republic of Congo. But preliminary studies have been carried out on the biochemical and microbiological composition of fermented cassava tubers [8-10, 12]. We find that the cassava pieces fermented with all these isolates were better than the naturally fermented cassava pieces. An improvement of the softness in texture of cassava pieces fermented with the isolates is evident in less time than in both cases of natural fermentation.

According to the literature, *Bacillus* provide undeniable added value to fermented foods during and after fermentation [39]. These bacteria remain alive or in the spore state in food regardless of treatment (cooking temperature and others) and will continue to promote in the consumer's digestive tract the growth of the microbiome in the digestive tract of the consumer that will help strengthen the immune system and intervene in the prevention and treatment of several diseases [44-46]. *Bacillus*-based starters should have different biological activities that improve not only the quality of the product but also the health of consumers. Thus, fermented cassava made by bacteria of the genus *Bacillus* will be taken as a functional food with all the benefits that these bacteria confer.

Today, the progress of industrialization generates an increased development of starters to ensure mass production, which we cannot achieve by empirical methods. The latter not only provide non standardized products, but also those that pose a risk to consumers and even lead to economic losses

due to the lack of control of the variety of microorganisms involved [47].

The association of bacteria in this work contributed to the reduction of fermentation time while accelerating the process. Coculture fermentation was better compared to fermentation with pure isolates. This clearly demonstrates various interactions including mutualism and symbiosis between strains, including their growth acceleration [48, 49]. Cocultured microorganisms have a promising potential to increase bioprocess efficiency and provide improved results [50]. Bader et al. (2009) also reported that coculture fermentations could lead to an increase in yield, and better control of product qualities [50]. Additionally, modification of raw materials during food production by cocultures would lead to an improvement in the texture, taste, and flavour of the fermented product as well as to stabilization of the microbial. The efficiency of *Bacillus* strains in coculture was tested to improve aflatoxin degradation [51-55].

The examination of the product of each fermentation was carried out and we noted the growth of yeasts in the product of the fermentation carried out with each pure isolate especially at 72 H; less in 48 H and a total absence of yeasts in 24 H. The total absence of yeasts in the product of 24 H fermentation could be explained by the mass secretion of certain antimicrobial substances of the *Bacillus* consortia preventing the development of yeasts. The co-culture fermentation here allowed for a domination by cells involved in the reactor, inhibiting the growth of other cells [51-55]. One of the particularities of the starter is the influence of the bacteria on each other during fermentation. The commercial *Bacillus* probiotic strains in use are *B. cereus, B. clausii, B. coagulans, B. licheniformis, B. polyfermenticus, B. pumilus,* and *B. subtilis*. Many of them had been uste as starter [56-60]. Therefore, the coculture starter makes it possible to considerably increase the number of cells involved. The food made with this starter is generally of better quality and cleaner due to the improvement of the fermentation environment. A large population from the start, strong and numerous enough to start fermentation quickly, would give less chance for the development of unwanted yeasts and bacteria [49].

The *Bacillus* species identified in this work are frequently isolated from many traditionally fermented food products in Africa and around the world [4, 8, 10, 61, 62]. The most dominant species among the identified isolates is *B. subtilis* (3) followed by *B. safensis* (2). We also identified one strain as *B. tequilensis* and another as *B. pumilus.* Those species have been identified in previous works [32, 36, 56, 63].

5. Conclusion

In this work, different biotechnological ferments used in the optimisation of cassava retting were developed from bacteria of the genus *Bacillus*. *Bacillus* isolated from fermented cassava was characterized by microbiology, biochemistry, and molecular biology techniques. The different isolates can secrete essential enzymes for the biotransformation of cassava: amylases (63.80%), cellulases (56.19%) and proteases (41.90%). They also produce biosurfactants (38.09%).

Molecular identification was performed only on isolates with a good fermentation profile in less time. B. Subtilis (S22), B. Safensis (S5 and S57) and B. Pumilus (S96) were obtained by fibrinolytic enzyme PCR. 16S rRNA PCR yielded Bacillus subtilis (Gen bank: MT994787.1) corresponding to strain S26, B.subtilis (Gen bank: MT994789.1) corresponding to strain S42 and B. tequilensis (Gen bank: MT994788.1), including strain S46. The strain S81 was associated with Bacillus sp. More concretely, molecular identification reveals that the Bacillus strains isolated in this work, such as B. safensis, B. subtilis, B. pumilus, B. subtilis, B. subtilis and B. tequilensis, could be used as starters in cassava retting. Each of these strains constitutes a ferment capable of retting in 48 hours.

The consortium between (*B. safensis* + *B. subtilis*), (*B. safensis* + *B. subtilis)*, (B. safensis + B. subtilis, (B. safensis + B. tequilensis, (*B. tequilensis* + *Bacillus* sp.*)*, (*B. tequilensis* + *B. pumilus*), (*B. safensis* + *Bacillus* sp), (*B. safensis* + *B. pumilus*) created a stimulus in cell growth during the fermentation process of cassava tubers. The retting time was reduced from 48 to 24 H. With three consortia having the highest penetrometry indices: (*B. Safensis* + *B. Pumilus*), (*B. tequilensis* + *B. Pumilus*), and (*B. subtilis*+ *B. Safensis*). No screening and characterization of potential *Bacillus* starter cultures for fermenting cassava tubers have been proposed.

The application of ferments is necessary for the industrialisation of fermented foods. For all microorganisms of interest, it is imperative to check their status, because they must be incapable of causing disease or other harmful effects for fermented products and consumers. A starter based on microorganisms should shorten the fermentation time, inhibit the growth of pathogens, and reliably reproduce fermented products. The use of ferments in the Republic of Congo will free the population from hunger because cassava and its derivatives will be available, accessible, and constant.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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